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Abstract

Background: Recent data support the hypothesis that genetic damage occurring early in life during childhood can play an important role in the development of chronic diseases in adulthood, including cancer. Objectives: The objective of this paper, part of the MAPEC_LIFE project, is to describe the frequency of micronuclei and meta-nuclear alterations in exfoliated buccal cells of 6-8-year-old Italian children recruited in five Italian towns (i.e., Brescia, Torino, Pisa, Perugia and Lecce) with different air pollution levels. Methods: About 200 children per town were recruited from primary schools. Biological samples were collected twice from the same children, in two different seasons (winter 2014-15 and late spring 2015). Cytogenetic damage was evaluated by the buccal micronucleus cytome assay. Results: Overall, n = 1,046 children represent the final cohort of the MAPEC_LIFE study. On the whole, the results showed a higher mean MN frequency in winter (0.42 ± 0.54 ‰) than late-spring (0.22 ± 0.34 ‰). MN frequency observed among the five Italian towns showed a trend that follows broadly the levels of air pollution in Italy: the highest MN frequency was observed in Brescia, the lowest in Lecce (winter) and Perugia (late-spring). Conclusions: To the best of our knowledge, the number of recruited children included in the analysis (n = 1,046) is the highest compared to previous studies evaluating the frequency of MN in exfoliated buccal cells so far. MN frequency was associated with winter season and living in towns at various levels of air pollution, suggesting an important role of this exposure in determining early cytogenetic effects.

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Buccal micronucleus cytome assay in primary school children: a descriptive analysis of the MAPEC_LIFE multicenter cohort study.

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Abbreviations:

Micronuclei (MN), nuclear buds (NBUD), buccal micronucleus cytome assay (BMCyt), phosphate-buffered saline, pH 7.4 (PBS), basal cells (BC), binucleated cells (BNC), condensed chromatin cells (CCC), karyorrhectic cells (KHC), pyknotic cells (PYK), karyolitic cells (KYL), repair index (RI), standard deviations (SD), body mass index (BMI), Italian Mediterranean Index (IMI), ~~$PM_{2.5}$~~, ~~PM2.5~~, particulate matter with aerodynamic diameter ≤ 2.5 μm; ~~NO_2~~, nitrogen dioxide.

Abstract

Background: Recent data support the hypothesis that genetic damage occurring early in life during childhood can play an important role in the development of chronic diseases in adulthood, including cancer.

Objectives: The objective of this paper, part of the MAPEC_LIFE project, is to describe the frequency of micronuclei and meta-nuclear alterations in exfoliated buccal cells of 6-8-year-old Italian children recruited in five Italian towns (i.e., Brescia, Torino, Pisa, Perugia and Lecce) with different air pollution levels.

Methods: About 200 children per town were recruited from primary schools. Biological samples were collected twice from the same children, in two different seasons (winter 2014-15 and late spring 2015). Cytogenetic damage was evaluated by the buccal micronucleus cytome assay.

Results: Overall, n = 1,046 children represent the final cohort of the MAPEC_LIFE study. On the whole, the results showed a higher mean MN frequency in winter (0.42 ± 0.54 ‰) than late-spring (0.22 ± 0.34 ‰). MN frequency observed among the five Italian towns showed a trend that follows broadly the levels of air pollution in Italy: the highest MN frequency was observed in Brescia, the lowest in Lecce (winter) and Perugia (late-spring).

Conclusions: To the best of our knowledge, the number of recruited children included in the analysis (n = 1,046) is the highest compared to previous studies evaluating the frequency of MN in exfoliated buccal cells so far. MN frequency was associated with winter season and living in towns at various levels of air pollution, suggesting an important role of this exposure in determining early cytogenetic effects.

Keywords: Children; Air pollution; Socio-economic factors; Early biological effects; Buccal micronucleus cytome assay; MAPEC_LIFE study.

Introduction

Several studies have shown an increased susceptibility of children population to the effects of genotoxic agents from both environment and lifestyle (Neri et al., 2006; Neri et al., 2006; Merlo et al., 2007). Children are considered a high-risk group in terms of the health effects because of their different and unique pathways of exposure, their dynamic developmental physiology and their longer life expectancy (WHO, 2008). Moreover, recent data support the hypothesis that genetic damage occurring early in life during childhood can play an important role in the development of chronic diseases in adulthood, including cancer (Wild and Kleinjans, 2003; Landrigan, 2004; WHO, 2005; Bateson and Schwartz, 2008; Grigg, 2009). The higher susceptibility of children, with respect to adults, to the noxious effects of environmental pollutants might depend on smaller airways, immature detoxification and metabolic systems, as well as frequent exposure to outdoor air of children (Kurt et al., 2016).

In the last decades, numerous epidemiological studies have used a molecular approach to study health and disease conditions and related risk factors, for improving measurement of exposure and for early detection of health effects (Bennett and Waters, 2000). Biomonitoring of genotoxic hazards has been reported in several studies by the use of different genotoxicity endpoints, such as analysis of primary DNA damage (by the comet assay), or cytogenetic effects, such as micronuclei (MN), chromosome aberrations and sister chromatid exchanges. Among genotoxicity endpoints, MN is one of the most commonly used biomarker in molecular epidemiology studies to assess the presence and extent of chromosomal damage in human population exposed to genotoxic agents and for the identification of genetic and lifestyle factors able to affect genome stability (Fenech et al., 1999; Knudsen and Hansen, 2007). MN appear in the cytoplasm of interphasic cells as small additional nuclei, smaller than the main nucleus. MN typically generate during the anaphase from acentric chromosome fragments (chromosome breakage produced by clastogen agents) or whole chromosomes (chromosome malsegregation caused by aneugen agents). Acentric or whole chromosomes are left behind during mitotic cellular division and, consequently, are excluded from

both of the daughter nuclei (Fenech et al., 2011). Because of the ability to detect both clastogenic (e.g., chromosome breakage) and aneugenic (e.g., spindle disruption) effects, MN are considered biomarkers of early biological effect (NRC, 2006; Kirsch-Volders et al., 2011).

MN in peripheral blood lymphocytes have been extensively used in human biomonitoring studies to identify potential genotoxic exposures as well as chromosomal instability (Fenech, 2002; Fenech, 2002) and the frequency of MN in circulating lymphocytes is recognized to be a predictor of cancer risk in human populations (Bonassi et al., 2007; Murgia et al., 2008; Bonassi et al., 2011). Moreover, a significant increase in MN frequency in lymphocytes was found in patients with cancer or preneoplatic lesions (El-Zein et al., 2006; El-Zein et al., 2011; Maffei et al., 2014), neurodegenerative diseases (Migliore et al., 2011), cardiovascular diseases and diabetes (Andreassi et al., 2011).

In recent years, exfoliated cells from epithelial tissues have been increasingly used in the MN assay. The assessment of MN in (uncultured) exfoliated epithelial cells from oral mucosa has provided a complementary method for cytogenetic analyses in a easily accessible tissue without cell culture requirement (Fenech et al., 2011). Nowadays, the human buccal micronucleus cytome assay (BMCyt) is one of the most widely used techniques to measure genetic damage in human population studies (Bonassi et al., 2011; Fenech et al., 2011; Bolognesi et al., 2013). Moreover, MN frequency measured in peripheral blood lymphocytes and in buccal cells, even if occurring at different frequency, showed to be highly correlated, and hence to have a similar ability to detect effects of exposure to genotoxic agents (Ceppi et al., 2010).

Through the micronucleus cytome assay in buccal exfoliated cells it is possible to evaluate, aside to chromosomal and DNA damage markers (MN, and nuclear buds), cell proliferation markers (basal and binucleated cells), cell death/apoptosis markers (cells with condensed chromatin, or karyorrhectic, pyknotic and karyolytic cells), and repair index (Thomas et al., 2009; Thomas and Fenech, 2011). Moreover, the micronucleus cytome assay on exfoliated cells is particularly useful

in biomonitoring studies involving children to avoid traumatic and painful sampling procedures causing children any discomforts.

The objective of this paper, part of the MAPEC_LIFE project ("Monitoring Air Pollution Effects on Children for Supporting Public Health Policy"), is to describe the frequency of MN and meta-nuclear alterations in exfoliated buccal cells of 6-8-year-old Italian children recruited in five Italian towns (i.e., Brescia, Torino, Pisa, Perugia and Lecce). Cytogenetic data are presented in relation to children's characteristic, such as socio-demographic and anthropometric features, lifestyle, parent's characteristic and outdoor/indoor exposure to genotoxic agents.

Material and Methods

Study design. The MAPEC_LIFE project ("Monitoring Air Pollution Effects on Children for Supporting Public Health Policy"), is a prospective epidemiological cohort study funded by the European Life+ Programme (LIFE12 ENV/IT/000614), which aimed to investigate the association between air pollution exposure and early biological effects in children. Details of the study design have been described elsewhere (Feretti et al., 2014). Briefly, the study was conducted in five Italian towns (Figure 1) characterized by different levels and features of air pollution. Brescia and Torino are located in the Po Valley in Northern Italy, a highly industrialized area with unfavorable climate conditions, at the highest levels of air pollution in Europe; Pisa and Perugia are located in a medium-low polluted area in Central Italy, where air pollutants only occasionally exceed law limit values; Lecce is located in a very low polluted area, in Southern Italy, where air pollutants never exceed limit values. The five towns have also different demographic and socio-economic characteristics (Bagordo et al., 2017).

About 200 children per town were recruited from primary schools to evaluate, in their buccal mucosa (BM) cells, 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). Biological samples were collected twice from the same children, in two different seasons (winter 2014-15 and late spring 2015).

The children's parents were interviewed to gather information on exposure to air pollutants from both indoor and outdoor sources and children's lifestyle (Zani et al., 2015). 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 prosthetic, were excluded. Children whose parents correctly filled in consent forms and valid questionnaires were invited to provide biological samples. Informed consent, in the form of comic, was obtained from children themselves prior of BM cells sampling. Overall, of the invited children, n = 1,318 in winter (season I) and n = 1,149 in late-spring (season II) could be subjected to biological sampling.

Ethical aspects. For each participating unit, the study was approved by the competent Ethics Committee. Participation in this study was voluntary. Informed consent was obtained from both children's parents and children themselves, after explanation of the purpose of the study. All the data were anonymized and treated confidentially in accordance with current Italian legislation on the treatment of sensitive data.

Questionnaire. We used a validated questionnaire (Zani et al., 2015) to collect data on characteristics of the area of residence and on demographic, socio-economic and anthropometric variables, diet, physical activity and other aspects of children's lifestyle. The questionnaire contained 148 items related to personal details (e.g., sex, age, height and weight), exposure to second-hand smoke at home and to other indoor pollution sources (e.g., home heating systems), traffic intensity near home and school, child's health, physical activity and diet, and parent's

characteristics (e.g., birthplace, education, work and smoking habits). The dietary section was based on ARCA questionnaire (Barba et al., 2012) and contained 117 items on average frequency of food consumption.

Chemicals and media. All reagents used were of analytical grade. Ethanol was obtained from J.T. Baker® Chemicals (Deventer, The Netherlands); polyethylene glycol and DePex mounting medium were from VWR International PBI Srl (Milan, Italy). Acetic acid and methanol were purchased from Carlo Erba Reagents Srl (Milan, Italy). 18G needles were obtained from Becton Dickinson Italia SpA (Milan, Italy). Schiff's reagent and Light-green were provided by Sigma Aldrich Srl (Milan, Italy). Nylon filters (100 mm) were from Merck Spa (Milan, Italy). Trypan blue and phosphate-buffered saline (PBS) were purchased from Invitrogen Srl (Milan, Italy). Conventional microscope slides and cover-slips were supplied by LLG® Labware (Meckenheim, Germany). Distilled water was used throughout the experiments.

Collection of biological samples. Children were asked to rinse the mouth twice with mineral water. Small-headed toothbrush was used to collect epithelial buccal cells by gently scraping (10 times in a circular motion) the inner surface of both cheeks. The head of the brush was then dipped into tubes containing 15 ml of Saccomanno's fixative (50% ethanol, 2% polyethylene glycol, vol./vol.; solution diluted in water and stored at 4°C) and rotated repeatedly to dislodge and release the cells into the buffer. The epithelial buccal cells in Saccomanno's fixative were centrifuged and washed twice with PBS. Cell suspensions were then drawn up into a syringe using an 18G 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) and the samples (coded tubes) were shipped overnight (+4°C) to the Cytogenetics

Laboratory at the Unit of Public Health, University of Perugia, for subsequent processing (i.e., single-center slide preparation and scoring).

Buccal micronucleus cytome assay (BMCyt assay). The BMCyt assay was performed according to the procedure described by Thomas and Fenech (Thomas and Fenech, 2011), with minor modifications. For each subject, two slides were prepared by smearing 100 µL of cell suspension onto pre-cleaned slides (approximately 1×10^5 cells/slide). The slides were treated with Schiff's reagent, washed in running water and then rinsed well in deionized water. Subsequently, the slides were stained with 0.2% Light Green reagent, air dried, and finally mounted with DePex mounting medium.

Slide scoring. The coded slides were read blind by trained scorers by following the scoring scheme proposed by Thomas and Fenech (Thomas and Fenech, 2011). The slide preparations were scored initially to determine the frequency of all the various cell types in a minimum of 1,000 cells; cell types, anomalies associated with cell death, and nuclear abnormalities indicative of chromosomal instability or DNA damage were classified essentially according to established criteria (Bolognesi et al., 2013). The slides were then scored for cells with MN and nuclear buds (NBUD) among a minimum of 2,000 differentiated cells (1,000/slide) as respective measures of chromosomal and DNA damage. Proliferation, cytotoxicity and cell-death events were evaluated by counts among 1,000 differentiated cells. Cell proliferation was determined by recording the number of basal cells (BC), whereas binucleated cells (BNC) ~~cells~~ indicated cytokinesis defect (cytotoxicity); condensed chromatin cells (CCC), karyorrhectic (KHC), pyknotic (PYK) and karyolytic (KYL) cells are regarded as markers of early-to-late stages of apoptosis and cell death (Tolbert et al., 1992). Repair index (RI) was calculated as the sum of KHC and KYL cells divided by the sum of MN and NBUD (Ramirez and Saldanha, 2002) by applying the simple Freeman-Tukey root transformation for overdispersed near-to-zero counts (Freeman and Tukey, 1950; Hothorn et al., 2013).

Statistical analysis. Means and standard deviations (SD) and counts and percentages were reported for continuous and categorical variables, respectively. Approximation to the normal distribution of continuous variables was evaluated using the Kolmogorov-Smirnov test. All the considered cell types, or anomalies associated with cell death or chromosomal instability/DNA damage (i.e., cells with MN, NBUD, CCC, KHC, PYK, and KYL cells) and RI showed significant departures from the normal distribution even after logarithmic transformation. The differences among groups were investigated using the non-parametric Kruskal-Wallis *H* test. The differences between values observed in the same children in two seasons were assessed using the Wilcoxon test for paired data. The comparisons among proportions were performed using the Pearson's χ^2 test. Correlation between continuous variables was assessed using the Spearman's correlation coefficient. All the statistical tests were two-sided with $\alpha = 0.05$. The data analyses were performed using SPSS 20 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Study population. Lifestyles and socio-demographic features of the MAPEC_LIFE complete cohort have been described in detail elsewhere (Grassi et al., 2016; Zani et al., 2016; Bagordo et al., 2017). The final cohort reported in this paper included only subjects with suitable biological data in both seasons. Overall, slides suitable for microscope analysis were obtained from $n = 1{,}093$ children in winter (season I); of these, $n = 1{,}046$ were sampled also during late-spring (season II) and represent the final cohort of the MAPEC_LIFE study. The main characteristics of the study cohort are summarized in Table 1, according to town of residence. Children were aged 6-8 years, with a mean age of 6.83 ± 0.90 years, and 50.3% of them were boys. The majority of children's parents were

Italian, with a high education level and employment rate, with some difference between the towns. As regards smoking habits, 18.7% of the mothers and 27.2% of the fathers were current smokers, however, the proportion varied significantly among the towns. Body mass index (BMI) values and weight classes were similar in the five towns, with about 30% of children overweight or obese. Significant differences among towns were found for the Italian Mediterranean Index (IMI), which overall showed a low adherence of the children's diet to the Mediterranean diet. The other children's and parents' characteristics did not vary from season I (winter) to season II (late-spring), apart from children outdoor sport or play practice with an increased proportion of children from season I to season II, and high exposure to vehicular traffic emission referred by parents from 67.7% in winter to 57.8% in late-spring. Important differences were found for the fuels used in the heating systems among the towns: district heating was the most represented system in Brescia, present, though less, in Torino, while it was negligible in the other towns, where natural gas heating system were prevalent.

BMCyt assay. The results of chromosomal and DNA damage markers (MN and NBUD), cell proliferation markers (BC and BNC), cell death/apoptosis markers (CCC, KHC, PYK, and KYL), and repair index (RI) in children are summarized in Table 2. Overall, the mean MN frequency decreased significantly (~50%) from winter to late-spring (0.42 ± 0.54 ‰ and 0.22 ± 0.34 ‰, respectively; $p < 0.001$). Similarly, NBUD frequency decreased from 0.26 ± 0.48 ‰ in winter to 0.17 ± 0.36 ‰ in late-spring ($p < 0.001$). All the other markers of cell proliferation and death followed the same trend, with values slightly higher in winter than in late-spring, while the repair index (RI) was significantly higher in late-spring than in winter (6.69 ± 2.42 and 6.24 ± 2.55, respectively; $p < 0.05$). By comparing children residing in the five towns, significantly different MN frequencies were found in both seasons, with the highest values in Brescia in both winter (0.53 ± 0.61 ‰) and late-spring (0.28 ± 0.42 ‰), and the lowest in Lecce in winter (0.32 ± 0.44 ‰) and in Perugia in late-spring (0.17 ± 0.28 ‰). Children living in the five towns had also statistically

different frequencies of NBUD, with the highest values in Pisa (season I: 0.58 ± 0.70 ‰; season II: 0.39 ± 0.55 ‰), the lowest in Lecce (season I: 0.14 ± 0.31 ‰; season II: 0.09 ± 0.26 ‰). Similar differences were observed for the frequency of BC (in season I), BNC (in season II) and cell death/apoptosis markers (CCC, KHC, PYK, and KYL). Statistically significant differences were observed also for RI values: in winter, the highest RI value was observed in Brescia (7.10 ± 2.78), the lowest in Torino (5.69 ± 2.20); whereas, in late-spring the highest RI value was observed in Torino (7.22 ± 2.24), the lowest in Perugia (6.01 ± 1.96). The proportion of children showing at least one MN in exfoliated buccal cells was 52.0% in the winter season and 35.9% in late-spring ($p < 0.001$). In winter, the highest proportion of children with at least one MN was observed in Brescia (59.9%), the lowest in Lecce (44.6%); in late-spring, the highest value was observed in Brescia (40.1%), the lowest in Perugia (31.4%) (Figure 2). The proportion of children with at least one MN decreased significantly in late-spring compared to winter in all the towns except Lecce, which showed similar values in season I and II.

The frequency of MN in children's buccal cells according to town of residence and children's and parents' features are summarized in Table 3. No variable was associated with children's MN frequency apart from mother's smoking habits in season II: children with smoking mothers showed higher MN frequency than those with non-smoking mothers (0.30 ± 0.37 ‰ and 0.20 ± 0.33 ‰, respectively; $p < 0.001$). Accordingly, in season II the proportion of children with at least one MN in exfoliated buccal cells was 46.7% and 33.3% in children with smoking or non-smoking mothers, respectively ($p = 0.007$) (Supplement Table 1S).

Table 4 shows MN frequency according to children's BMI, diet and exercise patterns. BMI and outdoor sport and play were not associated with the presence of MN, whereas a high adherence to the Mediterranean diet (IMI) was associated with a significant reduction in MN frequency in season I (winter).

Table 5 summarizes MN frequency according to parents' reports of traffic near children's home and school and type of fuel used for domestic heating. MN frequency was analyzed with reference to

only electric/gas or fossil fuel/wood as heating systems, as district heating was not considered to contribute to indoor pollution. MN frequency did not vary significantly by heating system, even if children with fossil fuel/wood heating system showed a slightly higher mean value than children using electric/gas system.

According to common interpretation of correlation coefficients, negligible values of Spearman's *r* were found between MN frequency and the other investigated biomarkers, apart from a negative correlation with RI ($r = -0.548$, $p < 0.001$) (data not shown in table).

Discussion

The frequency of markers of chromosomal and DNA damage, cell proliferation, cell death/apoptosis, and repair index was evaluated in exfoliated buccal cells of 1,046 children residing in five Italian towns during two seasons of the year (winter and late-spring). The results showed a higher mean MN frequency in winter (0.42 ± 0.54 ‰) than late-spring (0.22 ± 0.34 ‰), with a corresponding proportion of children with at least one MN in exfoliated buccal cells decreasing from 52.0% in winter to 35.9% in late-spring.

These results are not properly comparable with those of other studies investigating the genotoxic effect of residential exposure to genotoxic xenobiotics in exfoliated buccal mucosa cells of children in recent years. Differences in the study design features, such as subjects' age, method used, type of exposure, sample size, may cause a large variability among different studies (Fenech and Bonassi, 2011). MN mean frequency observed in our study (0.42 ± 0.54 ‰ in winter) was lower than that found in 64 children with mean age of 7.3 years, living close to major freeways and arterial roads in Oakland, California (0.67 ± 1.44 ‰) (Huen et al., 2006), and that observed in 411 9-years-old children living in the chipboard manufacturing district of Viadana, Italy (1.2 ± 0.9 ‰) (Marcon et al., 2014). Accordingly, a Brazilian study showed, in a small sample of children aged ≤ 7 years,

higher mean MN frequency in children living in the urban, polluted area (1.20 ± 0.83 ‰) than those living in a rural area (0.19 ± 0.31 ‰) (Sisenando et al., 2012). Overall, the MN frequencies observed in this study are close (higher in winter, lower in late-spring) to the value of the lowest confidence interval of the estimated mean MN values in exfoliated BC of healthy controls (Bonassi et al., 2011); in this review, on the basis of the extensive database derived from studies conducted on both children and adults, the MN mean was estimated to be 0.74 ‰ with a range of 0.3–1.7 MN/1,000 cells.

In our study, no differences were observed according to gender in the frequency of MN and meta-nuclear alterations, suggesting that boys and girls are similarly susceptible to genotoxic agents. This observation is in agreement with published data, which reported similar buccal cell MN frequencies in males and females (Bonassi et al., 2011). The results of this study confirm the increased risk of chromosomal alterations in epithelial buccal cells associated with the presence of smokers in the children's house. MN frequency was positively associated with mother smoking habits in season II (late-spring), in the absence of residential heating; this result is in line with a 30% or more increase in MN frequency in children exposed to cigarette smoke with respect to those unexposed (Neri et al., 2003; Holland et al., 2011). A high adherence of children's dietary habits to Mediterranean diet was associated with lower MN frequency, supporting a possible association between dietary habits and MN frequency, which is still under discussion for both adults and children (Bonassi et al., 2011). Statistically significant differences in MN frequency were observed among the five Italian towns in both seasons, with a trend that follows broadly the levels of air pollution in our country (ISPRA, 2016): the highest MN frequency was observed in Brescia, located in one of the most polluted area in Europe ($PM_{2.5}$ annual average of 29 µg/m^3 and NO_2 annual average of 68 µg/m^3 in 2015), and the lowest in Lecce, in which very low levels of air pollutants are usually registered ($PM_{2.5}$ annual average of 13 µg/m^3 and NO_2 annual average of 30 µg/m^3 in 2015). Intermediate values of MN frequency were found in Pisa and Perugia, where air pollutants only occasionally exceed law limit values ($PM_{2.5}$ annual average of 17 and 20 µg/m^3 and NO_2 annual average of 37

and 28 µg/m^3 for Pisa and Perugia in 2015, respectively). Instead, Torino showed a relatively low mean MN frequency in children's cells, despite its high concentrations of air pollutants ($PM_{2.5}$ annual average of 27 µg/m^3 and NO_2 annual average of 68 µg/m^3 in 2015).

Overall, our findings suggest an important role of air pollution exposure in MN formation in children's buccal cells, the evaluation of which represent the main aim of the MAPEC_LIFE project. A detailed analysis of the associations between single air pollutant levels and biomarkers of early effects in children's buccal cells, according to socio-demographic and lifestyle factors, will be reported in a companion paper.

This study has various strengths. First, the sample size: to the best of our knowledge, the number of recruited children included in the analysis (n = 1,046) is the highest compared to previous studies evaluating the frequency of MN in exfoliated buccal cells so far (Lahiri et al., 2000; Ceretti et al., 2014; Demircigil et al., 2014; Mergener et al., 2014; Silva da Silva et al., 2015; Cavalcante et al., 2017), particularly in 6-8 years old children (Huen et al., 2006; Sisenando et al., 2012; Marcon et al., 2014). Moreover, the cells of the 1,046 children were collected twice in two different seasons, winter and late-spring, allowing a comparison between the genotoxic effects in different air pollution conditions in the same subjects. Furthermore, to avoid inter-laboratory biases, biological samples collected in the five towns were processed for the BMCyt assay (i.e., slide preparation and scoring) in a single laboratory. Finally, investigation of the role of demographic, socio-economic, and life-style factors, as possible modifiers of the effect of air pollution on human health, and strict inclusion (e.g., residence in the urban area of the five towns) and exclusion (e.g., severe diseases, therapy with antineoplastic agents or radiation therapy, exposure to X-rays, or use of dental brace) criteria allowed us to exclude important confounding factors and to evaluate other possible risk factors for cytogenetic damage.

Conclusions

In conclusion, this study showed that MN frequency in buccal cells of children was associated with passive smoking at home and low adherence to Mediterranean diet, confirming the impact of these factors on children's health. Furthermore, MN frequency was associated with winter season and living in towns at various levels of air pollution, suggesting an important role of this exposure in determining early cytogenetic effects.

These findings support the need of local, national, and global efforts to decrease the impact of environmental exposure and promote educational programs on lifestyle determinants of health.

Conflict of interest

The authors declare they have no actual or potential competing financial interests.

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Figure 1. Map of Italy showing the geographical position of the five towns participating the multicenter MAPEC_LIFE study.

Figure 2. Proportion of children with at least one micronucleus in exfoliated buccal cells, according to town of residence.

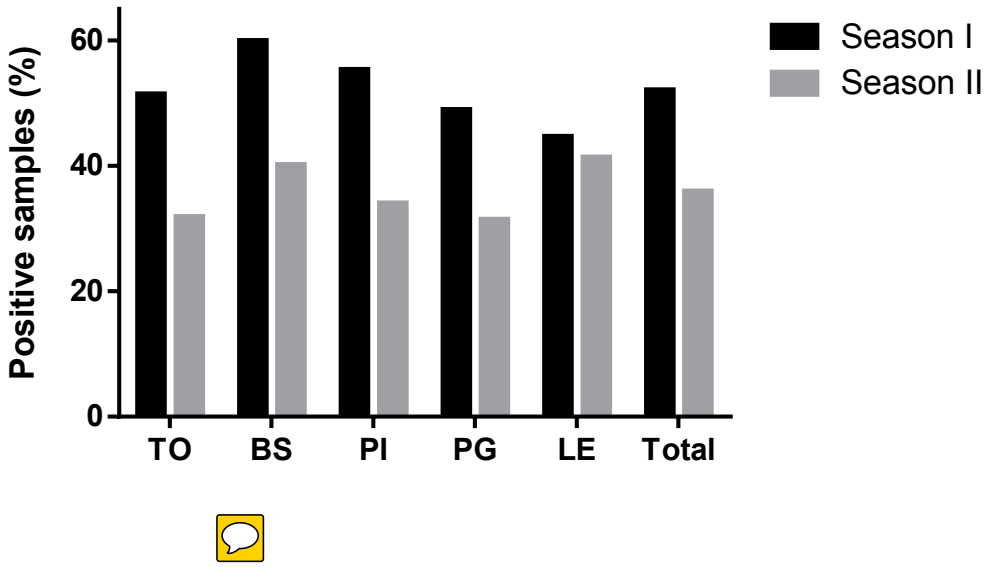

Table 1. Characteristics of children included in the study cohort: socio-demographic and anthropometric characteristics, life-style, parents' features, perceived traffic density and home heating systems.

Characteristics	Torino	Brescia	Pisa	Perugia	Lecce	Total	*p*-value
Children[1]	214 (20.5)	237 (22.7)	159 (15.2)	223 (21.3)	213 (20.4)	1.046 (100.0)	
Boys / Girls[2]	112 / 102 (1.10)	108 / 129 (0.84)	71 / 88 (0.81)	129 / 94 (1.37)	106 / 107 (0.99)	526 / 520 (1.01)	*0.048*[a]
Children's age[3]	6.76 ± 0.81	6.91 ± 0.88	6.69 ± 0.98	6.89 ± 0.93	6.88 ± 0.90	6.83 ± 0.90	*n.s.*[b]
Parents of Italian nationality[1]							
Mother	175 (81.8)	196 (82.7)	123 (77.8)	193 (86.5)	195 (91.5)	882 (84.4)	*0.003*[a]
Father	183 (86.7)	203 (87.9)	132 (84.1)	202 (92.2)	203 (95.8)	923 (89.6)	*0.001*[a]
Parents' level of education[1]							
Mother (high school or university)	169 (79.0)	188 (79.3)	136 (86.1)	203 (91.0)	180 (84.5)	876 (83.8)	*0.002*[a]
Father (high school or university)	159 (75.4)	159 (68.8)	117 (74.5)	185 (84.5)	171 (80.7)	791 (76.8)	*0.001*[a]
Parents' occupational status[1]							
Mother employed	166 (77.6)	178 (75.1)	117 (74.1)	172 (77.1)	131 (61.5)	764 (73.1)	*0.016*[a]

Father employed		190 (90.0)	216 (93.5)	136 (86.6)	201 (91.8)	178 (84.0)	921 (89.4)	*0.047*[a]
Parents' smoking habits[1]								
Mother current smoker		52 (24.3)	43 (18.1)	33 (20.9)	25 (11.2)	42 (19.7)	195 (18.7)	*0.010*[a]
Father current smoker		77 (36.5)	54 (23.4)	45 (28.7)	46 (21.0)	58 (27.4)	280 (27.2)	*0.004*[a]
Children's BMI[4]								
Season I	**Mean**	16.33 ± 2.53	16.29 ± 2.53	16.61 ± 2.32	16.83 ± 2.92	16.73 ± 2.88	16.55 ± 2.66	*n.s.*[b]
	UW[1]	14 (6.5)	19 (8.0)	7 (4.4)	11 (4.9)	11 (5.2)	62 (5.9)	*n.s.*[a]
	NW	147 (68.7)	161 (67.9)	105 (66.0)	145 (65.0)	135 (63.4)	693 (66.3)	
	OW	37 (17.3)	37 (15.6)	29 (18.2)	44 (19.7)	48 (22.5)	195 (18.6)	
	OB	16 (7.5)	20 (8.4)	18 (11.3)	23 (10.3)	19 (8.9)	96 (9.2)	
Season II	**Mean**	16.57 ± 3.12	16.28 ± 2.13	16.88 ± 3.03	16.71 ± 2.60	16.77 ± 2.60	16.62 ± 2.69	*n.s.*[b]
	UW[1]	12 (5.6)	10 (4.2)	9 (5.7)	7 (3.1)	11 (5.2)	49 (4.7)	*n.s.*[a]
	NW	138 (64.5)	173 (73.0)	106 (66.7)	151 (67.7)	133 (62.4)	701 (67.0)	

	OW	48 (22.4)	40 (16.9)	25 (15.7)	41 (18.4)	49 (23.0)	203 (19.4)	
	OB	16 (7.5)	14 (5.9)	19 (11.9)	24 (10.8)	20 (9.4)	93 (8.9)	
Children's IMI[5]								
Season I	**Mean**	3.64 ± 1.76	3.56 ± 1.63	3.33 ± 1.73	3.87 ± 1.80	3.08 ± 1.68	3.51 ± 1.74	*<0.001*[b]
	Low MD	101 (47.2)	123 (51.9)	93 (58.5)	99 (44.4)	135 (63.4)	551 (52.7)	
	Medium MD	82 (38.3)	80 (33.8)	47 (29.6)	85 (38.1)	58 (27.2)	352 (33.7)	*0.006*[a]
	High MD	31 (14.5)	34 (14.3)	19 (11.9)	39 (17.5)	20 (9.4)	143 (13.7)	
Season II	**Mean**	3.38 ± 1.61[c]	3.28 ± 1.75[c]	3.37 ± 1.66	3.51 ± 1.74[c]	2.97 ± 1.68	3.30 ± 1.70[c]	*0.013*[b]
	Low MD	122 (57.0)	139 (58.6)	88 (55.3)	127 (57.0)	140 (65.7)	616 (58.9)	
	Medium MD	67 (31.3)	73 (30.8)	56 (35.2)	62 (27.8)	57 (26.8)	315 (30.1)	*n.s.*[a]
	High MD	25 (11.7)	25 (10.5)	15 (9.4)	34 (15.2)	16 (7.5)	115 (11.0)	
Children's outdoor sport[1]								
Season I		51 (23.8)	76 (32.1)	43 (27.0)	71 (31.8)	55 (25.8)	296 (28.3)	*n.s.*[a]

Season II		61 (28.5)	99 (41.8)	55 (34.6)	92 (41.3)	79 (37.1)	386 (36.9)	*0.026*[a]
Children's outdoor play[1]								
Season I		74 (34.6)	87 (36.7)	67 (42.1)	62 (27.8)	55 (25.8)	345 (33.0)	*0.004*[a]
Season II		159 (74.3)	169 (71.3)	129 (81.1)	149 (66.8)	126 (59.2)	732 (70.0)	*<0.001*[a]
Traffic density[1]								
Season I	**Low**	59 (27.6)	81 (34.2)	80 (50.3)	39 (17.5)	79 (37.1)	338 (32.3)	*<0.001*[a]
	High	155 (72.4)	156 (65.8)	79 (49.7)	184 (82.5)	134 (62.9)	708 (67.7)	
Season II	**Low**	80 (37.4)	107 (45.1)	91 (57.2)	60 (26.9)	103 (48.4)	441 (42.2)	*<0.001*[a]
	High	134 (62.6)	130 (54.49)	68 (42.8)	163 (73.1)	110 (51.6)	605 (57.8)	
Heating systems[1]								
Electric or gas		128 (59.8)	74 (31.2)	153 (96.2)	178 (79.8)	195 (91.5)	728 (69.6)	
Fossil fuels/diesel or wood/pellet		25 (11.7)	16 (6.8)	6 (3.8)	42 (18.8)	18 (8.5)	107 (10.2)	*<0.001*[a]
District heating		61 (28.5)	147 (62.0)	--- (0.0)	3 (1.3)	--- (0.0)	211 (20.2)	

[1] Number of subjects and % (between brackets), respectively.

[2] Number of subjects and masculinity ratio (between brackets), respectively.

[3] Group mean ± standard deviation (age expressed in years).

[4] Group mean ± standard deviation [body mass index, BMI = weight (kg) divided by the square of the height (m)]. The children were classified as underweight (UW), normal weight (NW), overweight (OW), or obese (OB) on the basis of their BMI.

[5] Group mean ± standard deviation (IMI = Italian Mediterranean Index). IMI was calculated according to the intake of 6 typical Mediterranean and 4 non-Mediterranean foods. Based on IMI score, ranging from 0 to 10, adherence to Mediterranean diet (MD) was classified as low (≤ 3), medium (4-5) or high (≥ 6).

Statistical significance:

[a] Comparisons among proportions were performed using the Pearson's χ^2 test.

[b] Differences among the five towns were investigated using the non-parametric Kruskal-Wallis *H* test.

[c] ($p < 0.05$) differences between values observed in the same children in two seasons were assessed using the Wilcoxon test for paired data.

n.s.: not significant.

Table 2. Frequency of chromosome damage markers (MN and NBUD), cell proliferation markers (BC and BNC), cell death markers (CCC, KHC, PYK and KYL) and RI in exfoliated buccal cells of children according to town of residence; data summarized as the group mean ± standard deviation.

Biomarker[1]		Town					Total	*p*-value[a]
		Torino	Brescia	Pisa	Perugia	Lecce		
MN (‰)	Season I	0.39 ±0.48	0.53 ±0.61	0.46 ±0.59	0.40 ±0.57	0.32 ±0.44	0.42 ±0.54[b]	*0.002*
	Season II	0.18 ±0.29	0.28 ±0.42	0.24 ±0.37	0.17 ±0.28	0.24 ±0.32	0.22 ±0.34[b]	*0.036*
NBUDs (‰)	Season I	0.25 ±0.40	0.24 ±0.46	0.58 ±0.70	0.19 ±0.40	0.14 ±0.31	0.26 ±0.48[b]	*<0.001*
	Season II	0.11 ±0.27	0.17 ±0.37	0.39 ±0.55	0.15 ±0.29	0.09 ±0.26	0.17 ±0.36[b]	*<0.001*
BC (‰)	Season I	0.36 ±0.66	0.61 ±1.25	0.64 ±1.15	0.33 ±0.61	0.27 ±0.55	0.44 ±0.90[b]	*0.012*
	Season II	0.17 ±0.42	0.16 ±0.40	0.13 ±0.38	0.13 ±0.35	0.19 ±0.41	0.16 ±0.39[b]	*n.s.*
BNC (‰)	Season I	3.93 ±2.11	3.76 ±2.17	3.83 ±2.08	3.83 ±1.79	3.41 ±1.87	3.75 ±2.01[b]	*n.s.*
	Season II	3.28 ±1.92	3.78 ±1.99	4.06 ±1.85	3.51 ±1.80	3.26 ±1.88	3.56 ±1.91[b]	*<0.001*
CCC (‰)	Season I	28.55 ±17.64	35.68 ±21.41	25.35 ±15.21	27.46 ±20.99	26.85 ±19.30	29.10 ±19.61[b]	*<0.001*
	Season II	18.87 ±12.56	24.31 ±18.43	24.97 ±14.60	18.35 ±13.12	26.60 ±18.59	22.49 ±16.10[b]	*<0.001*
KHC (‰)	Season I	10.37 ±8.37	22.84 ±18.96	12.42±9.99	10.74 ±11.70	11.57 ±11.49	13.83 ±13.81[b]	*<0.001*
	Season II	11.61 ±11.42	11.89 ±10.95	9.25 ±7.16	8.72 ±7.10	13.29 ±13.90	11.04 ±10.69[b]	*<0.001*
PYK (‰)	Season I	0.15 ±0.48	0.37 ±0.69	0.27 ±0.55	0.27 ±0.69	0.08 ±0.28	0.23 ±0.57[b]	*<0.001*
	Season II	0.11 ±0.33	0.10 ±0.30	0.11 ±0.31	0.18 ±0.43	0.11 ±0.31	0.12 ±0.34[b]	*n.s.*
KYL (‰)	Season I	24.96 ±16.56	32.84 ±16.80	39.77 ±26.00	23.07 ±14.36	26.86 ±16.27	28.98 ±18.76	*<0.001*
	Season II	29.29 ±15.20	27.69 ±14.36	38.45 ±20.31	19.73 ±9.73	28.62 ±21.05	28.15 ±17.25	*<0.001*
RI	Season I	5.69 ±2.20	7.10 ±2.78	5.98 ±2.44	5.83 ±2.49	6.44 ±2.52	6.24 ±2.55[b]	*<0.001*

	Season II	7.22 ±2.24	6.62 ±2.50	6.60 ±2.30	6.01 ±1.96	7.04 ±2.81	6.69 ±2.42[b]	*<0.001*

[1] MN, micronuclei; NBUD, nuclear buds; BC, basal cells; BNC, binucleated cells; CCC, condensed chromatin cells; KHC, karyorrhectic cells; PYK, pyknotic cells; KYL, karyolytic cells; RI, repair index.

Statistical significance:

[a] differences among the five towns (Kruskal-Wallis *H* test).

[b] differences between values observed in all children in the two seasons (Wilcoxon test for paired data): MN, $p < 0.001$; NBUD, $p < 0.001$; BC, $p < 0.001$; BNC, $p = 0.033$; CCC, $p < 0.001$; KHC, $p < 0.001$; PYK, $p < 0.001$; KYL, n.s.; RI, $p < 0.001$.

n.s.: not significant.

Table 3. Frequency of MN in exfoliated buccal cells of children according to town of residence and in relation to children sex, and parents' nationality, level of education, occupational status, and smoking habits.

Characteristics		MN (‰)[1]					
		Torino	**Brescia**	**Pisa**	**Perugia**	**Lecce**	**Total**
Sex							
Season I	**M**	0.40 ± 0.48	0.54 ± 0.64	0.51 ± 0.57	0.40 ± 0.64	0.29 ± 0.39	0.42 ± 0.56
	F	0.38 ± 0.48	0.53 ± 0.58	0.41 ± 0.61	0.40 ± 0.47	0.35 ± 0.48	0.42 ± 0.53
Season II	**M**	0.17 ± 0.25	0.31 ± 0.43	0.22 ± 0.36	0.16 ± 0.31	0.23 ± 0.29	0.21 ± 0.33
	F	0.20 ± 0.32	0.27 ± 0.42	0.25 ± 0.39	0.19 ± 0.24	0.24 ± 0.34	0.23 ± 0.35
Mother nationality							
Season I	**Italian**	0.41 ± 0.50	0.52 ± 0.60	0.50 ± 0.62	0.40 ± 0.58	0.32 ± 0.42	0.43 ± 0.55
	Foreigner	0.28 ± 0.36	0.60 ± 0.65	0.33 ± 0.47	0.38 ± 0.49	0.31 ± 0.62	0.39 ± 0.53
Season II	**Italian**	0.18 ± 0.29	0.28 ± 0.43	0.22 ± 0.36	0.18 ± 0.29	0.24 ± 0.32	0.22 ± 0.34
	Foreigner	0.19 ± 0.30	0.29 ± 0.39	0.27 ± 0.39	0.12 ± 0.22	0.19 ± 0.25	0.22 ± 0.33
Father nationality							
Season I	**Italian**	0.40 ± 0.48	0.53 ± 0.60	0.47 ± 0.61	0.39 ± 0.56	0.33 ± 0.44	0.42 ± 0.54
	Foreigner	0.34 ± 0.45	0.57 ± 0.68	0.43 ± 0.51	0.44 ± 0.61	0.11 ± 0.33	0.42 ± 0.56
Season II	**Italian**	0.19 ± 0.29	0.28 ± 0.43	0.21 ± 0.34	0.18 ± 0.29	0.23 ± 0.32	0.22 ± 0.34

	Foreigner	0.16 ± 0.31	0.30 ± 0.42	0.36 ± 0.49	0.12 ± 0.22	0.33 ± 0.35	0.25 ± 0.38
Mother level of education[2]							
Season I	**High**	0.39 ± 0.46	0.52 ± 0.61	0.47 ± 0.62	0.40 ± 0.59	0.29 ± 0.40	0.41 ± 0.54
	Low	0.39 ± 0.54	0.60 ± 0.61	0.41 ± 0.45	0.40 ± 0.42	0.45 ± 0.58	0.47 ± 0.55
Season II	**High**	0.16 ± 0.28	0.28 ± 0.43	0.25 ± 0.38	0.18 ± 0.29	0.23 ± 0.30	0.22 ± 0.34
	Low	0.26 ± 0.33	0.29 ± 0.40	0.14 ± 0.28	0.10 ± 0.21	0.27 ± 0.40	0.23 ± 0.35
Father level of education[2]							
Season I	**High**	0.37 ± 0.42	0.55 ± 0.66	0.46 ± 0.54	0.41 ± 0.60	0.31 ± 0.42	0.42 ± 0.54
	Low	0.43 ± 0.62	0.49 ± 0.48	0.48 ± 0.75	0.34 ± 0.34	0.37 ± 0.49	0.43 ± 0.55
Season II	**High**	0.18 ± 0.29	0.29 ± 0.43	0.26 ± 0.39	0.18 ± 0.29	0.24 ± 0.30	0.23 ± 0.34
	Low	0.21 ± 0.30	0.28 ± 0.41	0.15 ± 0.30	0.16 ± 0.24	0.23 ± 0.39	0.22 ± 0.35
Mother occupational status							
Season I	**Employed**	0.37 ± 0.44	0.46 ± 0.56	0.46 ± 0.56	0.35 ± 0.58	0.31 ± 0.45	0.38 ± 0.52
	Unemployed	0.41 ± 0.51	0.58 ± 0.63	0.46 ± 0.62	0.45 ± 0.56	0.32 ± 0.43	0.45 ± 0.56
Season II	**Employed**	0.17 ± 0.30	0.26 ± 0.43	0.18 ± 0.31	0.18 ± 0.27	0.21 ± 0.28	0.20 ± 0.32
	Unemployed	0.19 ± 0.28	0.30 ± 0.42	0.27 ± 0.40	0.17 ± 0.30	0.25 ± 0.33	0.24 ± 0.36
Father occupational status							

Season I	**Employed**	0.38 ± 0.46	0.54 ± 0.62	0.49 ± 0.61	0.39 ± 0.57	0.32 ± 0.41	0.42 ± 0.55
	Unemployed	0.43 ± 0.58	0.50 ± 0.46	0.32 ± 0.50	0.44 ± 0.52	0.33 ± 0.53	0.39 ± 0.52
Season II	**Employed**	0.18 ± 0.30	0.27 ± 0.40	0.24 ± 0.38	0.17 ± 0.29	0.23 ± 0.31	0.22 ± 0.34
	Unemployed	0.20 ± 0.25	0.45 ± 0.62	0.20 ± 0.35	0.19 ± 0.25	0.28 ± 0.34	0.26 ± 0.38
Mother smoking habits							
Season I	**Current smoker**	0.44 ± 0.57	0.52 ± 0.49	0.32 ± 0.46	0.30 ± 0.43	0.46 ± 0.57	0.43 ± 0.52
	Non-smoker	0.37 ± 0.44	0.54 ± 0.63	0.50 ± 0.62	0.41 ± 0.59	0.28 ± 0.39	0.42 ± 0.55
Season II	**Current smoker**	0.21 ± 0.26	0.37 ± 0.42	0.29 ± 0.42	0.24 ± 0.33	0.37 ± 0.40	0.30 ± 0.37*
	Non-smoker	0.17 ± 0.29	0.27 ± 0.42	0.22 ± 0.36	0.16 ± 0.33	0.20 ± 0.29	0.20 ± 0.33*
Father smoking habits							
Season I	**Current smoker**	0.44 ± 0.51	0.47 ± 0.50	0.43 ± 0.57	0.30 ± 0.41	0.42 ± 0.51	0.42 ± 0.50
	Non-smoker	0.36 ± 0.46	0.55 ± 0.64	0.47 ± 0.61	0.42 ± 0.60	0.28 ± 0.40	0.42 ± 0.56
Season II	**Current smoker**	0.21 ± 0.32	0.35 ± 0.43	0.26 ± 0.43	0.20 ± 0.29	0.28 ± 0.38	0.26 ± 0.37
	Non-smoker	0.17 ± 0.27	0.27 ± 0.42	0.23 ± 0.35	0.17 ± 0.28	0.22 ± 0.29	0.21 ± 0.33

[1] MN, micronuclei; data summarized as the group mean ± standard deviation.

[2] The parents' level of education was defined on the basis of the answers reported in the questionnaire; a high level of education was assigned to parents reported to have a university degree or a high school diploma, a low level of education was assigned to parents having lower qualifications.

Statistical significance:

* $p < 0.05$, differences among the characteristics were investigated by the non-parametric Kruskal-Wallis *H* test.

Table 4. Frequency of MN in exfoliated buccal cells of children according to town of residence and in relation to child diet and exercise patterns.

Characteristics		MN (‰)[1]					
		Torino	**Brescia**	**Pisa**	**Perugia**	**Lecce**	**Total**
Children's BMI[2]							
Season I	**UW**	025 ± 0.38	0.53 ± 0.49	0.29 ± 0.39	0.82 ± 1.33	0.27 ± 0.34	0.44 ± 0.68
	NW	0.38 ± 047	0.52 ± 0.59	0.42 ± 0.50	0.39 ± 0.50	0.27 ± 0.39	0.40 ± 0.51
	OW	0.49 ± 0.48	0.61 ± 0.65	0.45 ± 0.62	0.36 ± 0.50	0.42 ± 0.49	0.46 ± 0.54
	OB	0.38 ± 0.59	0.53 ± 0.77	0.75 ± 0.96	0.35 ± 0.51	0.47 ± 0.59	0.49 ± 0.70
Season II	**UW**	0.21 ± 0.26	0.25 ± 0.35	0.17 ± 0.25	0.07 ± 0.19	0.27 ± 0.34	0.20 ± 0.29
	NW	0.17 ± 0.30	0.27 ± 0.42	0.27 ± 0.39	0.18 ± 0.27	0.25 ± 0.33	0.23 ± 0.35
	OW	0.19 ± 0.28	0.32 ± 0.47	0.18 ± 0.38	0.17 ± 0.36	0.18 ± 0.26	0.21 ± 0.35
	OB	0.25 ± 0.26	0.32 ± 0.37	0.16 ± 0.34	0.19 ± 0.25	0.27 ± 0.30	0.23 ± 0.30
Children's IMI[3]							
Season I	**Low MD**	0.43 ± 0.51	0.52 ± 0.58	0.49 ± 0.61	0.36 ± 0.49	0.30 ± 0.41	0.41 ± 0.52*
	Medium MD	0.45 ± 0.48	0.59 ± 0.67	0.44 ± 0.57	0.48 ± 0.69	0.34 ± 0.44	0.47 ± 0.59*
	High MD	0.11 ± 0.21	0.47 ± 0.55	0.37 ± 0.60	0.32 ± 0.47	0.40 ± 0.60	0.33 ± 0.50*
Season II	**Low MD**	0.18 ± 0.30	0.28 ± 0.41	0.18 ± 0.30	0.17 ± 0.24	0.25 ± 0.33	0.22 ± 0.33
	Medium MD	0.18 ± 0.27	0.32 ± 0.46	0.28 ± 0.39	0.15 ± 0.27	0.22 ± 0.30	0.23 ± 0.35

	High MD	0.18 ± 0.28	0.22 ± 0.36	0.40 ± 0.57	0.24 ± 0.41	0.22 ± 0.31	0.24 ± 0.39
Outdoor sport							
Season I	**No**	0.37 ± 0.48	0.57 ± 0.63	0.43 ± 0.59	0.44 ± 0.60	0.36 ± 0.46	0.43 ± 0.56
	Yes	0.43 ± 0.48	0.46 ± 0.56	0.55 ± 0.59	0.32 ± 0.50	0.21 ± 0.33	0.39 ± 0.51
Season II	**No**	0.19 ± 0.30	0.25 ± 0.41	0.21 ± 0.37	0.18 ± 0.25	0.25 ± 0.32	0.22 ± 0.33
	Yes	0.17 ± 0.27	0.33 ± 0.44	0.28 ± 0.37	0.17 ± 0.33	0.22 ± 0.32	0.24 ± 0.36
Outdoor play							
Season I	**No**	0.39 ± 0.45	0.46 ± 0.50	0.40 ± 0.50	0.43 ± 0.61	0.30 ± 0.40	0.40 ± 0.50
	Yes	0.39 ± 0.23	0.66 ± 0.74	0.54 ± 0.70	0.32 ± 0.44	0.36 ± 0.53	0.47 ± 0.62
Season II	**No**	0.19 ± 0.31	0.25 ± 0.43	0.23 ± 0.34	0.15 ± 0.24	0.21 ± 0.27	0.20 ± 0.32
	Yes	0.18 ± 0.28	0.30 ± 0.42	0.24 ± 0.38	0.18 ± 0.30	0.25 ± 0.34	0.23 ± 0.35

[1] MN, micronuclei; data summarized as the group mean ± standard deviation.

[2] Body mass index [BMI = weight (kg) divided by the square of the height (m)]. The children were classified as underweight (UW), normal weight (NW), overweight (OW), or obese (OB) on the basis of their BMI.

[3] Italian Mediterranean Index (IMI) was calculated according to the intake of 6 typical Mediterranean and 4 non-Mediterranean foods. Based on IMI score, ranging from 0 to 10, adherence to Mediterranean diet (MD) was classified as low (≤ 3), medium (4-5) or high (≥ 6).

Statistical significance:

* $p < 0.05$, differences among the characteristics were investigated by the non-parametric Kruskal-Wallis *H* test.

Table 5. Frequency of MN in exfoliated buccal cells of children according to the level of motor traffic near children's homes and schools and the type of fuel used for domestic heating.

		MN[1]					
Characteristics		**Torino**	**Brescia**	**Pisa**	**Perugia**	**Lecce**	**Total**
Traffic at child's home/school[2]							
Season I	**Low**	0.24 ± 0.35	0.55 ± 0.62	0.43 ± 0.50	0.29 ± 0.42	0.29 ± 0.46	0.38 ± 0.50
	High	0.45 ± 0.51	0.53 ± 0.61	0.49 ± 0.68	0.42 ± 0.60	0.34 ± 0.42	0.44 ± 0.56
Season II	**Low**	0.23 ± 0.32	0.28 ± 0.45	0.22 ± 0.36	0.22 ± 0.34	0.21 ± 0.29	0.23 ± 0.36
	High	0.16 ± 0.32	0.29 ± 0.40	0.26 ± 0.39	0.16 ± 0.26	0.26 ± 0.34	022 ± 0.33
Heating system at child's home							
Season I	**Electric or gas**	0.37 ± 0.45	0.51 ± 0.54	0.46 ± 0.60	0.38 ± 0.58	0.31 ± 0.44	0.39 ± 0.53
	Fossil fuels or wood	0.36 ± 0.47	0.69 ± 0.66	0.50 ± 0.55	0.46 ± 0.55	0.39 ± 0.40	0.46 ± 0.53
Season II	**Electric or gas**	0.18 ± 0.29	0.30 ± 0.44	0.22 ± 0.36	0.17 ± 0.29	0.24 ± 0.32	0.22 ± 0.33
	Fossil fuels or wood	0.18 ± 0.32	0.44 ± 0.54	0.67 ± 0.52	0.18 ± 0.24	0.17 ± 0.24	0.24 ± 0.36

[1] MN, micronuclei; data summarized as the group mean ± standard deviation and the percentage of children with at least one micronucleus (between brackets).

[2] Vehicular traffic density was defined as "low" (cars and trucks never/seldom passed the child's house and/or school) or "high" (cars or trucks passed frequently the child's house and/or school).

Table 1S. Proportion of children with at least one micronucleus in exfoliated buccal cells according to town of residence and in relation to parents' nationality, level of education, occupational status, and smoking habits.

Characteristics		MN[1]					
		Torino	**Brescia**	**Pisa**	**Perugia**	**Lecce**	**Total**
Mother nationality							
Season I	**Italian**	52.6	59.2	57.7	48.2	45.6	52.3
	Foreigner	46.2	63.4	45.7	53.3	33.3	50.3
Season II	**Italian**	31.4	39.3	32.5	32.6	38.9	35.8
	Foreigner	33.3	43.9	37.1	23.3	41.5	35.6
Father nationality							
Season I	**Italian**	52.5	59.6	55.3	48.0	46.3	52.1
	Foreigner	46.4	64.3	56.0	58.8	11.1	52.3
Season II	**Italian**	33.3	39.4	33.6	32.7	40.4	36.0
	Foreigner	25.0	42.9	40.0*	23.5	55.6	35.5
Mother level of education[2]							
Season I	**High**	52.7	58.5	53.7	47.8	42.8	50.9
	Low	46.7	65.3	63.6	60.0	54.5	57.4
Season II	**High**	28.4	39.4	35.3	32.5	40.6	35.3

	Low	44.4*	42.9	22.7	20.0	45.5	38.5
Father level of education[2]							
Season I	**High**	54.1	58.5	56.4	47.6	44.4	51.7
	Low	44.2	63.9	52.5	55.9	46.3	53.6
Season II	**High**	30.2	39.0	37.6	31.9	42.1	36.0
	Low	38.5	41.7	22.5	32.4	36.6	35.6
Mother occupational status							
Season I	**Employed**	50.9	53.8	56.1	42.3	43.0	48.8
	Unemployed	51.9	63.9	54.3	55.4	45.5	54.4
Season II	**Employed**	29.2	35.5	28.8	33.3	38.0	33.0
	Unemployed	34.3	43.1	37.0	29.5	43.3	38.0
Father occupational status							
Season I	**Employed**	51.6	59.3	58.3	47.7	46.2	52.5
	Unemployed	51.9	68.2	40.0	58.3	38.5	49.6
Season II	**Employed**	31.0	38.8	34.8	31.3	39.9	35.2
	Unemployed	40.7	50.0	28.0	37.5	46.2	40.9
Mother smoking habits							

Season I	**Current smoker**	55.8	65.1	45.5	44.0	52.4	53.8
	Non-smoker	50.0	58.8	57.6	49.5	42.7	51.5
Season II	**Current smoker**	38.5	53.5	39.4	40.0	59.5	46.7*
	Non-smoker	29.6	37.1	32.0	30.3	36.8	33.3*
Father smoking habits							
Season I	**Current smoker**	59.7	61.1	51.1	45.7	51.7	54.6
	Non-smoker	47.0	59.9	57.1	49.7	42.2	51.2
Season II	**Current smoker**	35.1	50.0	33.3	34.8	46.6	40.0
	Non-smoker	30.6	36.7	33.9	31.2	39.0	34.4

[1] MN, micronuclei; data summarized as the percentage of children with at least one micronucleus.

[2] The parents' level of education was defined on the basis of the answers reported in the questionnaire; a high level of education was assigned to parents reported to have a university degree or a high school diploma, a low level of education was assigned to parents having lower qualifications.

Statistical significance:

* $p < 0.05$, differences among proportions performed using the Pearson's χ^2 test.

Table 2S. Proportion of children with at least one micronucleus in exfoliated buccal cells according to town of residence and in relation to child diet and exercise patterns.

		MN[1]					
Characteristics		**Torino**	**Brescia**	**Pisa**	**Perugia**	**Lecce**	**Total**
Children's BMI[2]							
Season I	**UW**	35.7	68.4	42.9	54.5	45.5	51.6*
	NW	50.3	59.6	55.2	49.7	39.3	50.9*
	OW	64.9	64.9	48.3	47.7	56.2	56.4*
	OB	43.8	45.0	72.2	43.5	52.6	51.0*
Season II	**UW**	41.7	40.0	33.3	14.3	45.5	36.7
	NW	28.3	38.7	38.7	32.5	42.1	35.9
	OW	33.3	42.5	24.0	26.8	34.7	33.0
	OB	50.0	50.0	21.1	37.5	50.0	40.9
Children's IMI[3]							
Season I	**Low MD**	54.5	58.5	62.4	45.5	44.4	52.6
	Medium MD	58.5	63.7	48.9	55.3	43.1	55.1
	High MD	22.6	55.9	36.8	43.6	50.0	42.0
Season II	**Low MD**	31.1	39.6	29.5	32.3	42.9	35.7

	Medium MD	32.8	42.5	39.3	24.4	38.6	36.2
	High MD	32.0	36.0	40.0	35.3	37.5	35.7
Outdoor sport							
Season I	**No**	49.1	62.1	54.3	52.6	48.7	53.3
	Yes	58.8	55.3	58.1	40.8	32.7	48.6
Season II	**No**	32.0	35.5	28.8	34.4	44.8	35.3
	Yes	31.1	46.5	43.6	27.2	35.4	36.8
Outdoor play							
Season I	**No**	52.9	58.7	52.2	50.3	44.3	51.5*
	Yes	48.6	62.1	59.7	45.2	45.5	53.0*
Season II	**No**	32.7	32.4	36.7	28.4	40.2	34.1
	Yes	31.4	43.2	33.3	32.9	42.2	36.6

[1] MN, micronuclei; data summarized as the percentage of children with at least one micronucleus.

[2] Body mass index [BMI = weight (kg) divided by the square of the height (m)]. The children were classified as underweight (UW), normal weight (NW), overweight (OW), or obese (OB) on the basis of their BMI.

[3] Italian Mediterranean Index (IMI) was calculated according to the intake of 6 typical Mediterranean and 4 non-Mediterranean foods. Based on IMI score, ranging from 0 to 10, adherence to Mediterranean diet (MD) was classified as low (≤ 3), medium (4-5) or high (≥ 6).

Statistical significance:

* $p < 0.05$, differences among proportions performed using the Pearson's χ^2 test.

Table 3S. Proportion of children with at least one micronucleus in exfoliated buccal cells according to the level of motor traffic near children's homes and schools and the type of fuel used for domestic heating.

Characteristics		MN[1]					
		Torino	**Brescia**	**Pisa**	**Perugia**	**Lecce**	**Total**
Traffic at child's home/school[2]							
Season I	**Low**	35.6	59.3	57.5	41.0	35.4	47.0*
	High	57.4	60.3	53.2	50.5	50.0	54.4*
Season II	**Low**	38.8	35.5	31.9	38.3	38.8	36.5
	High	27.6	43.8	36.8	28.8	43.6	35.4
Heating system at child's home							
Season I	**Electric or gas**	51.6	63.5	54.9	46.6	43.6	50.1
	Fossil fuels or wood	44.0	75.0	66.7	54.8	55.6	56.1
Season II	**Electric or gas**	33.1	45.2	33.6	30.5	41.9	36.2
	Fossil fuels or wood	30.8	43.8	42.9	34.9	33.3	35.5

[1] MN, micronuclei; data summarized as the group mean ± standard deviation and the percentage of children with at least one micronucleus (between brackets).

[2] Vehicular traffic density was defined as "low" (cars and trucks never/seldom passed the child's house and/or school) or "high" (cars or trucks passed frequently the child's house and/or school).

Statistical significance:

* $p < 0.05$, differences among proportions performed using the Pearson's χ^2 test.


MAPEC
Life
Monitoring
Air
Pollution
Effects on
Children
Buccal micronucleus cytome assay
TORINO
MN‰: 0.38 0.19
PISA
MN‰: 0.46 0.24
BRESCIA
MN‰: 0.53 0.28
PERUGIA
MN‰: 0.40 0.17
LECCE
MN‰: 0.32 0.24
Winter
Late-Spring
(LIFE12 ENV/IT/000614)

Highlights

The buccal micronucleus cytome assay was applied in the MAPEC study

The frequency of MN and meta-nuclear alterations was evaluated in 1,046 children

The number of recruited children is the highest compared to previous studies

Overall, the mean MN frequency decreased significantly from winter to late-spring