Buccal micronucleus cytome assay in children living in an area with low anthropogenic pressure: the EFFE.BI.P. study

Francesco Bagordo[a], Alessandra Panico[a*], Antonella Zizza[b], Francesca Serio[a], Adele Idolo[c], Maria Rosaria Tumolo[d], Marcello Guido[a], Isabella Gambino[a], Tiziana Grassi[a]

[a] Department of Biological and Environmental Sciences and Technologies, University of Salento, Via Monteroni 165, 73100 Lecce, Italy. (FB: francesco.bagordo@unisalento.it; AP: alessandra.panico@unisalento.it; FS: francesca.serio@unisalento.it; MG: marcello.guido@unisalento.it; IG: isabella.gambino@unisalento.it; TG: tiziana.grassi@unisalento.it)

[b] Institute of Clinical Physiology, National Research Council, 73100 Lecce, Italy. (AZ: zizza@ifc.cnr.it)

[c] Department of Prevention, Local Health Authority (ASL LE), 73100 Lecce, Italy. (AI: adele.idolo@unisalento.it)

[d] Institute for Research on Population & Social Policies, National Research Council, Research Unit of Brindisi, c/o ex Osp. Di Summa, Piazza Di Summa, 72100 Brindisi, Italy. (MRT: mariarosaria.tumolo@irpps.cnr.it)

***Corresponding author**
e-mail address: alessandra.panico@unisalento.it (A. Panico)

Abstract

The study aimed to evaluate the micronucleus (MN) frequency in exfoliated buccal cells (EBCs) of 256 6-8-years-old schoolchildren living in an area of Salento peninsula (Southern Italy) with low anthropogenic pressure and with a normal rate of chronic diseases was carried out in order to determine the basal level of MN and identify which factors are able to influence it. Information about the personal data, lifestyles and dietary habits of the children were obtained by the administration of a questionnaire to their parents. The buccal micronucleus cytome assay was performed to evaluate the presence of early genotoxic effects. Individual and environmental conditions related to MN frequency were also assessed. In addition, the level of environmental exposure of the children was assessed by sampling atmospheric particulate fractions near the involved schools. The results showed that the children involved in the study had a mean MN frequency of 0.27 ± 0.43‰ with a 95% CI of 0.22-0.33 MN‰. This frequency was positively associated with vehicular traffic (OR=2.99; 95%CI=1.15-7.74) and negatively associated with a high educational level of the mother (OR=0.41; 95%CI=0.18-0.95) and physical exercise (OR=0.56; 95%CI=0.32-0.57). Data on genotoxic effects in buccal cells found in this study could be considered as the MN level in a pediatric population not exposed to environmental pollution.

Keywords: Buccal micronucleus cytome assay, children, air pollution, environmental exposure, lifestyle, early genotoxic effects.

1. Introduction

Micronuclei (MN) are additional small nuclei in the extranuclear vicinity, originating from aberrant mitosis, due to alterations of the chromosomal structure and oxidative stress [1]. MN can occur in several cells or tissues as a result of natural processes, such as aging, but they can be also induced by many environmental factors and unhealthy habits [2].

In general, MN are associated with the development of some chronic diseases, such as cancer [3,4], cardiovascular diseases [5], auto-immune disease [6], diabetes [7-9], etc. Their formation could be the first step in the pathological process leading to the disease [10] and occur much earlier than the clinical signs. Therefore, MN are considered as early DNA damage indicators and their frequency at the population level can detect the effect of certain risk factors [11,12].The appearance of the molecular alterations occur even in childhood or during pregnancy, highlighting a risk condition of developing chronic diseases in adulthood [13,14].

The MN frequency was widely studied in populations exposed to environmental pollution and was used in molecular epidemiological studies for measuring a quantifiable biological response directly attributed to an exposure [15].

When studying the effect of air pollution, micronucleated cells can be investigated in oral mucosa, especially exfoliated buccal cells (EBCs) which are in direct contact with inhaled and ingested genotoxic agents [16,17]. Nowadays, the human buccal micronucleus cytome (BMCyt) assay [18] is one of the most widely used techniques to measure genetic damage in studies on human population exposed to airborne contaminants [19].

Higher frequency of MN was observed in populations living in industrialized [20,21] or in heavily trafficked areas [22,23]. Several individual and behavioral factors (i.e. diet, physical activity, smoking habit), should be considered when setting up a molecular epidemiology study that assesses the presence of MN in population exposed to environmental pollutants since they could influence the molecular alterations.

MN are mainly studied in population living in highly polluted areas due to human activity with an elevated environmental impact and correlated with air pollutants, such as particulate matter (PM), benzene, ozone, polycyclic aromatic hydrocarbons, etc. [12,24,25], and in populations (or subgroups) characterized by a high incidence of chronic diseases [26,27]. Any risk condition is defined by comparing the frequency of MN in a population exposed to risk factors with a basal level. But currently doesn't exist a recognized normal range of MN frequency or a cut-off value to be used to identify the risk to develop chronic diseases.

Therefore, studies on MN frequency and related confounding factors should be conducted in populations not exposed to significant levels of air pollution in order to identify basal levels of MN which could be used as a reference range in studies on populations living in polluted areas.

This paper reported the results of the "Early Biological Effects associated with Environmental Pollution and lifestyles in the Population of the Union of Municipalities of Terre d'Oriente" (EFFE.BI.P.) Study, a research project aimed to evaluate the frequency of MN in EBCs of children living in an area of Salento peninsula (Southern Italy) with low anthropogenic pressure and with a normal rate of chronic diseases to determine the basal level of MN and identify which factors are able to influence it.

2. Materials and methods

2.1.Study design

The EFFE.BI.P. (Early Biological Effects associated with Environmental Pollution and lifestyles in the Population of the Union of Municipalities of Terre d'Oriente) Study was a Local Research Project funded by the Interprovincial University Consortium of Salento (CUIS) aimed to evaluate the frequency of the DNA damage in the buccal mucosa of children living in five municipalities (Cursi, Giurdignano, Muro Leccese, Otranto and Uggiano La Chiesa) in the Province of Lecce.
The study followed the MAPEC_LIFE (Monitoring Air Pollution Effects on Children for Supporting Public Health Policy) project protocol [28] which provided for: the recruitment of children attending primary school in a central-eastern area of Salento Peninsula; the administration of a questionnaire to the parents to obtain information about their children's lifestyle; the sampling of children's EBCs; the assessment of genotoxic damage in the cells by the BMCyt assay; the collection of PM samples near the selected schools; the analysis of data to identify any association between DNA damage and environmental or individual factors. The biological and atmospheric samplings were made in May 2018 for minimizing the effect of seasonal variability and to compare the results with those of other studies conducted in the same period of different years in other areas of the Apulia region.

2.2.Study area

The children involved in the study lived in five small villages in the Province of Lecce: Cursi, Giurdignano, Muro Leccese, Otranto, and Uggiano la Chiesa (Figure 1), which in 2018 counted a total population of 21,177 inhabitants and a 6-8-year-old population of 518 children [29]. These villages are located in the central-eastern area of Salento Peninsula, bordering the Adriatic Sea, and are characterized by a predominantly agricultural economy. In this part of the Apulia Region agriculture is mainly extensive and is represented by many small farms. The prevalence of farms in these villages (26.7%) is higher than in the whole Province (14.1%), while the prevalence of manufacturing activities is lower (6.7% vs 8.9%) [30]. Overall, in this territory the utilized agricultural area (UAA) was 88.8% of the total agricultural area (TAA) and mostly cultivated with olive groves (66.7%). A smaller proportion (29.2%) is represented by arable land mainly used for the cultivation of vegetables [29]. Previous studies [31-33], conducted in this territory to evaluate a possible groundwater pollution, highlighted widespread microbiological contamination attributable to the disposal of domestic sewage but absence of toxic substances.
Considering health data from these five villages between 2006 and 2009, the observed all-cause mortality resulted slightly lower than the expected mortality, showing a standardized mortality ratio (SMR) of 97.7; as far as all-cancer mortality, the observed rate is also lower than the expected one (SMR=98.1) [34].
Considering these factors and the absence of an industrial zone nearby the five villages involved in the study, this area can be considered with low anthropogenic pressure with particular reference to the release of toxic substances.

[Figure 1 near here]

2.3.Recruitment

The recruitment of subjects was made among children attending the first, second, and third classes in the primary schools of the five villages in the school year 2017-2018. Preliminarily, the research team carried out a presentation meeting with children's parents in each involved school to explain to them the study aims and methods and to promote their children's participation. The parents who joined the study signed the Consent form, after verifying that their children did not fall into exclusion criteria (age below 6 years or equal/above 9, residence in cities other than those involved in the study, presence of serious illness, exposure to radiotherapy or chemotherapy in the 12 months preceding the investigation, exposure to radiographic testing in the month preceding the investigation, use of dental braces).

2.4.Questionnaire administration

Before the biological and environmental sampling, the parents who accepted their children participating in the study filled in a questionnaire, developed by Zani et al. [35], to collect demographic, socio-economic, and lifestyle information on children. All the questionnaires were checked to exclude those incomplete or reporting positive answers for the exclusion criteria. An alphanumeric code was assigned to each valid questionnaire, which also unequivocally identified the biological samples.

The questionnaire included questions about children's demographic data (age, sex), health status, exposures to outdoor (perceived vehicular traffic) and indoor (wood fireplaces and stoves, cooking fumes, second-hand smoke) air pollution, and behavioral features such as physical activity and dietary habits, including the consumption of food known to contain genotoxic compounds (foods cooked on the griddle, barbecued and smoked foods, red/processed meat) [36,37] which could contribute to the MN formation. In order to make the perceived traffic level as uniform as possible, the following definitions were included in the questionnaire: a "low-traffic road" was classified as a secondary road, with low traffic consisting mainly of cars of resident people and rarely travelled by heavy vehicles. On the contrary, a "heavy-traffic road" was classified as a main road, with continuous traffic at peak times, with the possibility of queues at crossroads, often traveled by heavy vehicles. Also, some parent's characteristics (nation of birth, education level, occupational status) were included in the questionnaire.

The dietary section was based on the ARCA questionnaire [38], modified with the addition of some questions regarding breakfast and weekly fast food-frequency. It contained a total of 109 items on the frequency of food consumption. This section was used to determine the level of adherence to the Mediterranean Diet (MD) of children, using the "Mediterranean Diet Quality Index for children and adolescents" (KIDMED) [39]. It is based on 16 questions: those indicating a negative connotation for the MD were assigned a value of -1 (going more than once a week to a fast-food restaurant, skipping breakfast, having commercially baked goods or pastries for breakfast, taking sweets and candy several times every day); those with a positive aspect were assigned a value of +1 (taking a fruit or fruit juice every day, having a second fruit every day, having fresh or cooked vegetables regularly once a day, having fresh or cooked vegetables more than once a day, consuming fish at least 2-3 times per week, eating pulses more than once a week, consuming pasta or rice 5 or more times per week, having cereals or grains for breakfast, consuming nuts at least 2-3 times per week, using olive oil at home, having a dairy product for breakfast, taking two yogurts and/or some cheese daily). The total score ranges from -4 to 12 and there are three levels of adherence to the MD: ≥8, high adherence; 4-7, moderate adherence; ≤3, low adherence. The dietary habits of this cohort were previously reported in Grassi et al. [40].

2.5.Anthropometric Measurements and Biological Sampling

Anthropometric measurements (weight and height) and biological sampling were performed at schools during the morning hours before the break time. Weight and height were taken according to WHO recommendations [41] and used to calculate the children's body mass index (BMI) (weight

[kg]/height [m] squared). This was used in turn to assess whether the child was underweight (UW), of normal weight (NW), overweight (OW), or obese (OB), according to the cut-off points established by World Obesity Federation (WOF) [42].
The biological sampling of EBCs from children was performed by gently scraping the inner surface of both cheeks with a small-headed toothbrush as described in Idolo et al. [27]. The cells were stored at 4 °C in Saccomanno's fixative (50% ethanol, 2% polyethylene glycol, v/v; solution diluted in water) and processed in 7 days.

2.6.Buccal Micronucleus Cytome (BMCyt) Assay

The BMCyt assay was performed according to the procedure described by Thomas and Fenech [44]. The EBCs in Saccomanno's fixative were centrifuged, washed twice with PBS (Invitrogen Srl, Milan, Italy), filtered through a 100 µm nylon filter (Merck Spa, Milan, Italy), and centrifuged again. The cells were then fixed with ice-cold Carnoy's fixative (methanol and glacial acetic acid 3:1), stored at -20 °C, and processed one month later. For each subject, two slides were prepared by smearing 100 µL of cell suspension onto pre-cleaned slides. The slides were fixed in ethanol (Carlo Erba, Milan, Italy), washed with distilled water, and treated in 5M HCl (Carlo Erba, Milan, Italy). After washing, the slides were stained with Schiff's reagent (Sigma-Aldrich, Milan, Italy), further washed, and then counterstained with 0.2% Light Green reagent (Sigma-Aldrich, Steinheim, Germany). The slides were air-dried and finally mounted with DePex mounting medium (VWR International PBI Srl, Milan, Italy).
Overall, two thousand differentiated cells were scored for each subject (one thousand for each slide) by two trained scorers (each scorer read one slide from each subject) using fluorescence microscopy (Eclipse 50i, Nikon, Tokyo, Japan) following the scoring scheme proposed by Thomas and Fenech [44]. Cell types were classified according to Bolognesi et al. [44]: cells with micronuclei (MN) and nuclear buds (NBUD), as markers of nuclear damage, basal cells (BC) and binucleated cells (BNC), as cell proliferation markers, condensed chromatin cells (CCC), karyorrhectic (KHC), pyknotic (PYK) and karyolytic (KYL), as cell death markers, were scored and their frequency was reported as mean of MN in 1000 differentiated cells (‰).

2.7.Collection of particulate matter samples

Child exposure to air pollution was evaluated by collecting PM through a high-volume air sampler equipped with a multistage cascade impactor (AirFlow PM10 HVS sampler, AMS Analitica Srl, Pesaro, Italy). The sampler was placed in the courtyard of each involved school, in an open and paved point, away from relevant obstacles (walls, trees, etc.) and punctual sources of atmospheric pollution (chimneys, vent pipes, etc.). The particle size fractions, collected on fiberglass filters for 72 consecutive hours (three 24h periods), were of 10.0-7.2, 7.2-3.0, 3.0-1.5, 1.5-0.95, 0.95-0.49, and <0.49 µm ($PM_{0.5}$). All filters were pre- and post-conditioned and singularly weighed at controlled temperature and humidity.

2.8.Data analysis

All the information obtained from the biological and environmental surveys as well as the questionnaire administration were entered into a Microsoft Excel database (Microsoft Corporation, Redmond, Washington, USA) and statistically processed using MedCalc Software ver. 12.3 (MedCalc Software BVBA, Ostend, Belgium).

A descriptive statistical analysis was performed for both quantitative and qualitative variables. Approximation to the normal distribution of continuous variables was evaluated using the Kolmogorov-Smirnov test. Frequencies of MN showed significant departures from the normal distribution, even after logarithmic transformation. Therefore, the differences among groups were analyzed using the nonparametric Kruskal-Wallis H test. For the other continuous variables, one-way ANOVA test was used. The comparison among proportions was performed using Pearson's chi-squared test. Differences were considered significant at $p<0.05$.
In order to examine the possible association between individual, socio-economic, behavioral, and environmental factors (independent variables) and positivity for the BMCyt assay (dependent variable), a multivariate logistic regression analysis, with the corresponding odds ratio (OR) and 95% confidence interval (CI), was performed.

2.9.Ethical aspects

The study was approved by the Ethical Committee of the Lecce Local Health Authority (ASL/LE) on February 5, 2018, with deliberation n° 15. Participation in this study was voluntary. All parents of children received oral and written information about the project and expressed their approval by signing the informed consent form. Even the children expressed their approval to participate in the study through an assent form prepared *ad hoc*. All data were collected and analyzed confidentially in accordance with Italian laws (Legislative Decree n. 196 of 30/6/2003, and subsequent additions), only for research purposes.

3. Results

3.1.Recruitment

Overall, the parents of 455 children living in the selected villages were invited to participate in the study. Three hundred and ten (68.1%) parents provided their agreement by signing the consent form and, subsequently, filled in the questionnaire. The final sample, excluding the subjects falling into the exclusion criteria or the incomplete questionnaire (9.0%), was of 282 children, corresponding to 54.3% of the population aged 6-8 years living in the five villages involved in the study. Of these, 256 were sampled because 26 children were not at school during the biological monitoring (Table 1).

Villages	Invited children (n)	Consents (n)	Excluded children (n)	Valid questionnaires (n)	Absent from school (n)	Sampled children (n)
Cursi	102	80	15	65	7	58
Muro Leccese	83	54	5	49	1	48
Giurdignano	62	36	4	32	1	31
Otranto	106	69	2	67	14	53
Uggiano La Chiesa	102	71	2	69	3	66
Total	**455**	**310**	**28**	**282**	**26**	**256**

Table 1. Results of recruitment and questionnaire administration activities.

3.2.Study population

The characteristics of the study population were summarized in Table 2. All children were born in Italy, and 55.9% were males. The mean age was of 7.60±0.81 years, the mean weight was 28.2±7.0 kg, the mean height of 128.0±7.20 cm, and the mean BMI of 17.0±3.0 kg/m^2. According to the WOF

cut-offs, 60.9% of the children were of normal weight, 20.3% were overweight, 10.2% were obese, while 8.6% were underweight. Regarding the children's health status, 20.3% of children suffered from respiratory diseases beyond the common cold. As for physical activity, 53.1% of children played sports regularly at least 3 times a week.
According to the KIDMED score, 29.3% of children showed a high adherence to the MD, 41.4% a moderate adherence, and 29.3% a low adherence.

Characteristics	**n (%)**
Born in Italy	256 (100)
Males	143 (55.9)
6 years old	71 (27.7)
7 years old	89 (34.8)
8 years old	96 (37.5)
Underweight	22 (8.6)
Normal weight	156 (60.9)
Overweight (without obese)	52 (20.3)
Obese	26 (10.2)
Respiratory problems[1]	52 (20.3)
Sport (≥3 times/week)	136 (53.1)
High adherence to MD	75 (29.3)
Moderate adherence to MD	106 (41.4)
Low adherence to MD	75 (29.3)

Table 2. Individual characteristics of the recruited children.
[1] beyond the common cold.

3.3. Characteristics of parents

In Table 3 were reported the characteristics of children's parents. Most of them were born in Italy (86.7% of mothers and 90.2% of fathers); only 19.2% of mothers and 9.4% of fathers were graduated; almost half of the mothers (47.8%) and the majority of fathers (86.6%) were employed, and 13.7% of mothers and 31.5% of fathers were habitual smokers.

Characteristics	**n (%)**
Mother born in Italy	221 (86.7)
Father born in Italy	229 (90.2)
Graduated mother	49 (19.2)
Graduated father	24 (9.4)
Employed mother	122 (47.8)
Employed father	220 (86.6)
Smoking mother	35 (13.7)
Smoking father	80 (31.5)

Table 3. Characteristics of the children's parents.

3.4. Risk factors related to household environment and children lifestyle

Table 4 shows the risk factors investigated among recruited children and related to household environment and lifestyle. Twenty-two (8.6%) children lived in area perceived from their parents as highly trafficked. Considering the different kind of heating that may be possible sources of indoor

pollution, in the month preceding the survey, fireplaces were used in 35.0% of children's homes (with an average use of 4.9 days per month per family) and wood or pellet stoves in 6.3% (with an average use of 1.1 days per month per family). Fifty children (19.5%) stayed often in the kitchen during the food preparation and thirteen (5.1%) stayed frequently close to smokers in an indoor environment.
In the month preceding the survey, the children often consumed food potentially containing genotoxic compounds: 55.5% barbecued foods, 52.0% food cooked on the griddle, 18.8% smoked foods, and 27.7% red/processed meat.

Risk factors	**n (%)**
Living in a high-traffic area	22 (8.6)
Use of fireplaces or wood/pellet stoves[1]	68 (26.6)
Frequent presence in the kitchen during food cooking	50 (19.5)
Staying close to smokers in an indoor environment	13 (5.1)
Consumption of barbecued foods (wood/charcoal)[2]	142 (55.5)
Consumption of foods cooked on the griddle[2]	133 (52.0)
Consumption of smoked foods[2]	48 (18.8)
Consumption of red/processed meat[3]	71 (27.7)

Table 4. Risk factors investigated among recruited children and related to household environment and lifestyle. [1]more than 10 days in the month preceding the survey; [2]in the month preceding the survey; [3]more than once a day.

3.5.Air quality

Table 5 shows the concentrations of PM fractions detected using an air sampler placed in the schools' courtyards. Data were reported as the mean value of three samples collected in each school. The mean values ranged from 9.80 ± 2.57 μg/m^3 to 15.40 ± 0.51 μg/m^3 for PM_{10} and from 2.28 ± 0.87 μg/m^3 to 4.61 ± 1.82 μg/m^3 for $PM_{0.5}$ with a total average of 11.6 ± 3.9 μg/m^3 and 3.16 ± 1.40 μg/m^3 respectively. No significant differences were found among the villages.

Villages	**Samples (n)**	**PM_{10} (μg/m^3)**	**$PM_{0.5}$ (μg/m^3)**
Cursi	3	9.80 ± 2.57	4.61 ± 1.82
Giurdignano	3	15.40 ± 0.51	2.28 ± 0.87
Muro Leccese	3	10.07 ± 6.16	2.73 ± 1.21
Otranto	3	9.94 ± 4.18	3.67 ± 1.36
Uggiano La Chiesa	3	12.73 ± 3.13	2.51 ± 0.88
Total	15	11.6 ± 3.93	3.16 ± 1.40
*p-value**		0.35021	0.22328

Table 5. Mean concentrations (± SD) of PM fractions in the study area. * one-way ANOVA

3.6.BMCyt Assay

The BMCyt assay was performed on the EBCs of 256 children participating in the study. All samples were suitable for cell counting as the number of cells was sufficient in each slide. The mean frequency of chromosomal and DNA damage markers (MN, and NBUD), cell proliferation markers (BC and BNC) and cell death/apoptosis markers (CCC, KHC, PYK, and KYL) in the scored samples was reported in Table 6.

Biomarkers*	Frequency (‰ ± SD)
MN	0.27 ± 0.43
NBUD	0.04 ± 0.16
BC	2.28 ±0.65
BNC	2.83 ± 0.67
CCC	21.67 ± 2.22
KHC	7.77 ± 1.62
PYK	2.02 ±0.58
KYL	4.78 ± 1.04

Table 6. Frequency (‰ ± SD) of chromosome damage markers (MN and NBUD), cell proliferation markers (BC and BNC), and cell death markers (CCC, KHC, PYK and KYL) in exfoliated buccal cells (EBC) of children involved in the EFFE.BI.P. study. (*MN, micronuclei; NBUD, nuclear buds; BC, basal cells; BNC, binucleated cells; CCC, condensed chromatin cells; KHC, karyorrhectic cells; PYK, pyknotic cells; KYL, karyolytic cells).

Overall, 37.1% of samples had at least one MN and the mean MN frequency in the EBC cells of studied population was 0.27 ± 0.43 MN‰ with a 95% CI of 0.22-0.33 MN‰. No significant differences were found among the children living in different villages (Table 7).

Villages	Sampled children n	Samples with MN n (%)	MN frequency MN‰ ± SD
Cursi	58	22 (37.9)	0.29 ± 0.43
Giurdignano	31	12 (38.7)	0.32 ± 0.50
Muro Leccese	48	24 (50.0)	0.40 ± 0.50
Otranto	53	13 (24.5)	0.17 ± 0.31
Uggiano La Chiesa	66	24 (36.4)	0.23 ± 0.36
Total	256	95 (37.1)	0.27 ± 0.43
p-value		*0.1318**	*0.1000***

Table 7. Micronuclei positive samples and mean frequency in EBCs (exfoliated buccal cells) of children (* chi-square test; ** Kruskal Wallis test).

A multivariate logistic regression analysis was carried out considering individual, socio-economic, lifestyle, and environmental exposure factors, in order to identify their possible effect on MN frequency (Table 8). Living in a high-traffic area (OR=2.99; 95%CI=1.15-7.74) was the only variable positively associated with MN frequency ($p<0.05$); while practicing sport activity (OR=0.56; 95%CI=0.32-0.57) and having a graduated mother (OR=0.41; 95%CI=0.18-0.95) were negatively associated ($p<0.05$). Considering the odds ratios, other variables can be of note: staying close to smokers in an indoor environment and consuming red/processed meat had respectively OR=1.60 (95%CI=0.48-5.30) and OR=1.51 (95%CI=0.62-3.65), while the high adherence to Mediterranean Diet had OR=0.50 (95%CI=0.20-1.26).

Variables	Odds Ratio	95%CI	*p-value*

Normal weight	0.94	0.54-1.64	*0.8264*
Respiratory problems[1]	1.23	0.63-2.39	*0.5513*
Living in a high-traffic area	2.99	1.15-7.74	*0.0243*
High adherence to Mediterranean Diet[2]	0.50	0.20-1.26	*0.1438*
Sport activity	0.56	0.32-0.57	*0.0398*
Staying close to smokers in an indoor environment	1.60	0.48-5.30	*0.4420*
Use of fireplaces or wood/pellet stoves[3]	1.14	0.62-2.11	*0.6756*
Frequent presence in the kitchen during food cooking	1.36	0.69-2.68	*0.3774*
Consumption of barbecued foods (wood/charcoal)[4]	1.22	0.70-2.13	*0.4855*
Consumption of foods cooked on the griddle[4]	1.12	0.64-1.97	*0.6905*
Consumption of smoked food[4]	1.15	0.96-1.39	*0.1379*
Consumption of red/processed meat[5]	1.51	0.62-3.65	*0.3613*
Graduated mother	0.41	0.18-0.95	*0.0383*
Graduated father	1.22	0.44-3.36	*0.6964*
Employed mother	1.36	0.76-2.42	*0.3003*
Employed father	1.02	0.46-2.27	*0.9589*

Table 8. Multivariate logistic regression analyses, with the corresponding odds ratio (OR) and 95% confidence interval (CI) of MN frequency associated with individual and environmental factors. [1]beyond the common cold; [2]according to KIDMED score; [3]more than 10 days in the month preceding the survey; [4]in the month preceding the survey; [5]more than 4 times a week.

4. Discussion

In this study, the frequency of MN in EBCs of 256 schoolchildren living in five villages of Salento Peninsula was assessed.

This area is characterized by the absence of an industrial zone, a predominantly agricultural economy, a previously recognized absence of toxic substances in groundwater, and an all-cancer SMR <1, so it could be considered with low anthropogenic pressure.

The data relating to the air pollution levels in the villages involved in the study confirmed this hypothesis. The PM concentration detected using an air sampler in the schools' courtyards was lower than that observed in towns with high anthropogenic pressure [45-47]. Moreover, during the study period, the PM level was always under either the daily limit of 50 µg/m^3 indicated in the European Directive 2008/50/EC on air quality and the threshold concentration of 20 µg/m^3 proposed by the WHO.

The results of the biological survey showed that the children living in this area had a mean MN frequency of 0.27 ± 0.43‰ with a 95%CI of 0.22-0.33 MN‰.

A frequency of this magnitude would seem to be low when compared with that of other studies conducted on the EBCs of children of the same age. As we know, MN can occur as a result of natural processes, but their frequency can be increased by many environmental factors such as living in high impacted areas [48].

Previous studies investigated the presence of MN in EBCs of 6-8-years-old children living in other areas of the Salento peninsula in the same period of the year (month of May) in which the present research was carried out. In the children living in Lecce, a city with low anthropogenic pressure, the mean MN frequency was 0.24 ± 0.32 MN‰ [49]; in the children living in a central area of Salento with moderate industrial activities and high lung cancer SMR, it was 0.49 ± 0.65 MN‰ [26]; in the children living near an important industrial area, it was 0.66 ± 0.61 MN‰ [21].

Other studies performed in polluted areas showed higher frequencies of this biomarker: 0.67 ± 1.44 MN‰ in children of about 7 years old living close to highly trafficked areas in Oakland, California

[50]; 1.20 ± 0.90 MN‰ in 9-year-old children [20] and 0.53 ± 0.61 MN‰ in 6-8-year-old children [49] living in two different towns of the Po Valley, a known highly polluted area in northern Italy.
Conversely, data similar to ours were reported for two groups of Brazilian children aged ≤7 years and 8-9 years living in a rural nonpolluted area, with a mean frequency of 0.19 ± 0.31 MN‰ and 0.29 ± 0.46 MN‰, respectively [25].

Therefore, the MN frequency found in this study could be used as a reference in studies performed with similar conditions (i.e., sampling and analysis methods, period of the year) on populations with comparable characteristics (i.e., age, socio-economic factors) but with different environmental exposures.

Despite the low MN frequency, the statistical analysis conducted in this study had identified some factors responsible for its variability. Vehicular traffic probably wasn't comparable to what can be found in a large city, but nevertheless, it was associated with an increase in the MN frequency showing to be a possible sensitive indicator of this environmental stress. This finding is consistent with previous research that observed a high MN frequency in a population living in heavily trafficked areas [22,23]. Presumably, even if the total amount of vehicular traffic was not high, in the sites with more intense traffic (i.e. crossroads, main roads, queues at traffic lights), the level of airborne pollutants could vary significantly compared to the less trafficked sites. However, more accurate studies could be conducted in order to verify the chemical content of PM in these areas.

Also, individual or behavioral factors could influence the MN formation [2]. In the present study, the physical activity and educational level of the mother seemed to be protective against MN formation. Other studies reported a reduction of MN frequency in EBCs associated with moderate exercise [21], while an increase of MN was observed when practicing exhaustive physical activity [51].

Parental education was recognized to be associated with MN frequency with a significant decrease when the parents were graduated [12]. A high level of parental education could ensure healthier choices regarding the education and care of the children during all the childhood as well as the adoption of correct behaviors and lifestyles [52-54]. In addition, it is often correlated with a greater level of socio-economic well-being, which entails access to goods and services able to create a protective environment against many risk factors [55] including those that affect the formation of MN. The results of the present study confirm the evidence regarding the decisive role of maternal education in building such a protective environment towards the children [56,57].

Considering the odds ratios, other variables, although not statistically significant, could be considered noteworthy: the high adherence to Mediterranean Diet, with a potential protective role against MN formation, staying close to smokers in an indoor environment, and consuming red or processed meat, with a potential promoting role.

In general, a diet with a balanced intake of nutrients, vitamins, and antioxidants, such as MD, is correlated with a low level of MN [3]. Moreover, studies investigating the direct role of MD also showed a negative association between the frequency of MN and the high adherence to MD [12]. Overall, the studied population showed a level of adherence to the MD higher than that observed in previous Italian surveys [35,58-60]. Further studies should be performed considering populations with different dietary patterns in order to determine their actual contribution to MN formation.

Regarding the smoking habits of children's parents, the fathers smoked slightly more (31.5%) than Italian men overall (29.9%); while the mothers smoked less (13.7%) compared to Italian women (21.6%) [61]. Many studies demonstrated the direct association of MN frequency with second-hand smoking [62,63].

Lastly, previous observational studies on the genotoxic effect of a diet rich in meat demonstrated a

significant positive relationship between meat consumption and MN formation [26,64].
Cobanoglu et al. [65] suggested that other factors could be involved in the formation of micronuclei in EBCs, including direct exposure to pesticides used in agriculture. In our study, however, this factor may have had little significant effects on the MN frequency of children. Firstly, the analyzed subjects did not have direct exposure to these toxic substances. Furthermore, in this area, as well as in the whole province of Lecce, agriculture is extensive and mainly represented by the cultivation of olive trees which use fewer pesticides than other crops. This is confirmed by the data on the consumption of pesticides which in 2018 in the Province of Lecce was equal to 6.68 kg/ha UAA, while in the whole region it was 9.36 kg/ha UAA and Italy it was 8.82 kg/ha UAA [29]. In addition, the surveys conducted from 2016 to 2018 by the Regional Agency for the Environmental Protection (ARPA) of the Apulia Region demonstrated the absence of pesticide residues in the groundwater circulating in the study area [33].
The present study has several strengths. Firstly, a consolidated method was used to detect MN in EBCs and this allows for the comparison with other molecular epidemiological studies. In addition, this study involved a large number of 6-8-years old children (n=256) and provided an indication regarding a reference basal range of MN that could be used as a comparison in studies involving populations of the same age exposed to environmental contaminants. Finally, identification of the variables related to lifestyle able to influence MN frequency in a population living in a low anthropogenic pressure area was provided.
However, this study is not without its limitations. First of all, the recruitment was conducted on a voluntary basis, therefore, a selection bias due to motivational factors cannot be excluded. Then, data related to personal, residential, and behavioral factors may have been underestimated or overestimated depending on the subjective perception of the participants. In particular, the level of traffic was considered on the basis of personal perception although the definition of the different traffic level was given in the questionnaire. However, since the villages involved in the study had a similar number of inhabitants, they had also a presumably no different traffic amount. Therefore, the perception of the various traffic levels by the inhabitants of different villages can be considered comparable. Lastly, other factors (i.e. electromagnetic waves, drinking water, other environmental factors), not considered in this study, may have had some effect on the MN frequency.

5. Conclusions

Children of 6-8 years living in a rural area of Salento peninsula showed a MN frequency of 0.27 ± 0.43‰. Since this area was characterized by non-significant anthropogenic pressures, the MN frequency found in the EBCs of children could be considered as MN level in pediatric populations not exposed to environmental pollution.
In these conditions, the variables that influence the base-line MN frequency would seem to be vehicular traffic (positively associated) as well as on the high educational level of mother and regular physical activity of children (negatively associated).
Buccal micronucleus cytome assay could be a sensitive method to detect early biological effects due to environmental exposures but it should be used taking into consideration other variables such as characteristics and behaviors of the population (socio-economic features, lifestyle, eating habits, indoor exposures, etc.). Further and deeper studies should be performed in order to individually investigate the role of each factor involved in MN occurrence, including chemical composition of PM.

Declaration of interest: none.

Funding: This work was supported by Interprovincial University Consortium of Salento (CUIS) (prot. n. 6 of 26/06/2016). The funder had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

References

[1] M. Fenech. The in vitro micronucleus technique. Mutat. Res. 455 (2000) 81-95.

[2] S. Sommer, I. Buraczewska, M. Kruszewski. Micronucleus assay: The state of art, and future directions. Int. J. Mol. Sci. 21 (2020) 1534.

[3] M. Fenech, S. Bonassi. The effect of age, gender, diet and lifestyle on DNA damage using micronucleus frequency in human peripheral blood lymphocytes. Mutagenesis. 26 (2011) 43-9.

[4] A.L.G. da Silva, M.J. Bresciani, T.E. Karnopp, A.F. Weber, J.H. Ellwanger, J.A. Henriques, A.R. Valim, L.G. Possuelo. DNA damage and cellular abnormalities in tuberculosis, lung cancer and chronic obstructive pulmonary disease. Multidiscip. Respir. Med. 10 (2015) 38.

[5] E. Murgia, V. Maggini, R. Barale, A. M. Rossi. Micronuclei, genetic polymorphisms and cardiovascular disease mortality in a nested case-control study in Italy. Mutat. Res. 621 (2007) 113-118.

[6] M. Kirsch-Volders, C. Bolognesi, M. Ceppi, M. Bruzzone, M. Fenech. Micronuclei, inflammation and auto-immune disease. Mutat. Res. 786 (2020) 108335.

[7] M. Guido, A. Zizza, M. R. Tumolo, G. Stefanelli, M. D'Alba, A. Idolo, F. Bagordo, F. Serio, T. Grassi, A. De Donno. Using the cytokinesis-block micronucleus cytome assay to evaluate chromosomal DNA damage in chronic renal patients undergoing bicarbonate haemodialysis and haemodiafiltration. J Prev Med Hyg. 57 (2016) E178-E184.

[8] M. Salimi, B. Broumand, H. Mozdarani. Association of elevated frequency of micronuclei in peripheral blood lymphocytes of type 2 diabetes patients with nephropathy complications. Mutagenesis. 31 (2016) 627-633.

[9] M. Witczak, T. Ferenc, E. Gulczyńska, D. Nowakowska, D. Łopaczyńska, J. Wilczyński. Elevated frequencies of micronuclei in pregnant women with type 1 diabetes mellitus and in their newborns. Mutat. Res. 763 (2014) 12-17.

[10] S. Bonassi, D. Ugolini, M. Kirsch-Volders, U. Strömberg, R. Vermeulen, J.D. Tucker. Human population studies with cytogenetic biomarkers: review of the literature and future prospective. Environ. Mol. Mutagen. 45 (2005) 258-70.

[11] M. Fenech, S. Knasmueller, C. Bolognesi, N. Holland, S. Bonassi, M. Kirsch-Volders. Micronuclei as biomarkers of DNA damage, aneuploidy, inducers of chromosomal hypermutation and as sources of pro-inflammatory DNA in humans. Mutat. Res. 786 (2020) 108342.

[12] E. Ceretti, F. Donato, C. Zani, M. Villarini, M. Verani, A. De Donno, S. Bonetta, D. Feretti, A. Carducci, A. Idolo, E. Carraro, L. Covolo, M. Moretti, G. Palomba, T. Grassi, A. Bonetti, S. Bonizzoni, A. Biggeri, U. Gelatti, the MAPEC_LIFE Study Group. Results from the European Union MAPEC_LIFE cohort study on air pollution and chromosomal damage in children: are public health policies sufficiently protective? Environ. Sci. Eur. 32 (2020) 1-11.

[13] R. Barouki, P.D. Gluckman, P. Grandjean, M. Hanson, J.J. Heindel. Developmental origins of non-communicable diseases: implications for research and public health. Environ. Health. 11 (2012) 42.

[14] C.M. Boney, A. Verma, R. Tucker, B.R. Vohr. Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics. 115, (2005) e290-e296.

[15] D.A. Bennett, M.D. Waters. Applying biomarker research. Environ. Health Perspect. 108 (2000) 907-910.

[16] N. Holland, C. Bolognesi, M. Kirsch-Volders, S. Bonassi, E. Zeiger, S. Knasmueller, M. Fenech. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage. The HUMN project perspective on current status and knowledge gaps. Mutat. Res. 659 (2008) 93-108.

[17] E. Ceretti, D. Feretti, G.C.V. Viola, I. Zerbini, R.M. Limina, C. Zani, M. Capelli, R. Lamera, F. Donato, U. Gelatti. DNA Damage in Buccal Mucosa Cells of Pre-School Children Exposed to High Levels of Urban Air Pollutants. PLoS ONE. 9 (2014) e96524.

[18] P. Thomas, N. Holland, C. Bolognesi, M. Kirsch-Volders, S. Bonassi, E. Zeiger, S. Knasmueller, M. Fenech. Buccal micronucleus cytome assay. Nat. Protoc. 4 (2009) 825-837.

[19] S. Bonassi, E. Coskun, M. Ceppi, C. Lando, C. Bolognesi, S. Burgaz, N. Holland, M. Kirsh-Volders, S. Knasmueller, E. Zeiger, D. Carnesoltas, D. Cavallo, J. da Silva, V.M. de Andrade, G.C. Demircigil, A. Domínguez Odio, H. Donmez-Altuntas, G. Gattas, A. Giri, S. Giri, B. Gómez-Meda, S. Gómez-Arroyo, V. Hadjidekova, A. Haveric, M. Kamboj, K. Kurteshi, M.G. Martino-Roth, R. Montero Montoya, A. Nersesyan, S. Pastor-Benito, D.M. Favero Salvadori, A. Shaposhnikova, H. Stopper, P. Thomas, O. Torres-Bugarín, A.S. Yadav, G. Zúñiga González, M. Fenech. The Human MicroNucleus project on eXfoLiated buccal cells (HUMNxl): the role of life-style, host factors, occupational exposure, health status, and assay protocol. Mutat. Res. 728 (2011) 88-97.

[20] A. Marcon, M.E. Fracasso, P. Marchetti, D. Doria, P. Girardi, L. Guarda, G. Pesce, V. Pironi, P. Ricci, R. De Marco. Outdoor formaldehyde and NO2 exposures and markers of genotoxicity in children living near chipboard industries. Environ. Health Perspect. 122 (2014) 639-645.

[21] A. Panico, T. Grassi, F. Bagordo, A. Idolo, F. Serio, M.R. Tumolo, M. De Giorgi, M. Guido, M. Tutino, A. De Donno. Micronucleus frequency in exfoliated buccal cells of children living in an industrialized area of Apulia (Italy). Int. J. Environ. Res. Public Health. 17 (2020) 1208.

[22] A. De Donno, T. Grassi, E. Ceretti, G.C.V. Viola, S. Levorato, S. Vannini, T. Salvatori, A. Carducci, M. Verani, Sa. Bonetta, E. Carraro, S. Bonizzoni, A. Bonetti, F. Bagordo, F. Serio, A. Idolo, U. Gelatti & MAPEC_life study group. Air pollution biological effects in children living in Lecce (Italy) by buccal micronucleus cytome assay (the MAPEC_LIFE study). Int. J. Sust. Develop. Plan. 11 (2016) 500-510.

[23] É.P. Domingues, G.G. Silva, A.B. Oliveira, L.M. Mota, V.S.V. Santos, E.O. Jr de Campos, B.B. Pereira. Genotoxic effects following exposure to air pollution in street vendors from a high-traffic urban area. Environ. Monit. Assess. 190 (2018) 215.

[24] A. Rossnerova, M. Spatova, A. Pastorkova, N. Tabashidze, M. Jr. Veleminsky, I. Balascak, I. Solansky, R.J. Sram. Micronuclei levels in mothers and their newborns from regions with different types of air pollution. Mutat. Res. 715 (2011) 72-78.

[25] H.A. Sisenando, S.R. Batistuzzo de Medeiros, P. Artaxo, P.H. Saldiva, S. Hacon Sde. Micronucleus requency in children exposed to biomass burning in the Brazilian Legal Amazon region: a control case study. BMC Oral Health. 12 (2012) 6.

[26] A. Idolo, T. Grassi, F. Bagordo, A. Panico, M. De Giorgi, F. Serio, M. Guido, P. Piscitelli, G. De Filippis, A. Raho, A. De Donno. Micronuclei in Exfoliated Buccal Cells of Children Living in a Cluster Area of Salento (Southern Italy) with a High Incidence of Lung Cancer: The IMP. AIR Study. Int. J. Environ. Res. Public Health. 15 (2018) 1659.

[27] C. Bolognesi, S. Bonassi, S. Knasmueller, M. Fenech, M. Bruzzone, C. Lando, M. Ceppi. Clinical application of micronucleus test in exfoliated buccal cells: A systematic review and metanalysis. Mutat. Res. Rev. Mutat. Res. 766 (2015) 20-31.

[28] D. Feretti, E. Ceretti, A. De Donno, M. Moretti, A. Carducci, S. Bonetta, M.R. Marrese, A. Bonetti, L. Covolo, F. Bagordo, M. Villarini, M. Verani, T. Schilirò, R.M. Limina, T. Grassi, S. Monarca, B. Casini, E. Carraro, C. Zani, G. Mazzoleni, R. Levaggi, U. Gelatti, the MAPEC_LIFE Study Group. Monitoring air pollution effects on children for supporting public health policy: the protocol of the prospective cohort MAPEC study. BMJ Open. 4 (2014) e006096.

[29] National Statistics Institute (ISTAT). Resident Population on 1st January. http://dati.istat.it (accessed 4.06.2021).

[30] Lecce Chamber of Commerce. The Companies of the Municipalities. http://www.le.camcom.gov.it/P42A0C157S69/Le-imprese-dei-comuni.htm (accessed 4.06.2021).

[31] F. Lugoli, M. I. Leopizzi, F. Bagordo, T. Grassi, M. Guido, A. De Donno. Widespread microbiological groundwater contamination in the South-eastern Salento (Puglia-Italy). J. Environ. Monit. 13 (2011) 192-200.

[32] T. Grassi, F. Bagordo, A. Idolo, F. Lugoli, G. Gabutti, A. De Donno. Rotavirus detection in environmental water samples by tangential flow ultrafiltration and RT-nested PCR. Environ. Monit. Assess. 164 (2010) 199-205.

[33] Regional Agency for Environmental Protection (ARPA Puglia). Acque sotterranee triennio 2016-2018. https://www.arpa.puglia.it/pagina3105_acque-sotterranee-triennio-2016-2018.html (accessed 14.10.2021).

[34] Apulia Regional Epidemiological Observatory, Mortality tables by cause. https://www.sanita.puglia.it/web/oer/tavole-mortalita-per-causa (accessed 15.06.2021).

[35] C. Zani, E. Ceretti, S. Grioni, G.C.V. Viola, F. Donato, D. Feretti, A. Festa, S. Bonizzoni, A. Bonetti, S. Monarca, M. Villarini, S. Levorato, A. Carducci, M. Verani, B. Casini, A. De Donno, T. Grassi, F. Bagordo, E. Carraro, Si. Bonetta, Sa. Bonetta, U. Gelatti, the MAPEC_LIFE Study group. Are 6–8 year old Italian children moving away from the Mediterranean diet? Ann. Ig. 28 (2016) 339-348.

[36] M. Jägerstad, K. Skog. Genotoxicity of heat-processed foods. Mutat. Res. 574 (2005) 156-172.

[37] International Agency for Research on Cancer (IARC). Red meat and processed meat. IARC Monographs on the Evaluation of Carcinogenic Risk to Humans. Volume 114, Lyon, France, 2018.

[38] G. Barba, S. Sieri, M.D. Russo, E. Donatiello, A. Formisano, F. Lauria, S. Sparano, A. Nappo, P. Russo, F. Brighenti, V. Krogh, A. Siani. Glycaemic index and body fat distribution in children: The results of the ARCA project. Nutr. Metab. Cardiovasc. Dis. 22 (2012) 28-34.

[39] L. Serra-Majem, L. Ribas, J. Ngo, R.M. Ortega, A. Garcìa, C. Pèrez-Rodrigo, J. Aranceta. Food, youth and the Mediterranean diet in Spain. Development of KIDMED, Mediterranean Diet Quality Index in children and adolescents. Public Health Nutr. 7 (2004) 931-935.

[40] T. Grassi, F. Bagordo, A. Panico, M. De Giorgi, A. Idolo, F. Serio, M.R. Tumolo, A. De Donno. Adherence to Mediterranean diet of children living in small Southern Italian villages. Int. J. Food Sci. Nutr. 71 (2020) 490-499.

[41] World Health Organization (WHO) Expert Committee. Physical Status: The Use and Interpretation of Anthropometry, in: WHO Technical Report Series 854; World Health Organization Expert Committee: Geneva, Switzerland, 1995.

[42] T.J. Cole, T. Lobstein. Extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity. Pediatr. Obes. 7 (2012) 284-294.

[43] P. Thomas, M. Fenech. Buccal micronucleus cytome assay. Methods Mol. Biol. 682 (2011) 235-248.

[44] C. Bolognesi, S. Knasmueller, A. Nersesyan, P. Thomas, M. Fenech..The HUMNxl scoring criteria for different cell types and nuclear anomalies in the buccal micronucleus cytome assay - An update and expanded photogallery. Mutat. Res. 753 (2013) 100-113.

[45] M. Masiol, S. Squizzato, G. Formenton, R. M. Harrison, C. Agostinelli. Air quality across a European hotspot: Spatial gradients, seasonality, diurnal cycles and trends in the Veneto region, NE Italy. Sci. Total Environ. 576 (2017) 210-224.

[46] Y. L. Zhang, F. Cao. Fine particulate matter (PM 2.5) in China at a city level. Sci. Rep. 5 (2015), 1-12.

[47] S. Bonetta, S. Bonetta, T. Schilirò, E. Ceretti, D. Feretti, L. Covolo, S. Vannini, M. Villarini, M. Moretti, M. Verani, A. Carducci, F. Bagordo, A. De Donno, S. Bonizzoni, A. Bonetti, C. Pignata, E. Carraro, U. Gelatti. Mutagenic and genotoxic effects induced by PM0. 5 of different Italian towns in human cells and bacteria: The MAPEC_LIFE study. Environ. Pollut. 245 (2019) 1124-1135.

[48] C. Federico, V. Vitale, N. La Porta, S. Saccone. Buccal micronucleus assay in human populations from Sicily (Italy) exposed to petrochemical industry pollutants. Environ. Sci. Pollut. Res. 26 (2019) 7048-7054.

[49] M. Villarini, S. Levorato, T. Salvatori, E. Ceretti, S. Bonetta, A. Carducci, T. Grassi, S. Vannini, F. Donato, Si. Bonetta, M. Verani, A. De Donno, S. Bonizzoni, A. Bonetti, M. Moretti, U. Gelatti. Buccal micronucleus cytome assay in primary school children: A descriptive analysis of the MAPEC_LIFE multicenter cohort study. Int. J. Hyg. Environ. Health. 221 (2018) 883-892.

[50] K. Huen, L. Gunn, P. Duramad, M. Jeng, R. Scalf, N. Holland. Application of a geographic information system to explore associations between air pollution and micronucleus frequencies in African American children and adults. Environ. Mol. Mutagen. 47 (2006) 236-246.

[51] R. Sharma, G. Gandhi. Pre-cancerous (DNA and chromosomal) lesions in professional sports. J. Cancer Res. Ther. 8 (2012) 578.

[52] C. E. Ross, J. Mirowsky. The interaction of personal and parental education on health. Social Science & Medicine. 72 (2011) 591-599.

[53] L. Lissner, T.M.A. Wijnhoven, K. Mehlig, A. Sjöberg, M. Kunesova, A. Yngve, A. Petrauskiene, V. Duleva, A.I. Rito, J. Breda. Socioeconomic inequalities in childhood overweight: Heterogeneity across five countries in the WHO European Childhood Obesity Surveillance Initiative (COSI-2008). Int. J. Obes. 40 (2016) 796-802.

[54] M. Balaj, H.W. York, K. Sripada, E. Besnier, H.D. Vonen, A. Aravkin, J. Friedman, M. Griswold, M.R. Jensen, T. Mohammad, E.C. Mullany, S. Solhaug, R. Sorensen, D. Stonkute, A. Tallaksen, J. Whisnant, P. Zheng, E. Gakidou, T.A. Eikemo. Parental education and inequalities in child mortality: a global systematic review and meta-analysis. The Lancet. 398 (2021) 608-620.

[55] A. Lamerz, J. Kuepper-Nybelen, C. Wehle, N. Bruning, G. Trost-Brinkhues, H. Brenner, J. Hebebrand, B. Herpertz-Dahlmann. Social class, parental education, and obesity prevalence in a study of six-year-old children in Germany. Int. J. Obes. 29 (2005) 373-380.

[56] T. Grassi, A. De Donno, F. Bagordo, F. Serio, P. Piscitelli, E. Ceretti, C. Zani, G.C.V. Viola, M. Villarini, M. Moretti, S. Levorato, A. Carducci, M. Verani, G. Donzelli, Sa. Bonetta, Si. Bonetta, E.

Carraro, S. Bonizzoni, A. Bonetti, U. Gelatti. Socio-Economic and environmental factors associated with overweight and obesity in children aged 6–8 years living in five Italian cities (the MAPEC_LIFE cohort). Int. J. Environ. Res. Public Health. 13 (2016) 1002.

[57] A.P. Vermeiren, M. Willeboordse, M. Oosterhoff, N. Bartelink, P. Muris, H. Bosma. Socioeconomic multi-domain health inequalities in Dutch primary school children. Eur. J. Public Health. 28 (2018) 610-616.

[58] R. Roccaldo, L. Censi, L. D'Addezio, E. Toti, D. Martone, D. D'Addesa, A. Cernigliaro, the ZOOM8 Study group. Adherence to the Mediterranean diet in Italian school children (The ZOOM8 Study). Int. J. Food Scie. Nutr. 65 (2014) 621-628.

[59] F. Bagordo, A. De Donno, T. Grassi, M. Guido, G. Devoti, E. Ceretti, C. Zani, D. Feretti, M. Villarini, M. Moretti, T. Salvatori, A. Carducci, M. Verani, B. Casini, S. Bonetta, E. Carraro, T. Schilirò, S. Bonizzoni, A. Bonetti, U. Gelatti, the MAPEC_LIFE Study group. Lifestyles and socio-cultural factors among children aged 6–8 years from five Italian towns: The MAPEC_LIFE study cohort. BMC Public Health. 17 (2017) 233.

[60] D. Pierannunzio, M. Buoncristiano, P. Nardone, L. Lauria, A. Spinelli, Gruppo OKkio alla SALUTE 2016. Attività fisica e comportamenti sedentari nei bambini, in: P. Nardone, A. Spinelli, M. Buoncristiano, L. Lauria, D. Pierannunzio, D. Galeone, (Eds.), Il Sistema di Sorveglianza OKkio Alla SALUTE: Risultati 2016. Istituto Superiore di Sanità, Rome, Italy, 2018, pp. 29-36.

[61] Epicentro. The PASSI Survey: Italian Data about Smoking Habits. https://www.epicentro.iss.it/passi/dati/fumo (accessed 11.06.2021).

[62] D.N. Cavalcante, J.C. Sposito, B.D. Crispim, A.V. do Nascimento, A.B. Grisolia. Genotoxic and mutagenic effects of passive smoking and urban air pollutants in buccal mucosa cells of children enrolled in public school. Toxicol. Mech. Methods. 27 (2017) 346-351.

[63] R. Chandirasekar, K. Suresh, R. Jayakumar, R. Venkatesan, B.L. Kumar, K. Sasikala. XRCC1 gene variants and possible links with chromosome aberrations and micronucleus in active and passive smokers. Environ. Toxicol. Pharmacol. 32 (2011) 185-192.

[64] S. Pastor, S. Gutiérrez, A. Creus, A. Cebulska-Wasilewska, R. Marcos. Micronuclei in peripheral blood lymphocytes and buccal epithelial cells of Polish farmers exposed to pesticides. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 495 (2001) 147-156.

[65] H. Cobanoglu, M. Coskun, M. Coskun, A. Çayir. Results of buccal micronucleus cytome assay in pesticide-exposed and non-exposed group. Environ. Sci. Pollut. Res. 26 (2019) 19676-19683.