Comet assay in salivary leukocytes: evaluation of early effects of air pollution exposure in pre-school children

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RESPIRA study
(Rischio ESPosizione Inquinamento aRia Atmosferica)
• most important health impacts of air pollution are due to PM.
• associations between PM and mortality at levels well below the current annual WHO air quality guideline level for PM2.5 (10 µg/m³).
• no threshold below which PM has no adverse health effects.
• more than 400,000 premature deaths attributable to ambient air pollution in Europe in 2010 and 2012.
• air pollution increases the incidence of respiratory, cardiovascular and cancer diseases, with both long- and short-term health effects.
• exposure in early life can significantly affect childhood development and trigger disease later in life.
• exposure to air pollution during pregnancy has been associated with adverse birth outcomes.
IARC: Outdoor air pollution a leading environmental cause of cancer deaths

Lyon/Geneva, 17 October 2013 – The specialized cancer agency of the World Health Organization, the International Agency for Research on Cancer (IARC), announced today that it has classified outdoor air pollution as carcinogenic to humans (Group 1). ¹

After thoroughly reviewing the latest available scientific literature, the world’s leading experts convened by the IARC Monographs Programme concluded that there is sufficient evidence that exposure to outdoor air pollution causes lung cancer (Group 1). They also noted a positive association with an increased risk of bladder cancer.

Particulate matter, a major component of outdoor air pollution, was evaluated separately and was also classified as carcinogenic to humans (Group 1).

The IARC evaluation showed an increasing risk of lung cancer with increasing levels of exposure to particulate matter and air pollution. Although the composition of air pollution and levels of exposure can vary dramatically between locations, the conclusions of the Working Group apply to all regions of the world.

A major environmental health problem
Air pollution is already known to increase risks for a wide range of diseases, such as respiratory and heart diseases. Studies indicate that in recent years exposure levels have increased significantly in some parts of the world, particularly in rapidly industrializing countries with large populations. The most recent data indicate that in 2010, 223 000 deaths from lung cancer worldwide resulted from air pollution. ²
Air quality in Europe – 2014 EEA Report
PM10 and PM2.5 levels in Europe in 2012

Brescia, Italy – 2012

PM 10 annual mean 41 µg/m³
106 days with PM10 values > 50 µg/m³
AIR POLLUTION EFFECTS

- Symptoms or diseases (traditional epidemiology)
- Biomarkers of early effect (molecular epidemiology)

- Detectable before disease development
- Valuable for exposure to low doses and mixture of compounds
- Associated with an increase in cancer risk

Carcinogenesis vol.28 no.3 pp.625–631, 2007
doi:10.1093/carcin/bg1177
Advance Access publication September 14, 2006

An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans

Stefano Bonassi¹, Ariana Znaor³, Marcello Ceppi¹
The RESPIRA study aim

TO ASSESS

EARLY BIOLOGICAL EFFECTS
(DNA damage and presence of micronuclei)

IN BUCCAL MUCOSA CELLS
OF
PRE-SCHOOL CHILDREN

EXPOSED TO
HIGH LEVEL OF
AIR POLLUTANTS
Study design

**PRE-SCHOOLS**

**CHILD (3-6 years)**

**COLLECTION OF BIOLOGICAL SAMPLES**
- buccal mucosa cells

**QUESTIONNAIRE ADMINISTRATION**

**COLLECTION OF ENVIRONMENTAL SAMPLES**
- PM0.5

**GENOTOXICITY TESTS**
- AMES TEST (S. typhimurium)
- COMET ASSAY (human leukocytes)

**GENOTOXICITY TEST**
- MICRONUCLEUS TEST (A. cepa)

**MICRONUCLEUS TEST**
- epithelial buccal cells

**COMET ASSAY**
- salivary leukocytes

**CHEMICAL ANALYSIS**
- PAHs
- METALS
- ARPA Agency (PM10, PM2.5, O3, CO, NO2, SO2, Benzene)
- Routine chemical analysis
Genetic damage, caused by environmental pollutants, viruses and lifestyle factors, occurring early in life can increase the risk of carcinogenesis in adulthood (Holland et al., 2011)
BIOLOGICAL SAMPLES

COLLECTION
OF
BUCCAL MUCOSA
CELLS

rinsing of mouth with mineral water and collection of saliva

scraping of cheeks using interdental brush

LEUKOCYTES

EPITHELIAL CELLS

COMET ASSAY

MN TEST
COMET ASSAY

rinsing of mouth with mineral water → collection of saliva in saline solution → cells in PBS → cells in LMA (0.7%) → overnight lysis (pH 10) → 20-minute unwinding pH > 13 → 20-minute electrophoresis 0.8 V/cm, 300 mA, pH > 13 → neutralization

comet analysis (Komet5, Kinetic Imaging Ltd) % DNA in tail → staining with EtBr (10 µg/ml) → neutralization

neutralization
RESULTS

sampling period: January – March 2012

3 pre-schools involved

147 children

3 PM0.5 samples

The results presented during ICAW Conference were preliminary results referred to children attending three of the six schools involved in the RESPIRA study. Final results will be published soon.
RESULTS

data from Regional Agency for Environmental Protection

Directive 2008/50/EC on Ambient Air Quality and Cleaner Air for Europe:
PM10 daily limit value = 50 µg/m³, not to be exceeded on more than 35 days per year;
PM10 annual limit value = 40 µg/m³;
PM2.5 annual target value = 25 µg/m³.
<table>
<thead>
<tr>
<th>Measured PAHs</th>
<th>IARC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenanthrene</td>
<td>3</td>
</tr>
<tr>
<td>Anthracene</td>
<td>3</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>3</td>
</tr>
<tr>
<td>Pyrene</td>
<td>3</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td>2B</td>
</tr>
<tr>
<td>Chrysene</td>
<td>2B</td>
</tr>
<tr>
<td>Benzo(b)fluoranthene</td>
<td>2B</td>
</tr>
<tr>
<td>Benzo(k)fluoranthene</td>
<td>2B</td>
</tr>
<tr>
<td>Benzo(j)fluoranthene</td>
<td>2B</td>
</tr>
<tr>
<td>Benzo(e)pyrene</td>
<td>3</td>
</tr>
<tr>
<td>Benzo(a)pyrene*</td>
<td>1</td>
</tr>
<tr>
<td>Perylene</td>
<td>3</td>
</tr>
<tr>
<td>Indeno(1,2,3-cd)pyrene</td>
<td>2B</td>
</tr>
<tr>
<td>Dibenzo(a,h)anthracene</td>
<td>2A</td>
</tr>
<tr>
<td>Benzo(g,h,i)perylene</td>
<td>3</td>
</tr>
<tr>
<td>Dibenzo(a,l)pyrene</td>
<td>2A</td>
</tr>
<tr>
<td>Dibenzo(a,e)pyrene</td>
<td>3</td>
</tr>
</tbody>
</table>

• PAHs levels similar in PM0.5 of the 3 schools

• Annual target value of 1 ng/m³ for *benzo(a)pyrene in PM10 fraction (Directive 2008/50/EC)

**IARC CLASSIFICATION:**
Group 1 = carcinogenic to humans
Group 2A = probably carcinogenic to humans
Group 2B = possibly carcinogenic to humans
Group 3 = not classifiable as to its carcinogenicity to humans
Group 4 = probably not carcinogenic to humans
RESULTS
chemical analysis (PM0.5)

• several elements derived from industrial activity and fossil fuel combustion

• high level of some metals in PM0.5 of school 1

• annual target value in PM10 fraction only for few elements (Directive 2008/50/EC):
  arsenic: 6.0 ng/m³
cadmium: 5.0 ng/m³
nickel: 20.0 ng/m³
lead: 0.5 ng/m³

<table>
<thead>
<tr>
<th>Measured metals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium</td>
</tr>
<tr>
<td>Beryllium (Group 1)</td>
</tr>
<tr>
<td>Aluminium</td>
</tr>
<tr>
<td>Vanadium</td>
</tr>
<tr>
<td>Chromium (Group 3)</td>
</tr>
<tr>
<td>Iron</td>
</tr>
<tr>
<td>Manganese</td>
</tr>
<tr>
<td>Cobalt (Group 2B)</td>
</tr>
<tr>
<td>Nickel (Group 2B)</td>
</tr>
<tr>
<td>Copper</td>
</tr>
<tr>
<td>Zinc</td>
</tr>
<tr>
<td>Arsenic (Group 1)</td>
</tr>
<tr>
<td>Selenium (Group 3)</td>
</tr>
<tr>
<td>Cadmium (Group 1)</td>
</tr>
<tr>
<td>Silver</td>
</tr>
<tr>
<td>Tin</td>
</tr>
<tr>
<td>Antimony</td>
</tr>
<tr>
<td>Mercury (Group 3)</td>
</tr>
<tr>
<td>Lead (Group 2B)</td>
</tr>
</tbody>
</table>
RESULTS

In vitro tests on PM0.5 organic extracts

<table>
<thead>
<tr>
<th>PM0.5 (µg/m³)</th>
<th>school 1</th>
<th>school 2</th>
<th>school 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.0</td>
<td>17.6</td>
<td>21.1</td>
<td></td>
</tr>
</tbody>
</table>

**AMES TEST**

PM0.5 induced point mutation in *Salmonella typhimurium* TA98 strain, with a dose-response relationship

Mutagenic activity was similar for 3 samples

**COMET ASSAY on peripheral blood cells**

PM0.5 induced DNA damage in human leukocytes in vitro, with a dose-response relationship

Genotoxic activity was similar for 3 samples
PRELIMINARY RESULTS
comet assay in salivary leukocytes

152 recruited children
147 saliva samples
47 subjects with very few nucleoids (< 60 in two slides)

100 subjects included in result analysis

% DNA in tail
mean   6.14 ± 4.76 % 
median 5.65 %
range  0.59 – 30.80 %
Among all the child features and information collected with the questionnaire, we found that DNA damage in child cells was significantly ($p=0.04$) associated with only truck traffic in home area (self-reported data). No other characteristic, including sex, age, parent education, indoor exposure and second hand smoke, was correlated with percentage of DNA in tail.
PRELIMINARY RESULTS
comet assay in salivary leukocytes

p = 0.038
RESULTS

Air pollution exposure in sampling periods
data from Regional Agency for Environmental Protection

![Graph showing air pollution exposure in sampling periods]
RESULTS

Air pollution exposure in the schools

<table>
<thead>
<tr>
<th>School</th>
<th>PM0.5 (µg/m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>School 1</td>
<td>14.0</td>
</tr>
<tr>
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<td>17.6</td>
</tr>
<tr>
<td>School 3</td>
<td>21.1</td>
</tr>
</tbody>
</table>

p = 0.0001
RESULTS

**MN test** in buccal epithelial cells of children

**DNA Damage in Buccal Mucosa Cells of Pre-School Children Exposed to High Levels of Urban Air Pollutants**

Elisabetta Ceretti¹, Donatella Feretti¹*, Gaia C. V. Viola¹, Ilaria Zerbini¹, Rosa M. Limina¹, Claudia Zani¹, Michela Capelli², Rossella Lamera², Francesco Donato¹, Umberto Gelatti¹

¹ Unit of Hygiene, Epidemiology and Public Health, Department of Medical and Surgical Specialities, Radiological Sciences and Public Health, University of Brescia, Brescia, Italy, ² Post-Graduate School of Public Health, University of Brescia, Brescia, Italy

**Abstract**

Air pollution has been recognized as a human carcinogen. Children living in urban areas are a high-risk group, because genetic damage occurring early in life is considered able to increase the risk of carcinogenesis in adulthood. This study aimed to investigate micronuclei (MN) frequency, as a biomarker of DNA damage, in exfoliated buccal cells of pre-school children living in a town with high levels of air pollution. A sample of healthy 3-6-year-old children living in Brescia, Northern Italy, was investigated. A sample of the children’s buccal mucosa cells was collected during the winter months in 2012 and 2013. DNA damage was investigated using the MN test. Children's exposure to urban air pollution was evaluated by means of a questionnaire filled in by their parents that included items on various possible sources of indoor and outdoor pollution, and the concentration of fine particulate matter (PM10, PM2.5) and NO₂ in the 1-3 weeks preceding biological sample collection. 181 children (mean age ± SD: 4.3 ± 0.9 years) were investigated. The mean ± SD MN frequency was 0.29 ± 0.13%. A weak, though statistically significant, association of MN with concentration of air pollutants (PM10, PM2.5 and NO₂) was found, whereas no association was apparent between MN frequency and the indoor and outdoor exposure variables investigated via the questionnaire. This study showed a high MN frequency in children living in a town with heavy air pollution in winter, higher than usually found among children living in areas with low or medium-high levels of air pollution.
CONCLUSIONS about STUDY RESULTS

• % DNA in tail significantly different in children from the 3 schools

• % DNA in tail followed the trend of the concentration and the genotoxic properties of PM0.5 samples collected near the schools

• children of Brescia are exposed to high concentration of pollutants during the whole winter season

• no comparison has been possible for the level of DNA damage
CONCLUSIONS about COMET PROTOCOL

Salivary leukocytes for comet assay:

+ easily collectable
+ directly exposed to air pollutant passage

- variable concentration
- presence of epitheial buccal cells
THANKS TO

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Q-TECH RESEARCH AND STUDY CENTRE
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