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

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1672368> since 2018-08-08T12:04:45Z

Published version:

DOI:10.1016/j.ijheh.2018.05.014

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(Article begins on next page)

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3 **Buccal micronucleus cytome assay in primary school children: a descriptive analysis of the**
4 **MAPEC_LIFE multicenter cohort study.**
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9 Milena Villarini^a, Sara Levorato^a, Tania Salvatori^a, Elisabetta Ceretti^b, Sara Bonetta^c, Annalaura
10 Carducci^d, Tiziana Grassi^e, Samuele Vannini^a, Francesco Donato^b, Silvia Bonetta^c, Marco Verani^d,
11 Antonella de Donno^e, Silvia Bonizzoni^f, Alberto Bonetti^g Massimo Moretti^{a,*}, Umberto Gelatti^b, the
12 MAPEC_LIFE Study Group[§]
13
14
15
16
17
18

19
20 ^a Department of Pharmaceutical Sciences, University of Perugia, Via del Giochetto, 06122 Perugia,
21 Italy.
22

23
24 ^b Department of Medical and Surgical Specialties, Radiological Science and Public Health,
25 University of Brescia, 11 Viale Europa, 25123 Brescia, Italy.
26

27
28 ^c Department of Public Health and Pediatrics, University of Torino, 94 Piazza Polonia, 10126
29 Torino, Italy.
30

31
32 ^d Department of Biology, University of Pisa, 13 Via Ghini, 56126 Pisa, Italy.
33

34
35 ^e Department of Biological and Environmental Science and Technology, University of Salento, 165
36 Via Provinciale Lecce-Monteroni, 73100 Lecce, Italy.
37

38
39 ^f Comune di Brescia, 1 Piazza Repubblica, 25100 Brescia, Italy.
40

41
42 ^g Centro Servizi Multisetoriale e Tecnologico – CSMT Gestione S.c.a.r.l., 45 Via Branze, 25123
43 Brescia, Italy.
44
45

46
47
48 [§]MAPEC-LIFE Study Group: University of Perugia: Cristina Fatigoni, Silvano Monarca; University
49 of Brescia: Loredana Covolo, Donatella Feretti, Andrea Festa, Gaia Claudia Viviana Viola, Claudia
50 Zani, Ilaria Zerbini; University of Torino: Giorgio Gilli, Elisabetta Carraro, Tiziana Schilirò,
51 Cristina Pignata, Marta Gea, Valeria Romanazzi; University of Pisa: Gabriele Donzelli, Giacomo
52 Palomba; University of Salento: Francesco Bagordo, Mattia De Giorgi, Marcello Guido, Adele
53
54
55
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57
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59

60
61
62 Idolo, Alessandra Panico, Francesca Serio, Tiziano Verri; Comune di Brescia: Camilla Furia;
63
64 CSMT Gestione S.c.a.r.l.: Paolo Colombi, Roberta Codenotti, Licia Zagni.
65
66
67

68
69 * Corresponding author at: Department of Pharmaceutical Sciences (Unit of Public Health),
70
71 University of Perugia, Via del Giochetto, 06122 Perugia, Italy.
72

73 *E-mail address:* massimo.moretti@unipg.it (M. Moretti)
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81 Abbreviations:
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83 Micronuclei (MN), nuclear buds (NBUD), buccal micronucleus cytome assay (BMCyt), phosphate-
84 buffered saline, pH 7.4 (PBS), basal cells (BC), binucleated cells (BNC), condensed chromatin cells
85 (CCC), karyorrhectic cells (KHC), pyknotic cells (PYK), karyolytic cells (KYL), repair index (RI),
86 standard deviations (SD), body mass index (BMI), Italian Mediterranean Index (IMI), ~~PM_{2.5}~~,
87 ~~PM_{2.5}~~, particulate matter with aerodynamic diameter $\leq 2.5 \mu\text{m}$; ~~NO₂~~, nitrogen dioxide.
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121 **Abstract**
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123 Background: Recent data support the hypothesis that genetic damage occurring early in life during
124 childhood can play an important role in the development of chronic diseases in adulthood, including
125 cancer.
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129 Objectives: The objective of this paper, part of the MAPEC_LIFE project, is to describe the
130 frequency of micronuclei and meta-nuclear alterations in exfoliated buccal cells of 6-8-year-old
131 Italian children recruited in five Italian towns (i.e., Brescia, Torino, Pisa, Perugia and Lecce) with
132 different air pollution levels.
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138 Methods: About 200 children per town were recruited from primary schools. Biological samples
139 were collected twice from the same children, in two different seasons (winter 2014-15 and late
140 spring 2015). Cytogenetic damage was evaluated by the buccal micronucleus cytome assay.
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144 Results: Overall, $n = 1,046$ children represent the final cohort of the MAPEC_LIFE study. On the
145 whole, the results showed a higher mean MN frequency in winter ($0.42 \pm 0.54 \%$) than late-spring
146 ($0.22 \pm 0.34 \%$). MN frequency observed among the five Italian towns showed a trend that follows
147 broadly the levels of air pollution in Italy: the highest MN frequency was observed in Brescia, the
148 lowest in Lecce (winter) and Perugia (late-spring).
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155 Conclusions: To the best of our knowledge, the number of recruited children included in the
156 analysis ($n = 1,046$) is the highest compared to previous studies evaluating the frequency of MN in
157 exfoliated buccal cells so far. MN frequency was associated with winter season and living in towns
158 at various levels of air pollution, suggesting an important role of this exposure in determining early
159 cytogenetic effects.
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172 **Keywords:** Children; Air pollution; Socio-economic factors; Early biological effects; Buccal
173 micronucleus cytome assay; MAPEC_LIFE study.
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239 **Introduction**
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241 Several studies have shown an increased susceptibility of children population to the effects of
242 genotoxic agents from both environment and lifestyle (Neri  al., 2006; Neri et al., 2006; Merlo et
243 al., 2007). Children are considered a high-risk group in terms of the health effects because of their
244 different and unique pathways of exposure, their dynamic developmental physiology and their
245 longer life expectancy (WHO, 2008). Moreover, recent data support the hypothesis that genetic
246 damage occurring early in life during childhood can play an important role in the development of
247 chronic diseases in adulthood, including cancer (Wild and Kleinjans, 2003; Landrigan, 2004; WHO,
248 2005; Bateson and Schwartz, 2008; Grigg, 2009). The higher susceptibility of children, with respect
249 to adults, to the noxious effects of environmental pollutants might depend on smaller airways,
250 immature detoxification and metabolic systems, as well as frequent exposure to outdoor air of
251 children (Kurt et al., 2016).
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265 In the last decades, numerous epidemiological studies have used a molecular approach to study
266 health and disease conditions and related risk factors, for improving measurement of exposure and
267 for early detection of health effects (Bennett and Waters, 2000). Biomonitoring of genotoxic
268 hazards has been reported in several studies by the use of different genotoxicity endpoints, such as
269 analysis of primary DNA damage (by the comet assay), or cytogenetic effects, such as micronuclei
270 (MN), chromosome aberrations and sister chromatid exchanges. Among genotoxicity endpoints,
271 MN is one of the most commonly used biomarker in molecular epidemiology studies to assess the
272 presence and extent of chromosomal damage in human population exposed to genotoxic agents and
273 for the identification of genetic and lifestyle factors able to affect genome stability (Fenech et al.,
274 1999; Knudsen and Hansen, 2007). MN appear in the cytoplasm of interphasic cells as small
275 additional nuclei, smaller than the main nucleus. MN typically generate during the anaphase from
276 acentric chromosome fragments (chromosome breakage produced by clastogen agents) or whole
277 chromosomes (chromosome malsegregation caused by aneugen agents). Acentric or whole
278 chromosomes are left behind during mitotic cellular division and, consequently, are excluded from
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298 both of the daughter nuclei (Fenech et al., 2011). Because of the ability to detect both clastogenic
299 (e.g., chromosome breakage) and aneugenic (e.g., spindle disruption) effects, MN are considered
300 biomarkers of early biological effect (NRC, 2006; Kirsch-Volders et al., 2011).
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304 MN in peripheral blood lymphocytes have been extensively used in human biomonitoring studies to
305 identify potential genotoxic exposures as well as chromosomal instability (Fenech, 2002; Fenech,
306 2002) and the frequency of MN in circulating lymphocytes is recognized to be a predictor of cancer
307 risk in human populations (Bonassi et al., 2007; Murgia et al., 2008; Bonassi et al., 2011).
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311 Moreover, a significant increase in MN frequency in lymphocytes was found in patients with cancer
312 or preneoplastic lesions (El-Zein et al., 2006; El-Zein et al., 2011; Maffei et al., 2014),
313 neurodegenerative diseases (Migliore et al., 2011), cardiovascular diseases and diabetes (Andreassi
314 et al., 2011).
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318 In recent years, exfoliated cells from epithelial tissues have been increasingly used in the MN assay.
319 The assessment of MN in (uncultured) exfoliated epithelial cells from oral mucosa has provided a
320 complementary method for cytogenetic analyses in a easily accessible tissue without cell culture
321 requirement (Fenech et al., 2011). Nowadays, the human buccal micronucleus cytome assay
322 (BMCyt) is one of the most widely used techniques to measure genetic damage in human
323 population studies (Bonassi et al., 2011; Fenech et al., 2011; Bolognesi et al., 2013). Moreover, MN
324 frequency measured in peripheral blood lymphocytes and in buccal cells, even if occurring at
325 different frequency, showed to be highly correlated, and hence to have a similar ability to detect
326 effects of exposure to genotoxic agents (Ceppi et al., 2010).
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330 Through the micronucleus cytome assay in buccal exfoliated cells it is possible to evaluate, aside to
331 chromosomal and DNA damage markers (MN, and nuclear buds), cell proliferation markers (basal
332 and binucleated cells), cell death/apoptosis markers (cells with condensed chromatin, or
333 karyorrhectic, pyknotic and karyolytic cells), and repair index (Thomas et al., 2009; Thomas and
334 Fenech, 2011). Moreover, the micronucleus cytome assay on exfoliated cells is particularly useful
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357 in biomonitoring studies involving children to avoid traumatic and painful sampling procedures
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359 causing children any discomforts.
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361 The objective of this paper, part of the MAPEC_LIFE project (“Monitoring Air Pollution Effects on
362 Children for Supporting Public Health Policy”), is to describe the frequency of MN and meta-
363 nuclear alterations in exfoliated buccal cells of 6-8-year-old Italian children recruited in five Italian
364 towns (i.e., Brescia, Torino, Pisa, Perugia and Lecce). Cytogenetic data are presented in relation to
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366 children’s characteristic, such as socio-demographic and anthropometric features, lifestyle, parent’s
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368 characteristic and outdoor/indoor exposure to genotoxic agents.
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381 **Material and Methods**

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385 **Study design.** The MAPEC_LIFE project (“Monitoring Air Pollution Effects on Children for
386 Supporting Public Health Policy”), is a prospective epidemiological cohort study funded by the
387 European Life+ Programme (LIFE12 ENV/IT/000614), which aimed to investigate the association
388 between air pollution exposure and early biological effects in children. Details of the study design
389 have been described elsewhere (Feretti et al., 2014). Briefly, the study was conducted in five Italian
390 towns (Figure 1) characterized by different levels and features of air pollution. Brescia and Torino
391 are located in the Po Valley in Northern Italy, a highly industrialized area with unfavorable climate
392 conditions, at the highest levels of air pollution in Europe; Pisa and Perugia are located in a
393 medium-low polluted area in Central Italy, where air pollutants only occasionally exceed law limit
394 values; Lecce is located in a very low polluted area, in Southern Italy, where air pollutants never
395 exceed limit values. The five towns have also different demographic and socio-economic
396 characteristics (Bagordo et al., 2017).
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416 About 200 children per town were recruited from primary schools to evaluate, in their buccal
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418 mucosa (BM) cells, biomarkers indicative of DNA damage (*i.e.*, micronuclei and/or nuclear buds),
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420 cellular proliferation potential (*i.e.*, basal and/or binucleated cells), and/or cell death (*i.e.*, condensed
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422 chromatin, karyorrhectic, pyknotic, and karyolytic cells). Biological samples were collected twice
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424 from the same children, in two different seasons (winter 2014-15 and late spring 2015).

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427 The children's parents were interviewed to gather information on exposure to air pollutants from
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429 both indoor and outdoor sources and children's lifestyle (Zani et al., 2015). Children with severe
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431 diseases and those who had been exposed to antineoplastic agents, had undergone radiation therapy
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433 or X-rays in the previous 12 months, or had a dental prosthetic, were excluded. Children whose
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435 parents correctly filled in consent forms and valid questionnaires were invited to provide biological
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437 samples. Informed consent, in the form of comic, was obtained from children themselves prior of
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439 BM cells sampling. Overall, of the invited children, $n = 1,318$ in winter (season I) and $n = 1,149$ in
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441 late-spring (season II) could be subjected to biological sampling.
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446 ***Ethical aspects.*** For each participating unit, the study was approved by the competent Ethics
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448 Committee. Participation in this study was voluntary. Informed consent was obtained from both
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450 children's parents and children themselves, after explanation of the purpose of the study. All the
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452 data were anonymized and treated confidentially in accordance with current Italian legislation on
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454 the treatment of sensitive data.
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459 ***Questionnaire.*** We used a validated questionnaire (Zani et al., 2015) to collect data on
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461 characteristics of the area of residence and on demographic, socio-economic and anthropometric
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463 variables, diet, physical activity and other aspects of children's lifestyle. The questionnaire
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465 contained 148 items related to personal details (e.g., sex, age, height and weight), exposure to
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467 second-hand smoke at home and to other indoor pollution sources (e.g., home heating systems),
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469 traffic intensity near home and school, child's health, physical activity and diet, and parent's
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475 characteristics (e.g., birthplace, education, work and smoking habits). The dietary section was based
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477 on ARCA questionnaire (Barba et al., 2012) and contained 117 items on average frequency of food
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479 consumption.
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483 **Chemicals and media.** All reagents used were of analytical grade. Ethanol was obtained from J.T.
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485 Baker[®] Chemicals (Deventer, The Netherlands); polyethylene glycol and DePex mounting medium
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487 were from VWR International PBI Srl (Milan, Italy). Acetic acid and methanol were purchased
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489 from Carlo Erba Reagents Srl (Milan, Italy). 18G needles were obtained from Becton Dickinson
490
491 Italia SpA (Milan, Italy). Schiff's reagent and Light-green were provided by Sigma Aldrich Srl
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493 (Milan, Italy). Nylon filters (100 mm) were from Merck Spa (Milan, Italy). Trypan blue and
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495 phosphate-buffered saline (PBS) were purchased from Invitrogen Srl (Milan, Italy). Conventional
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497 microscope slides and cover-slips were supplied by LLG[®] Labware (Meckenheim, Germany).
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499 Distilled water was used throughout the experiments.
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505 **Collection of biological samples.** Children were asked to rinse the mouth twice with mineral water.
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507 Small-headed toothbrush was used to collect epithelial buccal cells by gently scraping (10 times in a
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509 circular motion) the inner surface of both cheeks. The head of the brush was then dipped into tubes
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511 containing 15 ml of Saccomanno's fixative (50% ethanol, 2% polyethylene glycol, vol./vol.;;
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513 solution diluted in water and stored at 4°C) and rotated repeatedly to dislodge and release the cells
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515 into the buffer. The epithelial buccal cells in Saccomanno's fixative were centrifuged and washed
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517 twice with PBS. Cell suspensions were then drawn up into a syringe using an 18G needle, filtered
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519 through a 100 µm nylon filter, and centrifuged again. Cell pellets were resuspended in ice-cold
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521 PBS, aliquots (10 µL) of cell suspensions were diluted 1:1 with a 0.4% Trypan Blue solution and
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523 cell count was performed using a hemocytometer or a Countess automated cell counter (Invitrogen
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525 Srl, Milan, Italy). Buccal cells were then fixed with ice-cold Carnoy's fixative (methanol and glacial
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527 acetic acid 3:1) and the samples (coded tubes) were shipped overnight (+4°C) to the Cytogenetics
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534 Laboratory at the Unit of Public Health, University of Perugia, for subsequent processing (i.e.,
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536 single-center slide preparation and scoring).
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541 ***Buccal micronucleus cytome assay (BM Cyt assay).*** The BM Cyt assay was performed according to
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543 the procedure described by Thomas and Fenech (Thomas and Fenech, 2011), with minor
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545 modifications. For each subject, two slides were prepared by smearing 100 μ L of cell suspension
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547 onto pre-cleaned slides (approximately 1×10^5 cells/slide).  The slides were treated with Schiff's
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549 reagent, washed in running water and then rinsed well in deionized water. Subsequently, the slides
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551 were stained with 0.2% Light Green reagent, air dried, and finally mounted with DePex mounting
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553 medium.
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557 ***Slide scoring.*** The coded slides were read blind by trained scorers by following the scoring scheme
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559 proposed by Thomas and Fenech (Thomas and Fenech, 2011). The slide preparations were scored
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561 initially to determine the frequency of all the various cell types in a minimum of 1,000 cells; cell
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563 types, anomalies associated with cell death, and nuclear abnormalities indicative of chromosomal
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565 instability or DNA damage were classified essentially according to established criteria (Bolognesi et
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567 al., 2013). The slides were then scored for cells with MN and nuclear buds (NBUD) among a
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569 minimum of 2,000 differentiated cells (1,000/slide) as respective measures of chromosomal and
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571 DNA damage. Proliferation, cytotoxicity and cell-death events were evaluated by counts among
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573 1,000 differentiated cells. Cell proliferation was determined by recording the number of basal cells
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575 (BC), whereas binucleated cells (BNC) ~~cells~~ indicated cytokinesis defect (cytotoxicity); condensed
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577 chromatin cells (CCC), karyorrhectic (KHC), pyknotic (PYK) and karyolytic (KYL) cells are
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579 regarded as markers of early-to-late stages of apoptosis and cell death (Tolbert et al., 1992). Repair
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581 index (RI) was calculated as the sum of KHC and KYL cells divided by the sum of MN and NBUD
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583 (Ramirez and Saldanha, 2002) by applying the simple Freeman-Tukey root transformation for
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585 overdispersed near-to-zero counts (Freeman and Tukey, 1950; Hothorn et al., 2013).
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595 **Statistical analysis.** Means and standard deviations (SD) and counts and percentages were reported
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597 for continuous and categorical variables, respectively. Approximation to the normal distribution of
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599 continuous variables was evaluated using the Kolmogorov-Smirnov test. All the considered cell
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601 types, or anomalies associated with cell death or chromosomal instability/DNA damage (i.e., cells
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603 with MN, NBUD, CCC, KHC, PYK, and KYL cells) and RI showed significant departures from the
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605 normal distribution even after logarithmic transformation. The differences among groups were
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607 investigated using the non-parametric Kruskal-Wallis H test. The differences between values
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609 observed in the same children in two seasons were assessed using the Wilcoxon test for paired data.
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611 The comparisons among proportions were performed using the Pearson's χ^2 test. Correlation
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613 between continuous variables was assessed using the Spearman's correlation coefficient. All the
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615 statistical tests were two-sided with $\alpha = 0.05$. The data analyses were performed using SPSS 20
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617 statistical package (SPSS Inc., Chicago, IL, USA).
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627 **Results**

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631 **Study population.** Lifestyles and socio-demographic features of the MAPEC_LIFE complete cohort
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633 have been described in detail elsewhere (Grassi et al., 2016; Zani et al., 2016; Bagordo et al., 2017).
634
635 The final cohort reported in this paper included only subjects with suitable biological data in both
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637 seasons. Overall, slides suitable for microscope analysis were obtained from $n = 1,093$ children in
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639 winter (season I); of these, $n = 1,046$ were sampled also during late-spring (season II) and represent
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641 the final cohort of the MAPEC_LIFE study. The main characteristics of the study cohort are
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643 summarized in Table 1, according to town of residence. Children were aged 6-8 years, with a mean
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645 age of 6.83 ± 0.90 years, and 50.3% of them were boys. The majority of children's parents were
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652 Italian, with a high education level and employment rate, with some difference between the towns.
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654 As regards smoking habits, 18.7% of the mothers and 27.2% of the fathers were current smokers,
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656 however, the proportion varied significantly among the towns. Body mass index (BMI) values and
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658 weight classes were similar in the five towns, with about 30% of children overweight or obese.
659
660 Significant differences among towns were found for the Italian Mediterranean Index (IMI), which
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662 overall showed a low adherence of the children's diet to the Mediterranean diet. The other
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664 children's and parents' characteristics did not vary from season I (winter) to season II (late-spring),
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666 apart from children outdoor sport or play practice with an increased proportion of children from
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668 season I to season II, and high exposure to vehicular traffic emission referred by parents from
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670 67.7% in winter to 57.8% in late-spring. Important differences were found for the fuels used in the
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672 heating systems among the towns: district heating was the most represented system in Brescia,
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674 present, though less, in Torino, while it was negligible in the other towns, where natural gas heating
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676 system were prevalent.
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682 ***BMCyt assay.*** The results of chromosomal and DNA damage markers (MN and NBUD), cell
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684 proliferation markers (BC and BNC), cell death/apoptosis markers (CCC, KHC, PYK, and KYL),
685
686 and repair index (RI) in children are summarized in Table 2. Overall, the mean MN frequency
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688 decreased significantly (~50%) from winter to late-spring (0.42 ± 0.54 ‰ and 0.22 ± 0.34 ‰,
689
690 respectively; $p < 0.001$). Similarly, NBUD frequency decreased from 0.26 ± 0.48 ‰ in winter to
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692 0.17 ± 0.36 ‰ in late-spring ($p < 0.001$). All the other markers of cell proliferation and death
693
694 followed the same trend, with values slightly higher in winter than in late-spring, while the repair
695
696 index (RI) was significantly higher in late-spring than in winter (6.69 ± 2.42 and 6.24 ± 2.55 ,
697
698 respectively; $p < 0.05$). By comparing children residing in the five towns, significantly different
699
700 MN frequencies were found in both seasons, with the highest values in Brescia in both winter (0.53
701
702 ± 0.61 ‰) and late-spring (0.28 ± 0.42 ‰), and the lowest in Lecce in winter (0.32 ± 0.44 ‰) and
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704 in Perugia in late-spring (0.17 ± 0.28 ‰). Children living in the five towns had also statistically
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711 different frequencies of NBUD, with the highest values in Pisa (season I: 0.58 ± 0.70 ‰; season II:
712 0.39 ± 0.55 ‰), the lowest in Lecce (season I: 0.14 ± 0.31 ‰; season II: 0.09 ± 0.26 ‰). Similar
713
714 differences were observed for the frequency of BC (in season I), BNC (in season II) and cell
715
716 death/apoptosis markers (CCC, KHC, PYK, and KYL). Statistically significant differences were
717
718 observed also for RI values: in winter, the highest RI value was observed in Brescia (7.10 ± 2.78),
719
720 the lowest in Torino (5.69 ± 2.20); whereas, in late-spring the highest RI value was observed in
721
722 Torino (7.22 ± 2.24), the lowest in Perugia (6.01 ± 1.96). The proportion of children showing at
723
724 least one MN in exfoliated buccal cells was 52.0% in the winter season and 35.9% in late-spring (p
725
726 < 0.001). In winter, the highest proportion of children with at least one MN was observed in Brescia
727
728 (59.9%), the lowest in Lecce (44.6%); in late-spring, the highest value was observed in Brescia
729
730 (40.1%), the lowest in Perugia (31.4%) (Figure 2). The proportion of children with at least one MN
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732 decreased significantly in late-spring compared to winter in all the towns except Lecce, which
733
734 showed similar values in season I and II.

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739 The frequency of MN in children's buccal cells according to town of residence and children's and
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741 parents' features are summarized in Table 3. No variable was associated with children's MN
742
743 frequency apart from mother's smoking habits in season II: children with smoking mothers showed
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745 higher MN frequency than those with non-smoking mothers (0.30 ± 0.37 ‰ and 0.20 ± 0.33 ‰,
746
747 respectively; $p < 0.001$).  Accordingly, in season II the proportion of children with at least one MN
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749 in exfoliated buccal cells was 46.7% and 33.3% in children with smoking or non-smoking mothers,
750
751 respectively ($p = 0.007$) (Supplement Table 1S).

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753
754 Table 4 shows MN frequency according to children's BMI, diet and exercise patterns. BMI and
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756 outdoor sport and play were not associated with the presence of MN, whereas a high adherence to
757
758 the Mediterranean diet (IMI) was associated with a significant reduction in MN frequency in season
759
760 I (winter).

761
762 Table 5 summarizes MN frequency according to parents' reports of traffic near children's home and
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764 school and type of fuel used for domestic heating. MN frequency was analyzed with reference to
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770 only electric/gas or fossil fuel/wood as heating systems, as district heating was not considered to
771
772 contribute to indoor pollution. MN frequency did not vary significantly by heating system, even if
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774 children with fossil fuel/wood heating system showed a slightly higher mean value than children
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776 using electric/gas system.
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779 According to common interpretation of correlation coefficients, negligible values of Spearman's r
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781 were found between MN frequency and the other investigated biomarkers, apart from a negative
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783 correlation with RI ($r = -0.548, p < 0.001$) (data not shown in table).
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791 **Discussion**

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793 The frequency of markers of chromosomal and DNA damage, cell proliferation, cell
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795 death/apoptosis, and repair index was evaluated in exfoliated buccal cells of 1,046 children residing
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797 in five Italian towns during two seasons of the year (winter and late-spring). The results showed a
798
799 higher mean MN frequency in winter ($0.42 \pm 0.54 \text{ ‰}$) than late-spring ($0.22 \pm 0.34 \text{ ‰}$), with a
800
801 corresponding proportion of children with at least one MN in exfoliated buccal cells decreasing
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803 from 52.0% in winter to 35.9% in late-spring.
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807 These results are not properly comparable with those of other studies investigating the genotoxic
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809 effect of residential exposure to genotoxic xenobiotics in exfoliated buccal mucosa cells of children
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811 in recent years. Differences in the study design features, such as subjects' age, method used, type of
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813 exposure, sample size, may cause a large variability among different studies (Fenech and Bonassi,
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815 2011). MN mean frequency observed in our study ($0.42 \pm 0.54 \text{ ‰}$ in winter) was lower than that
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817 found in 64 children with mean age of 7.3 years, living close to major freeways and arterial roads in
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819 Oakland, California ($0.67 \pm 1.44 \text{ ‰}$) (Huen et al., 2006), and that observed in 411 9-years-old
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821 children living in the chipboard manufacturing district of Viadana, Italy ($1.2 \pm 0.9 \text{ ‰}$) (Marcon et
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823 al., 2014). Accordingly, a Brazilian study showed, in a small sample of children aged ≤ 7 years,
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829 higher mean MN frequency in children living in the urban, polluted area ($1.20 \pm 0.83 \%$) than those
830 living in a rural area ($0.19 \pm 0.31 \%$) (Sisenando et al., 2012). Overall, the MN frequencies
831 observed in this study are close (higher in winter, lower in late-spring) to the value of the lowest
832 confidence interval of the estimated mean MN values in exfoliated BC of healthy controls (Bonassi
833 et al., 2011); in this review, on the basis of the extensive database derived from studies conducted
834 on both children and adults, the MN mean was estimated to be 0.74% with a range of 0.3–1.7
835 MN/1,000 cells.
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839 In our study, no differences were observed according to gender in the frequency of MN and meta-
840 nuclear alterations, suggesting that boys and girls are similarly susceptible to genotoxic agents. This
841 observation is in agreement with published data, which reported similar buccal cell MN frequencies
842 in males and females (Bonassi et al., 2011). The results of this study confirm the increased risk of
843 chromosomal alterations in epithelial buccal cells associated with the presence of smokers in the
844 children's house. MN frequency was positively associated with mother smoking habits in season II
845 (late-spring), in the absence of residential heating; this result is in line with a 30% or more increase
846 in MN frequency in children exposed to cigarette smoke with respect to those unexposed (Neri et
847 al., 2003; Holland et al., 2011). A high adherence of children's dietary habits to Mediterranean diet
848 was associated with lower MN frequency, supporting a possible association between dietary habits
849 and MN frequency, which is still under discussion for both adults and children (Bonassi et al.,
850 2011). Statistically significant differences in MN frequency were observed among the five Italian
851 towns in both seasons, with a trend that follows broadly the levels of air pollution in our country
852 (ISPRA, 2016): the highest MN frequency was observed in Brescia, located in one of the most
853 polluted area in Europe ($PM_{2.5}$ annual average of $29 \mu\text{g}/\text{m}^3$ and NO_2 annual average of $68 \mu\text{g}/\text{m}^3$ in
854 2015), and the lowest in Lecce, in which very low levels of air pollutants are usually registered
855 ($PM_{2.5}$ annual average of $13 \mu\text{g}/\text{m}^3$ and NO_2 annual average of $30 \mu\text{g}/\text{m}^3$ in 2015). Intermediate
856 values of MN frequency were found in Pisa and Perugia, where air pollutants only occasionally
857 exceed law limit values ($PM_{2.5}$ annual average of 17 and $20 \mu\text{g}/\text{m}^3$ and NO_2 annual average of 37
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888 and 28 $\mu\text{g}/\text{m}^3$ for Pisa and Perugia in 2015, respectively). Instead, Torino showed a relatively low
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890 mean MN frequency in children's cells, despite its high concentrations of air pollutants ($\text{PM}_{2.5}$
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892 annual average of 27 $\mu\text{g}/\text{m}^3$ and NO_2 annual average of 68 $\mu\text{g}/\text{m}^3$ in 2015).
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895 Overall, our findings suggest an important role of air pollution exposure in MN formation in
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897 children's buccal cells, the evaluation of which represent the main aim of the MAPEC_LIFE
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899 project. A detailed analysis of the associations between single air pollutant levels and biomarkers of
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901 early effects in children's buccal cells, according to socio-demographic and lifestyle factors, will be
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903 reported in a companion paper.
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906 This study has various strengths. First, the sample size: to the best of our knowledge, the number of
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908 recruited children included in the analysis ($n = 1,046$) is the highest compared to previous studies
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910 evaluating the frequency of MN in exfoliated buccal cells so far (Lahiri et al., 2000; Ceretti et al.,
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912 2014; Demircigil et al., 2014; Mergener et al., 2014; Silva da Silva et al., 2015; Cavalcante et al.,
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914 2017), particularly in 6-8 years old children (Huen et al., 2006; Sisenando et al., 2012; Marcon et
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916 al., 2014). Moreover, the cells of the 1,046 children were collected twice in two different seasons,
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918 winter and late-spring, allowing a comparison between the genotoxic effects in different air
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920 pollution conditions in the same subjects. Furthermore, to avoid inter-laboratory biases, biological
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922 samples collected in the five towns were processed for the BMCyt assay (i.e., slide preparation and
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924 scoring) in a single laboratory. Finally, investigation of the role of demographic, socio-economic,
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926 and life-style factors, as possible modifiers of the effect of air pollution on human health, and strict
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928 inclusion (e.g., residence in the urban area of the five towns) and exclusion (e.g., severe diseases,
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930 therapy with antineoplastic agents or radiation therapy, exposure to X-rays, or use of dental brace)
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932 criteria allowed us to exclude important confounding factors and to evaluate other possible risk
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934 factors for cytogenetic damage.
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947 **Conclusions**
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949 In conclusion, this study showed that MN frequency in buccal cells of children was associated with
950 passive smoking at home and low adherence to Mediterranean diet, confirming the impact of these
951 factors on children’s health. Furthermore, MN frequency was associated with winter season and
952 living in towns at various levels of air pollution, suggesting an important role of this exposure in
953 determining early cytogenetic effects.
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956 These findings support the need of local, national, and global efforts to decrease the impact of
957 environmental exposure and promote educational programs on lifestyle determinants of health.
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971 **Conflict of interest**
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973 The authors declare they have no actual or potential competing financial interests.
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981 **Funding**
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983 This study was part of the project “Monitoring Air Pollution Effects on Children for Supporting
984 Public Health Policy” (MAPEC_LIFE) financed by the European Commission Directorate General
985 Environment in the LIFE+ Programme for 2012, Environment Policy and Governance (grant
986 number: LIFE12 ENV/IT/000614).
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998 **Acknowledgments**
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1006 The authors are grateful to the children and their parents for their participation in this study. The
1007
1008 authors also wish to thank school directors, teachers and janitors for their very valuable help during
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1010 biological sampling sessions.
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1392 Bonetti, A., Monarca, S., Villarini, M., Levorato, S., Carducci, A., Verani, M., Casini, B.,
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1403 Bonetti, A., Monarca, S., Villarini, M., Levorato, S., Carducci, A., Verani, M., Casini, B.,
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1411 27 (4), 646-656.
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1 **Figure 1.** Map of Italy showing the geographical position of the five towns participating the multicenter
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3 MAPEC_LIFE study.
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1 **Figure 2.** Proportion of children with at least one micronucleus in exfoliated buccal cells, according to
2 town of residence.
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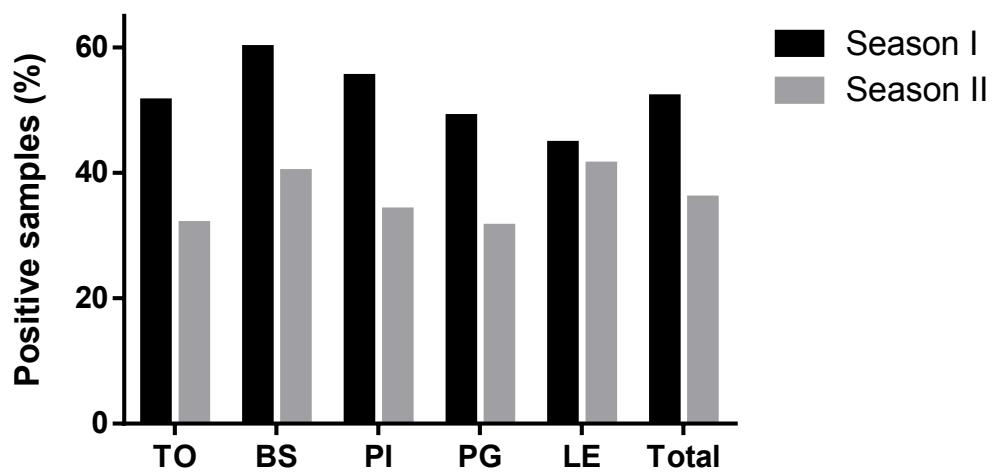


Table 1. Characteristics of children included in the study cohort: socio-demographic and anthropometric characteristics, life-style, parents' features, perceived traffic density and home heating systems.

| Characteristics | Torino | Brescia | Pisa | Perugia | Lecce | Total | <i>p</i>-value |
|---|---------------------|---------------------|-------------------|--------------------|---------------------|---------------------|---------------------------|
| Children¹ | 214 (20.5) | 237 (22.7) | 159 (15.2) | 223 (21.3) | 213 (20.4) | 1.046 (100.0) | |
| Boys / Girls² | 112 / 102 (1.10) | 108 / 129 (0.84) | 71 / 88 (0.81) | 129 / 94 (1.37) | 106 / 107 (0.99) | 526 / 520 (1.01) | <i>0.048</i> ^a |
| Children's age³ | 6.76 ± 0.81 | 6.91 ± 0.88 | 6.69 ± 0.98 | 6.89 ± 0.93 | 6.88 ± 0.90 | 6.83 ± 0.90 | <i>n.s.</i> ^b |
| Parents of Italian nationality¹ | | | | | | | |
| Mother | 175 (81.8) | 196 (82.7) | 123 (77.8) | 193 (86.5) | 195 (91.5) | 882 (84.4) | <i>0.003</i> ^a |
| Father | 183 (86.7) | 203 (87.9) | 132 (84.1) | 202 (92.2) | 203 (95.8) | 923 (89.6) | <i>0.001</i> ^a |
| Parents' level of education¹ | | | | | | | |
| Mother (high school or university) | 169 (79.0) | 188 (79.3) | 136 (86.1) | 203 (91.0) | 180 (84.5) | 876 (83.8) | <i>0.002</i> ^a |
| Father (high school or university) | 159 (75.4) | 159 (68.8) | 117 (74.5) | 185 (84.5) | 171 (80.7) | 791 (76.8) | <i>0.001</i> ^a |
| Parents' occupational status¹ | | | | | | | |
| Mother employed | 166 (77.6) | 178 (75.1) | 117 (74.1) | 172 (77.1) | 131 (61.5) | 764 (73.1) | <i>0.016</i> ^a |

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| Father employed | | 190 (90.0) | 216 (93.5) | 136 (86.6) | 201 (91.8) | 178 (84.0) | 921 (89.4) | <i>0.047^a</i> |
| Parents' smoking habits¹ | | | | | | | | |
| Mother current smoker | | 52 (24.3) | 43 (18.1) | 33 (20.9) | 25 (11.2) | 42 (19.7) | 195 (18.7) | <i>0.010^a</i> |
| Father current smoker | | 77 (36.5) | 54 (23.4) | 45 (28.7) | 46 (21.0) | 58 (27.4) | 280 (27.2) | <i>0.004^a</i> |
| Children's BMI⁴ | | | | | | | | |
| 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 | <i>n.s.^b</i> |
| | UW¹ | 14 (6.5) | 19 (8.0) | 7 (4.4) | 11 (4.9) | 11 (5.2) | 62 (5.9) | |
| | 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) | <i>n.s.^a</i> |
| | 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 | <i>n.s.^b</i> |
| | UW¹ | 12 (5.6) | 10 (4.2) | 9 (5.7) | 7 (3.1) | 11 (5.2) | 49 (4.7) | |
| | NW | 138 (64.5) | 173 (73.0) | 106 (66.7) | 151 (67.7) | 133 (62.4) | 701 (67.0) | <i>n.s.^a</i> |

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| 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 IMI⁵

| | | | | | | | | |
|------------------|------------------|--------------------------|--------------------------|--------------|--------------------------|---------------|--------------------------|---------------------|
| 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 sport¹

| | | | | | | | |
|-----------------|--------------|--------------|--------------|--------------|--------------|---------------|-------------------|
| Season I | 51 (23.8) | 76 (32.1) | 43 (27.0) | 71 (31.8) | 55 (25.8) | 296 (28.3) | n.s. ^a |
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| Season II | | 61 (28.5) | 99 (41.8) | 55 (34.6) | 92 (41.3) | 79 (37.1) | 386 (36.9) | <i>0.026^a</i> |
| Children's outdoor play¹ | | | | | | | | |
| Season I | | 74 (34.6) | 87 (36.7) | 67 (42.1) | 62 (27.8) | 55 (25.8) | 345 (33.0) | <i>0.004^a</i> |
| Season II | | 159 (74.3) | 169 (71.3) | 129 (81.1) | 149 (66.8) | 126 (59.2) | 732 (70.0) | <i><0.001^a</i> |
| Traffic density¹ | | | | | | | | |
| Season I | Low | 59 (27.6) | 81 (34.2) | 80 (50.3) | 39 (17.5) | 79 (37.1) | 338 (32.3) | <i><0.001^a</i> |
| | 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) | <i><0.001^a</i> |
| | High | 134 (62.6) | 130 (54.49) | 68 (42.8) | 163 (73.1) | 110 (51.6) | 605 (57.8) | |
| Heating systems¹ | | | | | | | | |
| 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) | <i><0.001^a</i> |
| District heating | | 61 (28.5) | 147 (62.0) | --- (0.0) | 3 (1.3) | --- (0.0) | 211 (20.2) | |

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

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

³ Group mean \pm standard deviation (age expressed in years).

⁴ Group mean \pm 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 \pm 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 \pm standard deviation.

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

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|-----------|------------|------------|------------|------------|------------|-------------------------|--------|
| 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 |

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| | | 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 education² | | | | | | |
| | 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 education² | | | | | | |
| | 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 | | | | | | |

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| 85 | 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 |
| 86 | | 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 |
| 87 | | | | | | | | |
| 88 | 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 |
| 89 | | 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 |
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| 91 | | | | | | | | |
| 92 | | | | | | | | |
| 93 | Mother smoking habits | | | | | | | |
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| 95 | 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 |
| 96 | | 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 |
| 97 | | | | | | | | |
| 98 | 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* |
| 99 | | 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* |
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| 104 | Father smoking habits | | | | | | | |
| 105 | | | | | | | | |
| 106 | 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 |
| 107 | | 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 |
| 108 | | | | | | | | |
| 109 | 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 |
| 110 | | 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 |
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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.

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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 BMI² | | | | | | | |
| Season I | UW | 0.25 ± 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 ± 0.47 | 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 IMI³ | | | | | | | |
| 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.

| Characteristics | | MN ¹ | | | | | |
|---|-----------------------------|-----------------|-------------|-------------|-------------|-------------|-------------|
| | | Torino | Brescia | Pisa | Perugia | Lecce | Total |
| Traffic at child’s home/school² | | | | | | | |
| 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 | 0.22 ± 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 | | | | | Total |
|--|------------------|-----------------|---------|-------|---------|-------|-------|
| | | Torino | Brescia | Pisa | Perugia | Lecce | |
| 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 education² | | | | | | | |
| 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 |

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|--|--|-------------------|-------|------|------|------|------|------|
| | | Low | 44.4* | 42.9 | 22.7 | 20.0 | 45.5 | 38.5 |
| | Father level of education² | | | | | | | |
| | 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

| | | | | | | | | |
|-----|------------------------------|-----------------------|------|------|------|------|------|-------|
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| 85 | Season I | Current smoker | 55.8 | 65.1 | 45.5 | 44.0 | 52.4 | 53.8 |
| 86 | | Non-smoker | 50.0 | 58.8 | 57.6 | 49.5 | 42.7 | 51.5 |
| 87 | | | | | | | | |
| 88 | | | | | | | | |
| 89 | Season II | Current smoker | 38.5 | 53.5 | 39.4 | 40.0 | 59.5 | 46.7* |
| 90 | | Non-smoker | 29.6 | 37.1 | 32.0 | 30.3 | 36.8 | 33.3* |
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| 92 | | | | | | | | |
| 93 | Father smoking habits | | | | | | | |
| 94 | | | | | | | | |
| 95 | Season I | Current smoker | 59.7 | 61.1 | 51.1 | 45.7 | 51.7 | 54.6 |
| 96 | | Non-smoker | 47.0 | 59.9 | 57.1 | 49.7 | 42.2 | 51.2 |
| 97 | | | | | | | | |
| 98 | | | | | | | | |
| 99 | Season II | Current smoker | 35.1 | 50.0 | 33.3 | 34.8 | 46.6 | 40.0 |
| 100 | | Non-smoker | 30.6 | 36.7 | 33.9 | 31.2 | 39.0 | 34.4 |
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104 ¹ MN, micronuclei; data summarized as the percentage of children with at least one micronucleus.

105 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
106 parents reported to have a university degree or a high school diploma, a low level of education was assigned to parents having lower qualifications.

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108 Statistical significance:

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



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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.

| Characteristics | | MN ¹ | | | | | |
|-----------------------------------|------------------|-----------------|---------|------|---------|-------|-------|
| | | Torino | Brescia | Pisa | Perugia | Lecce | Total |
| Children's BMI² | | | | | | | |
| 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 IMI³ | | | | | | | |
| 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 |

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|--|-----------|----------------------|------|------|------|------|------|-------|
| | | 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.



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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 | | | | | Total |
|---|-----------------------------|-----------------|---------|------|---------|-------|-------|
| | | Torino | Brescia | Pisa | Perugia | Lecce | |
| Traffic at child’s home/school² | | | | | | | |
| 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:

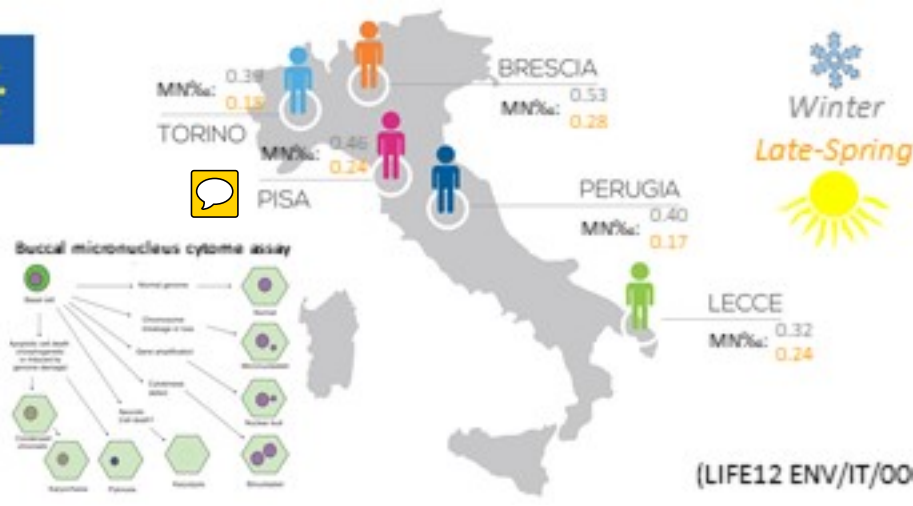
* $p < 0.05$, differences among proportions performed using the Pearson’s χ^2 test.



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Monitoring
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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