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ABSTRACT

Introduction: Genotoxic biomarkers has been studied largely in adult population, but few studies have investigated them in children exposed to air pollution so far. Children are a high-risk group as regards the health effects of air pollution and some studies suggest that early exposure during childhood can play an important role in the development of chronic diseases in adulthood.

Objectives: The objective of the project is to evaluate the associations between the concentration of urban air pollutants and biomarkers of early biological effect in children, and to propose a model for estimating the global risk of early biological effects due to air pollutants and other factors in children.

Methods and analysis: Two biomarkers of early biological effects, DNA damage by the comet assay and the micronuclei test, will be investigated in oral mucosa cells of 6-8-year-old children. Concurrently, some toxic airborne pollutants (PAH and nitro-PAH) and in vitro air mutagenicity and toxicity in ultra-fine air particulates (PM0.5) will be evaluated. Furthermore demographic and socio-economic variables, and other sources of exposures to air pollutants, and life-style variables will be assessed by a structured questionnaire. The associations between socio-demographic, environmental and other exposure variables and biomarkers of early biological effect using univariate and multivariate models will be analyzed. A tentative model for calculating the global absolute risk of having early biological effects caused by air pollution and other variables will be proposed.

Ethics and dissemination: The project has been approved by the Ethics Committees of the local Health Authorities. The results will be communicated to local Public Health Agencies, for supporting educational programs and health policy strategies.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

This project has some strengths compared with previous studies on the effects of air pollution on human health:

1) evaluation of both children's diet and lifestyle factors together with air pollution exposure to investigate how different habits can influence the adverse health effects of air pollution on children;
2) direct measures of air pollution exposure using data on daily concentration of fine particulate matter, including PM10 and PM2.5, nitrogen dioxide, ozone, carbon monoxide, sulphur dioxide and benzene;
3) direct measure of exposure using data on concentration of PAH and nitroPAH in fine particulate (PM0.5) collected in the same areas where children live using high-volume samplers;
4) measure of toxicity and mutagenicity of urban air using in-vitro mutagenicity and toxicity tests on PM0.5 extracts;
5) direct measures of biological effect in children's buccal mucosa cells, i.e. MN frequency and DNA damage, which have been shown to be predictive of cancer development later in life;
6) large sample size as 1,000 children are enrolled in various areas, in a population-based study, which is much higher than previous studies using early effect biomarkers;
7) the lack of some confounding factors which are common in studies on air pollution health effects on adults, including tobacco smoking and occupational exposures, due to the choice of enrolling children of 6-8 years of age;
8) the collection of buccal cells during two different seasons (winter and summer) with different level and types of air pollutants and in different towns characterized by high and low airborne pollutants concentration;

9) the intra-individual variability of early effect biomarkers will be evaluated by collecting further biological samples from 200 children enrolled in Brescia during two following winter season.

The main limitations of this study are:

1) the biomarkers examined are not specific, as DNA damage can be caused by numerous environmental and individual (genetic, metabolic) factors;
2) weather conditions not suitable for air sampling (rain, snow, strong wind) may be a limit but there is nothing that can be done about it.

INTRODUCTION

Air pollution is a global problem, especially in urban areas.[1] In particular, particulate matter (PM) has been studied intensely as regards its effects on human health. PM consists of breathable particles to which several compounds, such as heavy metals, polycyclic aromatic hydrocarbons (PAHs) and some volatile compounds, may adhere. Epidemiological studies have found a consistent association between exposure to airborne PM and incidence and mortality for cardiovascular disease and lung cancer and natural-cause mortality.[2-9] Recently, also diabetes and other chronic diseases have been associated with PM exposure, possibly through oxidative stress and inflammation.[10]

The finest fractions of particulate matter (PM with aerodynamic diameter <2.5 μm and less) play a major role in causing chronic diseases because they are retained in the alveolar regions of the lung and diffuse into the blood stream, inducing inflammation, oxidative stress, and blood coagulation.[7, 11-13] Extracts of urban air particles can induce cancer in animals,[14-15] and are mutagenic in bacteria, plant and mammalian cells in *in-vitro* tests.[16-22]

Urban air is a complex and variable mixture of many different chemical species.[1, 23] The effects of exposure to such a mixture are not merely the sum of the effects of each compound, because they can interact with synergistic effects; moreover, one or more chemicals can cause different effects and have multiple cellular targets.[24] It has been reported that even moderate or low levels of air pollution can contribute to carcinogenesis.[7, 23] Indeed, due to the very large number of people exposed to air pollutants, even a small increase in the risk of disease is a relevant public health issue.

Among the several adverse health effects associated with exposure to air pollutants, genetic damage has received a particular interest, especially because a high frequency of markers of chromosomal damage, such as chromosomal aberrations and micronuclei in peripheral blood lymphocytes, has been found to predict cancer occurrence in cohort studies.[25-27]

Genetic biomarkers have been studied largely in adult population, but only few studies have investigated genetic damage in children exposed to air pollution so far.[27-31] In recent years micronuclei frequency in mucosa buccal cells of children or young adults has been studied showing cytogenetic damage in the subjects who lived in areas with high concentration of PM or oxidant pollutants.[34-37]

Recently, some of us[37] have found a higher MN frequency than that observed in a pooled general population of the same age[27] in exfoliated buccal cells of pre-school children living in a highly polluted town in the Po Valley in Italy.

On the other hand, studies of genetic damage in children are of the utmost interest because children are a high-risk group as regards the short- and long-term health effects of air pollution.[38-43] Indeed, some studies suggest that early exposure during childhood can play an important role in the development of chronic diseases in adulthood: the earlier the exposure, the greater the risk of chronic disease, including cancer.[44]

The micronuclei test is a mutagenicity test widely used as a marker of early biological effects due to its ability to detect both clastogens and aneuploidy-inducing chemicals.[45] Micronuclei (MN) appear in the cytoplasm of interphasic cells as small additional nuclei, smaller than main nuclei. They are formed of acentric chromosomal fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division. MN induction therefore reflects clastogenic and/or aneugenic events.

The primary DNA damage may be studied also using the comet assay (single cell gel electrophoresis test), which is one of the genotoxicity tests that rapidly detects DNA damage in eukaryotic cells. In alkaline conditions, it detects single- and double-stranded breaks, alkali labile sites, incomplete repair sites and possibly also DNA-DNA and DNA-protein cross-links. The comet test shows, very early, reversible DNA damage that represents a marker of early biological effect.[46-49]

The micronuclei frequency and the primary DNA damage, as markers of early biological effect, may be studied in different types of cells, such as lymphocytes and exfoliated cells from nasal and buccal mucosa, and from urine.[30, 49] Buccal cells are certainly easier to collect than blood and do not require highly trained personnel. Moreover collection of mucosa buccal cells is considered more acceptable to children and then more useful in studies on paediatric population.

The main objective of the project is to analyse the associations between some air pollutions parameters (PM10, PM2.5, PM0.5, NOx, PAHs and nitro-PAHs) of air pollution and two biomarkers of early effects, and to propose a model for estimating the global risk of early biological effects due to air pollutants and other factors including demographic and socioeconomic factors, indoor exposures, diet, physical activity, body mass index (BMI) in school children. These data will provide information valuable for guiding policy-making and planning individual and community interventions to protect children from possible health effects of air pollutants. This objective will be achieved studying two biomarkers of DNA damage, the comet assay and the frequency of micronuclei, in oral mucosa cells of 6-8-year-old children, and the following exposure variables, as possible risk factors: airborne pollutants, in vitro air mutagenicity and toxicity, and exposures to other indoor/outdoor pollutants, demographic and socio-economic variables, and life-style variables. The project will also be useful for increasing the sensitivity of the people to the air pollution concern and promote the local authorities involvement in efforts to reduce urban air pollutants.

METHODS AND ANALYSIS

Study design

The MAPEC project runs three years: the project began January 1, 2014 and it will end December 31, 2016. The core of the study is a prospective epidemiological cohort study to evaluate and disseminate a method for monitoring biological effects of air pollution on children, with regard to biological parameters that can predict the occurrence of chronic diseases in adult age. Markers of

early biological effect, such as primary DNA damage evaluated with the comet assay and presence of micronuclei (MN), will be investigated in buccal mucosa cells taken from the 6-8-years children living in five Italian towns (Brescia, Turin, Pisa, Perugia, Lecce), with different levels of airborne particulates (PM10 annual average from 44 $\mu g/m^3$ measured in Torino to 20 $\mu g/m^3$ measured in Lecce).[50] Child exposure to urban air pollution will be evaluated by collecting ultra-fine particulate matter (PM0.5) samples in the school areas, in the same days of biological sampling. The particulate samples will be analysed for PAH and nitro-PAH concentration and for in vitro toxicity and mutagenicity/genotoxicity. In support of these analyses data on airborne pollution routinely analysed by local authorities responsible for the control will be collected, referring to the air monitoring units closest to the schools participating in the MAPEC project, in the five cities. Biological and air samples will be repeated in winter and spring, seasons characterized by different type and concentration of air pollutants. Other indoor and outdoor exposures will be investigated using an ad hoc questionnaire administrated to children's parents that includes questions on possible confounders and interaction factors, such as demographic and socioeconomic variables, children's lifestyle, with a focus on diet and physical activity. In conclusion the whole data set will be analysed to investigate the association between air pollution exposure measures (chemical pollutants and PM toxicity and mutagenicity/genotoxicity) and the early effect biomarkers with the aim of developing a model for calculating the global absolute risk of having early biological effects according to air pollution and the other possible exposures data obtained by the questionnaire.

Sample size

A total of 1,000 children (200 children per town) aged 6-8 years will be recruited from first grade schools. From two to four schools will be identified in each town and the teachers, school personnel and children's parents will be involved in meetings to explain the project and promote participation. The sample size of the study has been determined in order to detect statistically significant differences in the early effect biomarkers, i.e. primary DNA damage and MN, between children living in towns with high and low air pollution levels. Based on published studies comparing groups

at different levels of exposure to airborne mutagens, we expect to observe DNA damage in the comet test, evaluated as an average of 10% of DNA in comet tail (tail intensity, TI) among subjects with higher air pollution exposure levels compared to 7% in those at a lower exposure levels with a standard deviation of 10%. By enrolling a total of 1,000 children, about 40% of whom are supposed to live in highly polluted towns (Brescia and Turin which are located in the Po Valley), and assuming a log-normal distribution of the biomarkers of early effect, we expect to observe a statistically significant difference between the two groups, with an alpha error of 0.05 and a power higher than 95%, using a Student t-test for independent groups at two tails.

Estimating a loss of approximately 20%, because of incomplete or incorrectly questionnaires compiled and/or an insufficient number of cells collected through biological sampling, an oversampling of subjects will be necessary, therefore a total of 1,300 children will be recruited.

Inclusion/exclusion criteria

Only children living in the participating towns will be recruited. Children with severe diseases and those who have been exposed to antineoplastic agents, have undergone radiation therapy or X-rays in the previous 12 months or have a dental prosthetic will be excluded.

Questionnaires

An ad-hoc questionnaire will be designed including items on demographic and socio-economic variables, exposures to indoor and outdoor air pollution sources, characteristics of the area of residence, parents' smoking at home, BMI, children's respiratory symptoms and diseases, diet, physical activity and other aspects of children's lifestyle. The questionnaire will be based on others used in some international studies on children's respiratory diseases performed in recent decades.[51]

After a pre-testing phase, the reliability of the questionnaire will be assessed on about 100 subjects using the test-retest method.

The questionnaires will be distributed and collected with the help of the school personnel at two separate times, in winter and late spring (before school closing for summer holidays), from the

1,000 children enrolled (2 questionnaires per each child), in the weeks in which environmental and biological samplings will be performed.

In order to evaluate the intra-individual variability of biomarkers of early effect, a third environmental and biological sampling will be carried out in the following winter, restricted to the 200 children recruited in Brescia.

Collection of environmental samples

Ultra-fine particulate matter (PM0.5) will be collected near the schools involved in the research. A high-volume air sampler will be located near the schools for 72 hours during the days of biological sampling, both in winter and spring. Furthermore, air and biological samplings will be repeated in the following winter in Brescia. PM0.5 sampling will be carried out using fibreglass filters. All the filters will be weighed for gravimetric determination of PM0.5 and then will undergo to organic extraction using sonication to prepare the samples for in vitro mutagenicity/genotoxicity and toxicity tests and chemicals analysis.

Chemical analysis

The chemical analysis of PM0.5 extracts collected in each town for the determination of PAHs and nitro-PAHs will be performed at a single laboratory using High Pressure Liquid Chromatography.

Mutagenicity of ultra-fine particulate matter: Ames test

The mutagenicity of PM0.5 organic extracts collected in all towns will be evaluated using Ames test on bacteria. This test is a short-term mutagenicity test which detects point mutations (base substitution and frameshift mutations) in *Salmonella typhimurium* strains.[52, 53] The PM0.5 organic extracts, dissolved in a compatible solvent (DMSO), will be tested in duplicate at increasing doses with *Salmonella typhimurium* TA100 and TA98 strains, which are generally utilized for environmental studies,[18] and NR98 and YG1024 strains, which are particularly able to detect nitro-compound.[20, 54] The Ames test will be performed with and without metabolic activation (±S9), adding microsomal enzymes of rat liver to detect direct and indirect mutagens. Plates will be incubated at 37°C in the dark for 72 hours, after which revertant colonies will be counted and a

dose-response curve will be constructed. The net amount of revertants per cubic meter of air equivalent will be evaluated using a linear regression model. The Ames test will be performed on all samples from the same laboratory.

Genotoxicity of ultra-fine particulate matter

The genotoxicity of PM0.5 organic extracts collected in all towns will be evaluated using comet test and MN test on human pulmonary A549 cell line.

Comet assay

The Comet assay is a sensitive genotoxicity test which detects primary DNA damage in eukaryotic individual cells. This assay will be carried out on human cells of the respiratory system (A549), which represent the first type of tissue in contact with the airborne pollutants.

The organic extracts of PM0.5 will be transferred to a compatible solvent (DMSO) and tested (24 h at 37°C with 5% CO_2) in duplicate at increasing doses using A549 cells. The comet assay will be performed in alkaline conditions (pH>13) with the protocol to detect oxidative damage using endonuclease (formamidopyrimidine DNA glycosylase) incubation.[55, 56] The comet will be examined using an image analysis system (Comet Assay IV). Results will be expressed as genotoxic parameter per cubic meter of air equivalent. All samples will be analysed from a single laboratory.

Cytokinesis-block micronucleus test

The genotoxicity of PM0.5 organic extracts collected in the five towns will be evaluated using the cytokinesis-block micronucleus (CBMN) test. The CBMN test will be performed in accordance with the original method by Fenech[57] on human A549 cells treated in vitro with air extracts. At the end of the in vitro treatment, the medium will be replaced by fresh medium containing cytochalasin B to inhibit cell division after mitosis. The cells will be harvested by trypsinization and fixed with Carnoy's reagent, and the cell suspensions will be poured onto pre-cleaned frosted microscope slides. After drying, the slides will be stained, air-dried and mounted with Eukitt. Cells will be examined for MN at 400× magnification according to established criteria.[45] MN will be

scored in 1,000 binucleated cells for each concentration of each repeated experiment. Two independent evaluations will be performed for each sample. All samples will be analysed from a single laboratory.

Toxicity of ultra-fine particulate matter

Specific potential lung toxicity of organic extracts of PM0.5 will be assessed in vitro on a total of 10 unique samples, obtained by mixing the organic extracts from all the samples individually collected from each town and each season. Cell viability will be assessed by the use of two traditional colorimetric assays: the MTT dye-based assay[58], and the Neutral Red dye-based assay [59].

Organ-specific toxicity / non-genotoxic, tumor promoting potential of the samples will be evaluated by testing their influence on the gap-junctional function (GJIC) of suitable epithelial cells (e.g. primary culture of oral mucosae cells or highly junctionally-coupled cell line). According to the cell type selected, the scrape-loading technique [60] or the microinjection/dye-transfer assay [61] will be used. GJIC was chosen as a biological parameter in our study, since its evaluation still represents one of the most promising and sensitive endpoints for the mechanistic evaluation of organ-specific toxicity and/or tumor promoting potential of agents [62, 63]. This particular form of cell-cell communication is, in fact, a unique cell/tissue specific cellular function, with an unquestioned role in integrated regulation of growth, differentiation processes and functions of multicellular organisms and in tissue homeostatic control [64].

Toxicity tests on all PM0.5 extracts will be performed by a single laboratory.

Collection of urban air chemical data

Data regarding the main air pollutants for which routine measures are performed by local authorities (Regional Agency for Environmental Protection, ARPA Italian acronym for Agenzia Regionale per la Protezione dell'Ambiente), such as CO, NO_2, SO_2, benzene, O_3, PM10 and PM2.5, will be retrieved for each town.

Sampling of oral mucosa cells

Biological samples will be collected from all the children both in winter and in late spring (1,000 x 2 = 2,000 samples). Intra-individual variability of early effect biomarkers will be evaluated by collecting further biological samples from 200 children enrolled in Brescia during the following winter. In order to collect buccal mucosa cells, the children will rinse their mouths twice with mineral water and the mouthwashes will be collected in tubes containing 25 ml of saline solution (NaCl 0.9%) to obtain leukocytes for the comet assay.[65] Disposable cytobrush cell collectors will then be used to collect exfoliated buccal cells for the micronucleus test by scraping the inside of both cheeks gently and dipping the material into tubes containing 15 ml of PBS (phosphate buffered saline solution).

Early biological effects in children

Comet assay

An in vivo assessment of primary DNA damage will be performed on leukocytes of the children's buccal mucosa with the comet assay.[65] In order to evaluate primary DNA damage caused by exposure to air pollutants, the comet assay in alkaline conditions (pH>13) will be performed to show single and double strand breaks and alkali labile sites.[55] The comet assay will also be performed to detect oxidative damage using endonuclease (formamidopyrimidine DNA glycosylase) incubation.[56] All the slides will be dried and sent to the same laboratory for microscopic analysis. After rehydration and staining with a fluorochrome DNA intercalating fluorochrome (i.e. ethidium bromide, or SYBR green), the analysis will be performed using a fluorescence microscope at 400x magnification, equipped with a digital camera. An average of 100 randomly selected nuclei per subject will be acquired. The images, acquired in TIFF format, will be evaluated and the following damage parameters measured for each nucleoid: comet tail length (TL), percentage of detectable DNA in the tail (%TDNA), tail moment (TM) and Olive tail moment (OTM).

Micronucleus test

The micronucleus test will be performed on buccal mucosa cells from the children. For this purpose, the buccal mucosa scraped cells suspension will be used to prepare the slides according to Thomas et al.[66] The slides will be stained in light green using the Feulgen method. The dried slides will be mounted with Eukitt and sent to single laboratory for microscopic analysis. For microscope analysis, the slides will be examined under microscope at 400× magnification. According to proposed scoring criteria,[66, 67] before MN frequency calculation, BM cells will be gathered into categories (i.e. "normal" or "abnormal") on the basis of cytological and nuclear features indicating DNA damage, cytokinetic failure or cell death. MN frequency will be then assessed by two well-trained operators in at least 2,000 normal cells per subject.

Risk analysis based on environmental data

A quantitative estimate of carcinogenic risk will be produced using some risk analysis models, which are based on standard routinely air pollution parameters (PM10, NOx and others) collected by local authorities and the parameters investigated in this study (PM0.5 content of PAHs and nitro-PAHs). For carcinogenic substances, the risk (R) represents the probability of a rise, with respect to the usual conditions of life, in the number of cases of cancer in a person's lifetime caused by exposure to the substance. The risk analysis will be conducted in accordance with the RBCA (Risk-Based Corrective Action) procedure proposed by the American Society for Testing and Materials (ASTM) and will lead to an estimate of the carcinogenic risk (R) of exposure to polluted air. Afterwards, the model performance in predicting early biological effects in children, will be evaluated by comparing the estimates produced by the risk analysis models based on environmental data ("expected") and the actual level of biological effect detected in the children ("observed").

Statistical analysis and construction of a global risk model

The associations between levels of air pollutants, PM mutagenicity/genotoxicity and early effect biomarkers will be investigated using various types of regression models. Questionnaire data will also be analysed, including both demographic and socio-economic variables and indoor and outdoor exposure variables. Univariate and multivariate analyses will be performed, considering all the

variables of interest and confounding factors. Intra-individual variability of early effect biomarkers will be assessed by comparing the results observed in two samples taken from 200 children in two consecutive winter seasons. A comparison of the results of early effect biomarker tests will be performed between winter and summer seasons, among all towns and between those at higher (Brescia and Turin together) and lower (all the others) levels of PM10 in winter, using the common statistical techniques for the analysis of continuous variables, according to their distribution. The role of interaction factors will be assessed using both stratified analysis and statistical models. The associations between the standard (PM10, NOx, etc.) and investigated (PM0.5 content of PAHs and nitro-PAHs) parameters of air pollution and early effect biomarkers will be determined in order to evaluate the air pollution parameters as predictors of biological effect on children. Then a tentative model for calculating the global absolute risk of having early biological effects for air pollution and other variables together (demographic and socioeconomic factors, indoor exposures, physical activity, diet, body mass index, and others) will be proposed.

ETHICS AND DISSEMINATION

The project has been approved by the local Ethic Committee (Comitato Etico Provinciale della Provincia di Brescia) on January 15, 2014 and the local Health Authorities of each town involved in the study. Participation in the study will be voluntary. Informed consent will be obtained from the children's parents and the children themselves, after an adequate and understandable explanation of the intent of the study, of the possible results and their meaning. Only children with parents' informed consent signed and a complete questionnaire will undergo biological sampling. All the data collected will be treated confidentially in accordance with current Italian legislation on the treatment of sensitive data (privacy law).

Communication and dissemination activities are key issues for achieving the main objective of the MAPEC project. For successful dissemination of study results, multiple target audiences will be identified, each of them needs to be addressed in a different manner, using different media and with

different messages. The key message to be conveyed by MAPEC will be indications for addressing individual interventions and community policies to protect children from the health effects of air pollutants. A dissemination plan will be designed taking into account the specific language of interest and communication channel of possible stakeholders: health authorities, scientific community, teachers, children, parents, etc.

Scientific meetings and workshops will be periodically organized to show preliminary and final results, which will be presented at national and international conferences and then published in scientific journals. Also community, environmental and health authorities will be involved. MAPEC participatory integrated assessment is an object-oriented approach with the strong engagement of local, national and EU stakeholders. Furthermore, the results will be communicated to Public Health Agencies, and to the Mayor of each town for supporting health policy decisions. Annual meetings will be organized with representatives of national public health institutions and we will organize an international workshop at the end of the project.

Finally, general public will be involved. Presentation of the project objectives, information and results will need to be tailored to a public or non-scientific audience in order to ensure effective communication. Lay science articles in local and national newspapers will be produced with aim to reach out to and create a bridge between scientific and public audiences. Press conferences will be organized with local and national mass media to present the project and before all workshops. Moreover, a MAPEC newsletter will be developed and distributed to different stakeholders and a project website will be developed.

Local Public Health Authorities and teachers will be involved in developing information and educational packages, containing lesson plans, enrichment activities, ideas for encouraging students to adopt healthy lifestyles, which can be used by other schools throughout the country.

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2STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	4
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	7-8
Objectives	3	State specific objectives, including any prespecified hypotheses	9
Methods			
Study design	4	Present key elements of study design early in the paper	9-10
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	9-16
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	10-12
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	11-12
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	16-17
Bias	9	Describe any efforts to address potential sources of bias	16-17
Study size	10	Explain how the study size was arrived at	10-11
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	16-17
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	16-17
		(*b*) Describe any methods used to examine subgroups and interactions	16-17
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how loss to follow-up was addressed	
		(*e*) Describe any sensitivity analyses	
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	10-11
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9-12
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) Summarise follow-up time (eg, average and total amount)	11-12
Outcome data	15*	Report numbers of outcome events or summary measures over time	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	16-17
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	17
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	16-17
Discussion			
Key results	18	Summarise key results with reference to study objectives	
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Primary Subject Heading:	Public health
Secondary Subject Heading:	Epidemiology
Keywords:	PUBLIC HEALTH, air pollution, early biological effect, children, DNA damage, mucosa buccal cells

Monitoring Air Pollution Effects on Children for supporting Public Health Policy: the protocol of the prospective cohort MAPEC study.

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Keywords: air pollution, early biological effects, children, DNA damage, mucosa buccal cells.

World count: 3878

ABSTRACT

Introduction: Genotoxic biomarkers has been studied largely in adult population, but few studies have investigated them in children exposed to air pollution so far. Children are a high-risk group as regards the health effects of air pollution and some studies suggest that early exposure during childhood can play an important role in the development of chronic diseases in adulthood.

The objective of the project is to evaluate the associations between the concentration of urban air pollutants and biomarkers of early biological effect in children, and to propose a model for estimating the global risk of early biological effects due to air pollutants and other factors in children.

Methods and analysis: Two biomarkers of early biological effects, DNA damage by the comet assay and the micronuclei test, will be investigated in oral mucosa cells of 6-8-year-old children. Concurrently, some toxic airborne pollutants (PAH and nitro-PAH) and in vitro air mutagenicity and toxicity in ultra-fine air particulates (PM0.5) will be evaluated. Furthermore demographic and socio-economic variables, and other sources of exposures to air pollutants, and life-style variables will be assessed by a structured questionnaire. The associations between socio-demographic, environmental and other exposure variables and biomarkers of early biological effect using univariate and multivariate models will be analyzed. A tentative model for calculating the global absolute risk of having early biological effects caused by air pollution and other variables will be proposed.

Ethics and dissemination: The project has been approved by the Ethics Committees of the local Health Authorities. The results will be communicated to local Public Health Agencies, for supporting educational programs and health policy strategies.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

This project has some strengths compared with previous studies on the effects of air pollution on human health:

1) direct measures of air pollution exposure using data on daily concentration of fine particulate matter, including PM10 and PM2.5, nitrogen dioxide, ozone, carbon monoxide, sulphur dioxide and benzene and evaluation of toxicity and mutagenicity of urban air using in-vitro mutagenicity and toxicity tests on PM0.5 extracts;
2) direct measures of biological effect in buccal mucosa cells of 1000 children enrolled in various area and in two different seasons, i.e. MN frequency and DNA damage, which have been shown to be predictive of cancer development later in life.

The main limitations of this study are:

1) the biomarkers examined are not specific, as DNA damage can be caused by numerous environmental and individual (genetic, metabolic) factors;
2) weather conditions not suitable for air sampling (rain, snow, strong wind) may be a limit but there is nothing that can be done about it.

INTRODUCTION

Air pollution is a global problem, especially in urban areas.[1] In particular, particulate matter (PM) has been studied intensely as regards its effects on human health. PM consists of breathable particles to which several compounds, such as heavy metals, polycyclic aromatic hydrocarbons (PAHs) and some volatile compounds, may adhere. Epidemiological studies have found a consistent association between exposure to airborne PM and incidence and mortality for cardiovascular disease and lung cancer and natural-cause mortality.[2-9] Recently, also diabetes and other chronic diseases have been associated with PM exposure, possibly through oxidative stress and inflammation.[10]

The finest fractions of particulate matter (PM with aerodynamic diameter <2.5 μm and less) play a major role in causing chronic diseases because they are retained in the alveolar regions of the lung and diffuse into the blood stream, inducing inflammation, oxidative stress, and blood coagulation.[7, 11-13] Extracts of urban air particles can induce cancer in animals,[14-15] and are mutagenic in bacteria, plant and mammalian cells in *in-vitro* tests.[16-22]

Urban air is a complex and variable mixture of many different chemical species.[1, 23] The effects of exposure to such a mixture are not merely the sum of the effects of each compound, because they can interact with synergistic effects; moreover, one or more chemicals can cause different effects and have multiple cellular targets.[24] It has been reported that even moderate or low levels of air pollution can contribute to carcinogenesis.[7, 23] Indeed, due to the very large number of people exposed to air pollutants, even a small increase in the risk of disease is a relevant public health issue.

Among the several adverse health effects associated with exposure to air pollutants, genetic damage has received a particular interest, especially because a high frequency of markers of chromosomal damage, such as chromosomal aberrations and micronuclei in peripheral blood lymphocytes, has been found to predict cancer occurrence in cohort studies.[25-27]

Genetic biomarkers have been studied largely in adult population, but only few studies have investigated genetic damage in children exposed to air pollution so far.[27-31] In recent years micronuclei frequency in mucosa buccal cells of children or young adults has been studied showing cytogenetic damage in the subjects who lived in areas with high concentration of PM or oxidant pollutants.[34-37]

Recently, Ceretti and colleagues [37] have found a higher MN frequency than that observed in a pooled general population of the same age[27] in exfoliated buccal cells of pre-school children living in a highly polluted town in the Po Valley in Italy.

On the other hand, studies of genetic damage in children are of the utmost interest because children are a high-risk group as regards the short- and long-term health effects of air pollution.[38-43] Indeed, some studies suggest that early exposure during childhood can play an important role in the development of chronic diseases in adulthood: the earlier the exposure, the greater the risk of chronic disease, including cancer.[44]

The micronuclei test is a mutagenicity test widely used as a marker of early biological effects due to its ability to detect both clastogens and aneuploidy-inducing chemicals.[45] Micronuclei (MN) appear in the cytoplasm of interphasic cells as small additional nuclei, smaller than main nuclei. They are formed of acentric chromosomal fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division. MN induction therefore reflects clastogenic and/or aneugenic events.

The primary DNA damage may be studied also using the comet assay (single cell gel electrophoresis test), which is one of the genotoxicity tests that rapidly detects DNA damage in eukaryotic cells. In alkaline conditions, it detects single- and double-stranded breaks, alkali labile sites, incomplete repair sites and possibly also DNA-DNA and DNA-protein cross-links. The comet test shows, very early, reversible DNA damage that represents a marker of early biological effect.[46-49]

The micronuclei frequency and the primary DNA damage, as markers of early biological effect, may be studied in different types of cells, such as lymphocytes and exfoliated cells from nasal and buccal mucosa, and from urine.[30, 49] Buccal cells are certainly easier to collect than blood and do not require highly trained personnel. Moreover collection of mucosa buccal cells is considered more acceptable to children and then more useful in studies on paediatric population.

The main objective of the project is to analyse the associations between some air pollutions parameters (PM10, PM2.5, PM0.5, NOx, PAHs and nitro-PAHs) of air pollution and two biomarkers of early effects, and to propose a model for estimating the global risk of early biological effects due to air pollutants and other factors including demographic and socioeconomic factors, indoor exposures, diet, physical activity, body mass index (BMI) in school children. These data will provide information valuable for guiding policy-making and planning individual and community interventions to protect children from possible health effects of air pollutants. This objective will be achieved studying two biomarkers of DNA damage, the comet assay and the frequency of micronuclei, in oral mucosa cells of 6-8-year-old children, and the following exposure variables, as possible risk factors: airborne pollutants, in vitro air mutagenicity and toxicity, and exposures to other indoor/outdoor pollutants, demographic and socio-economic variables, and life-style variables. The project will also be useful for increasing the sensitivity of the people to the air pollution concern and promote the local authorities involvement in efforts to reduce urban air pollutants.

METHODS AND ANALYSIS

Study design

The MAPEC project runs three years (2014-2016). The core of the study is a prospective epidemiological cohort study to evaluate and disseminate a method for monitoring biological effects of air pollution on children, with regard to biological parameters that can predict the occurrence of chronic diseases in adult age. Markers of early biological effect, such as primary DNA damage

evaluated with the comet assay and presence of micronuclei (MN), will be investigated in buccal mucosa cells taken from the 6-8-years children living in five Italian towns (Brescia, Turin, Pisa, Perugia, Lecce), with different levels of airborne particulates (PM10 annual average from 44 $\mu g/m^3$ measured in Torino to 20 $\mu g/m^3$ measured in Lecce).[50] Child exposure to urban air pollution will be evaluated by collecting ultra-fine particulate matter (PM0.5) samples in the school areas, in the same days of biological sampling. The particulate samples will be analysed for PAH and nitro-PAH concentration and for in vitro toxicity and mutagenicity/genotoxicity. In support of these analyses data on airborne pollution routinely analysed by local authorities responsible for the control will be collected, referring to the air monitoring units closest to the schools participating in the MAPEC project, in the five cities. Biological and air samples will be repeated in winter and spring, seasons characterized by different type and concentration of air pollutants. Other indoor and outdoor exposures will be investigated using an ad hoc questionnaire administrated to children's parents that includes questions on possible confounders and interaction factors, such as demographic and socioeconomic variables, children's lifestyle, with a focus on diet and physical activity. In conclusion the whole data set will be analysed to investigate the association between air pollution exposure measures (chemical pollutants and PM toxicity and mutagenicity/genotoxicity) and the early effect biomarkers with the aim of developing a model for calculating the global absolute risk of having early biological effects according to air pollution and the other possible exposures data obtained by the questionnaire.

Sample size

A total of 1,000 children (200 children per town) aged 6-8 years will be recruited from first grade schools. From two to four schools will be chosen randomly from the list of schools located in areas, without adjacent air pollution emission sources, in each town. The teachers, school personnel and children's parents will be involved in meetings where members of the research team will explain the project and promote participation. Participation in the study will be voluntary and no incentive will be offered for this.

The sample size of the study has been determined in order to detect statistically significant differences in the early effect biomarkers, i.e. primary DNA damage and MN, between children living in towns with high and low air pollution levels. Based on published studies comparing groups at different levels of exposure to airborne mutagens, we expect to observe DNA damage in the comet test, evaluated as an average of 10% of DNA in comet tail (tail intensity, TI) among subjects with higher air pollution exposure levels compared to 7% in those at a lower exposure levels with a standard deviation of 10%. By enrolling a total of 1,000 children, about 40% of whom are supposed to live in highly polluted towns (Brescia and Turin which are located in the Po Valley), and assuming a log-normal distribution of the biomarkers of early effect, we expect to observe a statistically significant difference between the two groups, with an alpha error of 0.05 and a power higher than 95%, using a Student t-test for independent groups at two tails.

Estimating a loss of approximately 20%, because of incomplete or incorrectly questionnaires compiled and/or an insufficient number of cells collected through biological sampling, an oversampling of subjects will be necessary, therefore a total of 1,300 children will be recruited.

Inclusion/exclusion criteria

Only children living in the participating towns will be recruited. Children with severe diseases and those who have been exposed to antineoplastic agents, have undergone radiation therapy or X-rays in the previous 12 months or have a dental prosthetic will be excluded.

Questionnaires

An ad-hoc questionnaire will be designed including items on demographic and socio-economic variables, exposures to indoor and outdoor air pollution sources, characteristics of the area of residence, parents' smoking at home, BMI, children's respiratory symptoms and diseases, diet, physical activity and other aspects of children's lifestyle. The questionnaire will be based on others used in some international studies on children’s respiratory diseases performed in recent decades.[51]

After a pre-testing phase, the reliability of the questionnaire will be assessed on about 100 subjects using the test-retest method.

The questionnaires will be distributed and collected with the help of the school personnel at two separate times, in winter and late spring (before school closing for summer holidays), from the 1,000 children enrolled (2 questionnaires per each child), in the weeks in which environmental and biological samplings will be performed.

For children not providing a filled in questionnaire, further attempts will be performed on the next days. As biological samplings will go on for various days, some of the children absent from school on the established day for taking oral mucosa cells will be retrieved during the whole period of biological sampling.

In order to evaluate the intra-individual variability of biomarkers of early effect, a third environmental and biological sampling will be carried out in the following winter, restricted to the 200 children recruited in Brescia.

Collection of environmental samples

Ultra-fine particulate matter (PM0.5) will be collected near the schools involved in the research. A high-volume air sampler will be located near the schools for 72 hours during the days of biological sampling, both in winter and spring. Furthermore, air and biological samplings will be repeated in the following winter in Brescia. PM0.5 sampling will be carried out using fibreglass filters. All the filters will be weighed for gravimetric determination of PM0.5 and then will undergo to organic extraction using sonication to prepare the samples for in vitro mutagenicity/genotoxicity and toxicity tests and chemicals analysis.

Chemical analysis

The chemical analysis of PM0.5 extracts collected in each town for the determination of PAHs and nitro-PAHs will be performed at a single laboratory using High Pressure Liquid Chromatography.

Mutagenicity of ultra-fine particulate matter: Ames test

The mutagenicity of PM0.5 organic extracts collected in all towns will be evaluated using Ames test on bacteria. This test is a short-term mutagenicity test which detects point mutations (base substitution and frameshift mutations) in *Salmonella typhimurium* strains.[52, 53] The PM0.5 organic extracts, dissolved in a compatible solvent (DMSO), will be tested in duplicate at increasing doses with *Salmonella typhimurium* TA100 and TA98 strains, which are generally utilized for environmental studies,[18] and NR98 and YG1024 strains, which are particularly able to detect nitro-compound.[20, 54] The Ames test will be performed with and without metabolic activation (±S9), adding microsomal enzymes of rat liver to detect direct and indirect mutagens. Plates will be incubated at 37°C in the dark for 72 hours, after which revertant colonies will be counted and a dose-response curve will be constructed. The net amount of revertants per cubic meter of air equivalent will be evaluated using a linear regression model. The Ames test will be performed on all samples from the same laboratory.

Genotoxicity of ultra-fine particulate matter

The genotoxicity of PM0.5 organic extracts collected in all towns will be evaluated using comet test and MN test on human pulmonary A549 cell line.

Comet assay

The Comet assay is a sensitive genotoxicity test which detects primary DNA damage in eukaryotic individual cells. This assay will be carried out on human cells of the respiratory system (A549), which represent the first type of tissue in contact with the airborne pollutants.

The organic extracts of PM0.5 will be transferred to a compatible solvent (DMSO) and tested (24 h at 37°C with 5% CO_2) in duplicate at increasing doses using A549 cells. The comet assay will be performed in alkaline conditions (pH>13) with the protocol to detect oxidative damage using endonuclease (formamidopyrimidine DNA glycosylase) incubation.[55, 56] The comet will be examined using an image analysis system (Comet Assay IV). Results will be expressed as genotoxic parameter per cubic meter of air equivalent. All samples will be analysed from a single laboratory.

Cytokinesis-block micronucleus test

The genotoxicity of PM0.5 organic extracts collected in the five towns will be evaluated using the cytokinesis-block micronucleus (CBMN) test. The CBMN test will be performed in accordance with the original method by Fenech[57] on human A549 cells treated in vitro with air extracts. At the end of the in vitro treatment, the medium will be replaced by fresh medium containing cytochalasin B to inhibit cell division after mitosis. The cells will be harvested by trypsinization and fixed with Carnoy's reagent, and the cell suspensions will be poured onto pre-cleaned frosted microscope slides. After drying, the slides will be stained, air-dried and mounted with Eukitt. Cells will be examined for MN at 400× magnification according to established criteria.[45] MN will be scored in 1,000 binucleated cells for each concentration of each repeated experiment. Two independent evaluations will be performed for each sample. All samples will be analysed from a single laboratory.

Toxicity of ultra-fine particulate matter

Specific potential lung toxicity of organic extracts of PM0.5 will be assessed in vitro on a total of 10 unique samples, obtained by mixing the organic extracts from all the samples individually collected from each town and each season. Cell viability will be assessed by the use of two traditional colorimetric assays: the MTT dye-based assay[58], and the Neutral Red dye-based assay [59].

Organ-specific toxicity / non-genotoxic, tumor promoting potential of the samples will be evaluated by testing their influence on the gap-junctional function (GJIC) of suitable epithelial cells (e.g. primary culture of oral mucosae cells or highly junctionally-coupled cell line). According to the cell type selected, the scrape-loading technique [60] or the microinjection/dye-transfer assay [61] will be used. GJIC was chosen as a biological parameter in our study, since its evaluation still represents one of the most promising and sensitive endpoints for the mechanistic evaluation of organ-specific toxicity and/or tumor promoting potential of agents [62, 63]. This particular form of cell-cell communication is, in fact, a unique cell/tissue specific cellular function, with an unquestioned role

in integrated regulation of growth, differentiation processes and functions of multicellular organisms and in tissue homeostatic control [64].

Toxicity tests on all PM0.5 extracts will be performed by a single laboratory.

Collection of urban air chemical data

Data regarding the main air pollutants for which routine measures are performed by local authorities (Regional Agency for Environmental Protection, ARPA Italian acronym for Agenzia Regionale per la Protezione dell'Ambiente), such as CO, NO_2, SO_2, benzene, O_3, PM10 and PM2.5, will be retrieved for each town.

Sampling of oral mucosa cells

Biological samples will be collected from all the children both in winter and in late spring (1,000 x 2 = 2,000 samples). Intra-individual variability of early effect biomarkers will be evaluated by collecting further biological samples from 200 children enrolled in Brescia during the following winter. In order to collect buccal mucosa cells, the children will rinse their mouths twice with mineral water and the mouthwashes will be collected in tubes containing 25 ml of saline solution (NaCl 0.9%) to obtain leukocytes for the comet assay.[65] Disposable cytobrush cell collectors will then be used to collect exfoliated buccal cells for the micronucleus test by scraping the inside of both cheeks gently and dipping the material into tubes containing 15 ml of PBS (phosphate buffered saline solution).

Early biological effects in children

Comet assay

An in vivo assessment of primary DNA damage will be performed on leukocytes of the children's buccal mucosa with the comet assay.[65] In order to evaluate primary DNA damage caused by exposure to air pollutants, the comet assay in alkaline conditions (pH>13) will be performed to show single and double strand breaks and alkali labile sites.[55] The comet assay will also be performed to detect oxidative damage using endonuclease (formamidopyrimidine DNA glycosylase) incubation.[56] All the slides will be dried and sent to the same laboratory for

microscopic analysis. After rehydration and staining with a fluorochrome DNA intercalating fluorochrome (i.e. ethidium bromide, or SYBR green), the analysis will be performed using a fluorescence microscope at 400x magnification, equipped with a digital camera. An average of 100 randomly selected nuclei per subject will be acquired. The images, acquired in TIFF format, will be evaluated and the following damage parameters measured for each nucleoid: comet tail length (TL), percentage of detectable DNA in the tail (%TDNA), tail moment (TM) and Olive tail moment (OTM).

Micronucleus test

The micronucleus test will be performed on buccal mucosa cells from the children. For this purpose, the buccal mucosa scraped cells suspension will be used to prepare the slides according to Thomas et al.[66] The slides will be stained in light green using the Feulgen method. The dried slides will be mounted with Eukitt and sent to single laboratory for microscopic analysis. For microscope analysis, the slides will be examined under microscope at 400× magnification. According to proposed scoring criteria,[66, 67] before MN frequency calculation, BM cells will be gathered into categories (i.e. “normal” or “abnormal”) on the basis of cytological and nuclear features indicating DNA damage, cytokinetic failure or cell death. MN frequency will be then assessed by two well-trained operators in at least 2,000 normal cells per subject.

Risk analysis based on environmental data

A quantitative estimate of carcinogenic risk will be produced using some risk analysis models, which are based on standard routinely air pollution parameters (PM10, NOx and others) collected by local authorities and the parameters investigated in this study (PM0.5 content of PAHs and nitro-PAHs). For carcinogenic substances, the risk (R) represents the probability of a rise, with respect to the usual conditions of life, in the number of cases of cancer in a person's lifetime caused by exposure to the substance. The risk analysis will be conducted in accordance with the RBCA (Risk-Based Corrective Action) procedure proposed by the American Society for Testing and Materials (ASTM) and will lead to an estimate of the carcinogenic risk (R) of exposure to polluted air.

Afterwards, the model performance in predicting early biological effects in children, will be evaluated by comparing the estimates produced by the risk analysis models based on environmental data ("expected") and the actual level of biological effect detected in the children ("observed").

Statistical analysis and construction of a global risk model

The associations between levels of air pollutants, PM mutagenicity/genotoxicity and early effect biomarkers will be investigated using various types of regression models. Questionnaire data will also be analysed, including both demographic and socio-economic variables and indoor and outdoor exposure variables. Univariate and multivariate analyses will be performed, considering all the variables of interest and confounding factors. Intra-individual variability of early effect biomarkers will be assessed by comparing the results observed in two samples taken from 200 children in two consecutive winter seasons. A comparison of the results of early effect biomarker tests will be performed between winter and summer seasons, among all towns and between those at higher (Brescia and Turin together) and lower (all the others) levels of PM10 in winter, using the common statistical techniques for the analysis of continuous variables, according to their distribution. The role of interaction factors will be assessed using both stratified analysis and statistical models. The associations between the standard (PM10, NOx, etc.) and investigated (PM0.5 content of PAHs and nitro-PAHs) parameters of air pollution and early effect biomarkers will be determined in order to evaluate the air pollution parameters as predictors of biological effect on children. Then a tentative model for calculating the global absolute risk of having early biological effects for air pollution and other variables together (demographic and socioeconomic factors, indoor exposures, physical activity, diet, body mass index, and others) will be proposed.

ETHICS AND DISSEMINATION

The project has been approved by the local Ethic Committee (Comitato Etico Provinciale della Provincia di Brescia) on January 15, 2014 and the local Health Authorities of each town involved in the study. Participation in the study will be voluntary. Informed consent will be obtained from the

children's parents and the children themselves, after an adequate and understandable explanation of the intent of the study, of the possible results and their meaning. Only children with parents' informed consent signed and a complete questionnaire will undergo biological sampling. All the data collected will be treated confidentially in accordance with current Italian legislation on the treatment of sensitive data (privacy law).

An alphanumeric code (6 letter) will be randomly generated to identify both biological samples and questionnaires. In addition to the alphanumeric code the questionnaires will include the child's name in order to enroll only the children whose parents decide to participate in the research and return a written signed consent. Then this section with the child's name will be separated from the rest of the questionnaire and will be kept apart in a closed archive. Only the research staff will be allowed to see children's personal data.

All data will be analyzed in an aggregate and anonymous way for the preparation of scientific reports in which children will be not identifiable in any way.

Communication and dissemination activities are key issues for achieving the main objective of the MAPEC project. For successful dissemination of study results, multiple target audiences will be identified, each of them needs to be addressed in a different manner, using different media and with different messages. The key message to be conveyed by MAPEC will be indications for addressing individual interventions and community policies to protect children from the health effects of air pollutants. A dissemination plan will be designed taking into account the specific language of interest and communication channel of possible stakeholders: health authorities, scientific community, teachers, children, parents, etc.

Scientific meetings and workshops will be periodically organized to show preliminary and final results, which will be presented at national and international conferences and then published in scientific journals. Also community, environmental and health authorities will be involved. MAPEC participatory integrated assessment is an object-oriented approach with the strong

engagement of local, national and EU stakeholders. Furthermore, the results will be communicated to Public Health Agencies, and to the Mayor of each town for supporting health policy decisions. Annual meetings will be organized with representatives of national public health institutions and we will organize an international workshop at the end of the project.

Finally, general public will be involved. Presentation of the project objectives, information and results will need to be tailored to a public or non-scientific audience in order to ensure effective communication. Lay science articles in local and national newspapers will be produced with aim to reach out to and create a bridge between scientific and public audiences. Press conferences will be organized with local and national mass media to present the project and before all workshops. Moreover, a MAPEC newsletter will be developed and distributed to different stakeholders and a project website will be developed.

Local Public Health Authorities and teachers will be involved in developing information and educational packages, containing lesson plans, enrichment activities, ideas for encouraging students to adopt healthy lifestyles, which can be used by other schools throughout the country.

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De Donno A., Carducci A., Marrese M.R., Bonetti A., Monarca S., and Carraro E. have draft the article or revised it critically for important intellectual content;

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Monitoring Air Pollution Effects on Children for supporting Public Health Policy: the protocol of the prospective cohort MAPEC study.

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ABSTRACT

Introduction: Genotoxic biomarkers has been studied largely in adult population, but few studies have investigated them in children exposed to air pollution so far. Children are a high-risk group as regards the health effects of air pollution and some studies suggest that early exposure during childhood can play an important role in the development of chronic diseases in adulthood.

The objective of the project is to evaluate the associations between the concentration of urban air pollutants and biomarkers of early biological effect in children, and to propose a model for estimating the global risk of early biological effects due to air pollutants and other factors in children.

Methods and analysis: Two biomarkers of early biological effects, DNA damage by the comet assay and the micronuclei test, will be investigated in oral mucosa cells of 6-8-year-old children. Concurrently, some toxic airborne pollutants (PAH and nitro-PAH) and in vitro air mutagenicity and toxicity in ultra-fine air particulates (PM0.5) will be evaluated. Furthermore demographic and socio-economic variables, and other sources of exposures to air pollutants, and life-style variables will be assessed by a structured questionnaire. The associations between socio-demographic, environmental and other exposure variables and biomarkers of early biological effect using univariate and multivariate models will be analyzed. A tentative model for calculating the global absolute risk of having early biological effects caused by air pollution and other variables will be proposed.

Ethics and dissemination: The project has been approved by the Ethics Committees of the local Health Authorities. The results will be communicated to local Public Health Agencies, for supporting educational programs and health policy strategies.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

This project has some strengths compared with previous studies on the effects of air pollution on human health:

1) direct measures of air pollution exposure using data on daily concentration of fine particulate matter, including PM10 and PM2.5, nitrogen dioxide, ozone, carbon monoxide, sulphur dioxide and benzene and evaluation of toxicity and mutagenicity of urban air using in-vitro mutagenicity and toxicity tests on PM0.5 extracts;
2) direct measures of biological effect in buccal mucosa cells of 1000 children enrolled in various area and in two different seasons, i.e. MN frequency and DNA damage, which have been shown to be predictive of cancer development later in life.

The main limitations of this study are:

1) the biomarkers examined are not specific, as DNA damage can be caused by numerous environmental and individual (genetic, metabolic) factors;
2) weather conditions not suitable for air sampling (rain, snow, strong wind) may be a limit but there is nothing that can be done about it.

INTRODUCTION

Air pollution is a global problem, especially in urban areas.[1] In particular, particulate matter (PM) has been studied intensely as regards its effects on human health. PM consists of breathable particles to which several compounds, such as heavy metals, polycyclic aromatic hydrocarbons (PAHs) and some volatile compounds, may adhere. Epidemiological studies have found a consistent association between exposure to airborne PM and incidence and mortality for cardiovascular disease and lung cancer and natural-cause mortality.[2-9] Recently, also diabetes and other chronic diseases have been associated with PM exposure, possibly through oxidative stress and inflammation.[10]

The finest fractions of particulate matter (PM with aerodynamic diameter <2.5 μm and less) play a major role in causing chronic diseases because they are retained in the alveolar regions of the lung and diffuse into the blood stream, inducing inflammation, oxidative stress, and blood coagulation.[7, 11-13] Extracts of urban air particles can induce cancer in animals,[14-15] and are mutagenic in bacteria, plant and mammalian cells in *in-vitro* tests.[16-22]

Urban air is a complex and variable mixture of many different chemical species.[1, 23] The effects of exposure to such a mixture are not merely the sum of the effects of each compound, because they can interact with synergistic effects; moreover, one or more chemicals can cause different effects and have multiple cellular targets.[24] It has been reported that even moderate or low levels of air pollution can contribute to carcinogenesis.[7, 23] Indeed, due to the very large number of people exposed to air pollutants, even a small increase in the risk of disease is a relevant public health issue.

Among the several adverse health effects associated with exposure to air pollutants, genetic damage has received a particular interest, especially because a high frequency of markers of chromosomal damage, such as chromosomal aberrations and micronuclei in peripheral blood lymphocytes, has been found to predict cancer occurrence in cohort studies.[25-27]

Genetic biomarkers have been studied largely in adult population, but only few studies have investigated genetic damage in children exposed to air pollution so far.[27-31] In recent years micronuclei frequency in mucosa buccal cells of children or young adults has been studied showing cytogenetic damage in the subjects who lived in areas with high concentration of PM or oxidant pollutants.[34-37]

Recently, Ceretti and colleagues [37] have found a higher MN frequency than that observed in a pooled general population of the same age[27] in exfoliated buccal cells of pre-school children living in a highly polluted town in the Po Valley in Italy.

On the other hand, studies of genetic damage in children are of the utmost interest because children are a high-risk group as regards the short- and long-term health effects of air pollution.[38-43] Indeed, some studies suggest that early exposure during childhood can play an important role in the development of chronic diseases in adulthood: the earlier the exposure, the greater the risk of chronic disease, including cancer.[44]

The micronuclei test is a mutagenicity test widely used as a marker of early biological effects due to its ability to detect both clastogens and aneuploidy-inducing chemicals.[45] Micronuclei (MN) appear in the cytoplasm of interphasic cells as small additional nuclei, smaller than main nuclei. They are formed of acentric chromosomal fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division. MN induction therefore reflects clastogenic and/or aneugenic events.

The primary DNA damage may be studied also using the comet assay (single cell gel electrophoresis test), which is one of the genotoxicity tests that rapidly detects DNA damage in eukaryotic cells. In alkaline conditions, it detects single- and double-stranded breaks, alkali labile sites, incomplete repair sites and possibly also DNA-DNA and DNA-protein cross-links. The comet test shows, very early, reversible DNA damage that represents a marker of early biological effect.[46-49]

The micronuclei frequency and the primary DNA damage, as markers of early biological effect, may be studied in different types of cells, such as lymphocytes and exfoliated cells from nasal and buccal mucosa, and from urine.[30, 49] Buccal cells are certainly easier to collect than blood and do not require highly trained personnel. Moreover collection of mucosa buccal cells is considered more acceptable to children and then more useful in studies on paediatric population.

The main objective of the project is to analyse the associations between some air pollutions parameters (PM10, PM2.5, PM0.5, NOx, PAHs and nitro-PAHs) of air pollution and two biomarkers of early effects, and to propose a model for estimating the global risk of early biological effects due to air pollutants and other factors including demographic and socioeconomic factors, indoor exposures, diet, physical activity, body mass index (BMI) in school children. These data will provide information valuable for guiding policy-making and planning individual and community interventions to protect children from possible health effects of air pollutants. This objective will be achieved studying two biomarkers of DNA damage, the comet assay and the frequency of micronuclei, in oral mucosa cells of 6-8-year-old children, and the following exposure variables, as possible risk factors: airborne pollutants, in vitro air mutagenicity and toxicity, and exposures to other indoor/outdoor pollutants, demographic and socio-economic variables, and life-style variables. The project will also be useful for increasing the sensitivity of the people to the air pollution concern and promote the local authorities involvement in efforts to reduce urban air pollutants.

METHODS AND ANALYSIS

Study design

The MAPEC project runs three years (2014-2016). The core of the study is a prospective epidemiological cohort study to evaluate and disseminate a method for monitoring biological effects of air pollution on children, with regard to biological parameters that can predict the occurrence of chronic diseases in adult age. Markers of early biological effect, such as primary DNA damage

evaluated with the comet assay and presence of micronuclei (MN), will be investigated in buccal mucosa cells taken from the 6-8-years children living in five Italian towns (Brescia, Turin, Pisa, Perugia, Lecce), with different levels of airborne particulates (PM10 annual average from 44 μg/m^3 measured in Torino to 20 μg/m^3 measured in Lecce).[50] Child exposure to urban air pollution will be evaluated by collecting ultra-fine particulate matter (PM0.5) samples in the school areas, in the same days of biological sampling. The particulate samples will be analysed for PAH and nitro-PAH concentration and for in vitro toxicity and mutagenicity/genotoxicity. In support of these analyses data on airborne pollution routinely analysed by local authorities responsible for the control will be collected, referring to the air monitoring units closest to the schools participating in the MAPEC project, in the five cities. Biological and air samples will be repeated in winter and spring, seasons characterized by different type and concentration of air pollutants. Other indoor and outdoor exposures will be investigated using an ad hoc questionnaire administrated to children's parents that includes questions on possible confounders and interaction factors, such as demographic and socioeconomic variables, children's lifestyle, with a focus on diet and physical activity. In conclusion the whole data set will be analysed to investigate the association between air pollution exposure measures (chemical pollutants and PM toxicity and mutagenicity/genotoxicity) and the early effect biomarkers with the aim of developing a model for calculating the global absolute risk of having early biological effects according to air pollution and the other possible exposures data obtained by the questionnaire.

Sample size

A total of 1,000 children (200 children per town) aged 6-8 years will be recruited from first grade schools. From two to four schools will be chosen randomly from the list of schools located in areas, without adjacent air pollution emission sources, in each town. The teachers, school personnel and children's parents will be involved in meetings where members of the research team will explain the project and promote participation. Participation in the study will be voluntary and no incentive will be offered for this.

The sample size of the study has been determined in order to detect statistically significant differences in the early effect biomarkers, i.e. primary DNA damage and MN, between children living in towns with high and low air pollution levels. Based on published studies comparing groups at different levels of exposure to airborne mutagens, we expect to observe DNA damage in the comet test, evaluated as an average of 10% of DNA in comet tail (tail intensity, TI) among subjects with higher air pollution exposure levels compared to 7% in those at a lower exposure levels with a standard deviation of 10%. By enrolling a total of 1,000 children, about 40% of whom are supposed to live in highly polluted towns (Brescia and Turin which are located in the Po Valley), and assuming a log-normal distribution of the biomarkers of early effect, we expect to observe a statistically significant difference between the two groups, with an alpha error of 0.05 and a power higher than 95%, using a Student t-test for independent groups at two tails.

Estimating a loss of approximately 20%, because of incomplete or incorrectly questionnaires compiled and/or an insufficient number of cells collected through biological sampling, an oversampling of subjects will be necessary, therefore a total of 1,300 children will be recruited.

Inclusion/exclusion criteria

Only children living in the participating towns will be recruited. Children with severe diseases and those who have been exposed to antineoplastic agents, have undergone radiation therapy or X-rays in the previous 12 months or have a dental prosthetic will be excluded.

Questionnaires

An ad-hoc questionnaire will be designed including items on demographic and socio-economic variables, exposures to indoor and outdoor air pollution sources, characteristics of the area of residence, parents' smoking at home, BMI, children's respiratory symptoms and diseases, diet, physical activity and other aspects of children's lifestyle. The questionnaire will be based on others used in some international studies on children’s respiratory diseases performed in recent decades.[51]

After a pre-testing phase, the reliability of the questionnaire will be assessed on about 100 subjects using the test-retest method.

The questionnaires will be distributed and collected with the help of the school personnel at two separate times, in winter and late spring (before school closing for summer holidays), from the 1,000 children enrolled (2 questionnaires per each child), in the weeks in which environmental and biological samplings will be performed.

For children not providing a filled in questionnaire, further attempts will be performed on the next days. As biological samplings will go on for various days, some of the children absent from school on the established day for taking oral mucosa cells will be retrieved during the whole period of biological sampling.

In order to evaluate the intra-individual variability of biomarkers of early effect, a third environmental and biological sampling will be carried out in the following winter, restricted to the 200 children recruited in Brescia.

Collection of environmental samples

Ultra-fine particulate matter (PM0.5) will be collected near the schools involved in the research. A high-volume air sampler will be located near the schools for 72 hours during the days of biological sampling, both in winter and spring. Furthermore, air and biological samplings will be repeated in the following winter in Brescia. PM0.5 sampling will be carried out using fibreglass filters. All the filters will be weighed for gravimetric determination of PM0.5 and then will undergo to organic extraction using sonication to prepare the samples for in vitro mutagenicity/genotoxicity and toxicity tests and chemicals analysis.

Chemical analysis

The chemical analysis of PM0.5 extracts collected in each town for the determination of PAHs and nitro-PAHs will be performed at a single laboratory using High Pressure Liquid Chromatography.

Mutagenicity of ultra-fine particulate matter: Ames test

The mutagenicity of PM0.5 organic extracts collected in all towns will be evaluated using Ames test on bacteria. This test is a short-term mutagenicity test which detects point mutations (base substitution and frameshift mutations) in *Salmonella typhimurium* strains.[52, 53] The PM0.5 organic extracts, dissolved in a compatible solvent (DMSO), will be tested in duplicate at increasing doses with *Salmonella typhimurium* TA100 and TA98 strains, which are generally utilized for environmental studies,[18] and NR98 and YG1024 strains, which are particularly able to detect nitro-compound.[20, 54] The Ames test will be performed with and without metabolic activation (±S9), adding microsomal enzymes of rat liver to detect direct and indirect mutagens. Plates will be incubated at 37°C in the dark for 72 hours, after which revertant colonies will be counted and a dose-response curve will be constructed. The net amount of revertants per cubic meter of air equivalent will be evaluated using a linear regression model. The Ames test will be performed on all samples from the same laboratory.

Genotoxicity of ultra-fine particulate matter

The genotoxicity of PM0.5 organic extracts collected in all towns will be evaluated using comet test and MN test on human pulmonary A549 cell line.

Comet assay

The Comet assay is a sensitive genotoxicity test which detects primary DNA damage in eukaryotic individual cells. This assay will be carried out on human cells of the respiratory system (A549), which represent the first type of tissue in contact with the airborne pollutants.

The organic extracts of PM0.5 will be transferred to a compatible solvent (DMSO) and tested (24 h at 37°C with 5% CO_2) in duplicate at increasing doses using A549 cells. The comet assay will be performed in alkaline conditions (pH>13) with the protocol to detect oxidative damage using endonuclease (formamidopyrimidine DNA glycosylase) incubation.[55, 56] The comet will be examined using an image analysis system (Comet Assay IV). Results will be expressed as genotoxic parameter per cubic meter of air equivalent. All samples will be analysed from a single laboratory.

Cytokinesis-block micronucleus test

The genotoxicity of PM0.5 organic extracts collected in the five towns will be evaluated using the cytokinesis-block micronucleus (CBMN) test. The CBMN test will be performed in accordance with the original method by Fenech[57] on human A549 cells treated in vitro with air extracts. At the end of the in vitro treatment, the medium will be replaced by fresh medium containing cytochalasin B to inhibit cell division after mitosis. The cells will be harvested by trypsinization and fixed with Carnoy's reagent, and the cell suspensions will be poured onto pre-cleaned frosted microscope slides. After drying, the slides will be stained, air-dried and mounted with Eukitt. Cells will be examined for MN at 400× magnification according to established criteria.[45] MN will be scored in 1,000 binucleated cells for each concentration of each repeated experiment. Two independent evaluations will be performed for each sample. All samples will be analysed from a single laboratory.

Toxicity of ultra-fine particulate matter

Specific potential lung toxicity of organic extracts of PM0.5 will be assessed in vitro on a total of 10 unique samples, obtained by mixing the organic extracts from all the samples individually collected from each town and each season. Cell viability will be assessed by the use of two traditional colorimetric assays: the MTT dye-based assay[58], and the Neutral Red dye-based assay [59].

Organ-specific toxicity / non-genotoxic, tumor promoting potential of the samples will be evaluated by testing their influence on the gap-junctional function (GJIC) of suitable epithelial cells (e.g. primary culture of oral mucosae cells or highly junctionally-coupled cell line). According to the cell type selected, the scrape-loading technique [60] or the microinjection/dye-transfer assay [61] will be used. GJIC was chosen as a biological parameter in our study, since its evaluation still represents one of the most promising and sensitive endpoints for the mechanistic evaluation of organ-specific toxicity and/or tumor promoting potential of agents [62, 63]. This particular form of cell-cell communication is, in fact, a unique cell/tissue specific cellular function, with an unquestioned role

in integrated regulation of growth, differentiation processes and functions of multicellular organisms and in tissue homeostatic control [64].

Toxicity tests on all PM0.5 extracts will be performed by a single laboratory.

Collection of urban air chemical data

Data regarding the main air pollutants for which routine measures are performed by local authorities (Regional Agency for Environmental Protection, ARPA Italian acronym for Agenzia Regionale per la Protezione dell'Ambiente), such as CO, NO_2, SO_2, benzene, O_3, PM10 and PM2.5, will be retrieved for each town.

Sampling of oral mucosa cells

Biological samples will be collected from all the children both in winter and in late spring (1,000 x 2 = 2,000 samples). Intra-individual variability of early effect biomarkers will be evaluated by collecting further biological samples from 200 children enrolled in Brescia during the following winter. In order to collect buccal mucosa cells, the children will rinse their mouths twice with mineral water and the mouthwashes will be collected in tubes containing 25 ml of saline solution (NaCl 0.9%) to obtain leukocytes for the comet assay.[65] Disposable cytobrush cell collectors will then be used to collect exfoliated buccal cells for the micronucleus test by scraping the inside of both cheeks gently and dipping the material into tubes containing 15 ml of PBS (phosphate buffered saline solution).

Early biological effects in children

Comet assay

An in vivo assessment of primary DNA damage will be performed on leukocytes of the children's buccal mucosa with the comet assay.[65] In order to evaluate primary DNA damage caused by exposure to air pollutants, the comet assay in alkaline conditions (pH>13) will be performed to show single and double strand breaks and alkali labile sites.[55] The comet assay will also be performed to detect oxidative damage using endonuclease (formamidopyrimidine DNA glycosylase) incubation.[56] All the slides will be dried and sent to the same laboratory for

microscopic analysis. After rehydration and staining with a fluorochrome DNA intercalating fluorochrome (i.e. ethidium bromide, or SYBR green), the analysis will be performed using a fluorescence microscope at 400x magnification, equipped with a digital camera. An average of 100 randomly selected nuclei per subject will be acquired. The images, acquired in TIFF format, will be evaluated and the following damage parameters measured for each nucleoid: comet tail length (TL), percentage of detectable DNA in the tail (%TDNA), tail moment (TM) and Olive tail moment (OTM).

Micronucleus test

The micronucleus test will be performed on buccal mucosa cells from the children. For this purpose, the buccal mucosa scraped cells suspension will be used to prepare the slides according to Thomas et al.[66] The slides will be stained in light green using the Feulgen method. The dried slides will be mounted with Eukitt and sent to single laboratory for microscopic analysis. For microscope analysis, the slides will be examined under microscope at 400× magnification. According to proposed scoring criteria,[66, 67] before MN frequency calculation, BM cells will be gathered into categories (i.e. "normal" or "abnormal") on the basis of cytological and nuclear features indicating DNA damage, cytokinetic failure or cell death. MN frequency will be then assessed by two well-trained operators in at least 2,000 normal cells per subject.

Risk analysis based on environmental data

A quantitative estimate of carcinogenic risk will be produced using some risk analysis models, which are based on standard routinely air pollution parameters (PM10, NOx and others) collected by local authorities and the parameters investigated in this study (PM0.5 content of PAHs and nitro-PAHs). For carcinogenic substances, the risk (R) represents the probability of a rise, with respect to the usual conditions of life, in the number of cases of cancer in a person's lifetime caused by exposure to the substance. The risk analysis will be conducted in accordance with the RBCA (Risk-Based Corrective Action) procedure proposed by the American Society for Testing and Materials (ASTM) and will lead to an estimate of the carcinogenic risk (R) of exposure to polluted air.

Afterwards, the model performance in predicting early biological effects in children, will be evaluated by comparing the estimates produced by the risk analysis models based on environmental data ("expected") and the actual level of biological effect detected in the children ("observed").

Statistical analysis and construction of a global risk model

The associations between levels of air pollutants, PM mutagenicity/genotoxicity and early effect biomarkers will be investigated using various types of regression models. Questionnaire data will also be analysed, including both demographic and socio-economic variables and indoor and outdoor exposure variables. Univariate and multivariate analyses will be performed, considering all the variables of interest and confounding factors. Intra-individual variability of early effect biomarkers will be assessed by comparing the results observed in two samples taken from 200 children in two consecutive winter seasons. A comparison of the results of early effect biomarker tests will be performed between winter and summer seasons, among all towns and between those at higher (Brescia and Turin together) and lower (all the others) levels of PM10 in winter, using the common statistical techniques for the analysis of continuous variables, according to their distribution. The role of interaction factors will be assessed using both stratified analysis and statistical models. The associations between the standard (PM10, NOx, etc.) and investigated (PM0.5 content of PAHs and nitro-PAHs) parameters of air pollution and early effect biomarkers will be determined in order to evaluate the air pollution parameters as predictors of biological effect on children. Then a tentative model for calculating the global absolute risk of having early biological effects for air pollution and other variables together (demographic and socioeconomic factors, indoor exposures, physical activity, diet, body mass index, and others) will be proposed.

ETHICS AND DISSEMINATION

The project has been approved by the local Ethic Committee (Comitato Etico Provinciale della Provincia di Brescia) on January 15, 2014 and the local Health Authorities of each town involved in the study. Participation in the study will be voluntary. Informed consent will be obtained from the

children's parents and the children themselves, after an adequate and understandable explanation of the intent of the study, of the possible results and their meaning. Only children with parents' informed consent signed and a complete questionnaire will undergo biological sampling. All the data collected will be treated confidentially in accordance with current Italian legislation on the treatment of sensitive data (privacy law).

An alphanumeric code (6 letter) will be randomly generated to identify both biological samples and questionnaires. In addition to the alphanumeric code the questionnaires will include the child's name in order to enroll only the children whose parents decide to participate in the research and return a written signed consent. Then this section with the child's name will be separated from the rest of the questionnaire and will be kept apart in a closed archive. Only the research staff will be allowed to see children's personal data.

All data will be analyzed in an aggregate and anonymous way for the preparation of scientific reports in which children will be not identifiable in any way.

Communication and dissemination activities are key issues for achieving the main objective of the MAPEC project. For successful dissemination of study results, multiple target audiences will be identified, each of them needs to be addressed in a different manner, using different media and with different messages. The key message to be conveyed by MAPEC will be indications for addressing individual interventions and community policies to protect children from the health effects of air pollutants. A dissemination plan will be designed taking into account the specific language of interest and communication channel of possible stakeholders: health authorities, scientific community, teachers, children, parents, etc.

Scientific meetings and workshops will be periodically organized to show preliminary and final results, which will be presented at national and international conferences and then published in scientific journals. Also community, environmental and health authorities will be involved. MAPEC participatory integrated assessment is an object-oriented approach with the strong

engagement of local, national and EU stakeholders. Furthermore, the results will be communicated to Public Health Agencies, and to the Mayor of each town for supporting health policy decisions. Annual meetings will be organized with representatives of national public health institutions and we will organize an international workshop at the end of the project.

Finally, general public will be involved. Presentation of the project objectives, information and results will need to be tailored to a public or non-scientific audience in order to ensure effective communication. Lay science articles in local and national newspapers will be produced with aim to reach out to and create a bridge between scientific and public audiences. Press conferences will be organized with local and national mass media to present the project and before all workshops. Moreover, a MAPEC newsletter will be developed and distributed to different stakeholders and a project website will be developed.

Local Public Health Authorities and teachers will be involved in developing information and educational packages, containing lesson plans, enrichment activities, ideas for encouraging students to adopt healthy lifestyles, which can be used by other schools throughout the country.

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Feretti D., Ceretti E. and Gelatti U. gave substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data;

De Donno A., Carducci A., Marrese M.R., Bonetti A., Monarca S., and Carraro E. have draft the article or revised it critically for important intellectual content;

Moretti M., Bonetta S., Covolo L., Bagordo F., Villarini M., Verani M., Schilirò T., Limina R.M.,

Grassi T., Casini B., Zani C., Mazzoleni G., Levaggi R. gave final approval of the version to be published.

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2STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	4
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	7-8
Objectives	3	State specific objectives, including any prespecified hypotheses	9
Methods			
Study design	4	Present key elements of study design early in the paper	9-10
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	9-16
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	10-12
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	11-12
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	16-17
Bias	9	Describe any efforts to address potential sources of bias	16-17
Study size	10	Explain how the study size was arrived at	10-11
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	16-17
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	16-17
		(*b*) Describe any methods used to examine subgroups and interactions	16-17
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how loss to follow-up was addressed	
		(*e*) Describe any sensitivity analyses	
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	10-11
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9-12
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) Summarise follow-up time (eg, average and total amount)	11-12
Outcome data	15*	Report numbers of outcome events or summary measures over time	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	16-17
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	17
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	16-17
Discussion			
Key results	18	Summarise key results with reference to study objectives	
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.