Environmental Prints in Children Nasal Epithelium Living at the Attica Basin

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Abstract: - The metropolitan area of Athens is facing to a serious air pollution problem. The mucosa of the nasal epithelium is highly exposed through breathing to all the airborne irritating agents that exist in the urban air. Therefore, the morphological pattern of this mucosa is greatly affected. The study aimed to investigate histopathological alterations in the nasal mucociliary respiratory epithelium of healthy children, in relation to the lodging at the Attica basin. Samples were collected at schools from three different municipalities, north and near to the banks of the Kifissos River, and from a control population who lived in a low-polluted area, apart from Attica basin. The children's age ranged from 11 to 12 years. 49 samples were collected from exposed areas (11from the Municipality of Philadelphia, 19 from the Municipality of Kifisia, and 19 samples from the Municipality of Kryoneri) and 12 from the control area. Immunoistochemical staining was used to evaluate the expression of p53 protein. The results were analyzed with IBM SPSS Statistics 20 for Windows. Pearson's Chi-square and Friedman test were used to compare nasal epithelium changes between exposed and control areas. No statistically significant differences of the histopathology parameters were observed between exposed groups. Pearson Chi-square test shows a significant difference in the positive immunoreaction of p53 between control and exposed areas (p = 0,000). Friedman test shows that, from the thirteen examinee parameters for each municipality, eight of them were related with a confidence coefficient p = 0,000. None of the 49 exposed children to the Athens environment had normal nasal epithelium, in contrast with the control area. The fact that no statistical differences between the histopathology parameters and the municipalities was observed may confirm that people living at the Attica basin have the same probability of developing alterations in nasal epithelium.

Key-Words: - Nasal epithelium, nasal biopsies, children exposure, schools, Attica basin, Attica municipalities, Kifissos River, air pollution, histopathology parameters, p53.

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Statement of approval: - This study was approved by the Ministry of Education, Lifelong Learning and Religious Affairs of Greece and by the Bioethical Committee of the Medical School of the University of Athens (NKUA). Also, the parents of the children gave written consent for the research, after being clearly informed about the objectives of the study.

1 Introduction

The metropolitan area of Athens (MAA) extends over 600 km^2 and includes 61 municipalities. It is inhabited by 4.5 million people (census 2010) and it has many installations of human activities, including extensive anthropogenic interventions, agricultural and industrial activities which have been recorded in the north zone of the city, around the Kifissos River. The tropospheric air of the city is a complex mixture of air pollutants such as ozone, particulate matter and nitrogen oxides that frequently exceed the air quality guidelines. Natural sources (e.g. transport of dust from deserts) and local conditions (i.e. topography and climatic conditions) also have a major impact on the air quality of the city. Air quality is in turn strongly influenced by pollutants trapped due to thermal inversions caused from sea/land breezes and internal thermal boundary layers.

The human nasal epithelium (that can be used as an indicator of exposure to air pollution) (Calderón-Garcidueñas L., Rodriguez-Alcaraz A. et al., 1998), is located in the superior region of the nasal cavity and covers a portion of the septum and superior turbinates, and rests upon a thick layer of collagen fibrils, which is referred as the basement membrane. The nasal epithelium is pseudostratified with basal, globet and columnar cells (ciliated or nonciliated). Globet cells are arranged perpendicular to the epithelial surface and columnar cells are short and slender fingerlike cytoplasmic expansions that increase the surface area of the epithelial cells, promoting exchange processes across the epithelium (Baroody F.M., 2012). Mucociliary clearance is the most important mechanism by which particles and secretions are cleared from the nose, but it is not the only one. The filtering nasal activity provides protection to the lower respiratory tract from certain air pollutants.

p53 is a protein which contain 393 amino acids and enhances the rate of transcription known genes that carry out in a cell. This protein is kept at a low concentration, having a short half-time (about 20 min.) (Levine A., 1997). Dispatch the prototypic tumor suppressor gene that is well suited as a molecular link between the causes of cancer (chemical, physical or microbiological agents) and the development of cancer. The expression of p53 can reflect the characteristics and geographical distributions of carcinogens through several types of DNA damage, and this result in a rapid increase of concentration and activation of p53 as a transcription factor (Levine A., 1997). The p53 alterations in the early phases of squamous cell carcinoma suggest that p53 abnormalities can be used as a biomarker who indicates a predisposition of toward malignancy (Calderón-Garcidueñas L., Rodriguez-Alcaraz A. et al., 2001).

No epidemiological reports addressing specifically the incidence of damages to nasal epithelium or nasal neoplasms for the children who live in Athens have been recorded. The aim of this study was to evaluate the degree of cell alterations in the nasal mucociliary respiratory epithelium in healthy children, living at three different Attica municipalities, north and near to the banks of the Kifissos River, aged between 11 and 12 years old.

2 Materials and methods

2.1 The study area

The study area was the north zone of the city of Athens, near to the Kifissos River. The intensive urbanization and the uncontrolled activities of industries represent a major threat to the environment of this area. Three schools from three different municipalities of Athens were chosen. Two of them (Kifisia and Philadelphia), have an extensive industrial activities with higher environmental air and water pollutants. The third school is at the municipality of Kryoneri, having lower anthropogenic activity, transport, noise and citizens. The three municipalities have the same atmospheric conditions such as sunshine, winds and temperature. The control area was a rural area, apart from Attica basin, having the same atmospheric conditions, with lower anthropogenic activities and lower air pollution.

Air quality data was provided by an automated surface network of 3 monitoring stations in the exposed area; hourly near-surface measurement of monitored pollutants, such as ozone (O_3), particulate matter 10μm (PM_{10}), sulfur dioxide (SO_2), nitrogen dioxide and monoxide (NO, NO_2), and carbon monoxide (CO). Air quality data at the control area was provided by 1 monitoring station.

2.2 Study population

The study proposed was approved by the Ministry of Education, Lifelong Learning and Religious Affairs and the Bioethical Committee of the Medical School of the University of Athens (NKUA). The parents of the children gave written consent for the research, after being clearly informed about the objectives of the study. Nasal samples were collected from healthy children aged from 11 to 12 years, 49 living at the exposed area, and 12 from the rural area. From the 49 samples, 11 were collected from the school of the Municipality of Philadelphia, 19 from the school of the Municipality of Kifisia and 19 were collected from the school of the Municipality of Kryoneri. Children participation had the following characteristics: (*1*) nonsmoking households and negative personal smoking history and environmental tobacco exposure; (*2*) lifelong residency in the study and control area; (*3*) no known exposures to local sources of air pollutants; *(4)* physically active children. From each municipality of the exposed area, children had the same socioeconomic level and living at the same neighborhood, near to the school.

A clinical history was obtained from the parents of each child, answering a small questionnaire specially designed in order to obtain important information, regarding in their age, sex, outdoor exposure time, food ingestion, health conditions and medications. The questionnaire was adapted in order to make it friendlier and easier for parents. Parents' occupational histories were also included.

2.3 Nasal specimens

Nasal specimens were taken from the areas, using a head light source and a nasal speculum in order to prevent any accidental contact with the squamous epithelium of the nasal vestibule. Specimen collection was painless, although tearing and sneezing was observed in a few cases. The biopsies were taken under direct visual inspection, with a disposable plastic curette, from the middle third of the inferior nasal turbinate. The tip of the plastic curette was immediately immersed into a vial containing 15 ml preservative solution (comprised ethyl-alcohol, polyethylene glycol, methyl alcohol and formaldehyde). The nasal samples were processed with cytoblock preparation system. The first step was to vortex the vial with the specimen for about 10 minutes at 2.500 rpm. Gelling reagents was added into the sample and mixed by vortexing. For the assembly of the cassette, a cytofunnel (a specially designed tube which enabled deposition of a randomized sample of cells onto the cassette by centrifugation) was placed over the cassette and a metal clip holder was used to guarantee the right orientation. Each mixed sample was placed into the cytofunnel assembly and then into the centrifuge. Each sample was centrifuged for 5 min at 1500 rpm at a low acceleration. This generated slides with a rectangular 22 x 14.75 mm sample, ready for conventional cytological screening. Sections were cut at 4 μm and dried overnight, deparaffinized in xylene, rehydrated through graded alcohols, rinsed two times in phosphate-buffered saline and allowed to dry. All samples were stained with haematoxylin-eosin (HE) and covered by a coverslip.

The expression of p53 protein was studied by immunochemistry. Immunostaining was performed by the Avidin–Biotin complex method (Hsu S., Raine L. and Fanger H., 1981). Samples were incubated for 45 min with a normal rabbit serum diluted 1:40 in 0.5 M phosphate buffer saline (PBS), rinsed three times with PBS for 5 min. and incubated overnight in anti p53 (DO7) monoclonal antibody (Menarini Diagn. Italy), at dilution 1:100.

Samples were examined and photographed using bright-field light microscopy. All cytology specimens were evaluated blindly and independently by two experienced observers. The observers had no access to the data related to the geographic source of the biopsies at any point of the evaluation. Each sample was assessed based on the following histology criteria: *(1)* impaired orientation, *(2)* absence of cilia, *(3)* alteration of epithelial cell orientation, *(4)* lack of cells cohesion, *(5)* presence of mucus, *(6)* presence of squamous metaplasia, *(7)* cell atypia, *(8)* presence of cylindrical cells, *(9)* nuclear disorder, *(10)* presence of eosinophilic and neutrophilic infiltrates in the nasal epithelium, *(11)* anisocytosis, *(12)* presence of nucleoli and (13) lymphocytes.

The severity of the selected parameters was assessed semi quantitatively from 0 to 3 ("0" for no pathologic change; "1" for small pathologic change, "2" for moderate pathologic change and "3" for the most severe pathologic change). The p53 expression was assessed from 0 to 1 ("0" for negative expression and "1" for positive expression).

2.4 Statistical analysis

Statistical evaluation was performed with IBM SPSS Statistics 20 for Windows. Pearson's Chi-square test and Friedman test were used to compare nasal epithelium changes. A p value <0.05 was considered significant.

3 Results

From the 49 samples examined of the exposed area, three (3) were excluded from the study because there was no sufficient material to evaluate the histopathological parameters. At the sampling time, all children volunteer no nasal or respiratory symptoms were reported. There were no differences in the number of hours spend outdoors for exposed and control children, as well as for boys and girls.

From the questionnaires, no references of serious children health problems were register. In respect with the respiratory system, 2/19 (10%) children from the municipality of Kryoneri, 3/19 (16%) from Kifisia and 1/11 (9%) from Philadelphia suffer pollinosis at spring time. Only one child has all the year allergic symptoms and another one has only at winter time (from Kifisia). Two children (one from Kifisia and one from Philadelphia) are subjected to medications. No references of respiratory symptoms were registered at the volunteers from the control area.

The most important findings from the samples of exposed area are illustrated in Fig. 1. Samples from these areas were characterized by an abnormal nasal epithelium, in contrast with the rural (control) area. Distorted orientation (Fig. 2) was greater at the municipality of Kifisia in regard with the municipalities of Kryoneri and Philadelphia (84,2%, 73,7% and 87,5% respectively). Patchy loss of cilia was noted in 7/8 cases from Philadelphia, in 13/19 cases from Kifisia and in 12/19 cases from Kryoneri. Same number of samples from Kifisia and Kryoneri (14/19) and all from Philadelphia shows alteration of epithelial cell orientation. High lack of cellular cohesion shows all the samples of Kifisia, 12 from Kryoneri and 8 from Philadelphia. A great presence of mucus was observed in the samples from both areas.

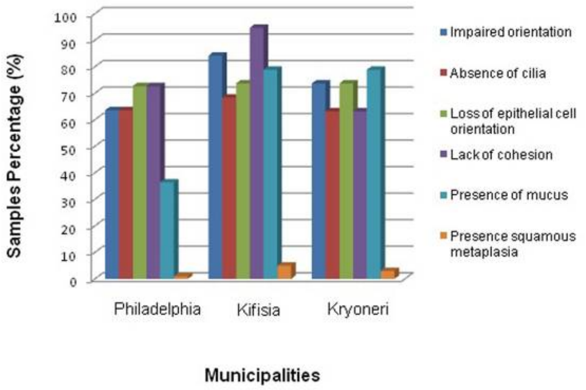

Fig. 1 Cytopathologic alterations of nasal mucosa

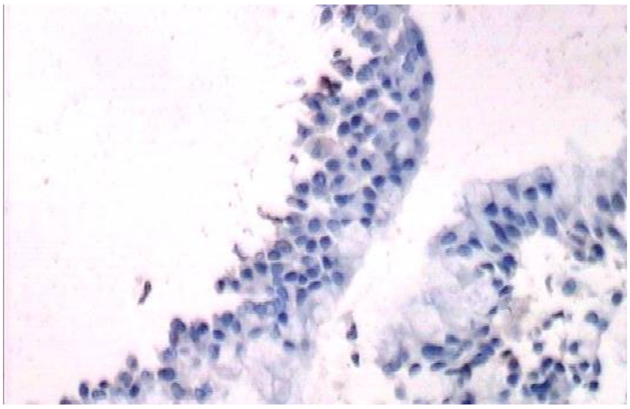

Fig. 2 Distorted orientation

Serious squamous metaplasia was observed in 26,3% samples from Kifisia (5/19), 15,8% samples from Kryoneri (3/19) and 9,1% from Philadelphia (1/8); see Fig. 3. The majority of cases showed no cellular atypia, and no presence of cylindrical epithelium (between 72,7 and 100%). Nuclear disorder was observed in 3 cases (1 at Kifisia and 2 at Kryoneri), while in the majority of the cases (39/46) was not observed any polymorphonuclear leucocytes, eosinophils or neutrophils. In 40 cases were not observed anisocytosis and presence of nucleoli. A wide divergence of lymphocytes between samples was found, where 14 had a small number (2 at Philadelphia, 7 at Kifisia and 5 at Kryoneri), 11 moderate (1 at Philadelphia, 5 at Kifisia and 5 at Kryoneri) and 3 had a high number of lymphocytes (1 at Kifisia and 2 at Kryoneri) (Fig. 4).

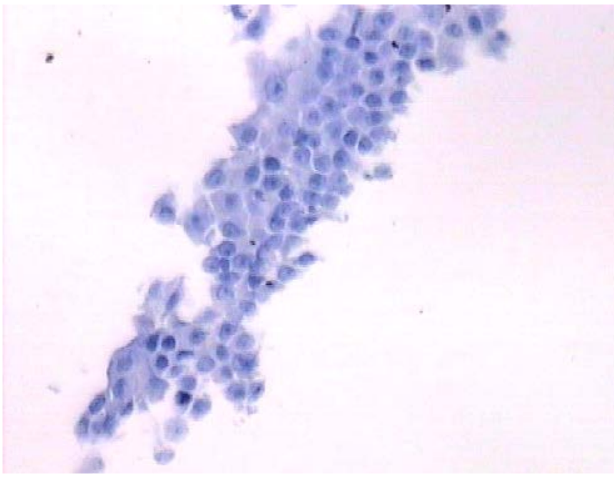

Fig. 3 Squamous metaplasia

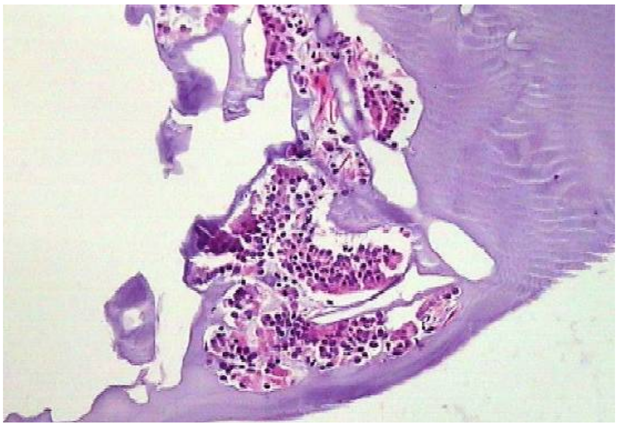

Fig. 4 Presence of mucus and lymphocytes in children nasal epithelium

Nasal biopsies from the control area are characterized by normal pseudostratified ciliated epithelium, which maintain the cellular cohesion, with basal, globet and columnar cells (ciliated) (Fig. 5). The orientation of the cells is almost perpendicular to the basement membrane and there is no presence of polymorphonuclear, eosinophils or neutrophils.

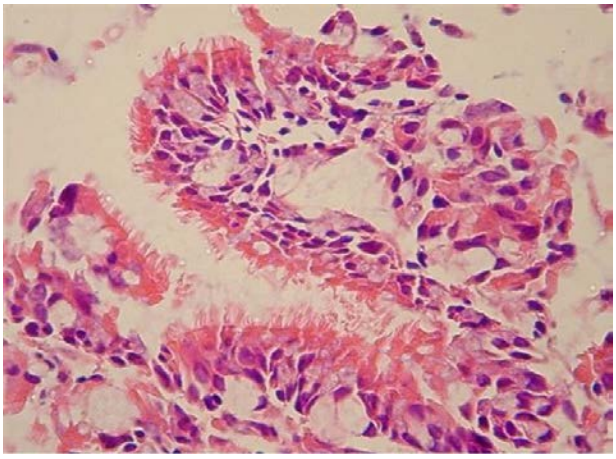

Fig. 5 Rural sample (control area)

Daily mean concentrations of PM_{10}, nitrogen dioxide (NO_2), and ozone (O_3) at the exposed area frequently exceed the air quality guidelines for those pollutants. Specially, the ozone concentrations at the municipality of Kryoneri were greater than those from the other two areas. The concentrations of these air pollutants at the control area were lower than at the exposed area.

Pearson chi-square test shows a significant difference in the expression of p53 between exposed and control areas (p=0,000). No statistically differences were observed in the expression of p53 and squamous metaplasia between Kifisia and Kryoneri (p=0,465), and Piladelphia and Kryoneri (p=0,462). Also, no statistically significant differences were observed between the histopathology parameters and the samples that were taken from the exposed areas. For each municipality, the Friedman test shows that the impaired orientation, the absence of cilia, the alteration of the perpendicular axis to the base membrane, the lack of cell cohesion, the presence of mucus, the presence of squamous metaplasia and the nuclear disorder were related with a confidence coefficient p = 0,000. No abnormal results of biopsies such as nasal polyps, cancer, necrotizing granuloma or nasal tumors were identified.

4 Discussions

The resident populations of MAA are potentially exposed to air pollution, and a great part of them were potentially exposed to ozone daily concentration greater than 100 $\mu g/m^3$ (who are the current long-term objective air quality guideline of WHO, 2006) and 120 $\mu g/m^3$ (the EU limit). The target value averaged for the period 2011-to-2013 was exceeded a maximum of 122 days (EEA, 2014). Concentrations of air pollutants in Athens depend to a great extent on its geomorphologic and climatologic conditions. It is well known that the area has those topographic and climatic conditions that favour (especially during the warm period) the formation of photochemical pollutants, leading to episodes of high pollution levels (Kassomenos P.A. and Koletsis I.G., 2005), in contrast with the control area.

As the first point of contact of the respiratory apparatus with airborne chemicals in the environment, the nose can be seen as the "window to the respiratory system", a window that is readily accessible, that can be easily monitored, and which once altered may be compromised in its ability to protect the lower respiratory tract from exposure to air pollutants (Leopold D.A., 1994 and Proctor D.F., 1995).

Under normal and healthy conditions, a nose with an intact mucociliary apparatus acts as an extremely effective filter of inhaled gases and largest size particles (Hastings L. and Miller M.L., 2003). This activity can be influenced due to exposure to air pollutants. Air pollutant such as ozone (who is a pollutant with high oxidant properties), is capable of producing free oxygen radicals by several mechanisms and can cause significant alterations in nasal ciliary mobility (Min Y.G., Ohyama M., Lee K.S., et al., 1999). Significant inflammation can be detected at ambient ozone levels, both in controlled human exposures and in animal studies (Joad J.P., et al., 2000; Bell M.L., Dominici F. and Samet J.M., 2005). Short-term ozone inhalation induces a diffuse inflammation of the entire respiratory tract, including the most susceptible parts of airways which are the nasal cavity and the transition zone between conducting and gas-exchange airways (Gryparis A., et al., 2004). Long-term exposure of rodents to ozone (O_3) causes marked changes in nasal surface epithelial components (Harkema J.R., Hotchkiss J.A., Barr E.B., et al., 1999). Oxidation of the airway epithelial lining fluid (ELF) by ozone may generate bioactive compounds, such as ozonides and aldehydes, which have the potential to elicit inflammation and cell damage (Cross C.E., et al., 1998). Also, airflow velocity influences the interactions between ozone and ELF (Levine A., 1997). The geometry of the respiratory tract, malnutrition and pre-existing pulmonary disease, such as chemical and physical properties of air components, can influence the ozone toxicity (Sarangapani R et al., 2003). The variations in airway size and the tissue surface of the conducting airways make absorption higher in children and females (Bush ML, et al., 1996).

Calderón-Garcidueñas L., Valencia-Salazar G. et al., 2001 reported that the integrity of the mucociliary epithelium can influence the distribution of lung parenchymal lesions induced by air pollutants. When epithelium and specially the mucociliar system are affected, then the retention of toxic substances by the mucosa is reduced, increasing the burden for the rest of the respiratory system. The most common forms of damage within the nasal cavity produced by exposure to air pollution are shortening and partial or total loss of the olfactory cilia, which arise from the dendritic knobs of the olfactory neurons and along where the olfactory receptors are located. With the loss of cilia, the canals of the Bowman´s glands are affected and the nasal epithelium regenerates more slowly and eventually may even cease to do so (Evans M.J. and Plopper G.G., 1988; Hastings L. and Miller M.L., 2003; Eccles R., 1995). In our study, loss of cilia was observed at the exposed epithelium (73,9%) of the three municipalities of Athens. Also, Calderón-Garcidueñas L, Valencia-Salazar G., Garcia R, et al. 1999 found that nasal biopsies in the adult population exhibit histopathological changes such as presence of mucus, decreases in the number of ciliated and globet cells, squamous metaplasia after their exposure to a mixture of air pollutants. Presence of mucus (73,9%) and squamous metaplasia (21,8%) were observed at the samples from exposed Athenian children. Squamous metaplasia is the earliest recognizable morphological change in the metaplasia-dysplasia-carcinoma sequence and may be a helpful instrument in the identification of severe lesions of the nasal epithelium. Squamous metaplasia was seeing in the respiratory systems smoking or exposed to tobacco smoke individuals (Stanley PJ, et al. 1986).

In exposed nasal epithelium to ozone has been observed loss of cohesion among cells (Calderón-Garcidueñas L, Valencia-Salazar G., Rodriguez-Alcazar A, et al., 2001). Loss of cohesion among cells (82,6%) as well as a wide divergence of lymphocytes was observed in the biopsies of the children living at the Attica basin. Wide divergence of lymphocytes over a wide area may play an important role in the formation of local IgA (and possible IgE) defense antibodies as well as cell-mediated immunologic responses producing allergic and hypersensitive conditions (Jafek B.W., 1983).

Activation of p53 protein by some stressful stimuli such as DNA damage, metabolic changes and certain cytokines can in turn drives to a series of events that culminate either in cell cycle arrest or apoptosis (programmed cell death) (Levine A., 1997; Hall P.A, Meek D., and Lane D.P., 1996). If the physiological role of p53 is to prevent the formation of tumors, then the damage to the *p53* gene itself would predispose to tumor formation (Steele R.J.C., Thompson A.M., Hall P.A., et al. 1998). However, p53 protein accumulation may occur in the absence of mutation of the *p53* gene and may correlate with epithelial proliferative activity (Ingle R., Setzen G., Koltai P., et al. 1997). Because the p53 antigen level in normal cells is extremely low, immunohistochemical detection of p53 protein does not always coincide with the presence of the *p53* gene mutation, and it has been used as a marker of *p53* gene abnormalities (Chalastras T, Athanassiadou P, Patsouris E,·et al. 2010). In our study, expression of p53 was observed only at the samples from exposed area (47,4%).

Further children may be especially predisposed to the toxic effects of air pollutants due to the anatomy of their airways, an increase in minute ventilation rates, nasal respiratory pattern changes during growth and their limited awareness of potentially harmful ambient exposures (Spier C.E., Little D.E., Trim S.C., et al. 1992; Laine-Alava M.T., and Minkkinen U.K., 1997).

5 Conclusions

Air pollution driven by local emissions affect the Attica basin which is an area closed by mountains and where restricted air movement concentrates the suspended pollutants. Due to its altitude and latitude, the city of Athens receives intense solar radiation a condition that, together with less efficient combustion, promotes photochemical formation of secondary pollutants such as ground level ozone and secondary particulate matter.

The last report of the Greek Ministry of Environment and Climate Change of Greece confirms the main air pollution problem in Athens is tropospheric ozone, a product of the combination of intense sunshine with considerable emissions of ozone precursors. Particulate matter (PM) with aerodynamic diameter 10μm (PM_{10}) shows higher concentrations at Philadelphia and Kifisia in relation with Kryoneri. Also, nitrogen dioxide (NO_2) and nitrogen monoxide (NO) concentrations at the study area were double in regard with the control area. None of the other air pollutants, such as sulfur dioxide and carbon monoxide, had an increasing tendency at the control area.

The advantages that nasal microbiopsies have for biomonitoring, e.g. the easy and noninvasive procedure, make this type of analysis an attractive substrate for children screening. It is one of the most useful methods for preliminary investigations or for continuous monitoring especially for school children. The evaluation of lung pathology in children is difficult, since it requires the use of invasive techniques. However, a clear limitation of this study is given by the minute nasal tissue amounts obtained with the disposable plastic curette.

Continuously air pollutants exposure can alter the structure of normal nasal epithelium that may become with histopathology differentiations until to an increased cell proliferation and carcinomas (Calderón-Garcidueñas L, Valencia-Salazar G., Rodriguez-Alcazar A, et al., 2001). An important finding in this study was that none of the exposed children living at the study area had normal nasal cytomorphological characteristics. It is clear that the nasal epithelium of children living at the Attica basin is fundamentally disordered and probably their nasal mucociliary defense mechanisms no longer function optimally. A compromised nasal epithelium has a diminished ability to protect itself and the lower respiratory tract. Some of the most common forms of damages to nasal epithelium which are described in different studies due to the exposure to air pollutants, such as partial or total loss of the olfactory cilia, loss of cohesion among cells, and squamous metaplasia were observed in this study. The abundant mucous who was observed may be an indirect evidence of attempts to restore and protect the epithelium against the oxidative effects of continuous exposure to inhaled pollutants. Furthermore loss of epithelial cell orientation was observed in our study that is not described in any study to our knowledge from the literature. Worries cause the fact of positive immunohistochemistry of p53 who was observed in samples from children aged between 11-12 years old, living at the exposed area, because can indicate a predisposition of toward malignancy.

The fact that no statistical differences between the histopathology parameters of the exposed area was observed, may confirm that people living in the Attica basin potentially have the same probability of developing damages to nasal epithelium. Further extended research is needed in the city of Athens for the identification of high risk groups among the exposed population and for the formulation of effective policies in order to help and protect the citizen's health. Furthermore, it is extremely important that pediatricians advise parent accordingly and that public health experts are aware of the danger posed to exposed children in developing further nonrepairable histological lesions.

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