AIR POLLUTION BIOLOGICAL EFFECTS IN CHILDREN LIVING IN LECCE (ITALY) BY BUCCAL MICRONUCLEUS CYTOME ASSAY (THE MAPEC_LIFE STUDY)

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ABSTRACT

The aim of the MAPEC_LIFE (Monitoring Air Pollution Effects on Children for Supporting Public Health Policy) study is to evaluate the associations between the concentrations of air pollutants and early biological effects in children living in five Italian towns (Brescia, Torino, Lecce, Perugia and Pisa) characterised by varying levels of air pollution. This paper presents the results of micronucleus cytome assays performed on the oral mucosa cells of subjects living in Lecce (Puglia, Italy) and their relationship to factors associated with indoor/outdoor exposure and lifestyles. The study was conducted on 6-8-year-old schoolchildren living in Lecce. The micronucleus cytome assay was performed on exfoliated buccal cells collected from the oral mucosa of children using a soft-bristled toothbrush. Micronuclei were evaluated only in normal differentiated cells. Overall, 43.0% of the samples tested were positive, with an average frequency of 0.28 MN/1000 differentiated cells. Data analysis shows positive associations between the frequency of MN in the children's buccal mucosa cells and obesity, heavy traffic and smoking mothers, while outdoor sports seem to have the opposite effect. These data will be integrated with data from the other cities involved in the MAPEC_LIFE study and could be used to build a model for estimating global genotoxic risk.
Keywords: air pollution, children, early biological effects, MAPEC_LIFE study, micronucleus cytome assay.

1 INTRODUCTION

Air pollution is one of the most important health problems in the world. Several studies have found a consistent association between exposure to air pollution, especially particulate matter, and the incidence of several chronic diseases such as lung cancer, cardiovascular disease and diabetes [1–3]. Among the mechanisms responsible for these adverse effects, genotoxic damage is of particular concern. Although the whole population is exposed to air pollution, certain people, particularly children, are at a higher risk of suffering the health consequences of airborne chemicals, for various reasons. First, children have higher levels of physical activity, spend more time outside and have a higher air intake than adults. Second, children are more vulnerable to the adverse effects of air pollution due to their small body size, fast growth rate and relatively immature organs, body functions, immune system and cell repair

mechanisms [4–8]. Lastly, recent data suggest that genetic damage occurring early in life can increase the risk of carcinogenesis in adulthood [9].

Molecular epidemiology represents a modern approach to the assessment of the health effects of exposure to environmental pollutants. It is based essentially on understanding the interactions between genes and the environment and on the study of risk prediction models. The study of early biological effects makes use of "effect biomarkers" that can measure a directly quantifiable biological response attributed to an exposure. The biological response is determined by measuring morphological and/or functional effects observable in target cells and tissues following environmental exposure which could constitute the first step in a pathological process that leads to disease [10]. Micronuclei (MN) are key biomarkers of early biological effects linked to DNA damage, since they highlight the presence of alterations in chromosomal structure and oxidative stress caused by atmospheric pollutants. The MN test has recently been proposed as an effective biomarker for the study of airborne pollutants and is widely used in studies conducted in various parts of the world for measuring genotoxic damage in exposed populations [11–13]. An interesting target site for monitoring early genotoxic events, as a consequence of the entrance of potential mutagens following environmental and/or occupational exposure, is buccal mucosa (BM). In this regard, MN testing of BM cells [10, 14] is an effective tool for assessing chromosomal damage arising from environmental exposure. It is clear that any response detected using this method must necessarily be interpreted with reference to conditions of exposure, which are individually variable depending on lifestyle, air quality and the general state of health of the exposed subjects, as well as being variable over time and space [10].

Genetic biomarkers have been studied largely in adult populations. Few studies so far have investigated genetic damage in children exposed to air pollution [15–19]. In recent years, studies of MN frequency in the BM cells of children and young adults have shown cytogenetic damage in participants living in areas with high concentrations of particulate matter (PM) or oxidant pollutants [20–25]. Recently, Ceretti *et al.* [25] found a higher frequency of MN in the exfoliated buccal cells (EBCs) of pre-school children living in a highly polluted town in the Po Valley in Italy than was observed in a pooled general population of the same age.

On the basis of these considerations, the MAPEC_LIFE (Monitoring Air Pollution Effects on Children for Supporting Public Health Policy) project is a multicentre cohort study that seeks (a) to assess the association between concentrations of certain atmospheric pollutants and early biological effects in children aged 6–8 living in areas with varying levels of air pollution and (b) to build a global model for estimating global genotoxic risk. The study protocol [26] envisages: recruitment of about 1,000 primary schoolchildren in five Italian cities (Brescia, Lecce, Perugia, Pisa and Torino); sampling in two seasons of EBCs and salivary leukocytes; assessment of genotoxic damage in the sampled cells by micronucleus cytome assay and comet assay respectively; and atmospheric monitoring near the schools involved in order to determine the concentrations of genotoxic contaminants and the in vitro toxicity of PM. In order to evaluate the confounding role of other factors to which the subject may be exposed, the parents of the children participating in the study were asked to fill in an ad hoc questionnaire preliminarily validated [27]. This paper presents the results of micronucleus cytome assays performed on the oral mucosa cells of subjects living in Lecce and describes their relationship to factors associated with indoor/outdoor exposure and lifestyles.

2 METHODS

2.1 Recruitment

After having selected potentially suitable schools (located in the urban area of Lecce, distant from sources of industrial pollution, with a high number of pupils) and after having obtained formal approval from the school authorities, meetings were held with teachers and parents in order to illustrate the study.

After the project presentation, in each school, a project parcel was distributed to children attending first, second and third classes. This parcel contained (a) a fact sheet to inform the children's parents about the project, its objectives and methods; (b) the informed consent form for the children's parents' approval; (c) the assent form for the children's approval. After a few days, parents' consent and children's assent forms were gathered and checked.

2.2 Filling in the questionnaire

In the weeks preceding the biological and environmental sampling the parents who had agreed to participate in the study were asked to fill in the questionnaire.

The questionnaire was composed of 148 questions, subdivided into various sections: information on the investigation including its objectives and treatment of data; criteria for exclusion from the study (age below six years or above nine, residence in cities other than Lecce, the 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); the child's personal information (gender, date and nation of birth); the child's weight and height; information on the child's health status (respiratory problems and consumption of medicines); domestic environment (intensity of traffic near the home, fuel used for heating and cooking, presence of gas boilers, stoves and fireplaces inside the dwelling, presence of smokers inside the dwelling, use of solvents for hobbies); information on the child's lifestyle (exercise, consumption of dishes cooked in certain ways); parents' characteristics (nation of birth, level of education, occupation, smoking habits); information on the child's eating habits. All the questions were of the "closed answer" type.

The questionnaire was distributed twice, during the environmental and biological investigations in Lecce in February-March (season I) and April-May 2015 (season II).

2.3 Sampling of EBCs

Biological samples were collected from children living in Lecce in February-March and April-May 2015. Before collecting EBCs, the children rinsed their mouths twice with mineral water. Disposable soft-bristled toothbrushes were then used to collect EBCs for the MN test by scraping the inside of both cheeks gently and dipping the material into tubes containing 15 mL of phosphate buffered saline solution.

2.4 MN cytome assay

The MN cytome assay was performed on EBCs from the children in accordance with Thomas *et al.* [28]. For this purpose, a suspension of scraped BM cells from each sample was prepared [25]. The test tubes containing the cellular suspension were kept at −20°C and sent on ice to the University of Perugia which saw to their staining, fixing on slides and microscopic

analysis. The slides were stained in light green using the Feulgen method. In microscopic analysis, the slides were examined under a microscope at 1,000x magnification. In accordance with the proposed scoring criteria [28, 29], before the MN frequency calculation, EBCs were divided into: basal cells, normal differentiated cells, apoptotic/necrotic cells and binucleated cells. MN frequency was then assessed by two well-trained operators in at least 2,000 normal differentiated cells per participant and the result was expressed as MN/1,000 cells.

2.5 Statistical analysis

The data from the MN tests and the questionnaires were added to Microsoft Excel spreadsheets for processing. The data were processed for the definitive sample, based on those children for which were possible to collect samples in both seasons. The exact age at the moment of testing was calculated from the date of birth given on the questionnaire. The data for weight and height given by the parents were used to calculate the children's body mass index (BMI), which was used in turn to assess whether the child was underweight (UW), of normal weight (NW), overweight (OW) or obese (OB). The cut-offs for the OW and OB categories were set at the 85th and 95th BMI percentiles, respectively [30] while the cut-off for the UW category was set at the "-2 z-score" [31]. The data thus obtained were statistically processed using MedCalc software (Ver. 12.3). The samples were divided into two groups depending on results of MN test: when MN=0 they were considered "negative"; when MN>0 they were considered "positive". The results of the MN tests (frequency of positive results and the average concentration of MNs in each sample) were analysed by comparing their distribution in the context of the variables investigated via the questionnaire. The data on MN concentrations were compared using the Mann-Whitney test and the data on the frequency of positive results using the chi-square test. Differences were considered significant at *p<0.05*.

2.6 Ethical aspects

The study was approved by the Ethical Committee of the Lecce Local Health Authority (ASL). All the data were gathered and analysed in accordance with Decree DL 196 of 30/6/2003 ("protection of personal data") and subsequent additions, for the purposes of research.

3 RESULTS

Parents of children attending first, second and third classes in the selected schools were given 750 parcels; 343 consents were received (45.7%) and the same number of questionnaires was compiled. Of these, 270 (78.7%) were valid, after excluding those that were either incomplete (59) or did not meet the criteria for inclusion (34). Overall, in the first season, 266 children were sampled (98.5%) (four children were absent at the moment of sampling). The same parents were asked to fill in the questionnaire again in the second season. At this time 242 valid questionnaires were gathered (90.6%) and 241 children were sampled (Table 1).

Overall, in the micronucleus tests, samples from 213 children were legible in both the first and second seasons (final cohort). The final cohort was composed (Table 2) of 106 (49.8%) boys and 107 (50.2%) girls. At the moment of recruitment, 88 children (41.3%) were six years of age, 59 (27.7%) were seven, and 66 (31.0%) were eight.

183 samples tested were positive (43.0%). Among these, the concentration of micronucleated cells (MN) ranged from one to three cells every one thousand differentiated cells, while

Table 1: Data on the recruitment of children, compilation of questionnaires and biological sampling in the first and second seasons.

	Distributed parcels	Informed consents/ distributed questionnaire	Completed questionnaire	Eligible children	Sampled children
Season I	750	343	284	270	266
Season II	-	266	249	242	241

Table 2: Final cohort composition (213 children sampled in both seasons).

Age (years)	Male (n)	Female (n)	Total (n)
6	45	43	88
7	30	29	59
8	31	35	66
Total (6–8)	106	107	213

the average was 0.28 MN/1,000 cells. Regarding the association of the frequency of micronuclei (positive samples and the average concentration of micronucleated cells) and the variables investigated via the questionnaire compiled by the parents in the two seasons, the results are shown in Table 3.

The percentage of positive samples went from 44.6% in the first season to 41.3% in the second season, with an average frequency of 0.32 MN/1,000 differentiated cells in season I and 0.24 MN/1,000 cells in season II. Regarding gender, 42% of the boys' samples were positive in the MN test, with an average frequency of 0.26 MN/1,000 cells, while among the girls 43.5% of the samples were positive with an average concentration of 0.30 MN/1,000 cells. In addition, 44.0% (0.30 MN/1,000) of the samples from children aged six at the moment of sampling were positive, 43.1% (0.29 MN/1,000) of the samples from children aged seven and 41.7% (0.25 MN/1,000) of the samples from children aged eight.

Regarding weight, a significant difference ($p<0.05$) was found between obese children (53.1% of positive samples; 0.38 MN/1000 cells) and children of normal weight (40.4% of positive samples; 0.26 MN/1,000 cells). Concerning exercise, significant differences ($p<0.05$) emerged between children who do outdoor sports (34.3% of samples positive; 0.21 MN/1,000 cells) and those who do not (46.9% of samples positive; 0.31 MN/1,000 cells).

Regarding the perceived density of traffic near the children's homes, among those that live in areas with a high level of traffic, the positivity rate in the MN test (51.3%) and the average frequency (0.33 MN/1,000 cells) were significantly greater ($p<0.05$) than among children who live in areas with a moderate or low level of traffic (39.7%, 0.26 MN/1,000 cells).

In addition, analysis of the results obtained with the MN test highlighted the presence of MN in 55.0% of samples from children with smoking mothers, with an average frequency of 0.43 MN/1,000 cells, while children with mothers who did not smoke had a positivity rate of 40.2% and an average frequency of 0.25 MN/1,000 cells. Among children with smoking fathers the positivity rate (47.4%) and the average frequency of MN (0.33 MN/1,000 cells) were also greater than among children with fathers who did not smoke (41.3%, 0.26 MN/1,000 cells) but the differences were significant ($p<0.05$) only for data on the mothers. Lastly,

Table 3: Positive samples (%) and the average frequency of MN in EBCs (MN/1,000 cells ± SD) of sampled children with respect to the variables investigated by the questionnaire.

Variables	Positive samples (%)	Average frequency of MN (MN/1,000 cells ± SD)
Season		
I	44.6	0.32 ± 0.44
II	41.3	0.24 ± 0.32
p-value	*0.4933*	*0.1651*
Gender		
M	42.0	0.26 ± 0.34
F	43.5	0.30 ± 0.42
p-value	*0.8340*	*0.6203*
Age (years)		
6	44.0	0.30 ± 0.40
7	43.1	0.20 ± 0.39
8	41.7	0.25 ± 0.36
p-value	*0.9189*	*0.3845*
Weight Status Category		
NW	40.4	0.26 ± 0.36
OB	53.1	0.38 ± 0.49
p-value	*0.0471*	*0.0491*
Sport (≥3 times/week)		
YES	42.9	0.29 ± 0.41
NO	43.1	0.27 ± 0.35
p-value	*0.9597*	*0.9830*
Outdoor sports (≥3 times/week)		
YES	34.3	0.21 ± 0.32
NO	46.9	0.31 ± 0.41
p-value	*0.0197*	*0.0172*
Traffic density		
LOW	39.7	0.26 ± 0.37
HIGH	51.3	0.33 ± 0.42
p-value	*0.0311*	*0.0478*
Mother smoker		
YES	55.0	0.43 ± 0.50
NO	40.2	0.25 ± 0.34
p-value	*0.0221*	*0.0028*
Father smoker		

Table 3: (Continued)

YES	47.4	0.33 ± 0.42
NO	41.3	0.26 ± 0.37
p-value	*0.2622*	*0.1642*
Mother's level of education		
LOW	50.8	0.35 ± 0.46
HIGH	41.6	0.27 ± 0.36
p-value	*0.1735*	*0.1909*
Father's level of education		
LOW	43.5	0.31 ± 0.44
HIGH	42.8	0.27 ± 0.37
p-value	*0.8997*	*0.7769*

although the result was not statistically significant, a greater frequency of MN was found among children whose parents have a low level of education (middle school or lower) than among children whose parents had a high level of education (high school or superior).

4 CONCLUSIONS

The study conducted made it possible to assess the presence of MN in the BM cells of children recruited in the city of Lecce. Overall, the analysis of data showed the presence of MN in 43.0% of samples, while the average frequency of MN in the EBCs of the studied population was 0.28 MN/1,000 differentiated cells.

According to an extensive database derived from studies conducted on both children and adults [32], the range of spontaneous frequency of micronuclei in the EBCs of populations composed of healthy individuals is 0.3–1.7 MN/1,000 cells. The average frequency of MN in the EBCs of the population observed in this study is below the lower limit of this range and it may thus be concluded that the population of children in Lecce aged six to eight does not present worrying levels of early genotoxic damage. This result may be the effect of various factors, including the low level of atmospheric pollutants usually recorded in the city of Lecce [33].

However, it is possible to detect associations between the variables, linked to the home environment and lifestyles, investigated by means of questionnaires, and the frequency of MN in the subjects participating in the study, taking into consideration that the administered questionnaire, although validated, contains subjective responses for which it is not possible to verify the validity.

Regarding the individual characteristics of the children, no significant differences in relation to gender or age were detected, while a higher positivity rate and a greater concentration of MN were seen in obese children than children of normal weight. This positive correlation is confirmed by the literature for both peripheral lymphocytes [34] and BM cells [35] in school-age children.

Another significant factor is exposure to vehicular traffic. Children living in areas of high traffic density had a higher positivity rate and MN concentration than children living in areas with low traffic density. Urban areas characterised by heavy traffic have a high concentration of pollutants derived from tailpipe emissions (atmospheric particulate, carbon monoxide, sulphur dioxide, nitrogen dioxide, PAHs) and pose a high risk of genotoxic damage to the resident population that has been extensively documented [36].

As described in the literature [16], exposure to passive smoking also seems to affect the frequency and concentration of MN in the oral mucosa cells of children. In our study, the association between smoking mothers and genotoxic damage in children was particularly strong, probably due to the close bond between mothers and small children in terms of the time spent with them and activities carried out together.

Regarding the children's lifestyles, an inverse relation between practising sport, especially outdoor sport, and the MN frequency was observed. However, in the literature the association between physical exercise and MN appears to be controversial. Some studies [37] have observed a fall in MN in peripheral blood leukocytes in association with moderate exercise but an increase in the presence of extreme physical stress. Other studies however have stressed the need for further research in order to properly assess the true influence of exercise, including outdoor sports in areas characterised by high atmospheric pollution, on the frequency of DNA damage [38].

The data presented in this paper will be integrated with those from the other cities involved in the MAPEC_LIFE study, and with the chemical-physical and toxicological data from the environmental sampling. In this way, cohorts of subjects living in varying conditions of pollution can be compared, and the effects of the various concentrations of atmospheric pollutants on the DNA of children aged six to eight years can be investigated in greater detail. In addition, it will be possible "to build a model for estimating global genotoxic risk" starting from the analysis of certain biomarkers. This model will provide an estimate of the level of risk to which the population is exposed as an effect on air quality and can serve as a tool to support decision making in terms of environmental and public health policies.

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