SAPIENZA
UNIVERSITÀ DI ROMA

FACOLTÀ DI FARMACIA E MEDICINA

XXIV CICLO DEL DOTTORATO DI RICERCA IN
SCIENZE DI SANITÀ PUBBLICA E MICROBIOLOGIA
CURRICULUM: SCIENZE DELLA SALUTE

AIR POLLUTION AND PAEDIATRIC AGE: HOW AN ENVIRONMENTAL HEALTH RESEARCH CAN SUPPORT PUBLIC HEALTH CHOICES

L'INQUINAMENTO DELL'ARIA E L'ETÀ PEDIATRICA: COME UN'INDAGINE DI IGIENE AMBIENTALE PUÒ SUPPORTARE LE SCELTE IN SANITÀ PUBBLICA

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ANNO ACCADEMICO 2010-2011

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CHAPTER 1

INTRODUCTION TO THE RESEARCH: LIGHTS AND SHADOWS OF THE RELATIONSHIP BETWEEN AIR POLLUTION AND HUMAN HEALTH

My doctoral thesis presents the main findings of a research started in 2006 as a project funded by the National Institute for Occupational Safety and Prevention (ISPESL), in order to assess some aspects of air pollution in relationship with the possible adverse effects on human health.

Air pollution, both indoor and outdoor, is a well-known risk factor for human health, studied for many years, but still being researched because of the complexity of the issue. Current scientific evidences highlight some critical points which contribute to the difficulties of the studies regarding the health impact of air pollution:

1. several airborne contaminants can cause different adverse effects on human health (US EPA 2010); the present research was focused principally on benzene. Benzene is one of the most important air pollutant both in terms of quality and quantity:
   a. recognized as a class I carcinogen (sufficient evidences of carcinogenicity in humans) by the International Agency for Research on Cancer (IARC 1982);
   b. described as an "ubiquitous" environmental pollutant by the World Health Organization (inhalation is the main pathway of human exposure);
   c. considered a "health-based European Union priority substance" by the European Union (Bruinen de Bruin et al. 2008).

2. The concentration of air pollutants may experience significant temporal and spatial changes: in addition to the known daily and seasonal variations in atmospheric contaminants levels (Fuselli et al. 2002; Fuselli et al. 2010), differences in pollutants concentrations depend also on the considered environments; for instance, pollutants arising from motor vehicle traffic, such as particulate air pollutants and volatile organic compounds may reach levels up to ten fold higher in the autovehicles than in outdoor air. Likewise, the concentration of air pollutants can be higher in indoor rather that in outdoor air (WHO 2006a,b; 2010). This evidence is even more significant considering that, globally, people spend the great part of their time in indoor environments (Hellweg et al. 2009).

The scientific interest for indoor air pollution and the link with the possible adverse effects on human health has grown considerably in recent years. To support this assertion, different editions of the air quality guidelines published by the World Health Organization can be mentioned: in the first edition (WHO 1987), indoor air pollution was only discussed in a chapter on radon and in a compendium on tobacco smoke; in the second edition (WHO 2000), a specific section dedicated to indoor pollution was added, while in a successive global update for some selected air pollutants (WHO 2006b) there were several sections related to specific indoor contaminants (WHO 2010). In addition, the results of a recent systematic review (Prüss-Ustün et al. 2010), performed to estimate the health impact of global exposure to some environmental contaminants, showed that, in 2004, 4.9 million deaths could be attributed to the exposure to environmental pollutants. The greatest contribution was determined by the contaminants present in indoor environments, produced by heating and cooking activities (40.8%), outdoor pollutants (24.5%) and passive smoking (12.2%).

As regard to benzene, several studies showed that the mean indoor concentrations are generally higher (until two times) than those in outdoor air (Weisel et al. 2010). The indoor concentrations of benzene derived from the sum of atmospheric benzene and the amount of the substance produced in confined spaces. Outdoor benzene concentrations are mainly due to traffic sources, while indoor benzene levels are related principally to cigarette smoke, and to other sources of emission such as building materials and furniture, heating and cooking systems, stored solvents and various human activities (WHO 2010).

3. The assessment of human exposure to air pollution and related health effects in epidemiological studies is the weakest step of the risk assessment and management process. Several strategies may be adopted, but each of them presents critical aspects. Proximity models assume that closer proximity is equal to greater exposure, while inhalation models and biomarker estimates are most effective in assessing personal exposure, but are too expensive for large study populations. Relevant advantages could be obtained through the development of 'hybrid' models (Jerret et al. 2005; Zou et al. 2009), which use the positive

characteristics of the existing methods integrated with geospatial information technologies.

4. There are different degrees of susceptibility for different population groups: some subgroups have been recognized as particularly vulnerable to the effects of air pollutants, such as patients with diseases, elderly persons, pregnant women and children. Children, in particular, are the general population group that spends more time in confined environments; besides, the World Health Organization Task Force for the environmental health of the children said that "children are not little adults" but they should be considered "unique". Therefore, specific studies are needed to estimate the risk of adverse effect on children health resulting from air pollutants exposure (Anderson et al. 2000; WHO 2005).

The review of the scientific literature showed that, over the years, numerous researches have been conducted to evaluate the health impact of benzene exposure both for occupational settings and for adults not professionally exposed, but there are lack evidences related to benzene exposure in children and dedicated research to explore this issue are required.

The overall aim of the present doctoral thesis was to characterize a profile of exposure to air pollution, with specific reference to benzene, in a sample of Italian children.

CHAPTER 2

DESCRIPTION OF THE RESEARCH STEP BY STEP

The research was conducted according to different phases:

Preliminary knowledge phase

This phase consisted in the search of the scientific literature on "non-occupational exposure to benzene" in the electronic databases MEDLINE via PubMed, TOXNET, and Web of Science. The recovered literature was then accurately examined in order to identify

- the most appropriate methodology for the assessment of exposure to low doses of benzene;
- the main factors affecting benzene exposure in the general population, particularly in paediatric age.

The results of the literature review evidenced that the best method to evaluate benzene exposure in general population was by biological monitoring, in particular the detection in urine (u) samples of unmodified benzene (UB) and its two main metabolites, trans,trans-muconic acid (t,t-MA) and S-phenylmercapturic acid (SPMA) (Johnson et al. 2007).

As regard to the main factors influencing the exposure to benzene in non-occupational scenarios, scientific literature showed that the degree of air pollution in residence area and cigarette smoke were the most important source of exposure.

Development of a questionnaire "ad hoc" to investigate possible factors associated with benzene exposure in childhood

The questionnaire was designed to obtain information on the socio-demographic characteristics of the children and their families, the activities of the participant during the sampling day and during a "typical" day of the last year, household characteristics, and information about cohabitants' smoking habits and precautions taken by at-home smokers.

The questionnaire, previously validated, was filled out by each child's parents.

Study area

The research was conducted in three areas of central Italy whose urbanization characteristics allowed us to classify as follows: very urban, fairly urban and non urban. The choice of the areas was based on relevant urbanization indicators from national databases (National Institute of Statistics, Italian Automobile Club) from 2007-2008-2009 (the years in which the present study took place).

The selected urbanization indicators were:

– Resident population: total number of persons who usually live in the area.
– Population density: number of individuals living in the area divided by its surface area.
– Green area density: percentage of green areas in relation to total municipal territory.
– Motorization rate: number of auto vehicles and two wheeled vehicles per 100 inhabitants.

Areas were also selected on the basis of the airborne concentrations of some indicators of urban air pollution, as available from the environmental monitoring program performed by the Regional Environmental Protection Agency (ARPALAZIO): Carbon Monoxide (CO) and Nitrogen Dioxide (NO_2).

Sample enrollment

In each area, a district primary school was recruited; 348 children attended the very urban school, 150 children attended the fairly urban school, and 166 children attended the non urban school.

The measurement campaigns were conducted on Wednesdays during the winter of the academic years 2007-2008 and 2008-2009.

All of the students attending each school and their parents received information about the goals and plans of the research and were invited to take part in the cross-sectional study. Formation meetings for all children and their parents on the modalities to compile the questionnaire and to collect and store urine sample were carried on just before sampling days.

One urine sample for each participant was collected in the evening (just before bedtime) in a benzene-free polypropylene bottle with hermetic closure, and immediately stored in the refrigerator at 4°C. The next morning, the sample was placed into a polystyrene cooler containing an ice pack and delivered together with the compiled questionnaire to the research team.

Biological monitoring

Spot urine samples were divided into two aliquots: a 14-mL aliquot was poured into a 20-mL glass vial previously added with 4 g of NaCl, promptly closed with a rubber lid with a polyperfluoroethylene lining, and crimped with an aluminum seal for u-UB determination; and about 2 mL of specimen was partitioned into multiple plastic tubes for u-t,t-MA, u-SPMA, u-cotinine, and u-creatinine determinations. All samples were coded and then frozen at -20°C until analysis. Samples were analyzed within 30 days from sampling.

Analytical determinations were performed as follows:

- determination of u-UB: headspace solid phase micro extraction (SPME) followed by gas chromatography-mass spectrometry (GC-MS) (Vitali et al. 2006).
- determination of u-t,t-MA, u-SPMA, u-cotinine: microcapillary high-performance liquid chromatography (LC MS / MS) (Manini et al. 2008);
- determination of u-creatinine: Jaffé method (Henry 1974).

Statistical analyses

Information resulting from questionnaires were coded and entered into a database specifically created for the research. Statistical analyses were carried out using SPSS software (version 14.0 for Windows, Chicago, IL).

Main findings step by step

In total, 501 out of 665 children, aged between 5 and 11 years, were included in the study. The average rate of participation in the monitoring campaigns amounted to 75.5%.

Firstly, statistical elaborations were performed on data derived from the fairly urban and non urban monitoring campaigns. The results evidenced that among all the information investigated by the questionnaires, and evaluated as potential predictors of benzene exposure, variables that significantly influenced the urinary excretion of benzene biomarkers were the degree of urbanization of residence area and the exposure to passive smoking (also called environmental tobacco smoke - ETS or secondhand smoke - SHS).

The first interesting results on exposure to benzene as urban pollutant: the median levels of all urinary analytes (u-UB, u-t,t-MA and u-SPMA) of children were about 1.5-fold higher in children living in fairly urban area than in those in the non urban group. These differences became more clear in the group of children unexposed to ETS. As regard to passive smoking exposure, u-UB was the only biomarker able to discriminate exposure in both fairly urban and non urban children. These results are also confirmed by the significant positive correlation between u-UB and u-cotinine (the "gold standard" biomarker of exposure to active and passive smoking), both in all children and in children exposed to passive smoking.

The influence of passive smoke exposure - and smoking habits in general - on biological indices of benzene exposure is commonly considered negative, especially when these biomarkers must be used to assess benzene exposure in the workplace. The contribution of smoke to benzene excretion alters the role of biomarkers as indices of exposure due to the chemicals present in the workplace; for this reason, the guidelines and research in the field have judged u-t,t-MA and u-SPMA to be the best biological indices of benzene exposure (ACGIH 2009) and suggested a careful evaluation of smoking exposure when u-UB is used (Barbieri et al. 2008; Lovreglio et al. 2010). The influence of passive smoking exposure on u-UB excretion and the suitability of u-UB to differentiate between children exposed to passive smoking and those who were unexposed might be considered an advantageous characteristic of this biomarker, especially because it could permit one to assess exposure to low concentrations of benzene and passive exposure at the same time. U-UB could represent a good "carcinogen-derived biomarker" of exposure to passive smoking, specifically related to benzene, because it is a known carcinogen present in tobacco

smoke. A review of carcinogen-derived biomarkers and their application in studies of human exposure to passive smoking examined data on u-t,t-MA as a biomarker of benzene uptake derived from ETS exposure. The article concludes that, collectively, "benzene uptake in humans is not consistently associated with passive smoking exposure" (Hecht 2004). On the contrary, our results suggest that there is a strong association between human benzene uptake and passive smoking exposure, especially in children.

For this reasons, we went on with the research to study in depth two new issues:

- the evaluation of air pollution impact on exposure to benzene in children by using the biomarkers u-t,t-MA and u-SPMA that resulted not affected by passive smoking exposure;
- the evaluation of the relationship between ETS exposure of children and related benzene intake.

1. Evaluation of air pollution impact on exposure to benzene in children by using the biomarkers u-t,t-MA and u-SPMA, that resulted not affected by passive smoking exposure

Human biomonitoring of exposure is a mandatory method to evaluate personal exposure to air pollution, and to provide data on profile exposure of general population to support environmental and public health policies. Given the well-known differences of exposure to air pollution between children and adults in terms of magnitude of exposure and susceptibility to adverse effects, it is necessary to perform separate studies for the assessment of exposure to air pollutants for children and adults. Despite a great number of researches performed on urban benzene exposure for adult population is available, very few data are available for children.

As regard to u-SPMA, results show that its levels increase following the increase of the degree of urbanization of residence area:

- u-SPMA levels of children living in very urban area are, on average, 3.3 and 4.8 times higher than those of children living in fairly and non urban area, respectively;

- u-SPMA levels of children living in fairly urban area are, on average, 1.5 times higher than those of children living in non urban area.

As regard to u-t,t-MA, differences in excretion levels related to the degree of urbanization of residence area in children living in very urban and fairly urban areas were revealed only by the multivariate analyses. The lack of specificity of u-t,t-MA in adult population exposed to very low concentration of urban benzene was demonstrated by previous researches (Pezzagno et al. 1999; Renner et al. 1999; Weaver et al. 2000; Aprea et al. 2008; Weisel et al. 2010; Lovreglio et al. 2011). The most common explanation of this result is the influence of the diet on u-t,t-MA excretion: u-t,t-MA is also a metabolite of sorbic acid, a common food additive.

The other important source of benzene exposure for general population not occupationally exposed to benzene is tobacco smoke. The present study evidences no significant differences in children exposed to ETS respect to children unexposed, according to the results of other previous researches performed to assess benzene exposure in children (Weaver et al. 1996; Amodio-Cocchieri et al. 2001; Bahrami and Edward 2006). These findings contribute to consider u-SPMA and u-t,t-MA as good biomarkers for assessing urban benzene exposure in childhood.

In conclusion, both u-SPMA and u-t,t-MA are able to assess urban benzene exposure in childhood, even if u-SPMA should be taken in higher consideration because u-t,t-MA confirmed its less specificity for benzene exposure in the magnitude of sub-ppm exposures (general population scenario).

2. Evaluation of the relationship between ETS exposure of children and related benzene intake.

Scientific literature highlights some critical points about the issues "passive smoking exposure" and "passive smoking-derived benzene exposure":

- passive smoking is a significant risk factor for health, especially for children (US PHS 2006);

- passive smoking contains over 5,000 compounds, including many substances that are toxics for human health or carcinogens, such as benzene (IARC 2004);
- household environment is the main source of exposure to passive smoking for children; even some researchers have hypothesized that bans on smoking in public places could adversely affect children' health by shifting smoking into the domestic environment;
- benzene is present in tobacco smoke in sufficient concentrations to explain up to half of the estimated cases of acute myeloid leukaemia (IARC 2004);
- much research has been conducted to assess the links between youth benzene exposure, passive smoking exposure and the risk of lymphohematopoietic cancer in childhood; however this research has yielded contradictory findings (Chang 2009). On the other hand, considering the prolonged latency of the disease and the early initiation of exposure, the possibility of cancers in adulthood after ETS exposure during childhood cannot be excluded;
- actually, there is a gap of research dedicated to the issue "ETS-related benzene exposure levels in childhood".

Thus, we tried to answer to two questions remain unresolved: 1) Can u-UB be used as tobacco-related carcinogen biomarker? 2) How can smoking behaviours of cohabitant(s) smoker(s) impacts on the benzene exposure of children?

For this purpose, we considered just the u-UB data and information derived from the monitoring campaign performed on children living in the non urban area (to avoid as much as possible the influence of benzene present in atmospheric air due to motor vehicle traffic). The results showed significant differences in u-UB levels between passive smoking exposed and unexposed children. Besides, the excretion of u-UB increased significantly in parallel to increased ETS exposure as follows: unexposed to ETS < cohabitant(s) smoker(s) not smoking inside the home < cohabitant(s) smoking inside the home only when children are out < cohabitant(s) smoking inside the home even when children are in.

These findings strongly highlight that:

- u-UB may be a good indicator of benzene exposure specifically due to passive smoking;
- the smoking is a health threat for smokers and for others with whom they share an environment, regardless the household "precautions" of smokers;
- urinary benzene levels of children living in non urban areas at low traffic density but exposed to passive smoking may result higher than those of children living in urban areas but unexposed to passive smoking. This fact may nullify the benefits of living far from traffic pollutants;
- children passive smoking exposure increases in parallel with incorrect household smoking behaviors of cohabitants smokers.

The last mentioned point highlights that the precaution measures "smoking at home only when children are not there" or "smoking only outside the home" are not actual precaution measures. This consideration led us to further investigate the impact of the behaviours and influence of "home smoking policy" in a large sample of children using u-cotinine, defined as the gold standard biological marker of exposure to passive smoking. For this purpose, several statistical elaborations were performed on all the children participant to the research (n = 501).

The main findings showed that:

- over one third of the smokers cohabiting with study participants do not observe any home-smoking restrictions and smoke inside the home even in the presence of children;

- children's u-cotinine levels increase in a parallel pattern as the home-smoking restrictions adopted by cohabitants decrease:

    - the u-cotinine levels of children living with smokers who do not smoke at home are 1.4 times higher than those of children who do not have any cohabitants who smoke;

    - the u-cotinine levels of children living with smokers who smoke at home only when that child is out are 1.7 times higher than those of children whose cohabitants smoke, but never at home;

- the u-cotinine levels of children living with smokers who smoke at home even when that child is home are 1.4 times higher than those of children whose cohabitants smoke at home only when that child is out.

Consequently, the habit of smoking at home only when children are not there or smoking only outside the home decreases the exposure to passive smoking, but do not correspond to a complete lack of exposure. This argument can be related to the "thirdhand" smoke (THS) - so called to distinguish it from SHS. SHS is defined as "the combination of smoke emitted from the burning end of a cigarette or other tobacco products and smoke exhaled by the smoker" (WHO 2007). Thus, SHS exposure consists of an unintentional inhalation of smoke that occurs close to people smoking and/or in indoor environments where tobacco was recently used. THS is the residue from tobacco smoke that persists on the clothing and hair of smokers, on environmental surfaces, and in dust long after a cigarette has been extinguished (Invernizzi et al. 2007; Winickoff et al. 2009); this complex phenomenon is significant because it demonstrates that many components of tobacco smoke, including benzene, can persist in an indoor environment beyond the period of active smoking. THS is a major public health concern because it highlights the impossibility to maintaining a safe level of exposure to tobacco smoke and also because nicotine residues in the domestic environment can react with ambient nitrous acid to form new tobacco-specific, carcinogenic nitrosamines (Destaillats et al. 2006; Sleiman et al. 2010; Matt et al. 2011a,b; Burton 2011; Petrick et al. 2011).

The differences in passive smoking exposure are presumably attributable to a combination of SHS and THS, which likely contribute to the total ETS exposure in variable proportions, depending on the habits of and the precautions adopted by cohabitant smokers.

CHAPTER 3

BENZENE EXPOSURE IN CHILDHOOD: ROLE OF LIVING ENVIRONMENTS AND ASSESSMENT OF AVAILABLE TOOLS

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Environment International 2010; 36: 779-787

ABSTRACT

Introduction Benzene is a widespread air pollutant and a well-known human carcinogen. Evidence is needed regarding benzene intake in the pediatric age group. We investigated the use of urinary (u) trans,trans-muconic acid (t,t-MA), S-phenylmercapturic acid (SPMA), and unmodified benzene (UB) for assessing exposure to low concentrations of environmental benzene and the role of living environment on benzene exposure in childhood.

Material and Methods u-t,t-MA, u-SPMA, u-UB and u-cotinine were measured in urine samples of 243 Italian children (5-11 years) recruited in a cross-sectional study. Analytical results were compared with data obtained from questionnaires about participants' main potential exposure factors.

Results u-UB, u-t,t-MA and u-SPMA concentrations were about 1.5-fold higher in children living in urban areas than in those in the rural group. Univariate analyses showed that u-UB was the only biomarker able to discriminate secondhand smoke (SHS) exposure in urban and rural children (medians = 411.50 and 210.50 ng/L, respectively); these results were confirmed by the strong correlation between u-UB and u-cotinine in the SHS-exposed group and by multivariate analyses. A regression model on u-SPMA showed that the metabolite is related to residence area ($p < 0.001$), SHS exposure ($p = 0.048$) and gender ($p = 0.027$).

Conclusion u-UB is the best marker of benzene exposure in children in the present study, and it can be used as a good carcinogen-derived biomarker of exposure to passive smoking, especially related to benzene, when urine sample is collected at the end of the day. In addition, it is important to highlight that SHS resulted the most important contributor to benzene exposure, underlining the need for an information campaign against passive smoking exposure.

INTRODUCTION

In recent decades, the potential adverse effects on human health of pollution of living environments have caused great concern worldwide (WHO 2006a). In this context, one of the most important health-based European Union priority substances is benzene (Bruinen de Bruin et al. 2008), a well-known human carcinogen classified in group 1 (carcinogenic to humans) by the International Agency for Research on Cancer (IARC) since 1982. Strong evidence links benzene exposure with lymphohematopoietic cancers, particularly acute myeloid leukemia (IARC 1982; Lamm et al. 2009).

For this reason the use of benzene - an organic compound historically employed in numerous production and synthesis processes - has been progressively reduced and is rigorously regulated by occupational exposure limits and by air quality standards set by the European legislation for the general environment (European Commission 2000).

However, benzene exposure still occurs today because of its presence in petrochemical solvents, automobile gasoline, fuel, and their emissions.

Environmental tobacco smoke (ETS) is another important source of benzene (Johnson et al. 2007).

Benzene is a widespread, diffused air pollutant in outdoor and indoor environments. Its adverse health effects for the general population cannot be neglected, especially in the context of exposure to low concentrations for a prolonged period.

Children are considered a high-risk population for both acute and chronic effects of environmental hazards because they are much more susceptible than adults are (Weaver et al. 1998; Barton et al. 2005; Duderstadt 2006; van Leeuwen et al. 2008). Children's increased vulnerability is due to several factors, such as exposure, physiological characteristics, and pharmacokinetics. With regard to exposure, children absorb more from their surroundings than adults, even when exposed to the same concentrations of environmental contaminants. For example, per kilogram bodyweight, the daily intake of air has been estimated to be 2.3 times higher in

children than in adults, intake of fluids 4.8 times higher, and intake of food 6.1 times higher (Armstrong et al. 2002).

Moreover, one must consider differences in the biologically effective doses that reach target organs in children and adults. Thus, if the exposure to environmental pollution starts during childhood, the risk of adverse health effects with long latency becomes very high (Wild and Kleinjans 2003).

For these reasons, the World Health Organization (WHO) Task Force for the Protection of Children's Environmental Health, in the Bangkok statement, declared that children cannot be considered "little adults" with regard to the risk of adverse health effects resulting from exposure at an early age and exposure assessment tools (Anderson et al. 2000).

Research has yielded conflicting findings with respect to the link between benzene exposure in childhood and the risk of lymphohematopoietic cancer. However, in these studies, researchers considered only secondhand smoke (SHS) exposure, not other sources of benzene, and the possibility of cancers in adulthood after SHS exposure during childhood (Chang 2009).

Several exposure assessment studies have shown that children are exposed to low environmental concentrations of benzene. Many studies conducted on adults who are not professionally exposed to benzene suggest that urinary trans,trans-muconic acid (u-t,t-MA), urinary S-phenylmercapturic acid (u-SPMA) and urinary unmodified benzene (u-UB) serve as good exposure markers for benzene (Waidyanatha et al. 2001; Fustinoni et al. 2005; Johnson et al. 2007; Barbieri et al. 2008; Lovreglio et al. 2010), although their abilities to discriminate different levels of exposure (especially at low levels) are currently under evaluation.

Only a few research studies are available in the literature on the specific magnitude of children's exposure to benzene (Minoia et al. 1996; Weaver et al. 1996; Duarte-Davidson et al. 2001; Amodio- Cocchieri et al. 2001; Kouniali et al. 2003; Adgate et al. 2004; Bahrami and Edwards 2006; Ruchirawat et al. 2007). These studies were conducted using predictive models of daily benzene intake in different exposure scenarios or by monitoring levels of benzene in the air and/or biological exposure

indices, such as u-UB, u-t,t-MA, u-SPMA, and urinary phenol. At present, no research on children's exposure to low doses of benzene has been performed using u-UB, u-t,t-MA, and u-SPMA as biomarkers.

Due to the current lack of research in this area, there is a significant need to better evaluate benzene exposure during childhood and to determine which tools to use for assessment purposes.

The objectives of the present research were:

- To evaluate the abilities of u-UB, u-t,t-MA, and u-SPMA to assess exposure to low concentrations of environmental benzene in the pediatric age group; and
- To investigate the impacts of living environment and cohabitants' habits on benzene exposure in childhood.

MATERIALS AND METHODS

Study area

The research was conducted in two areas of central Italy whose urbanization characteristics allowed us to classify one as urban and the other as rural. The choice of the areas was based on relevant urbanization indicators from national databases (National Institute of Statistics, Italian Automobile Club) from 2007, the year in which the present study took place. The selected urbanization indicators were:

- Resident population: total number of persons who usually live in the area.
- Population density: number of individuals living in the area divided by its surface area.
- Green area density: percentage of green areas in relation to total municipal territory.
- Motorization rate: number of motor vehicles per 100 inhabitants.

Summary information about these urbanization indicators for the urban and rural areas is reported in Table 1.

Table 1. Summary information on relevant urbanization indicators of the selected urban and rural areas in 2007

	Resident population (n)	Population density (persons per km^2)	Green area density (% of total municipal territory)	Motorization rate (number of vehicles per 100 inhabitants)
Urban area	32,886	395	< 85	76
Rural area	3,308	120	> 85	66

In each area, a district primary school was recruited; 150 children attended the urban school, and 166 children attended the rural school.

Study population and design

All of the students attending each school and their parents received information about the goals and plans for the research and were invited to take part in the cross-sectional study. The overall participation rate was 76% (urban: 81% and rural: 73%, respectively).

Study subjects were 243 apparently healthy children between 5 and 11 years of age who were presumably exposed to benzene as a pollutant.

Detailed information about socio-demographic characteristics, activities engaged in on the sampling day, living environment, and lifestyle factors of the investigated subjects was obtained from a questionnaire completed by their parents.

The measurement campaigns were conducted on Wednesdays during the winter of 2007.

Before the monitoring day, we conducted formation meetings for all children and their parents on the modalities to compile the questionnaire and to collect and store urine sample.

One urine sample for each participant was collected in the evening (just before bedtime) in a benzene-free Polypropylene bottle with hermetic closure, and immediately stored in the refrigerator at 4 °C.

The next morning, the sample was placed into a polystyrene cooler containing an ice pack and was delivered to the research team.

Biological monitoring

Spot urine samples were divided into two aliquots: a 14-mL aliquot was poured into a 20-mL glass vial previously added with 4 g of NaCl, promptly closed with a rubber lid with a polyperfluoroethylene lining, and crimped with an aluminum seal; and about 2 mL of specimen was partitioned into multiple plastic tubes for u-t,t-MA, u-SPMA, urinary cotinine (u-cotinine), and u-creatinine determinations.

All samples were coded and then frozen at -20 °C until analysis.

A total of 243 urine samples were collected; 18 samples were rejected due to unsatisfactorily closure; besides, the volume of some other samples was not enough to carry out the whole set of analyses.

Consequently, analytical determinations were performed on 185 vials for u-UB and on 225 tubes for u-t,t-MA, u-SPMA, u-cotinine, and u-creatinine. Samples were analyzed within 30 days from sampling.

Determination of u-UB

u-UB was determined by headspace solid phase micro extraction (SPME) followed by gas chromatography-mass spectrometry (GC-MS) according to procedures outlined in Vitali et al. (2006). We used a 5973 GC-MS operating in Selected IonMonitoring (SIM)mode (Agilent, Santa Clara, CA, USA) equipped with a 30 m×0.25 mm×0.25 μm HP-VOC column (HP, Palo Alto, CA, USA). Pesticide-grade reagents, high-purity benzene and benzene d-6were supplied by Carlo Erba (Milan, Italy); all standards were used without further purification. The SPME apparatus, fitted with a 75-μm carboxen/polydimethylsiloxane fiber, was purchased from Supelco (Bellefonte, PA, USA).

Before analysis, the vials were conditioned at room temperature and then maintained at 60 °C for 1 h. The SPME fiber was held in the headspace for 10 min to reach the partition equilibrium, and then it was retracted into the needle and

immediately inserted into the GC injector for thermal desorption. No carry-over effects were observed.

The chromatographic conditions were as follows: splitless injection port (at 290 °C) with purge valve closed for 3 min; helium carrier gas at 1 mL/min; column temperature was maintained at 50 °C for 5 min and then increased at 15 °C/min to 200 °C; dwell time was set at 50 ms/ion; and monitored ions were 78 and 52 m/z for benzene and 84 m/z for benzene-d6.

Quantitative determination was conducted using benzene d-6 as the internal standard (IS). The linearity of the method was tested by spiking urine samples at 50, 100, 250, 500, 1000 and 2000 ng/L. The results showed good linearity, with a correlation coefficient of 0.998.

The coefficient of variation of the method (CV%) was below 9.8% for all intra- and inter-day determinations.

The limit of detection (LOD), calculated as the signal to noise ratio (S/N) N3, was 8 ng/L. All analytical determinations were above the corresponding limits of detection.

Determination of u-t,t-MA, u-SPMA, u-cotinine, and u-creatinine

The samples were analyzed using a methodology that has been previously described and extensively used in previous publications (Manini et al. 2008).

Adjustment for urinary creatinine. U-t,t-MA, u-SPMA and u-cotinine were adjusted for u-creatinine and expressed as μg/g creatinine. U-UB levels were not adjusted for u-creatinine because u-UB is excreted into urine through a concentration-dependent passive process that involves tubular reabsorption, while creatinine is eliminated through glomerular filtration and is not reabsorbed (Boeniger et al. 1993, Serdar et al. 2003).

Statistical analyses

Statistical analyses were carried out using SPSS software (version 14.0 for Windows, Chicago, IL).

The first data showed that the biomarkers' levels were not normally distributed. Therefore, parallel analyses were conducted with non-parametric techniques (Kolmogorov-Smirnov test, Mann-Whitney test, and Spearman's correlation coefficients) and corresponding parametric methods on natural log-transformed data (t-test for independent or paired samples and Pearson's correlation coefficients).

Descriptive statistical elaborations were performed on all selected children and on the following four subgroups, which were categorized based on the two most important exposure factors (urbanization of residence area and exposure to SHS):

1. Urban area children exposed to SHS
2. Rural area children exposed to SHS
3. Urban area children unexposed to SHS
4. Rural area children unexposed to SHS

All children were considered to be exposed to SHS if they lived in households where at least one person was a smoker.

Simple linear regression analyses were used to assess the relationship between u-UB, u-t,t-MA, u-SPMA and u-cotinine.

Forward multiple linear regression analysis was run on the entire sample to assess the role of SHS exposure status, residence area and other independent variables on selected urinary biomarkers (u-UB, u-t,t-MA, and u-SPMA). In every model, the natural log-transformed value of each urinary biomarker was included as a dependent variable, and the covariates were as follows: residence area (0 = rural area, 1 = urban area), SHS exposure status (0 = unexposed to SHS, 1 = exposed to SHS), gender (male = 0, female = 1), and age (0 = 1^{st}, 2^{nd}, or 3^{rd} grade of primary school, 1 = 4^{th} or 5^{th} grade of primary school).

The significance level for all tests was $p \leq 0.05$ (two-tailed). Linear regression analyses were run using a significance level of 0.05 for entry and 0.10 for removal from the model. The "goodness of fit" of the model was assessed using R^2 statistics.

RESULTS

Descriptive characteristics of the studied subjects are presented in Table 2.

Table 2. General characteristics of study individuals

		Total children	Urban area	Rural area
Gender (%)	**Male**	51.8	52.3	51.3
	Female	48.2	47.7	48.8
Grade of primary school (%)	**1st**	21.0	20.5	21.4
	2nd	18.8	19.6	17.9
	3rd	19.7	18.8	20.5
	4th	20.5	24.1	17.1
	5th	20.1	17.0	23.1
SHS exposure status (%)	**Exposed**	39.7	23.7	56.0
	Unexposed	60.3	76.3	44.0
Number of cohabitant smokers (only in SHS exposed group) (%)	**1**	78.6	67.2	70.7
	> 1	21.4	32.8	29.3
Number of cigarettes smoked daily by cohabitant smoker (mean)	**Total**	14.4	6.6	17.8
	At home	8.7	8.0	9.1
Home dimension (%)	**≤ 80 m²**	15.8	9.5	22.8
	> 80 m²- ≤120 m²	49.7	44.8	55.5
	> 120 m²	34.5	45.7	21.7
Proximity with high traffic area (%)	**No**	93.7	89.1	98.3
	Yes	6.3	10.9	1.7
Time (min) spent in different environments during sampling day until urine collection Mean ± SD	**At school (indoor environment)**	443.99 ± 75.85	461.54 ± 38.36	429.27 ± 90.38
	Other indoor environments	263.85 ± 97.79	240.09 ± 83.90	290.00 ± 102.29
	Outdoor environments	51.84 ± 60.83	58.47 ± 49.33	47.18 ± 68.53
	Motor vehicles	26.27 ± 43.68	21.69 ± 21.28	29.40 ± 52.00

The two groups were comparable with respect to gender and time spent in indoor and outdoor environments. The percentage of children who lived in a rural area who were exposed to SHS was greater than the percentage of SHS-exposed children in the urban group (56.0% versus 23.7%). In addition, Table 2 shows a wide range of time spent in motor vehicles on the sampling day between subjects, especially in rural children (mean = 21.29 min; SD = 52.00 min).

The ability of u-UB, u-t,t-MA, and u-SPMA levels to differentiate exposure due to air pollution and SHS was evaluated through univariate statistical analysis for all

children and for the four categorized subgroups. The same test was applied for u-cotinine, to demonstrate its role as a metabolite specific to SHS exposure (Benowitz 1996; Haufroid and Lison 1998; Keskinoglu et al. 2007). The results are reported in Table 3 (all children) and Table 4 (the four subgroups).

In addition, further potential sources of benzene (activities of the child in the last year, house characteristic, home heating devices, and possible indoor sources such as chemical containing benzene) were investigated by the questionnaire. All heating devices except two were feed by methane, while no significance was found for the other variables (data not shown).

Table 3. Summary statistics for urinary analytes in all children according to residence area (urban vs. rural area)

	AM[a]	ASD[b]	GM[c]	GSD[d]	Median	IQ Range[e]	5° - 95° percentile	Range	Min- Max	*p*[f]	*p*[g]
u-UB ng/L											
Urban	415.60	455.80	269.80	0.90	237.00	170.00 - 429.00	64.50 - 1820.80	2,058	36 - 2,094	*0.011*	*0.006*
Rural	293.32	320.50	184.76	0.96	180.00	87.25 - 347.25	45.45 - 1245.00	1,455	37 - 1,492		
u-t,t-MA µg/g creatinine											
Urban	132.96	99.45	110.56	0.60	114.95	66.29 - 166.98	45.66 - 253.78	821.66	24.84 - 846.50	*<0.001*	*<0.001*
Rural	80.21	81.37	64.15	0.61	65.04	45.62 - 89.56	26.81 - 180.22	659.07	13.76 - 672.83		
u-SPMA µg/g creatinine											
Urban	0.31	0.17	0.27	0.51	0.28	0.20 - 0.40	0.10 - 0.59	1.14	0.07 - 1.21	*0.001*	*0.001*
Rural	0.24	0.13	0.22	0.51	0.22	0.16 - 0.30	0.08 - 0.51	0.73	0.05 - 0.78		
u-cotinine µg/g creatinine											
Urban	3.75	4.29	2.59	0.81	2.55	1.50 - 4.12	0.84 - 13.25	26.53	0.43 - 26.95	*0.010*	*0.014*
Rural	5.37	7.89	3.42	0.85	3.40	2.04 - 4.59	0.10 - 18.10	51.95	0.24 - 52.20		

u-UB: urinary unmodified benzene; u-t,t-MA: urinary trans,trans-muconic acid; u-SPMA: urinary S-phenilmercapturic acid; u-cotinine: urinary cotinine
[a]AM: Arithmetic Mean
[b]SD: Arithmetic Standard Deviation
[c]GM: Geometric Mean
[d]GSD: Geometric Standard Deviation (calculated as the Standard Deviation of ln-trasformed data)
[e]IQ Range: Interquartile Range
[f]Mann–Whitney *U*-test was used to compare urban and rural areas
[g]Unpaired t-test was used to compare urban and rural areas (ln-trasformed data)

Table 4. Summary statistics for urinary analytes in children differentiated according to residence area and secondhand smoke (SHS) exposure

	u-UB ng/L			u-t,t-MA µg/g creatinine			u-SPMA µg/g creatinine			u-cotinine µg/g creatinine		
	Urban	**Rural**	***p***	**Urban**	**Rural**	***p***	**Urban**	**Rural**	***p***	**Urban**	**Rural**	***p***
Exposed to SHS												
AM[a]	746.83	493.26		156.58	67.53		0.35	0.25		6.96	7.48	
ASD[b]	659.33	367.16		155.11	31.87		0.21	0.13		6.97	10.33	
GM[c]	468.25	389.40		120.89	60.93		0.31	0.23		4.85	4.64	
GSD[d]	1.07	0.70		0.68	0.46		0.52	0.46		0.83	0.87	
Median	411.50	359.50	*0.394[h]*	121.18	63.41	*< 0.001[h]*	0.33	0.23	*0.007[h]*	4.36	3.77	*0.595[h]*
IQ Range[e]	234.25 - 1,188.50	267.75 - 629.50	*0.400[i]*	65.67 - 182.70	47.26 - 86.25	*< 0.001[i]*	0.23 -0.42	0.18 - 0.29	*0.011[i]*	2.72 - 7.08	2.61 - 7.12	*0.833[i]*
5° - 95° percentile	74.25 - 2,039.50	101.15 - 1,418.70		36.95 - 652.62	28.17 - 127.98		0.11 - 1.00	0.10 - 0.50		1.23 - 26.77	1.51 - 34.37	
Range	2,020	1,422		817.80	167.49		1.10	0.70		25.88	51.23	
Min - Max	74 - 2,094	70 - 1,492		28.70 - 846.50	22.52 - 190.01		0.11 - 1.21	0.12 - 0.35		1.10 - 26.95	0.97 - 52.20	
Unexposed to SHS												
AM	311.54	107.10		125.80	98.53		0.30	0.24		2.72	2.88	
SD	303.98	67.58		75.07	117.39		0.15	0.13		2.33	1.93	
GM	227.81	89.76		107.52	69.51		0.26	0.20		2.10	2.43	
GSD	0.78	0.60		0.57	0.77		0.51	0.58		0.70	0.59	
Median	210.50	92.50	*< 0.001[h]*	113.14	71.78	*< 0.001[h]*	0.27	0.20	*0.014[h]*	2.07	2.64	*0.144[h]*
IQ Range	167.25 - 329.50	51.25 - 141.50	*< 0.001[i]*	68.94 - 161.06	37.45 - 98.67	*< 0.001[i]*	0.18 - 0.38	0.14 - 0.30	*0.008[i]*	1.24 - 3.15	1.51 - 3.65	*0.233[i]*
5° - 95° percentile	56.40 - 914.45	37.10 - 275.70		45.13 - 250.62	23.17 - 410.42		0.10 - 0.58	0.07 - 0.54		0.61 - 7.02	0.86 - 6.13	
Range	1,914	259		480.20	659.07		0.75	0.60		13.50	11.81	
Min - Max	36 - 1,950	37 - 296		24.84 - 505.04	13.76 - 672.83		0.07 - 0.82	0.05 - 0.66		0.43 - 13.92	0.64 - 12.45	
p[f]	*0.003*	*< 0.001*		*0.486*	*0.409*		*0.159*	*0.281*		*< 0.001*	*< 0.001*	
p[g]	*0.001*	*< 0.001*		*0.386*	*0.279*		*0.159*	*0.204*		*< 0.001*	*< 0.001*	

u-UB: urinary unmodified benzene; u-t,t-MA: urinary trans,trans-muconic acid; u-SPMA: urinary S-phenilmercapturic acid; u-cotinine: urinary cotinine.

[a]AM: Arithmetic Mean; [b]ASD: Arithmetic Standard Deviation; [c]GM: Geometric Mean; [d]GSD: Geometric Standard Deviation; [e]IQ Range: Interquartile Range; [f]Mann–Whitney *U*-test was used to compare exposed and unexposed to SHS; [g]Unpaired t-test was used to compare exposed and unexposed to SHS (ln-trasformed data); [h]Mann–Whitney *U*-test was used to compare urban and rural areas; [i]Unpaired t-test was used to compare urban and rural areas (ln-trasformed data).

With regard to air pollution, levels of all urinary analytes were significantly different between the urban and rural children: u-UB, u-t,t-MA and u-SPMA concentrations were about 1.5-fold higher in the high-exposure group (urban) than in the low-exposure group (rural).

With respect to SHS exposure status, u-UB was the only biomarker able to discriminate SHS exposure in both urban and rural children (median = 411.50, $p < 0.003$ in the urban group; median = 210.50, $p < 0.001$ in the rural group).

The impact of SHS exposure status on u-UB, u-t,t-MA and u-SPMA concentrations also highlights the differences of biomarker levels between urban and rural children when differentiated according to SHS exposure status: significant differences between the median values of u-t,t-MA and u-SPMA were clear for urban and rural children in the SHS-exposed and -unexposed groups, while with u-UB the difference is only seen in the unexposed group.

Table 4 shows that u-cotinine excretion was significantly higher among the SHS-exposed group when compared with the unexposed group in both urban and rural children; this result confirms the reliability of the questionnaire to collect information on the smoking habits of the studied children's cohabitants.

Table 5 reports the results of simple regression analyses among biological markers in all of the investigated children and in children classified according to SHS status.

Table 5. Simple regression analyses between urinary biomarkers in children exposed and unexposed to secondhand smoke (SHS)

		Indipendent variable		
	Dependent variable	**u-t,t-MA**	**u-SPMA**	**u-cotinine**
All children	**u-UB**	y = 5.107 + 0.044 * x *p = 0.557 (n = 179)*	y = 5.232 - 0.064 * x *p = 0.393(n = 179)*	y = 5.087 + 0.259 * x *p < 0.01 (n = 179)*
	u-t,t-MA		y= 5.703 + 0.601 * x *p < 0.01 (n = 225)*	y= 4.236 + 0.083 * x *p = 0.215 (n = 225)*
	u-SPMA			y= - 1.594 + 0.110 * x *p = 0.098(n = 225)*
Unexposed to SHS	**u-UB**	y = 4.236 + 0.151 * x *p = 0.124 (n = 105)*	y = 4.945 - 0.049 * x *p = 0.620 (n = 105)*	y = 5.246 - 0.243 * x *p = 0.030 (n = 105)*
	u-t,t-MA		y = 5.835 + 0.596 * x *p < 0.01 (n = 131)*	y = 4.180 + 0.238 * x *p < 0.01 (n = 131)*
	u-SPMA			y = -1.647 + 0.222 * x *p < 0.01 (n = 131)*
Unexposed to SHS (with exclusion of 3 outliners)	**u-UB**	y = 4.341 + 0.142 * x *p = 0.155 (n = 102)*	y = 5.075 - 0.006 * x *p = 0.953 (n = 102)*	y = 5.224 - 0.152 * x *p = 0.128 (n = 102)*
	u-t,t-MA		y = 5.753 + 0.563 * x *p < 0.01 (n = 128)*	y = 4.429 + 0.222 * x *p = 0.012 (n = 128)*
	u-SPMA			y = -1.612 + 0.207 * x *p = 0.019 (n = 128)*
Exposed to SHS	**u-UB**	y = 6.015 + 0.000 * x *p = 1.000 (n = 65)*	y = 5.577 - 0.192 * x *p = 0.126 (n = 65)*	y = 5.294 + 0.474 * x *p < 0.01 (n = 65)*
	u-t,t-MA		y = 5.500 + 0.611 * x *p < 0.01 (n = 85)*	y = 3.879 + 0.177 * x *p = 0.105 (n = 85)*
	u-SPMA			y = -1.728 + 0.154 * x *p = 0.158 (n = 85)*

u-UB: urinary unmodified benzene; u-t,t-MA: urinary trans,trans-muconic acid; u-SPMA: urinary S-phenilmercapturic acid; u-cotinine: urinary cotinine.
The concentrations of all analytes were ln-transformed. U-UB is expressed as ng/L.
u-t,t-MA, u-SPMA and u-cotinine are expressed as a function of creatinine concentration (μg/g creatinine) for simple regression with u-UB. Simple regression between u-t,t-MA, u-SPMA and u-cotinine in the same sample were performed on concentrations expressed in μg/L.

Significant correlations were found between u-SPMA and u-t,t-MA both in all children and in exposed and unexposed SHS groups, with similar equations (u-t,t-MA = 5.703 + 0.601 * u-SPMA for all children, u-t,t-MA = 5.835 + 0.596 * u-SPMA for children unexposed to SHS, and u-t,t-MA = 5.500 + 0.611 * u-SPMA for children exposed to SHS). In addition, in the subgroup of children unexposed to SHS, u-SPMA and u-t,t-MA were most closely correlated to u-cotinine.

Good positive relations were found between u-UB and u-cotinine in all children and in the exposed to SHS group, while for these variables an inverse relationship was found among the unexposed subgroup (u-UB = 5.246 - 0.243 * u-cotinine; p = 0.03). However, the significant inverse relationship was lost when children considered not exposed to SHS with a very high value of u-cotinine (three children with u-cotinine > 10 µg/g creatinine) were excluded from the SHS unexposed group (u-UB = 5.224 - 0.152 * u-cotinine; p = 0.128).

Significant correlations were found between u-SPMA and u-t,t-MA both in all children and in exposed and unexposed SHS groups, with similar equations (u-t,t-MA = 5.703 + 0.601 * u-SPMA for all children, u-t,t-MA = 5.835 + 0.596 * u-SPMA for children unexposed to SHS, and u-t,t-MA = 5.500 + 0.611 * u-SPMA for children exposed to SHS). In addition, in the subgroup of children unexposed to SHS, u-SPMA and u-t,t-MA were most closely correlated to u-cotinine.

Fig. 1 shows the correlations between u-UB and u-cotinine in the samples of children exposed to SHS.

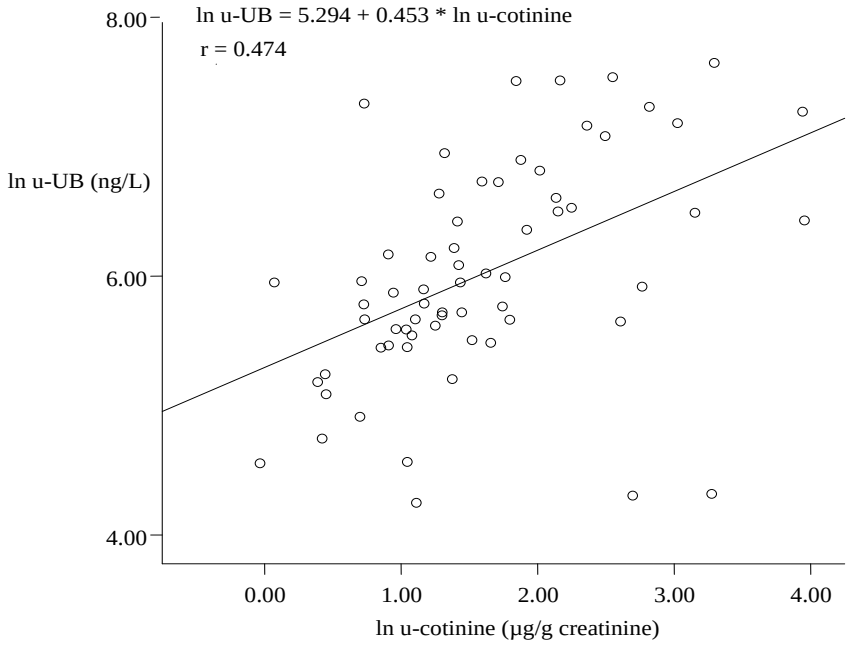

Figure 1. Relationships between urinary unmodified benzene (u-UB) and urinary cotinine (u-cotinine) in samples from children exposed to secondhand smoke (SHS) (number of sample = 65)

Finally, multiple linear regression analyses were run to assess the independent roles of age, gender, urban pollution, and SHS exposure on selected urinary biomarkers (u-UB, u-t,t-MA, and u-SPMA). Table 6 shows unstandardized and standardized regression coefficients, p and R^2 of the final models.

Table 6. Significant predictors of urinary concentration of benzene biomarkers (natural log-transformed data) in forward multiple linear regression models

Dependent variable	Independent variable	B[a]	SE[b]	β[c]	*p*	R^2 of the model
u-UB ng/L[e]	**Constant [d]**	4.684	0.110		< 0.001	0.337
	SHS exposure (exposed)	1.120	0.130	0.568	< 0.001	
	Residence area (urban)	0.685	0.127	0.357	< 0.001	
u-t,t-MA µg/g creatinine[e]	**Constant [d]**	4.170	0.059		< 0.001	0.159
	Residence area (urban)	0.529	0.085	0.404	< 0.001	
u-SPMA µg/g creatinine[e]	**Constant [d]**	-1.698	0.077		< 0.001	0.072
	Residence area (urban)	0.277	0.077	0.257	< 0.001	
	Gender (female)	0.164	0.074	0.153	0.027	
	SHS exposure (exposed)	0.157	0.079	0.143	0.048	

u-UB: urinary unmodified benzene; u-t,t-MA: urinary trans,trans-muconic acid; u-SPMA: urinary S-phenilmercapturic acid.
[a]B = unstandardized regression coefficients.
[b]SE = standard error.
[c]β = standardized regression coefficients.
[d]Constant = estimated intercept value.
[e]Variables considered: Residence area (urban vs. rural), SHS exposure status (exposed vs. unexposed), age (1st, 2nd, and 3rdgrade vs. 4th and 5th grade of primary school), gender (female vs. male).

The results confirmed an association of the u-UB concentrations with both SHS exposure ($\beta = 0.568$, $p < 0.001$) and residence area ($\beta = 0.357$, $p < 0.001$); the applied regression model explained up to 33.7% of the u-UB levels.

With respect to the other benzene biomarkers, u-t,t-MA was dependent only on residence area and explained 15.9% of the variance; in contrast, u-SPMA was associated with residence area, SHS exposure and female gender, even though the

regression model only explained a small fraction of variance of the metabolite (7.2%).

DISCUSSION

The present research studied the validity of u-UB, u-t,t-MA and u-SPMA as biomarkers to assess benzene exposure in children and to study in depth the effects of passive smoking and environmental exposure to low levels of benzene on the results. U-UB, u-t,t-MA and u-SPMA are the three biological markers of benzene exposure considered most suitable for detecting low exposure environmental benzene by experts in the field, but to our knowledge, this is the first study that has compared u-UB, u-t,t-MA and u-SPMA to establish the best biological indices of benzene exposure during childhood.

Urinary levels of benzene biomarkers present a great variability. This finding is in line with data reported in other recent studies on children and general population (Johnson et al. 2007). In our study, levels varied from a low of 36 to high of 2,094 ng/L for u-UB, from 13.76 to 672.83 μg/g creatinine for u-t,t-MA, and from 0.05 to 0.66 μg/g creatinine for u-SPMA, while other researches reported urinary concentrations ranges of 27-2,060 ng/L for u-UB (Waidyanatha et al. 2001), 28-840 μg/g creatinine for u-t,t-MA (Amodio-Cocchieri et al. 2001), and < 2-6 for u-SPMA (van Sittert et al. 1993).

Maximum levels of all biomarkers resulted for "worst cases" (urban children exposed to SHS). However, urinary biomarkers were found in all the analyzed samples, confirming the ubiquitous diffusion of benzene even in rural environments.

Significantly larger values of all analyzed biological indices were observed in children living in urban areas compared to the rural area group (Table 3), as already reported by other authors. Amodio-Cocchieri et al. (2001) observed mean urinary u-t,t-MA levels of 141.2 ± 145.4 μg/g creatinine in children who lived in cities and 109.8 ± 133.2 μg/g creatinine in those living in small towns. Similarly, Ruchirawat et al. (2007) reported that u-t,t-MA was more than twofold higher in Bangkok school

children than in school children living outside Bangkok. With regard to u-SPMA, research conducted in Australia showed a significant difference in metabolite concentrations in the urine of children living in homes situated more than 200 m from a main road compared to children living in homes situated less than 200 m from a main road (3.09 and 9.40 μg/g creatinine, respectively) (Bahrami and Edwards 2006). These results are hardly surprising considering that benzene is a known traffic-related pollutant. It is clear, in fact, that children living in urban areas are exposed to higher levels of air pollutants, such as benzene, than are children in rural areas, who are exposed to much less traffic congestion; however, the comparison of data between urban and rural groups permitted us to confirm the sensitivity of these biomarkers and their suitability for the assessment of environmental benzene exposure at and below ppm levels.

Apart from urban pollution, another important source of benzene exposure during childhood is passive smoking, especially in the household environment. In the univariate analysis, the u-UB levels are similar in urban and rural children exposed to SHS, while the median values of u-t,t-MA and u-SPMA are greater in urban than in rural children. This apparent anomaly could be explained by the strong influence of passive smoke on the u-UB, and its lower impact on u-t,t-MA and SPMA: same statistical analyses, performed on unexposed to SHS, show a significant difference between rural and urban groups, emphasizing the role of residence area on urinary excretion of benzene. Besides, our data show that only u-UB (out of the three biomarkers) distinguished between children exposed and not exposed to SHS both in the urban and rural groups; these results were also confirmed by the significant positive relationship between u-UB and u-cotinine in all samples and in the subgroup exposed to SHS (Table 5).

These results are in agreement with other previous studies. Minoia et al. (1996), for instance, evaluated u-UB as a biomarker of benzene exposure during childhood and found a significant increase of u-UB in the group exposed to SHS compared with unexposed subjects; on the contrary, other research carried out on u-t,t-MA (Weaver et al. 1996; Amodio-Cocchieri et al. 2001) and u-SPMA (Bahrami and

Edwards 2006) has reported no significant differences between children whose parents were smokers versus those who were non-smokers.

The influence of passive smoke exposure - and smoking habits in general - on biological indices of benzene exposure is commonly considered negative, especially when these biomarkers must be used to assess benzene exposure in the workplace. The contribution of smoke to benzene excretion could alter the role of biomarkers as indices of exposure due to chemicals present in the workplace; for this reason, the guidelines and research in the field have judged u-t,t-MA and u-SPMA to be the best biological indices of benzene exposure (ACGIH 2009) and suggested a careful evaluation of smoking exposure when u-UB is used (Barbieri et al. 2008; Lovreglio et al. 2010).

The influence of passive smoke exposure on u-UB excretion and the suitability of u-UB to differentiation between children exposed to SHS and those who were unexposed (as found in our study), might be considered an advantageous characteristic of this biomarker, especially because it could permit one to assess exposure to low concentrations of benzene and SHS exposure at the same time. Today, in addition to the need for a specific biomarker to assess benzene exposure in children, an index of passive smoking exposure is under evaluation (Florescu et al. 2009). In this context, u-cotinine is considered a very sensitive and specific method to assess ETS exposure as cotinine is a major metabolite of nicotine and presents a relatively long plasma half-life (about 20 hours) (Gourlay et al. 1996); our results confirmed this assumption. Despite this, it is noteworthy that u-cotinine concentration must be corrected for creatinine excretion, while benzene can be expressed directly as ng analyte/L of urine without the necessity of additional analysis for adjustment. Moreover, u-UB could represent a good "carcinogen-derived biomarker" of exposure to SHS, specifically related to benzene, because it is a known carcinogen present in tobacco smoke. A review of carcinogen-derived biomarkers and their application in studies of human exposure to SHS examined data on u-t,t-MA as a biomarker of benzene uptake derived from SHS exposure; on the basis of available data, the review concluded that, collectively, "benzene uptake in humans is not consistently associated with SHS exposure" (Hecht 2004). On the

contrary, our results suggest that there is a strong association between human benzene uptake and passive smoking exposure, especially in children. Carcinogen-derived biomarker evaluation might offer not only distinction between individuals exposed and unexposed to passive smoking but also information about the amount of carcinogen actually absorbed into the body and carcinogen metabolism in humans.

An apparent anomalous preliminary result is the significant inverse correlation between u-UB and u-cotinine in children unexposed to SHS. Statistical significance failed when three children with u-cotinine levels > 10 μg/g creatinine were excluded from analysis. These three children presented very low benzene concentrations (< 100 ng/L in all cases) and they were unexposed to SHS in domestic environment according to questionnaire. Against, the same children presented high values of u-cotinine and, even if there isn't an universal cut-off of u-cotinine to distinguish exposed and unexposed to SHS, these levels are probably due to an occasional exposure to passive smoke many hours preceding urine sampling.

The strong association between u-UB and SHS exposure status can be seen also from the results of the final multiple linear regression analysis (Table 6): the most important contributor to u-UB excretion in the present study was SHS exposure status ($\beta = 0.568$, $p < 0.001$). These results can be attributed to the choice of collecting urine samples during the evening, just before bedtime; it is known that unmodified benzene released in urine reflects exposure during the preceding hours because of its relatively short biological half-life in the human body (approximately a couple of hours) (Waidyanatha et al. 2001). Thus, since most of the studied population spent the afternoon and evening of the sampling day at home, probably due to the winter season, benzene intake in those hours was likely due to indoor benzene levels. As a matter of fact, one could assume that it is unlikely that the children in the present study smoke or are exposed to ETS at school, and benzene levels are highest in indoor environments, above all when smokers are present. With regard to the latter issue, the INDEX project of the European Commission (European Commission 2005) found that mean indoor levels of benzene were typically higher than the respective outdoor concentrations all over Europe. In

addition, when tobacco smoke was present in indoor air, the recovered quantity of benzene was two-fold higher than the outdoor levels.

The choice of collecting the urine samples in the evening could explain even the strong relationship of u-t,t-MA with u-SPMA but not with u-UB (Table 5). U-t,t-MA and u-SPMA present longer elimination half-lives than u-UB, but they are comparable with each other (about 5 and 9 h, respectively) (Boogaard and van Sittert 1996). The long elimination half-lives of metabolites could contribute to the lack of sensitivity of u-t,t-MA and u-SPMA as biomarkers of SHS exposure in the following way: the time elapsed since onset of exposure might have been insufficient for the excretion of a significant quantity of metabolites after exposure to passive smoking at home during the afternoon or evening.

The final multiple linear regression models (Table 6) summarize how the weights of residence area, SHS exposure, age and gender explain the variability of all studied analytes.

Unlike previous research (Fustinoni et al. 2005; Manini et al. 2008), we prefer to use the questionnaire as indicator of exposure to SHS both for the different half-life of cotinine and benzene and the reliability of questionnaire to distinguish children exposed and unexposed to SHS.

For u-UB, data show that residence area and SHS exposure are significant contributors to benzene exposure; in particular, SHS exposure represents the most important dependent variable, supporting the conclusion that benzene uptake from ETS is considerably higher than that from outdoor air.

With regard to u-t,t-MA, the selected regression model explained just 15.9% of its variance, and the only statistically correlated variable was residence in an urban area. The use of u-t,t-MA should be carefully evaluated because of its lack of specificity, yet it has been employed by previous studies (Pezzagno et al. 1999; Renner et al. 1999; Weaver et al. 2000; Aprea et al. 2008). In fact, t,t-MA, apart from metabolism of benzene, is also a metabolite of sorbic acid, an antimycotic commonly added to foods such as salad dressing, margarine, mayonnaise, cheese slices and spreads, refrigerated flavored drinks, sweet baked goods, and candy. In Europe, for instance,

its intake has been estimated at 6-30 mg/day (Ruppert et al. 1997). Its use as a biomarker may thus have to be partial; to limit this disadvantage, research on u-t,t-MA in urine samples should always be associated with an investigation of the individual's sorbic acid consumption during the sampling day. This kind of investigation is very difficult, especially when the studied population includes children, because questionnaires on children's dietary sorbic acid intake must be completed by parents. Presumably, parents do not know the kind and the amount of all foods ingested by their children each day.

An interesting result derived from multivariate analysis of u-SPMA was that this biomarker is related to residence in an urban area, SHS exposure and female gender. With respect to the influence of gender on excretion of u-SPMA, previous studies (Bergamaschi et al. 1999; Melikian et al. 2002; Cocco et al. 2003; Aprea et al. 2008) have found gender differences in excretion of u-t,t-MA and u-SPMA and hypothesized that higher excretion by females could be due to a higher blood/air partition coefficient and faster metabolism (> 23-26%) of benzene.

Aprea et al. (2008) observed differences between males and females only when u-t,t-MA concentrations were expressed in μg/g creatinine but not if they were expressed as μg/L. Consequently, the authors suggested that creatininuria is influenced by gender, probably because males have more muscle mass than females. Despite these findings, u-creatinine is an important correction factor for the compounds excreted from the renal pathway, and it is also used for biological limits of u-t,t-MA and u-SPMA reported by international bodies such as the American Conference of Governmental Industrial Hygienists (ACGIH) (Aprea et al. 2008).

All the shown regression models present a not very high adjusted R^2, the explanation could be the variability determined by other individual determinants of exposure, not considered in this study, such as body mass index, genetic polymorphism, etc.

This study has several limitations. First, urine samples were collected only at the end of the sampling day, so we cannot rule out the possibility of changes in biomarker excretion within that time. Second, we did not measure benzene concentrations in the living environments of selected children, so we cannot establish a correlation

between the benzene level in a specific environment and its intake. Third, urine samples were collected by parents just before bedtime and delivered to us the next morning, when samples were frozen. As described in literature (Weisel 2010), refrigeration and freezing phases could involve benzene loss. For this reason, we performed a series of preliminary tests on urine samples from adult donors smokers and no-smokers, stored in refrigerator (0 - +4 °C) for 12 and 24 h, and frozen (-20 °C) until 30 days. Calculated benzene losses resulted in average equal to 2.6% in the worst case (refrigeration for 24 h and freezing for 30 days). Finally, because the studied population was made up of children only, we could not examine associations between sorbic acid intake and excretion of u-t,t-MA.

CONCLUSIONS

In conclusion we found that, using the strategy to collect urine sample at the end of the day, u-UB is resulted the best marker of benzene exposure in children and that it could be used as a good carcinogen derived biomarker of exposure to passive smoking, specifically related to benzene. However, there is a need of further investigations to confirm or not that the good correlation between u-UB and SHS observed in children in this study is hold when urine samples are collected at other times of the day.

As regards to u-t,t-MA and u-SPMA, the high levels found in some children could reflect other sources of benzene out of SHS, other reasons such as genetic polymorphisms and body mass index, or sources of t-t-MA other than benzene.

Finally, it is important to highlight that passive smoking was the most important contributor to benzene exposure (40% of selected children can be considered exposed to SHS). This fact underlines the need for an information campaign discouraging tobacco use, with parents as its main target (Roncarolo et al. 2008).

REFERENCES ARE PRESENTED IN THE GENERAL REFERENCE LIST

CHAPTER 4

URINARY TRANS,TRANS-MUCONIC ACID AND S-PHENYLMERCAPTURIC ACID AS TRACERS OF EXPOSURE TO URBAN BENZENE POLLUTION IN CHILDHOOD

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To be submitted for publication

INTRODUCTION

Ambient air pollution is an established health risk factor worldwide, and it was estimated to be the eighth leading risk factor for mortality, associating with 2.5% of all deaths in high-income countries (Narayan et al. 2010).

Benzene is considered one of the most important airborne pollutants, released to the atmosphere mainly from gasoline vapors, automobile exhaust, chemical production and user facilities (Johnson et al. 2007; ATSDR 2007).

The relevance of benzene is due to its well-recognized link with adverse health effects. Benzene, in fact, has been known as a human carcinogen since 1982 (IARC 1982). These observations conducted to progressively reduce the use of benzene in many production and synthesis processes, and to regulate exposure by the introduction in European Union of standards limits for workplace scenarios (1 ppm or 3.25 mg/m^3) (European Commission 2004b) and general environment (1.5 ppb or 5 μg/m^3) (European Commission 2000, 2008).

Besides, since 1 January 2000 the amount of benzene concentration in fuels was reduced below 1% by volume by European legislation (European Commission 1998). Despite of these revised emission standards, the constant growth in traffic volumes involves the increase of total emissions of many air pollutants, including benzene, both in developed and developing countries (Krzyzanowski et al. 2005, Han and Naeher 2006). Thus, benzene exposure still occurs in general population, and it cannot be neglected in the light of actual scientific evidences on the association between benzene exposure and adverse health effects: it is no possible to determine a safe level of benzene exposure, and all kind of exposures represent a risk for human health (Smith 2010).

At today, one of the main objectives of epidemiological studies performed on this issue is to provide evidences for risk characterization and management of benzene exposure for the general population. The exposure assessment is an essential point - and often the weakest point - of this process (Weisel 2010).

General tools for assessing exposure to airborne benzene include elaboration of air pollution data from monitoring network, personal exposure monitoring, exposure

modeling, and human biomonitoring (Johnson et al. 2007; ATSDR 2007). The last cited method is the most significant health-related assessment tool for evaluating exposure to chemicals present in the environment, because it provides an actual measure of the chemical that gets into the body (CDC 2009). Besides, the application of human biomonitoring is more motivated by its recent adoption as Action 3 in the Environmental Action Plan 2004 - 2010 of the European Commission (European Commission 2004a).

An important debated question related to the biomonitoring of chemicals is the need to routinely collect human samples for large-scale surveys. For this purpose, non-invasively matrices is preferable, especially when the study population is constituted by most susceptible subjects such as children. Spot urine sampling is the most widely method to quantify exposure to environmental pollutants, because it represents a non-invasively matrix that permit sampling easily to repeat, with no risk, and without major ethical or practical limitations (Esteban and Castaňo 2009; Smolders et al. 2009). On the other hand, an undoubted disadvantage of urine samples is the possibility of variations in the volume and concentration of urine. Consequently, there is the necessity to adopt techniques to compensate differences in urine dilutions, i.e. taking into account their osmolality, gravity, relative density or creatinine content (Smolders et al. 2009).

Several studies were conducted on adults not occupationally exposed to benzene. The authors suggested the use of urinary trans,trans-muconic acid (u-t,t-MA) normalized for gram of creatinine, urinary S-phenylmercapturic acid (u-SPMA) normalized for gram of creatinine, and urinary unmodified benzene (u-UB) as biomarkers of benzene exposure (Waidyanatha et al. 2001; Fustinoni et al. 2005, 2010, 2012; Johnson et al. 2007; Barbieri et al. 2008; Lovreglio et al. 2010, 2011; Weisel et al. 2010; Campo et al. 2011). However, the feasibility of these biomarkers to discriminate different levels of urban benzene exposure - especially at very low airborne concentrations - is still under evaluation. One of the most stressed consideration is the possible influence of the active and/or passive smoking on the results: tobacco smoke, in fact, is another important source of benzene exposure (Johnson et al. 2007; Weisel 2010).

All the studies cited above were performed on adults. Since children cannot be considered "little adults" with regard nor to the risk of adverse health effects resulting from exposure at an early age and neither for the choice of the exposure assessment tools (Anderson et al. 2000), the results of "adults" researches cannot be used to estimate children exposure. For example, a detailed assessment on u-creatinine excretion of general population evidenced significant differences related to age, gender and ethnicity (Barr et al. 2005). Consequently, adults exposure profiles to chemicals obtained by values corrected for u-creatinine cannot be representative for children.

In our knowledge, few publications are available in literature on the assessment of benzene exposure by biomarkers monitoring in childhood (Minoia et al. 1996; Weaver et al. 1996; Amodio-Cocchieri et al. 2001; Kouniali et al. 2003; Bahrami and Edwards; 2006; Ruchirawat et al. 2007; Protano et al. 2010, Protano et al. 2011). Among the cited studies only our investigation (Protano et al. 2010) evaluated, in the same study population, the ability of u-UB, u-t,t-MA, and u-SPMA for this purpose. The results of this study showed that all investigated biomarkers were suitable assessment tools for benzene exposure in childhood: u-UB was mainly influenced by environmental tobacco smoke (ETS), and could be used as a good biomarker to predict ETS-derived benzene exposure levels, while u-t,t-MA and u-SPMA (both normalized for gram of creatinine) were lower affected by ETS and were able to discriminate differences in exposure to air pollution in children.

The aims of the present study were

To evaluate the feasibility of u-t,t-MA and u-SPMA as tracers of urban benzene pollution for human biomonitoring studies performed on children

To investigate the impact that the creatinine correction may have on classifying exposure status of children in the evaluation of benzene exposure by u-t,t-MA and u-SPMA.

MATERIALS AND METHODS

Study area

The research was conducted in three areas of central Italy whose urbanization characteristics allowed us to classify them as follows: very urban, fairly urban and non urban. The choice of the areas was based on some relevant urbanization indicators (resident population, population density, green area density, motorization rate for autovehicles and two wheeled vehicles) from national databases (National Institute of Statistics, Italian Automobile Club). In Table 1 are reported urbanization indicators data for each selected area in the years of the monitoring campaign.

Table 1. Summary information on relevant urbanization indicators of the three study areas in the year of monitoring campaigns (2007-2009)

	Resident population (n)	Population density (persons per km^2)	Green area density (% of total municipal territory)	Motorization rate	
				Number of autovehicles per 100 inhabitants	Number of two wheeled vehicles per 100 inhabitants
Very urban	2,743,796	2,098	< 85	69	15
Fairly urban	32,886	395	< 85	76	10
Non urban	3308	120	> 85	66	8

Besides, areas were selected on the basis of the airborne concentrations of some indicators of urban air pollution available from the environmental monitoring program performed by the Regional Environmental Protection Agency (ARPALAZIO): Carbon Monoxide (CO) and Nitrogen Dioxide (NO_2). Figure 1 shows the trends in the levels of CO and NO_2 in the three selected areas for the years 1999-2010.

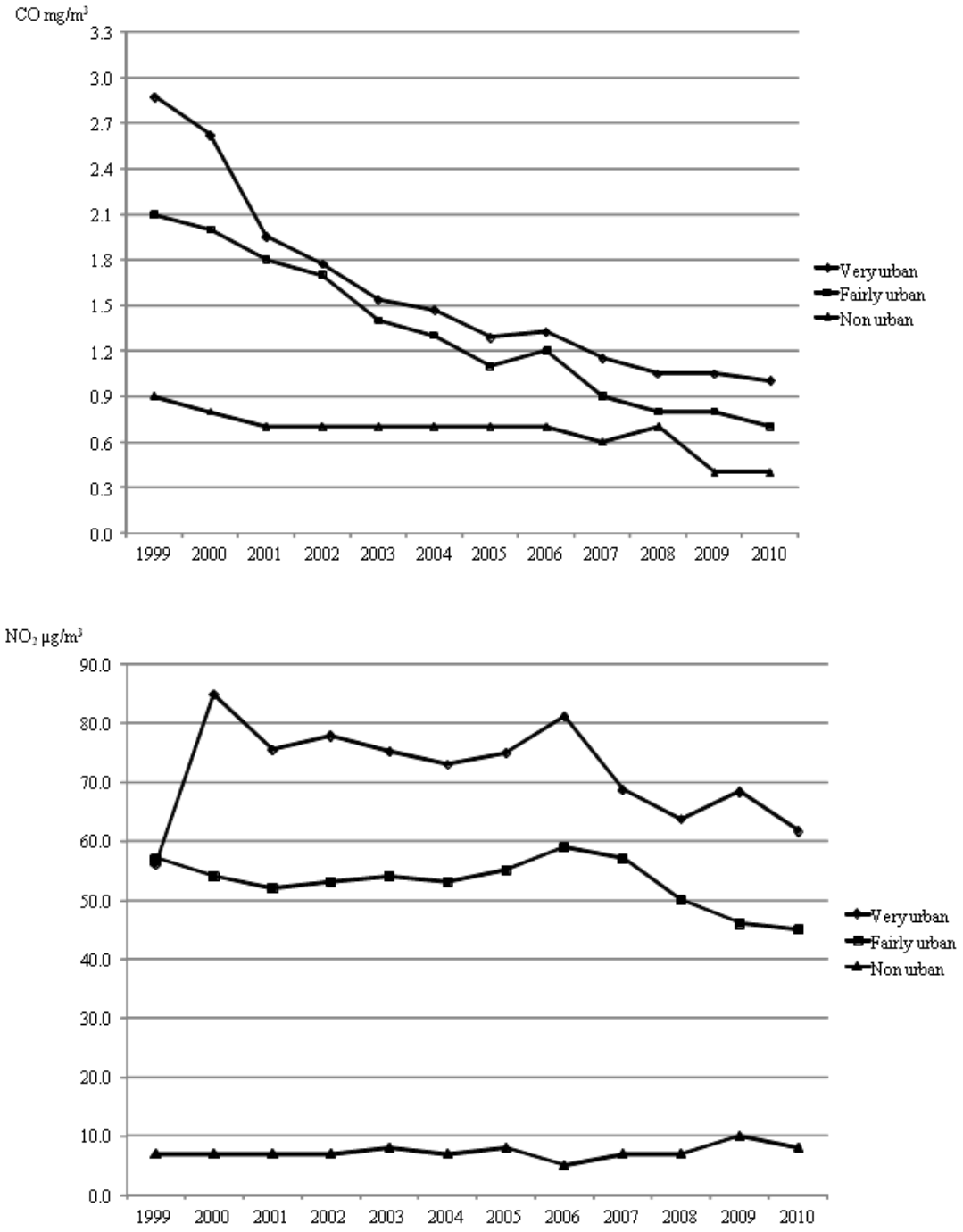


Figure 1. Temporal trend of monoxide carbon (CO) and nitrogen oxide (NO_2) levels monitored by local regulatory agency (ARPALAZIO) in the three selected area (very urban, fairly urban, non urban area). Years 1999 - 2010

Study population and design

The study population consisted of all of the students (655 children aged 5-11 years) frequenting three primary-school districts.

All of the students and their parents received information about the research goals and plans and were invited to take part in the cross-sectional study, which was conducted on Wednesdays (a typical weekday) during the winter seasons of the academic years 2007-08 and 2008-09.

Detailed information about socio-demographic characteristics, activities engaged in on the sampling day, lifestyle factors, and living environment, with particular reference to household characteristics, of the investigated subjects was obtained from a questionnaire completed by their parents. Formation meetings for all children and their parents on the modalities to compile the questionnaire and to collect and store urine sample were carried on just before sampling days.

One urine sample for each participant was collected in the evening (just before go to sleep) in a benzene-free polypropylene bottle with hermetic closure, and immediately stored in the refrigerator at 4°C. The next morning, the sample was placed into a polystyrene cooler containing an ice pack and was delivered to the research team.

Covariates gathered by questionnaire

The answers about the gender of the child and the age (as defined by the primary-school grade they were attending), were classified as 0 = male and 1 = female and as 0 = 1st, 2nd, or 3rd grade (the younger group) and 1 = 4th or 5th grade (the older group).

The practices of sport activities by participants during the sampling day were gathered using a simple Yes/No questions, then categorized as 0 = No and 1 = Yes.

With respect to the characteristics of the home environment, we asked about the home typology (detached/semi-detached unit housing or attached multi-unit housing) and the type of heating device used (open answer).

The questionnaire examined also other characteristics associated with potential sources of urban benzene in proximity of the home in which the participants live by using the questions "Are there petrol stations close to home?", "Are there factories close to home?" "Are there parking areas close to home?". The possible responses to each these questions were "Yes" and "No", then categorized as 0 = No and 1 = Yes.

Finally, the respondents were asked some questions about road traffic features close to home, with the following questions: "Is there traffic queues close to home?" "Is there passing of buses close to home?" "Is there passing of trucks close to home?". Possible answers to all these questions were "Frequently" or "Rarely/Not at all". The last question about road traffic in proximity of the home was "How intense is traffic close to home?" Possible responses were "No/Low" or "Moderate/High".

The presence of cohabitants smokers was assessed with the question "Are there smokers living with the child?" The possible responses were "Yes" and "No" (categorized as 0 = No and 1 = Yes). If the response was "Yes", the child was considered to be exposed to ETS.

Biological monitoring and analytical determinations

Spot urine samples were prepared and analyzed using a methodology that has been previously described and extensively used in previous publications (Manini et al. 2008; Protano et al. 2010).

Briefly, about 2 mL of urine for each sample was partitioned into multiple plastic tubes for u-t,t-MA, u-SPMA, and u-creatinine determinations. All samples were coded and then frozen at -20°C until analysis.

u-t,t-MA and u-SPMA were determined by isotopic dilution liquid chromatography tandem mass spectrometry (LC-MS-MS) using a PE-Sciex API 365 triple-quadrupole mass spectrometer (Applied Biosystems, Thornhill, Canada) equipped with a Ionspray interface for pneumatically assisted electrospray (ESI). U-Creatinine was determined by the method of Jaffé (Henry, 1974).

Samples were analyzed within 30 days from sampling.

Statistical analyses

Statistical analyses were carried out using SPSS software (version 14.0 for Windows, Chicago, IL).

Firstly, the normality of the distribution of analytes concentration was assessed by the one-sample Kolmogorov-Smirnov test: u-creatinine concentrations were normally distributed, while u-t,t,-MA and u-SPMA followed a normal distribution after natural log-transformation (both unadjusted or normalized data). Therefore, the statistical elaboration related to u-t,t,-MA and u-SPMA were performed with parallel analyses using non-parametric techniques (Mann-Whitney and Kruskal-Wallis tests) and corresponding parametric methods on natural log-transformed data (t-test for independent variables and one-way analysis of variance (ANOVA) with Bonferroni post-hoc tests). Simple linear regression analyses were run to assess the relationships between urinary analytes.

Finally, two forward multiple linear regression analyses for each metabolite were run to test the independent role of several variables - gender, age, degree of urbanization of residence area (dummy variable taking non urban area group as reference category), practice of sport during the sampling day, ETS exposure status - on natural-log transformed values of metabolites. In the first model, the dependent variable was the metabolite expressed as μg/g creatinine, while in the second model the dependent variable was the unadjusted metabolite, while u-creatinine concentration was included in the multiple regression as an independent variable, as suggested by Barr et al. (2005). The significance level for all tests was $p \leq 0.05$ (two-tailed). Linear regression analyses were run using a significance level of 0.05 for entry and 0.10 for removal from the model. The "goodness of fit" of the model was assessed using R^2 statistics.

RESULTS

In total, 501 out of 665 children took part in the research (response rate of 75%). However, 46 urine samples were rejected because of unsatisfactory sealing of sample containers; therefore, analytical determinations of t,t-MA, u-SPMA and u-

creatinine were performed for 455 samples. In addition, 59 children who had at least one parent who was not Italian were excluded from the data analysis to avoid interference from possible ethnic differences in the metabolism of benzene (US EPA, 1998). In the end, the analysis was conducted using the data of 396 children.

Descriptive characteristics of the studied subjects are presented in Table 2.

Table 2. General characteristics of study population (total sample and three groups distinguished according to the degree of urbanization of residence area)

		Degree of urbanization of residence area		
	Total children	**Very urban**	**Fairly urban**	**Non urban**
Gender n (%)				
Male	197 (52.2)	88 (52.6)	53 (52.3)	56 (51.3)
Female	180 (47.8)	81 (47.4)	48 (47.7)	51 (48.8)
Grade of primary school n (%)				
1st - 2nd - 3rd	212 (55.9)	90 (52.9)	63 (58.9)	59 (57.8)
4th 5th	167 (44.1)	80 (47.1)	44 (41.1)	43 (42.2)
Practice of sport activities during sampling day n (%)				
Yes	132 (34.1)	54 (32.1)	51 (45.9)	27 (25.0)
No	255 (65.9)	114 (67.9)	60 (54.1)	81 (75.0)
Home type n (%)				
Detached/Semi-detached unit housing	199 (52.1)	22 (13.5)	72 (65.5)	105 (96.3)
Attached Multi-unit housing	183 (47.9)	141 (86.5)	38 (34.5)	4 (3.7)
Factories close to home n (%)				
Yes	119 (31.2)	78 (47.6)	25 (22.9)	16 (14.7)
No	263 (68.8)	86 (52.4)	84 (77.1)	93 (85.3)
Petrol station close to home n (%)				
Yes	96 (25.0)	65 (38.7)	17 (15.6)	14 (13.1)
No	288 (75.0)	103 (61.3)	92 (84.4)	93 (86.9)
Parking close to home n (%)				
Yes	142 (37.0)	93 (55.0)	29 (26.9)	20 (18.7)
No	242 (63.0)	76 (45.0)	79 (73.1)	87 (81.3)
Traffic queues close to home n (%)				
Frequently	65 (17.0)	47 (28.1)	15 (13.9)	3 (2.8)
Rarely/Not at all	317 (83.0)	120 (71.9)	93 (86.1)	104 (97.2)
Truck passing close to home n (%)				
Frequently	94 (24.3)	40 (23.8)	21 (19.3)	33 (30.0)
Rarely/Not at all	239 (75.7)	128 (76.2)	88 (80.7)	77 (70.0)
Buses passing close to home n (%)				
Frequently	107 (27.7)	76 (45.2)	15 (13.8)	16 (14.7)
Rarely/Not at all	209 (72.3)	92 (54.8)	94 (86.2)	93 (85.3)
Traffic density close to home n (%)				
Moderate/High	189 (49.0)	122 (72.6)	46 (42.2)	21 (19.3)
No/Low	197 (51.0)	46 (27.4)	63 (57.8)	88 (80.7)
Environmental Tobacco Smoke (ETS) exposure status n (%)				
Yes	172 (45.1)	88 (52.7)	26 (24.1)	59 (56.0)
No	209 (54.9)	79 (47.3)	82 (75.9)	47 (44.0)
Time (min) spent in different environments during sampling day until urine collection (Mean ± SD)				
Time at school	447.1 ± 71.8	448.1 ± 73.3	461.5 ± 38.4	429.3 ± 90.4
Time in indoor environments	321.7 ± 148.9	380.9 ± 169.8	240.1 ± 83.9	290.0 ± 102.3
Time in outdoor environments	66.00 ± 91.7	78.9 ± 111.6	58.5 ± 49.3	47.2 ± 68.5
Time in motor vehicles	18.7 ± 35.5	12.1 ± 26.1	21.7 ± 21.3	29.4 ± 52.0

The three groups were comparable with respect to gender, age and time spent in indoor and outdoor environments, while it is notably a wide range of time spent in

motor vehicles on the sampling day between subjects, especially in non urban children (mean ± standard deviation = 29.4 ± 52.0 min). Apart from the school, the most common activity carried out by participants during the sampling day was the sport (34.1%), while a very few number of children were engaged in other activities, such as foreign language or music school.

In addition, Table 2 shows that the living environments' characteristics of participants were typically related to the degree of urbanization of residence area. First of all, the main typology of very urban and non urban houses was, respectively, a housing unit in a building and a detached or semi-detached unit housing. Likewise, the percentage of petrol stations, factories, parking area, frequently traffic queues, buses passing, and moderate/high traffic density close to home increases in parallel with the degree of urbanization of residence area. Even, the frequency of the category "high" of the variable "traffic density close to home" was 25.0% for children living in very urban area, 10.1% for those living in fairly urban area, and 1.8% for those living in non urban area.

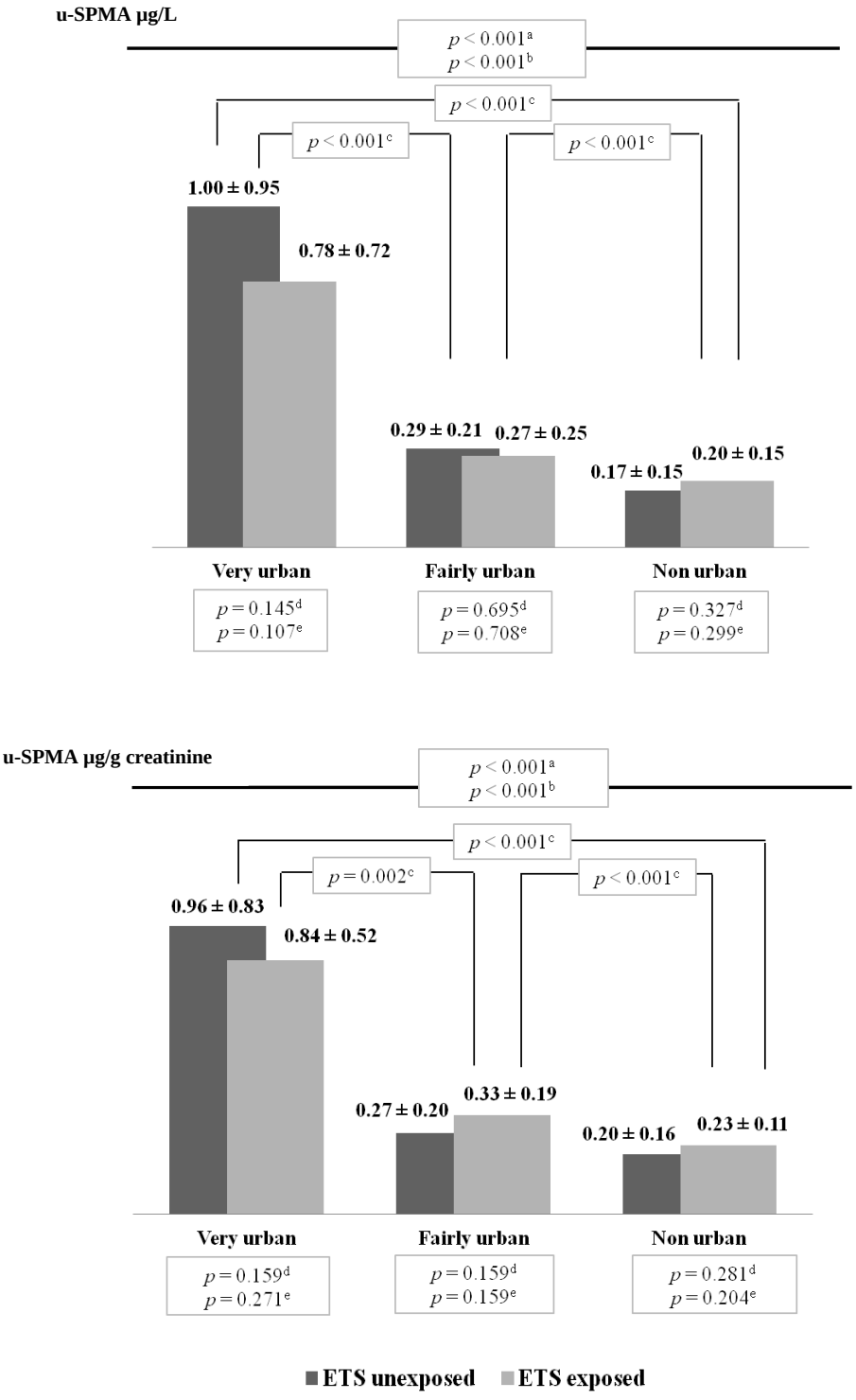
The only exception to these results is the frequency of trucks passing near the participants' domicile: 30.0, 23.8 and 19.3% in non urban, in fairly urban and very urban area.

Descriptive statistics of the metabolites u-SPMA and u-t,t-MA levels (unadjusted and as μg/g creatinine) of all children are reported in Table 3.

Table 3. Summary of descriptive statistics of urinary levels of S-phenylmercapturic (u-SPMA) and trans,trans-muconic acid (u-t,t-MA) in participants (total sample)

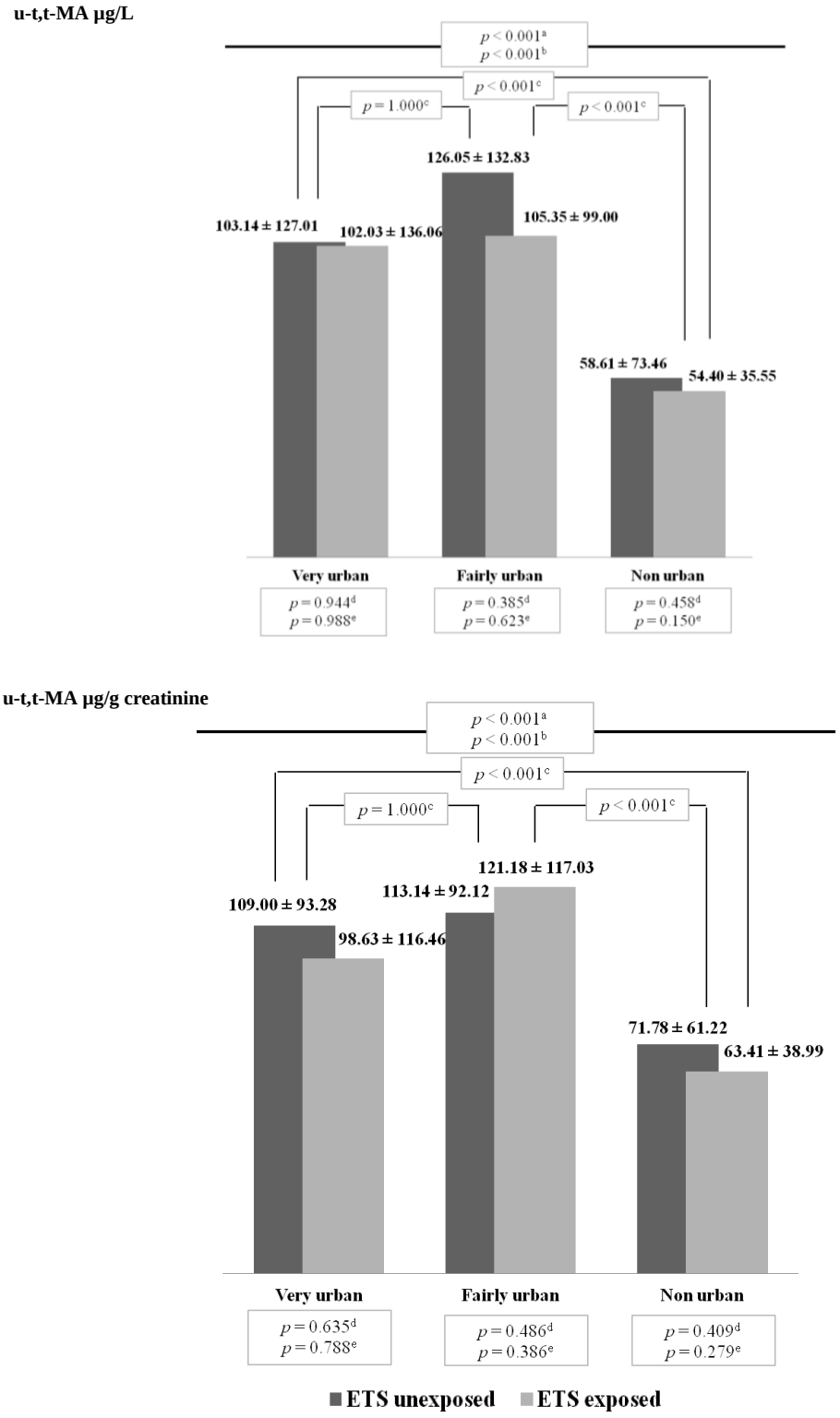
		SPMA μg/l	SPMAμg/g creatinine	t,t-MA μg/l	t,t-MA μg/g creatinine
N.		396	395	396	395
Mean		0.59	0.62	127.10	127.59
Standard Deviation		0.57	0.56	126.68	123.12
Minimum		0.03	0.06	5.50	13.76
Maximum		3.20	4.35	1159.70	972.918
Range		3.17	4.29	1154.20	959.16
Geometric Mean		0.40	0.44	88.25	96.03
Percentiles	5	0.10	0.11	20.87	30.78
	10	0.12	0.15	31.58	40.02
	25	0.21	0.23	50.80	59.39
	50	0.38	0.40	87.50	90.15
	75	0.78	0.84	159.90	149.29
	90	1.41	1.37	265.78	230.54
	95	1.82	1.83	358.26	363.47

Figure 2 (a and b) shows the distributions of median levels of u-SPMA and u-t,t-MA according to the degree of urbanization of residence areas and the ETS exposure status. In the same Figure are reported the results of univariate analyses performed to evaluate the impact of the degree of urbanization of residence area and ETS exposure (considering the children ETS-exposed and -unexposed of each of the three residence areas) on u-SPMA and u-t,t-MA levels.



[a]Kruskal-Wallis test was used to compare between groups according to the degree of urbanization of residence area
[b]One-way analysis of variance (ANOVA) with Bonferroni post hoc-tests was use to compare between groups according to the degree of urbanization of residence area (ln-data)
[c]Bonferroni post hoc tests
[d]Mann–Whitney U-test was used to compare exposed and unexposed to ETS
[e]Unpaired t-test was used to compare exposed and unexposed to ETS (ln-data)

Figure 2a. Urinary levels (median ± interquartile range) of S-phenylmercapturic (u-SPMA) in children differentiated according to the degree of urbanization of residence area and Environmental Tobacco Smoke (ETS) exposure



[a]Kruskal-Wallis test was used to compare between groups according to the degree of urbanization of residence area
[b]One-way analysis of variance (ANOVA) with Bonferroni post hoc-tests was use to compare between groups according to the degree of urbanization of residence area (ln-data)
[c]Bonferroni post hoc tests
[d]Mann–Whitney U-test was used to compare exposed and unexposed to ETS
[e]Unpaired t-test was used to compare exposed and unexposed to ETS (ln-data)

Figure 2b. Urinary levels (median ± interquartile range) of trans,trans-muconic acid (u-t,t-MA) in children differentiated according to the degree of urbanization of residence area and Environmental tobacco smoke (ETS) exposure

u-SPMA concentrations significantly increased in parallel to the increasing degree of urbanization of residence area, following sequence among the groups: non urban group (median = 0.19 μg/L; 0.22 μg/g creatinine) < fairly urban group (median = 0.28 μg/L; 0.28 μg/g creatinine) < very urban groups (median = 0.92 μg/L; 0.90 μg/g creatinine).

The results related to u-t,t-MA levels differ respect to those derived from u-SPMA concentrations: highest concentrations of u-t,t-MA were found for fairly urban group (median = 120.60 μg/L or 116.57 μg/g creatinine), medium concentrations for very urban group (median = 100.30 μg/L or 102.54 μg/g creatinine), and minimum concentrations for non urban one (median = 55.37 μg/L or 66.36 μg/g creatinine). These results were not significant at all: Bonferroni post-hoc tests showed that differences existed only between very urban versus non urban group ($p<0.001$) and fairly urban versus non urban group ($p<0.001$).

As regard to ETS exposure status, u-SPMA and u-t,t-MA levels of participants living in very urban, fairly urban or non urban area were not influenced by passive smoking.

Additional univariate analyses were conducted to evaluate the possible differences in u-SPMA and u-t,t-MA levels due to gender, age, characteristics of household environment, activities of child during the sampling day and in the last year, and other possible indoor sources. All the statistical elaborations were performed separately for the three areas, and all the variables resulted not significant predictors both for u-SPMA and u-t,t-MA (data not shown).

Table 4 shows the results of simple regression analyses among analytes for all of the investigated children and for children classified according to degree of urbanization of their residence area.

Table 4. Simple regression analyses between urinary biomarkers in all sample and in children according to degree of urbanization of residence area

		Indipendent variable	
	Dependent variable	**u-t,t-MA**	**u-creatinine**
All children	**u-SPMA**	y= -3.047 + 0.462 * x *p < 0.001 (n = 396)*	y= - 1.542 + 0.328 * x *p < 0.001 (n = 395)*
	u-t,t-MA		y= 3.545 + 0.507 * x *p < 0.001 (n = 395)*
Very urban	**u-SPMA**	y = - 1.447 + 0.376 * x *p < 0.001 (n = 173)*	y = - 0.815 + 0.488 * x *p < 0.001 (n = 172)*
	u-t,t-MA		y= 3.894 + 0.418 * x *p < 0.001 (n = 172)*
Fairly urban	**u-SPMA**	y = -2.722 + 0.480 * x *p < 0.001 (n = 111)*	y = - 1.767 + 0.442 * x *p < 0.001 (n = 111)*
	u-t,t-MA		y = 3.671 + 0.605 * x *p < 0.001 (n = 111)*
Non urban	**u-SPMA**	y = -3.299 + 0.554 * x *p < 0.001 (n = 112)*	y = - 2.348 + 0.547 * x *p < 0.001 (n = 112)*
	u-t,t-MA		y = 3.143 + 0.520 * x *p < 0.001 (n = 112)*

u-SPMA: urinary S-phenilmercapturic acid; u-t,t-MA: urinary trans,trans-muconic acid.
The concentrations of u-SPMA and u-t,t-MA were ln-transformed.

Significant correlations were found between u-t,t-MA, u-SPMA and u-creatinine both for all group and for children living in very urban, fairly urban and non urban areas, with similar equations.

The results of three multiple linear regression analyses are reported in Table 5.

Table 5. Significant predictors of urinary concentration of S-phenilmercapturic acid (u-SPMA) and trans,trans-muconic acid (u-t,t-MA) as natural log-transformed data expressed as μg/L and μg/g creatinine in forward multiple linear regression models

Independent variable		B[a]	SE[b]	β[c]	*p*	Adjusted R^2 of the model
Model 1 - u-SPMA μg/g creatinine[d]						
	Constant[e]	-1.492	0.055	-	<0.001	
Degree of urbanization of residence area	Non urban	Reference	-	-	-	0.616
	Fairly urban	0.263	0.076	0.137	0.001	
	Very urban	1.452	0.067	0.848	<0.001	
Practice of sport activity during sampling day	Yes	-0.159	0.060	-0.088	0.009	
Model 2 - u-SPMA μg/L[f]						
	Constant[e]	-2.264	0.72	-	<0.001	
Degree of urbanization of residence area	Non urban	Reference	-	-	-	0.681
	Fairly urban	0.326	0.073	0.163	<0.001	
	Very urban	1.502	0.064	0.837	<0.001	
	u-creatinine	0.567	0.055	0.314	<0.001	
Model 3 - u-t,t-MA μg/g creatinine[d]						
	Constant[e]	4.427	0.049		<0.001	
Degree of urbanization of residence area	Non urban	Reference	-	-	-	0.110
	Fairly urban	0.533	0.095	0.336	<0.001	
	Very urban	0.529	0.085	0.373	<0.001	
Model 4 - u-t,t-MA μg/L[f]						
	Constant[e]	3.162	0.099		<0.001	
Degree of urbanization of residence area	Non urban	Reference			-	0.358
	Fairly urban	0.564	0.101	0.289	<0.001	
	Very urban	0.568	0.089	0.325	<0.001	
	u-creatinine	0.856	0.076	0.587	<0.001	

[a]B = unstandardized regression coefficients.
[b]SE = standard error.
[c]β = standardized regression coefficients.
[d]Variables considered: degree of urbanization of residence area (dummy variables: non urban rural area as reference category, fairly urban area, very urban area), gender (female vs. male), age (1st, 2nd, and 3rdgrade vs. 4th and 5th grade of primary school), ETS exposure status (ETS exposed vs. ETS unexposed), practice of sport activity during sampling day (yes vs. no).
[e]Constant = estimated intercept value.
[f]Variables considered: degree of urbanization of residence area (dummy variables: non urban rural area as reference category, fairly urban area, very urban area), gender (female vs. male), age (1st, 2nd, and 3rdgrade vs. 4th and 5th grade of primary school), ETS exposure status (ETS exposed vs. ETS unexposed), practice of sport activity during sampling day (yes vs. no), u-creatinine.

First of all, multivariate analyses show the significant independent role of the degree of urbanization of residence area on the metabolites levels, previously showed by univariate analysis for u-SPMA and new-evidenced for u-t,t-MA. Secondly, an interesting result emerged from the model run for u-SPMA μg/g creatinine: the practice of a sport during the sampling day was a significant predictor of u-SPMA levels.

The regression models explained up to 35.8% of the variance of u-t,t-MA levels (unadjusted) or 11.0% (μg/g creatinine), and up to 68.1% (unadjusted) or 61.6% (μg/g creatinine) of the variance of u-SPMA levels.

DISCUSSION

Human biomonitoring of exposure is a mandatory method to evaluate personal exposure to air pollution, and to provide data on profile exposure of general population to support environmental and public health policies. Given the well-known differences of exposure to air pollution between children and adults in terms of magnitude of exposure and susceptibility to adverse effects, it is necessary to perform separate studies for the assessment of exposure to air pollutants for children and adults.

Despite a great number of researches performed on urban benzene exposure for adult population is available, very few data are available for children.

The first objective of the present research was aimed to evaluate the feasibility of u-SPMA and u-t,t-MA as tracers for assessing urban benzene exposure in a large sample of Italian children.

First of all, a great variability of studied metabolites concentrations was found, in line with other data available in literature for general population - both adults and children (Amodio-Cocchieri et al. 2001; Bahrami and Edward 2006; Johnson et al. 2007; Ruchirawat et al. 2007).

However, the concentrations of u-SPMA and u-t,t-MA were significantly higher in children living in the fairly/very urban areas respect to those living in no urban area.

As regard to u-SPMA, levels increase following the increase of the degree of urbanization of residence area:

- u-SPMA levels of children living in very urban area are, on average, 3.3 and 4.8 times higher than those of children living in fairly and non urban area, respectively;
- u-SPMA levels of children living in fairly urban area are, on average, 1.5 times higher than those of children living in non urban area.

According to the assumption that the degree of urbanization can be used as a proxy indicator for estimating exposure to traffic-related air pollutants (Rijnders et al. 2001), the findings of the study demonstrate that u-SPMA (unadjusted or μg/g creatinine) is able to discriminate differences in airborne benzene exposure of children, even in typical condition of general population exposure (very low concentrations of benzene).

As regard to u-t,t-MA, it is notable that differences in levels of excretion related to the degree of urbanization of residence area in children living in very urban and fairly urban areas were revealed only by the multivariate analyses. The lack of specificity of u-t,t-MA in adult population exposed to very low concentration of urban benzene was demonstrated by previous researches (Pezzagno et al. 1999; Renner et al. 1999; Weaver et al. 2000; Aprea et al. 2008; Weisel 2010; Lovreglio et al. 2011). The most common explanation of this result is the influence of the diet on u-t,t-MA excretion: t,t-MA is also a metabolite of sorbic acid, a common food additive for fruit juices, candies, etc, and the ingestion of sorbic acid could result more relevant in the increases of u-t,t-MA respect to sub-ppm exposure to airborne benzene. This reason is plausible as well as for adults and children; even, if the research on t,t-MA in urine samples of adults for monitoring benzene exposure could be associated with the examination of the individual consumption of sorbic acid during the sampling day, this kind of investigation results very hard when the study population is represented by children. It could be really difficult that a child recalls accurately what he ingested during a day.

The other important source of benzene exposure for general population not

occupationally exposed to benzene is tobacco smoke. It has been estimated that the benzene daily intake derived from the smoking habit is up to 85% of total benzene exposure, while the contribution of ETS to benzene exposure is approximately equal to 23% of total benzene exposure (Fruin et al. 2001; Weisel 2010). Different studies performed on adults exposed to very low concentrations of benzene evidenced the influence of active smoking on excretion of u-SPMA and u-t,t-MA (Fustinoni et al. 2005; Johnson et al. 2007; Manini et al. 2008; Lovreglio et al. 2011); the present study evidences no significant differences in children ETS-exposed respect to ETS-unexposed, according to the results of other previous researches performed to assess benzene exposure in children (Weaver et al. 1996; Amodio-Cocchieri et al. 2001; Bahrami and Edward 2006). These findings contribute to consider u-SPMA and u-t,t-MA as good biomarkers for assessing urban benzene exposure in childhood. However, the results of the present research should be interpreted in the light of the time of urine sample collection and the times of benzene metabolism: spot urine samples were collected in the evening, just before bedtime, and the elimination half-lives of u-t,t-MA and u-SPMA are about 5 and 9 hours, respectively (Boogaard and van Sittert 1996). Thus, it is comprehensible that the quantity of the metabolites found in the urine samples derived mainly from benzene taken into the body many hours before the sample collection. Considering that the major source of ETS exposure for children is the domestic environments (WHO 2007), and that the participants stayed at home during the afternoon or evening of the sampling day, the time elapsed since the intake of benzene from ETS exposure might have been insufficient for the formation of the related metabolites and their urinary excretion. It would be interesting to compare the results of analytical determination performed on urine samples of the same children collected at the end of a day and at the next morning.

The second objective of the present study was to investigate the role of the creatinine correction to normalize urinary values of the studied metabolites in children population.

Traditionally u-creatinine-corrected u-SPMA and u-t,t-MA were used as biomarkers of benzene exposure both in occupational and non occupational scenarios

(Waidyanatha et al. 2001; Fustinoni et al. 2005; Johnson et al. 2007; Barbieri et al. 2008; Lovreglio et al. 2010, 2011; Weisel 2010). Even, u-creatinine correction is also used for the biological exposure indices for u-t,t-MA and u-SPMA reported by international bodies such as the American Conference of Governmental Industrial Hygienists (ACGIH 2009). Despite of this, previous studies (Aprea et al., 2008; Protano et al. 2010) observed a significant influence of gender on benzene biomarkers concentrations, suggesting that the results could be distorted by the relationship between gender and creatininuria. Besides, other authors suggested that actual concentration of biomarkers of benzene exposure is a more appropriate metric to report data, because creatinine excretion rate of active subjects is not constant during the course of a day (Weisel 2010)

Thus, the results of all statistical elaborations performed on the two benzene biomarkers in the present study were presented both unadjusted and corrected for u-creatinine. As regard to the univariate analyses, results show a similar trend in the levels of metabolites, either expressed as unadjusted or as μg/g creatinine. As regard to the multiple linear regression analyses, an interesting result derives from the two models including u-SPMA as dependent variable, the first performed on creatinine-corrected u-SPMA levels, and the other carried out on unadjusted u-SPMA levels adding u-creatinine as an independent variable. In the first model, the practice of a sport activity during the sampling day resulted a significant predictor of u-creatinine-corrected u-SPMA levels (inverse relationship of the variables), while in the second model the same independent variable has not an independent role on unadjusted u-SPMA levels. This is the practical demonstration of the possibility to alter the results when an independent variable is unrelated to the chemical concentration itself, but related to the u-creatinine levels, in line with the considerations reported by Barr et al. (2005). Most likely, the practice of a sport activity during the sampling day does not reduce the u-SPMA levels, but increases the concentrations of u-creatinine after some hours respect to the practice of the sport. This last assertion is confirmed by the significant increase of creatininuria in children after two hours from the end of a physical exercise (Turgut et al. 2003).

Another interesting result is the positive influence of u-creatinine on both the

metabolites, just evidenced in recently studies on benzene biomarkers of exposure performed on adults (Fustinoni et al. 2010, 2012; Manini et al. 2010, Campo et al. 2011).

This study present some limitations. Firstly, only one spot urine sample was collected at the end of the sampling day. Consequently, we could not evaluate possible changes in biomarker excretion within the time. Secondly, benzene concentrations in the living environments of selected children were not measured, and air monitoring programs performed by local regulatory agency (ARPALAZIO) did not include the assessment of airborne benzene concentrations for all the selected areas; however, the temporal trends in the levels of CO and NO_2 demonstrated the differences in urban air pollution in the selected areas. Third, we could not examine the contribution of sorbic acid intake on excretion of u-t,t-MA. Finally, some possible individual determinants of exposure to benzene such as body mass index or genetic polymorphism were not evaluated in this study.

CONCLUSIONS

In conclusion, using the strategy to collect urine samples at the end of the day, both u-SPMA and u-t,t-MA were able to assess urban benzene exposure in childhood, even if u-SPMA should be taken in higher consideration because u-t,t-MA confirmed its less specificity for benzene exposure in the magnitude of sub-ppm exposures (general population scenario).

In addition, it is important to highlight that, in order to avoid the possible confounding effect of the creatinine correction, we think that it should be more correct to use u-creatinine as additional independent variable in multiple linear regression analyses performed to evaluate the independent role of covariate on the variability of u-t,t-MA and u-SPMA levels.

REFERENCES ARE PRESENTED IN THE GENERAL REFERENCE LIST

CHAPTER 5

A TOBACCO-RELATED CARCINOGEN: ASSESSING THE IMPACT OF SMOKING BEHAVIOURS OF COHABITANTS ON BENZENE EXPOSURE IN CHILDREN

Protano C, Andreoli R, Manini P, Guidotti M, Vitali M
Tobacco Control; doi:10.1136/tc.2010.039255

ABSTRACT

Background Secondhand smoke (SHS) represents a major preventable cause of morbidity for communities, especially for children, who are more susceptible than adults to the adverse effects of passive smoking. SHS contains several carcinogens, including benzene.

Objective To investigate the role of household characteristics and the smoking behaviours of cohabitants in predicting SHS-derived benzene exposure levels.

Methods In this cross-sectional study, 122 children (aged 5-11 years old) were selected from a school in rural Italy. Characteristics of their home environment and the smoking habits of the children's cohabitants were obtained via questionnaire, and urinary unmodified benzene (u-UB) and cotinine (a specific nicotine metabolite) levels were determined from spot urine samples.

Results Significant differences between SHS-exposed and SHS-unexposed children were found with respect to u-UB levels (median values 359.50 and 92.50 ng/litre, respectively; $p<0.001$). The excretion of u-UB increased significantly in parallel to increased SHS exposure as follows: unexposed to SHS (median value 92.50 ng/litre) < cohabitant(s) smoker(s) not smoking inside the home (282.00 ng/litre) < cohabitant(s) smoking inside the home only when children are out (314.50 ng/litre) < cohabitant(s) smoking inside the home even when children are in (596.00 ng/litre). The difference between groups was significant ($p = 0.019$).

Conclusions Although smoke-free legislation has transformed the smoking behaviours of some, domestic environments remain an important source of SHS exposure for children. This fact holds true even in the case of parents and other cohabitants who believe they are fully protecting children by smoking only outdoors or at home only when the children are not present. These findings should be included in Italian community-level health promotion interventions for discouraging tobacco use.

INTRODUCTION

In recent years, given the strong association of secondhand smoke (SHS) exposure and adverse health effects in humans (US PHS 2006), numerous countries have introduced restrictions or complete bans on smoking in the workplace and public areas for the sake of the health of non-smokers. Nevertheless, these bans do not protect those individuals exposed to SHS in their home environment; even greater concern exists for children, who are understood to be more susceptible than adults are to the adverse effects of SHS (Muller 2007), and whose exposure is strongly associated with parental smoking (McNabola and Gill 2009).

The link between passive smoking and disease is mediated by many chemicals in SHS (about 5000 compounds), including many compounds that have met the criteria for being a human or animal carcinogen as classified by various regulatory agencies (Environmental Protection Agency, International Agency for Research on Cancer, etc.) (Brownson et al. 2002; IARC 2004).

One of the major known human carcinogens in SHS is benzene, a well known leukaemogen (IARC 1982) present in tobacco smoke in sufficient concentrations to explain up to half of the estimated cases of acute myeloid leukaemia (IARC 2004). Much research has been conducted to assess the links between youth benzene exposure, SHS and the risk of lymphohaematopoietic cancer in childhood; however this research has yielded contradictory findings (Chang 2009). However, considering the prolonged latency of the disease and the early initiation of exposure, the possibility of cancers in adulthood after SHS exposure during childhood cannot be excluded.

Due to the possible adverse effects of benzene exposure in childhood, of which household SHS is the most important contributor (US EPA 2005), it is of relevant concern to investigate SHS-related benzene exposure levels in childhood, but, at present, there is a gap in the research in this area. Several previous studies conducted on subjects who are not professionally exposed to benzene (very low exposure levels) suggest that urinary unmodified benzene (u-UB) serves as good exposure marker for benzene for adults (Fustinoni et al. 2005; Lovreglio et al. 2010) and children (Protano et al. 2010).

The objective of this study was therefore to investigate the influence of the smoking behaviours of cohabitants in predicting passive-smoking derived benzene exposure levels of children, objectively measured by u-UB levels.

MATERIALS AND METHODS

Study population and design

The study was carried out in 122 apparently healthy children between 5-11 years of age, recruited from a primary district school located in a rural area in the province of Rieti (central Italy).

The rural area, far from freeways or highways, was selected to avoid interference from vehicular traffic fumes and other sources of environmental benzene on the children's exposure levels. The rural classification was based on urbanization characteristics (i.e., population density, green area density and motorization rate) obtained from national databases (i.e., National Institute of Statistics, Italian Automobile Club).

All attending students and their parents were invited to take part in this cross-sectional study and received information about its aims and design. Data collection was conducted on a Wednesday in the winter of 2007.

Methods to measure SHS exposure and u-UB levels

Questionnaires to investigate participants' characteristics and SHS exposure We collected detailed information on participants using a self-administered questionnaire filled by the parents. The following topics were investigated: sociodemographic characteristics, daily activities, living conditions and cohabitant smoking habits.

Based on the grades of primary school attended by the children, we coded age into two classes, 0 = first to third grades and 1 = fourth and fifth grades. We coded sex as 0 = male and 1 = female.

With respect to the characteristics of the home environment, we asked about the number of residents, the home dimensions, the number of floors in the residence and the type of heating device(s) used. These characteristics were classified in the following manner:

- Housing density: home dimension in m^2 divided by the number of people living in the home. The results were coded into two categories, 0 for < 30 m^2/inhabitant and 1 for ≥ 30 m^2/inhabitant.
- House floor: 0 = ground floor and 1 = first floor and higher. This characteristic was chosen in order to evaluate the possible influence of car-exhaust-related benzene on floor levels.
- Heating devices: respondents were permitted to provide an open answer. We classified different heating systems by potential sources of benzene: methane or electric heaters as 0 and oil or wood heaters as 1.

The questionnaire also examined other potential benzene exposure sources, such as parking areas, service stations or freeways near the house.

The presence of cohabitant(s) who smoked was determined by the question: 'Are there smokers living with children?' Possible responses here were yes or no. If the response was yes, the child was considered to have been exposed to SHS and the respondent was then invited to answer the following questions:

- 'Do cohabitant(s) smoke inside the home in which the child lives?' (yes/no);
- 'Do people smoke inside the home when the child is present?' (yes/no).

For yes/no questions, the response was coded as 0 = no and 1 = yes.

u-UB monitoring

One urine sample was collected from each participant at home in the evening (just before bedtime). The sample was immediately stored in the domestic refrigerator (about + 4°C). The next morning, all samples were collected, stored in transportable refrigerators and delivered to the laboratory, where they were each immediately coded and then frozen at - 18°C until analysis.

A total of 122 urine samples were collected: 10 samples were rejected because of unsatisfactory sample closure and 24 samples were of insufficient quantities for carrying out the analyses. Consequently, analytical tests for u-UB were performed on 88 vials, whereas tests for u-cotinine and u-creatinine were performed on 112 tubes.

u-Cotinine was included in the set of analytes because it is the main nicotine metabolite and thus its levels correlate strongly with active and passive tobacco smoke exposure (Haufroid and Lison 1998).

The analytical procedures used for u-UB, u-cotinine and u-creatinine determinations have been described in detail in other studies (Vitali et al. 2006; Manini et al. 2008). In brief, u-UB was determined by headspace solid phase microextraction followed by gas chromatography-mass spectrometry; the limit of detection, calculated as the signal to noise ratio > 3, was 8 ng/litre. U-Cotinine was determined by isotopic dilution liquid chromatography tandem mass spectrometry; the limit of detection, calculated as the signal to noise ratio > 3, was 0.2 μg/litre. U-Creatinine was determined using the method of Jaffé (Henry 1974).

Statistical analyses

Statistical analyses were carried out using SPSS software (V.14.0; SPSS, Chicago, Illinois, USA).

Our initial examination of the data showed that biomarkers levels were not normally distributed. Consequently, forward multiple linear regression (controlling for age, gender, house density and house floor) was conducted to predict the natural log-transformed values of u-UB levels on the basis of the smoking habits of some cohabitants. For this purpose, dummy variables were created for the categories of the cohabitants' smoking habits: 'habit of smoking only outdoors', 'habit of smoking inside the home only when child is out' and 'habit of smoking inside the home even when children are in'; no smoking represented the reference category.

The significance level chosen for all statistical tests was $p < 0.05$ (two tailed). Linear regression analyses were run using a significance level of 0.05 for entry and a 0.10

level for removal from the model. The goodness of fit for the model was assessed using R^2 statistics.

RESULTS

A summary of the participants' relevant characteristics and the relative u-UB and u-cotinine levels is presented in table 1.

Table 1. Urinary unmodified benzene (u-UB) levels of the participants categorised by general characteristics and smoking habits of cohabitant(s)

Variable		Frequency (%)	u-UB ng/L Median ± IQR	*p*	*u-cotinine µg/g creatinine* Median ± IQR	*p*
Gender	**Male**	57.1	233.00 ± 266.00	0.578[a]	3.44 ± 2.01	0.960[a]
	Female	42.9	162.50 ± 296.00		3.03 ± 22.30	
Grade in primary school	**1st, 2nd, 3rd**	60.7	181.00 ± 224.00	1.000[a]	3.38 ± 50.38	0.432[a]
	4th, 5th	39.3	178.00 ± 344.00		3.04 ± 51.95	
House density (m^2/habitant)	**< 30**	61.4	178.00 ± 394.00	0.668[a]	3.38 ± 51.95	0.500[a]
	≥ 30	38.6	244.00 ± 277.00		3.21 ± 15.73	
House floor	**ground**	46.8	220.00 ± 393.00	0.827[a]	3.38 ± 51.36	0.799[a]
	≥ 1	53.2	180.00 ± 278.00		3.21 ± 23.15	
ETS exposure status	**Exposed**	51.2	359.50 ± 362.00	<0.001[a]	1.89 ± 0.94	<0.001[a]
	Unexposed	48.8	92.50 ± 90.00		3.15 ± 33.65	
Habits of cohabitant(s) smoker(s)	**Habit of smoking only outdoor**	23.8	282.00 ± 131.00	0.019[b]	3.04 ± 4.19	<0.001[b]
	Habit of smoking inside the home only when children are out	28.5	314.50 ± 177.00		3.61 ± 21.92	
	Habit of smoking inside the home even when children are in	47.7	596.00 ± 548.00		5.95 ± 51.23	

[a] Value by independent t test using natural log-transformed values.
[b] Value by one-way analysis of variance (ANOVA) using natural log-transformed values.

About half of the children were considered to have been exposed to SHS, of which 47.7% were living with at least one adult who smoked inside the home even when children were present.

The u-UB mean concentrations were over four times greater among SHS-exposed children than among those unexposed ($p < 0.001$).

Further evidence of the association between u-UB and SHS exposure was given by the significant linear relationship between u-UB and u-cotinine (Pearson's $r = 0.765$; $p < 0.001$) in SHS exposed children, but not for SHS-unexposed children (Pearson's $r = 0.196$; $p < 0.232$), as shown in figure 1. These findings confirm the impact of passive smoking on the excretion of u-UB.

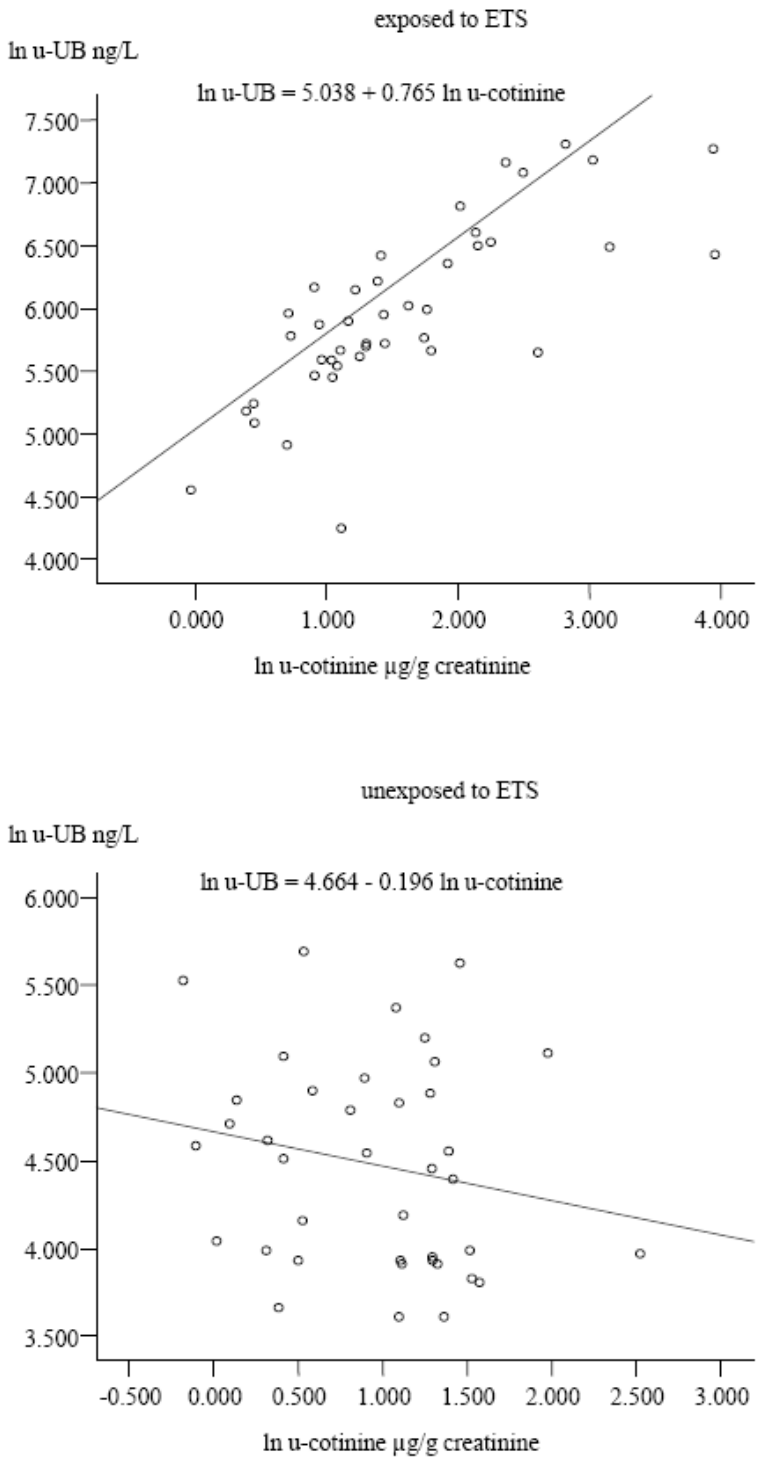


Figure 1. Relationships between urinary unmodified benzene (u-UB) and urinary cotinine (u-cotinine) in children exposed and unexposed to environmental tobacco smoke (ETS)

The excretion of u-UB increased significantly in parallel to increased SHS exposure. Levels of u-UB showed the following sequence among the groups: unexposed to

SHS (median = 92.50 ng/litre) < cohabitant(s) smoker(s) not smoking inside the home (median = 282.00 ng/litre) < cohabitant(s) smoking inside the home only when children are out (median = 314.50 ng/litre) < cohabitant(s) smoking inside the home even when children are in (median = 596.00 ng/litre). The difference between groups was significant with a *p* value of 0.019. The same differences were found in the same groups by using u-cotinine data, as shown in table 1.

The multivariate model for the natural log transformed u-UB levels of children, calculated after adjusting for the variables mentioned above, confirmed the results of the univariate analyses.

Compared with SHS-unexposed children, the u-UB levels (natural log data) of children increase, on average, by 1.01 ng/litre for the habit of smoking only outdoors, and by 1.36 ng/litre and 1.89 ng/litre for the habit of smoking inside the home (only when children are out or even when children are in, respectively). Data are shown in table 2.

Table 2. Significant predictors of unmodified benzene (u-UB) (natural log-transformed data) in forward multiple linear regression models

Variables	B [a]	SE [b]	β [c]	*p*	Adjusted R^2 of the model[d]
No smoking	Reference				
Habit of smoking only outdoor	1.010	0.272	0.287	$p < 0.001$	0.654
Habit of smoking inside the home only when children are out	1.363	0.242	0.439	$p < 0.001$	
Habit of smoking inside the home even when children are in	1.893	0.177	0.842	$p < 0.001$	

[a] B = 74nstandardized regression coefficients;
[b] SE = standard error;
[c] β = standardised regression coefficients;
[d] Model adjusted for gender (male vs. Female), age (1st, 2nd and 3rdgrade vs. 4th and 5th grade), house density (m^2/inhabitant < 30 vs. M^2/inhabitant ≥ 30), house floor (ground vs. ≥ 1).

DISCUSSION

This was a cross-sectional study carried out with the aim of detecting the impact of household smoking habits of cohabitant smokers on the passive smoking-derived benzene exposure levels of children.

After the introduction of smoking bans in public places, household environments became the focus of several studies on SHS exposure, in particular with regard to SHS exposure in children, which is mainly related to household parental smoking (IARC 2009; Kabir et al. 2010). Findings from these research projects showed that smoke-free public places seem to stimulate adoption of smoke-free homes, especially when smokers live with non-smoking adults or children (Soliman et al. 2004; Borland et al. 2006).

Borland et al (2006), for example, reported an increase of complete smoking restriction at home of 8.7%, 5.6%, 5.9% and 7.5% for Canada, USA, UK and Australia, respectively after seven months of smoke-free home policies in those countries.

Despite this, the same survey highlighted that in all investigated countries most children were inadequately protected, with a percentage of total bans from smoking at home ranging from 16% to 42%.

These results are similar to our findings that show a low prevalence of total smoking restriction inside the home (28.5%).

The impact of 'at home' smoking behaviours on children's exposure to benzene is marked by the significant continuous increase of u-UB in children unexposed to SHS to those exposed with cohabitant(s) not smoking inside the home, to those exposed with cohabitant(s) smoking inside the home only when children are out and finally to those with cohabitant(s) smoking inside the home even when children are in.

Our results concur with previous research (Matt et al. 2004) that reported children's exposure to SHS to be up to eight times higher in households of smokers who smoke indoors than that in households of smokers who smoke only outdoors. In

addition, exposure to SHS can still be up to seven times higher in households of smokers who smoke outdoors than that in the homes of non-smokers.

This argument can be related to the 'thirdhand' smoke (Invernizzi et al. 2007; Winickoff et al. 2009) (i.e., the remaining tobacco smoke contamination that persists after a cigarette is extinguished); this complex phenomenon is significant because it demonstrates that many components of tobacco smoke, including benzene, can persist in an indoor environment beyond the period of active smoking. Consequently, the habit of smoking outdoors or in different rooms of the home, or in the absence of children, can reduce the level of exposure but cannot assure adequate protection of children from tobacco smoke.

The only effective way to defend children from SHS is creating whole smoke-free living environments.

The present analyses and their interpretation are subject to several limitations. First of all, the classification of groups based on the smoking habits of cohabitants were determined by the responses to the questions 'Are there smokers living with children?', 'Do cohabitant(s) smoke inside the home in which the child lives?' and 'Do people smoke inside the home when the child is present?'. Parents could have been not completely sincere in their responses to the questionnaire; however, effective categorization of the groups was given good support by measured u-cotinine levels. Secondly, we did not consider the influence of household cigarette consumption of cohabitant smokers with respect to their smoking habits at home to predict differences in benzene excretion in the children studied. Nevertheless, it is plausible that lower tobacco dependence may have been one of the factors permitting smokers to avoid smoking at home, as previously shown by Jarvis et al. (2009). Thirdly, even if the multivariate model was adjusted for main benzene exposure factors for children living in a rural area (home dimension, inhabitants, house floor level, smoking habits at home), it explained 65.4% of the variance in benzene levels, suggesting that additional factors (eg, genetic polymorphisms, body mass index, etc.) may influence benzene urinary excretion; unfortunately, these additional factors were not investigated in the present study.

In conclusion, our findings show that although the smoke-free legislation introduced in many countries has transformed the smoking behaviours of some and increased the number of children living in smoke-free homes, household environments remain an important source of SHS exposure for children. This fact holds true even in the case of parents and other cohabitants who believe they are protecting children by smoking only outdoors or at home only when children are absent.

These findings strongly highlight that:

- Smoking is a health threat for smokers and for others with whom they share an environment, regardless of the 'precautions' taken by smokers.
- The SHS exposure level of children increases in parallel with the increased (i.e., less considerate) household smoking behaviours of their cohabitants.
- As reported by Protano et al (2010), urinary benzene levels of SHS exposed children living in rural areas at low traffic density may be higher than those of children living in urban areas but unexposed to SHS. This fact may nullify the health benefits of living far from traffic pollutants.

For these reasons, apart from the smoking bans at workplace and in public places, it is essential to promote educational intervention for parents with the aim to increase their awareness of the negative impacts of SHS exposure during childhood and to teach correct behaviours to protect the health of children.

As objectifiable data, these results should be included in Italian community-level health promotion interventions because they could contribute to a further persuasive discouragement of tobacco use.

REFERENCES ARE PRESENTED IN THE GENERAL REFERENCE LIST

CHAPTER 6

HOW HOME-SMOKING HABITS AFFECT CHILDREN: A CROSS-SECTIONAL STUDY USING URINARY COTININE MEASUREMENT IN ITALY

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International Journal of Public Health
DOI: 10.1007/s00038-012-0354-0
Accepted for publication

Abstract

Objectives To assess the impact of different home-smoking rules and smoking habits of cohabitant on environmental tobacco smoke (ETS) exposure of children.

Methods Information about 396 Italian children (5-11 years old) and cohabitants' smoking habits was collected by a questionnaire. Exposure assessment was performed by determination of urinary cotinine (u-cotinine).

Results Median u-cotinine concentrations in children significantly increased in a similar fashion as theoretical ETS exposure increase: cohabitants do not smoke (1.79 μg/g creatinine), cohabitant(s) smoker(s) never smoke at home (2.84), smoke at home only when children are out (3.90), and smoke at home even if children are in (6.02). Median u-cotinine levels of exposed children were associated to the strength of cohabitant' s smoking behaviours when smoker(s) consume daily a high number of cigarettes (≥ 20) respect to light consumption (1-9) (4.52 and 3.24 μg/g creatinine).

Conclusions The magnitude of ETS exposure in children is correlated with smoking habits and home-smoking precautions adopted by their cohabitants. Educational interventions on parents are essential to increase their awareness about ETS exposure and to teach correct behaviours to protect health of kids, especially in household environment.

INTRODUCTION

The well-known health problems associated with passive smoking, or environmental tobacco smoke (ETS) exposure (IARC 2004), have led numerous countries, Italy included, to introduce restrictions or complete bans on smoking in public areas. The Italian government established an official ban on smoking in any indoor public place on January 10, 2005.

However, this type of ban does not guarantee full protection from ETS exposure for non-smokers who live with smokers. This issue is of particular concern when the non-smoker is a child for two reasons: 1) in any community, children are the most susceptible population to the harmful health effects caused by ETS exposure (Adgent 2006; Muller 2007; Asomaning et al. 2008; Cheraghi and Salvi 2009); and 2) the greatest proportion of children's ETS exposure occurs in the household environment, because that is where they spend most of their time (McNabola and Gill 2009).

Some researchers have hypothesised that bans on smoking in public places could adversely affect children' health by shifting smoking into the domestic environment. Contrary to this assertion, Hyland et al. (2008) showed that at-home-smoking habits were similar in Ireland, which had introduced a smoking ban, and in the United Kingdom, which did not have such a ban at the time the research was conducted. In addition, several surveys conducted in the United Kingdom (O'Dowd 2005), Canada, the United States, and Australia (Borland et al. 2006) have shown an increase in the prevalence of smoke-free homes in recent years. The authors of these studies observed that making public places smoke-free seems to encourage smokers to also make their homes smoke-free or to at least adopt protective behaviours towards non-smoking adults or children, such as smoking at home only when non-smoking cohabitants are not home or are in separate rooms.

Further studies have been performed to evaluate the impacts of maintaining a smoke-free home and of adopting other smoking precautions in the household on the ETS exposure of children by measuring salivary and/or urinary cotinine (u-cotinine). Cotinine is the main metabolite of nicotine and is a proven biomarker for assessing passive-smoking exposure (Benowitz 1996; Haufroid and Lison 1998;

Keskinoglu et al. 2007). The results of these studies indicated that, for those who live with smokers, having a smoke-free home or a home where other protective measures are taken offers appreciable, but not complete, protection against passive smoking (Johansson et al. 2004; Jarvis et al. 2009).

These findings may be associated with the newly defined issue of "thirdhand" smoke (THS), which is the residue from tobacco smoke that persists on the clothing and hair of smokers, on environmental surfaces, and in dust long after a cigarette has been extinguished (Winickoff et al. 2009). THS contains many different chemicals that are re-emitted as a gas, either directly or as a result of reacting with oxidants or other compounds to form secondary contaminants, some of which are carcinogenic or toxic to humans (Destaillats et al. 2006; Sleiman et al. 2010; Burton 2011; Matt et al. 2011a; Petrick et al. 2011).

The term "thirdhand smoke" is derived from "secondhand smoke" (SHS), which is "the combination of smoke emitted from the burning end of a cigarette or other tobacco products and smoke exhaled by the smoker" (WHO 2007). Passive smoking is the combination of SHS and THS exposure (Protano and Vitali 2011).

To the best of our knowledge, no data exist regarding on the impact of policies adopted by Italian smokers for smoking at home on children's exposure to SHS and THS.

The present study was conducted with a group of Italian children (5-11 years old) using u-cotinine determination in order to:

- assess how the smoking habits of cohabitants predict ETS exposure levels;
- quantify the effectiveness of home-smoking policies adopted by smokers with respect to ETS exposure;
- identify the possible individual contributions of SHS and THS to overall ETS exposure.

METHODS

Study population and design

The study population consisted of all of the students in three primary-school districts located, respectively in northern, central, and southern area of Latium region (central Italy), comprising a total of 665 children aged 5-11 years.

All of the students and their parents received information about the research goals and plan and were invited to take part in the cross-sectional study, which was conducted on Wednesdays (a typical weekday) during the winter season of the academic years 2007-08 and 2008-09.

Information about cohabitants' smoking habits and precautions taken by at-home smokers as well as detailed information about the sociodemographic characteristics of the children and their families, the children's Wednesday activities, and household characteristics was collected using a self-administered questionnaire, previously validated, filled out by each child's parents.

Each participant's level of ETS exposure was estimated using an analytical determination of the cotinine level in a urine sample collected at the end of the sampling day. The urinary sample was taken at the last time of the day each participant urinated just before going to sleep in polypropylene bottle; then, the sample was immediately stored in the refrigerator at 4°C. The next morning, the sample was placed into a polystyrene cooler containing an ice pack and was delivered to the research team.

Covariates and ETS exposure data gathered by questionnaire

The answers to the first questions, which were about the gender of the child and the age (as defined by the primary-school grade they were attending), were classified as 0 = male and 1 = female and as 0 = first, second, or third grade (the younger group) and 1 = fourth or fifth grade (the older group). The sizes of the children's homes (expressed in cubic metres) were gathered using an open question.

The presence of cohabitant smokers was assessed with the question, "Are there smokers living with the child?" The possible responses were "Yes" and "No". If the

response was "Yes", the child was considered to be exposed to ETS, and the respondent was invited to answer some other questions:

"How many cohabitant smokers live with the child?" (An open numeric answer, which was categorised in the analysis as 0 = 1 cohabitant smoker and 1 = more than 1 cohabitant smoker);

"Do(es) the cohabitant smoker(s) smoke inside the home in which the child lives?" (Yes or no);

"Do cohabitants smoke inside the home when the child is present?" (Yes or no).

The formulation of these questions was nonspecific (avoiding terms such as "precautions" or "preventive measures") to encourage responses that would be as honest as possible. We classified the responses as 0 = no and 1 = yes.

The entire sample was divided into four groups on the basis of cohabitant smokers' behaviours and home-smoking rules:

- Children not living with smoker(s);
- Children living with smoker(s), with a total home-smoking-restriction (cohabitant smokers do not smoke at home);
- Children living with smoker(s), with a partial home-smoking-restriction (cohabitants smoke inside the home only if the child is out);
- Children living with smoker(s), with no home-smoking-restriction (cohabitants smoke inside the home even if the child is present).

Finally, the respondents were asked two questions about the average cigarettes consumption of each cohabitant smoker:

"On average, how many cigarettes are smoked by the cohabitant smoker(s) in the course of a weekday?" (Open numeric answer);

"On average, how many cigarettes are smoked by the cohabitant smoker(s) in the course of a weekday inside the home?" (Open numeric answer).

The responses to each of these questions were analysed as a single continuous variable; i.e., the smokers were added together for each question, but the questions were analysed separately (if there was more than one cohabitant smoker, the numbers of cigarettes consumed by all of the smokers were added together). In our evaluation of how the intensity of cohabitants' smoking habits affected the children's ETS exposure, the combined total daily consumption of cigarettes was categorised as:

- 0-9 cigarettes consumed daily by the cohabitant smokers: light-consumption;
- 10-19 cigarettes: moderate-consumption;
- ≥ 20 cigarettes: heavy-consumption.

ETS exposure level as measured by u-cotinine

u-Cotinine and urinary creatinine (u-creatinine) in the urine samples were measured using the procedures outlined in previous publications (Manini et al. 2008; Protano et al. 2010); in brief, about 2 mL of spot urine sample was partitioned into plastic tubes for u-cotinine and u-creatinine determinations. All samples were coded and then frozen at -20°C until analysis. The samples were analyzed within 30 days from sampling.

u-Cotinine was determined by isotopic dilution liquid chromatography tandem mass spectrometry (LC-MS-MS). Before analyses, urine samples were added with the internal standard (cotinine-d_3) and centrifuged at 3,000 g for 10 min. Chromatography was performed on an Atlantis®dC_{18} column (100 x 2.0 mm i.d., 3μm; Waters, Milford, MA, USA) using variable proportions of 10 mM aqueous formic acid (pH 3.75) and methanol. Elution program: 12% methanol, hold for 12 min; from 12% to 70% methanol in 2.5 min (linear gradient); 70% methanol, hold for 1 min. The flow-rate was 0.2 ml/min and the injection volume 30 μl. Analytes were ionized in positive-ion mode and the transitions chosen for selected reaction monitoring detection of cotinine and its internal standard were m/z 177 → 80 and m/z 180 → 101, respectively. The limit of detection was 0.2 μg/l (20 μl injected), the coefficient of variation of the method (expressed as %CV) was below 2% for all intra- and inter-day determinations.

u-Creatinine was measured by the method of Jaffe (Henry 1974). U-Cotinine concentrations were expressed in microgram/gram creatinine to adjust for urine dilution.

Statistical analyses

The statistical analyses were conducted using the SPSS software package (Version 14.0 for Windows, Chicago, IL).

As the u-cotinine results were not normally distributed, analyses were conducted using non-parametric techniques. Mann-Whitney tests were used to assess differences in the concentrations of u-cotinine between the following groups:

- children not living with smoker(s) and children living with smoker(s) groups;
- children living with one smoker and children living with more than one smoker groups.

Kruskal-Wallis test was used to explore differences in the u-cotinine levels for children living with smoker(s), on the basis of the different smoking rules at home and the daily cigarette consumption of cohabitant smoker(s).

Even, since the total number of cigarettes consumed in a day and at home were not normally distributed, Kruskal-Wallis test was used to examine differences in the amount of the total number of cigarettes consumed in a day between total home-smoking-restriction, partial home-smoking-restriction, and no home-smoking-restriction groups. Mann-Whitney test was used to assess the differences in the mean total numbers of cigarettes consumed at home between the partial home-smoking-restriction and the no home-smoking restriction groups.

In all, two forward multiple-linear-regression analyses were conducted to estimate the independent effects of cohabitant smokers' behaviours on the children's u-cotinine excretion. The first model was used to test the independent effects of home-smoking-restriction-related strategies adopted by cohabitant smokers (complete, partial or no restriction) on u-cotinine excretion, taking the children not living with smoker(s) group as the reference group. The second model (involving only the children living with smokers) was used to examine the contributions to

ETS exposure of home-smoking rules (taking the total home-smoking-restriction group as the reference group), number of cohabitant smokers, and intensity of cohabitant smokers' smoking habits (taking the light-daily-cigarette-consumption group as the reference group), as measured by the u-cotinine excretion. The two-tailed significance threshold chosen for all of the statistical tests was $p \leq 0.05$. Linear-regression analyses were conducted using a significance level of 0.05 for entry and a level of 0.10 for removal from the models. The goodness of fit of the models was assessed using adjusted R^2.

RESULTS

In total, out of 665 children 501 took part in the research, which constituted a response rate of 75%. However, 46 urine samples were rejected because of unsatisfactory sealing of sample containers; therefore, analytical determinations of u-cotinine and u-creatinine were performed for 455 samples.

In addition, we excluded 59 children who had at least one parent who was not Italian from the data analysis to avoid interference from well-known ethnic differences in the metabolism and excretion of u-cotinine (Perez-Stable et al. 1998; Benowitz et al. 1999). In the end, the analysis was conducted using the data on 396 children.

The descriptive characteristics of the study subjects are given in Table 1.

Table 1. Basic characteristics of the study population (Latium region, Italy; winter season of the academic years 2007-08 and 2008-09)

Characteristic		N.	Value
Gender (%)	Male	197	52.3
	Female	180	47.7
Grade in primary school (%)	1st, 2nd or 3rd	212	55.9
	4th or 5th	167	44.1
Time spent in indoor vs. Outdoor environments on the sampling day prior to the time of urine collection (Min; mean ± SD)	At school (indoor environment)	377	444.7 ± 74.2
	Home and other indoor environments	377	156.1 ± 147.1
	Outdoor environments	377	29.5 ± 52.1
Home size (m^3; mean ± SD)		340	312.4 ± 181.5
Children ETS-exposure status[a] (%)	Living with smoker(s)	172	45.1
	Not living with smoker(s)	209	54.9
At-home smoking rules of cohabitant(s)[b] (%)	Total home-smoking-restriction	58	33.7
	Partial home-smoking-restriction	48	27.9
	No home-smoking-restriction	66	38.4
Number of cohabitant smokers[b] (%)	1	107	63.3
	> 1	62	36.7
Number of cigarettes smoked in a day by cohabitants[b] (Mean ± SD)	Overall	163	16.6 ± 12.2
	At home	108	8.4 ± 7.1
Daily –cigarette consumption[b] (%)	Light (0-9 cigarettes)	49	30.1
	Moderate (10-19 cigarettes)	46	28.2
	Heavy (≥ 20 cigarettes)	68	41.7

[a] ETS: Environmental tobacco smoke
[b] Only includes responses for children exposed to ETS

The sample was well-balanced with respect to gender and age. Responses to the question regarding the children's activities on the sampling day revealed that they spent the greatest proportion of their time in indoor environments (school, home and other indoor settings).

The percentage of children who were exposed to ETS was similar to the percentage of unexposed children (45.1% vs. 54.9%). Approximately two-thirds of the children had cohabitant smokers who usually smoked inside the home. The mean overall cigarette-consumption level for cohabitant smokers was 16.6 per day, of which 34%, on average, was smoked inside the home.

A summary of the statistics on u-cotinine levels for all of the children combined and for each of the four ETS-exposure-status groups is given in Table 2.

Table 2. Summary of statistics on urinary-cotinine (u-cotinine) concentrations (expressed as μg/g creatinine) for all participants and for subgroups stratified according to environmental tobacco smoke (ETS) exposure and the smoking habits of their cohabitants (Latium region, Italy; winter season of the academic years 2007-08 and 2008-09)

		N	Missing values	Arithmetic mean	95% CI	Median	IQ range	*p value*
All children		396		4.37		2.59	1.51 - 4.23	
ETS-unexposed		209	15	2.40	2.14 - 2.66	1.79	1.30 - 2.88	< 0.001[a]
ETS-exposed		172		6.65	5.40 - 7.89	3.90	2.22 - 7.07	
Children living with smoker(s)		172						
No. Of cohabitant smokers	1	107	0	5.34	4.01 - 6.64	3.51	2.01 - 5.70	0.008[a]
	> 1	62		8.69	6.11 - 11.19	5.05	2.63 - 9.71	
1. Total home-smoking-restriction		58		3.34	2.60 - 4.08	2.84	1.56 - 3.86	
2. Partial home-smoking-restriction		48	3	5.49	4.29 - 6.70	3.90	2.66 - 6.41	< 0.001[b]
3. No home-smoking-restriction		66		10.40	7.54 - 13.26	6.02	2.88 - 12.30	
Daily cigarette consumption of cohabitant smoker(s)	1. Light (0-9)	49		5.18	3.51 - 6.86	3.24	1.84 - 5.39	
	2. Moderate (10-19)	46	9	5.05	3.35 - 6.74	3.73	2.23 - 5.71	0.022[b]
	3. Heavy (≥20)	68		8.76	6.01 - 11.31	4.52	2.64 - 9.03	

[a] Mann-Whitney test
[b] Kruskal-Wallis test

The median u-cotinine concentration value for the whole sample was 2.59 μg/g creatinine (interquartile (IQ) range = 1.51 - 4.23), whereas the median concentration for the children not living with smoker(s) and those living with smoker(s) taken separately were 1.79 and 3.90 μg/g creatinine, respectively ($p < 0.001$). u-Cotinine median levels increased significantly in a similar pattern as the levels of ETS exposure revealed by the questionnaire increased: children not living with smoker(s) << children living with smoker(s) total-home-smoking-restriction << partial-home-smoking-restriction << no-home-smoking-restriction.

The relationship between differences in the mean numbers of cigarettes consumed in a day and home-smoking rules was also examined. We found that the median total number of cigarettes consumed in a day by cohabitant smokers who did not smoke at home at all, by cohabitant smokers who smoked at home only when the child was not at home, and by cohabitants who smoked at home even when the child was there were 10.0, 15.0, and 17.5 respectively ($p = 0.034$). Every day, cohabitant smokers who smoked at home only when the child was out consumed, on average, 8.0 cigarettes at home, whereas cohabitants who smoked at home even when the child was there consumed, on average, 8.9 cigarettes at home ($p = 0.768$).

The results of the univariate analyses were confirmed by the first linear-regression model (Table 3).

Table 3. Differences in urinary-cotinine (u-cotinine) levels (ln u-cotinine expressed as μg/g creatinine) for children whose cohabitants have different at-home-smoking habits in comparison with children whose cohabitants do not smoke group (Model 1) and for different at-home-smoking habits among those children who live with smoker(s) (Model 2) (Latium region, Italy; winter season of the academic years 2007-08 and 2008-09)

Independent variable		B (regression coefficient) [a]	95% CI [a]	t statistic	*P value*
MODEL 1 [b]					
Children not living with smoker(s) (reference group)		1			
Children living with smoker(s) with total home-smoking-restriction		1.259	1.003 - 1.587	1.946	0.050
Children not living with smoker(s) with partial home-smoking-restriction		2.375	1.842 - 3.059	6.716	<0.001
Children not living with smoker(s) with no home smoking-restriction		3.307	2.672 - 4.092	11.045	<0.001
MODEL 2 [c]					
Home-smoking rules	Total home-smoking-restriction (reference group)	1			
	Partial home-smoking-restriction	1.797	1.251 - 2.581	3.200	0.002
	No home-smoking-restriction	2.425	1.747 - 3.367	5.340	< 0.001
Daily cigarette consumption	Heavy (≥ 20 cigarettes)	1.452	1.093 - 1.929	2.601	0.010

[a] Values were converted back to the original state by using the anti-log, EXP().

[b] Final forward linear-regression model with the participants' ages, genders, home sizes and the home-smoking habits of their cohabitants entered in step 1; constant = 1.891; final adjusted R equal to 0.307.

[c] Final forward linear-regression model with the participants' ages, genders, home sizes, the home-smoking habits of their cohabitants, the number of cohabitant smokers, and the cohabitants' daily cigarette consumption entered in step 1; constant = 2.065; final adjusted R equal to 0.218.

The various types of home-smoking behaviour were associated with significant increases in the children's u-cotinine levels in comparison with children not living with smoker(s).

In the second multivariate-regression model (also shown in Table 3), the significant predictors of higher levels of u-cotinine excretion were having partial (B = 1.797; p = 0.002) or no home-smoking-restriction (B = 2.425; $p < 0.001$) (in comparison with having a total home-smoking-restriction) and heavy daily cigarette consumption by cohabitants (B = 1.452; $p < 0.010$). Age, gender, home size and number of cohabitant smokers did not have significant effects on u-cotinine excretion. The presented models explained a percentage of the variability in u-cotinine levels equal to 31 and 21% (first and second model, respectively).

DISCUSSION

Principal findings and synthesis with prior research

In the present study, we found that only one-third of children living with smokers had a total restriction on smoking in the home is similar to the findings of studies conducted in other countries (Borland et al. 2006).

The second relevant result of this study relates to the impact of different home-smoking rules on the nicotine uptake of children. The impact of at-home-smoking practices on children's ETS exposure is highlighted by the significant and progressive increases in u-cotinine levels from children not living with smoker(s) to children living with smoker(s) who do not smoke at home to children living with smoker(s) who only smoke at home when the child is not there, and finally to children living with smoker(s) who smoke at home even if the child is in.

In addition, in comparing the three groups of children who living with smokers, we found that the ETS exposure was directly related to the home-smoking rules that parents reported in their responses to the questionnaires. The lowest levels of exposure were found among children living in domestic environments where there were complete smoking-restriction, children whose cohabitants observed partial

smoking-restriction evidenced median levels of exposure, and the highest levels of exposure were found among children living in homes without any smoking-restriction. This finding is in agreement with previous studies (Matt et al. 2004; Akhtar et al. 2009) and highlights two critical realities:

- The domestic environment is an important source of ETS exposure, even for Italian children.

- Smoking at home only when children are not there or smoking only outside the home gives smokers a false perception that they are fully protecting the children's health.

This last point is supported by evidence that children can be exposed not only to SHS but to THS as well. THS is a major public health concern because it highlights the impossibility to maintaining a safe level of exposure to tobacco smoke and also because nicotine residues in the domestic environment can react with ambient nitrous acid to form new tobacco-specific, carcinogenic nitrosamines (Sleiman et al. 2010).

Finally, we found that the u-cotinine levels of children living with smoker(s) increase in direct proportion to the intensity of the smoking habits of the cohabitant smokers; this finding is especially significant among children who live with smokers who consume a high number of cigarettes daily (≥ 20). This significant relationship shows that heavy smokers, in addition to risking adverse effects on their own health, are endangering people living in the same environment.

The possible contributions of SHS and THS to children's ETS exposure levels

The differences in u-cotinine levels that we found among the four groups of participants described above are presumably attributable to a combination of SHS and THS, which likely contribute to total ETS exposure in variable proportions, depending on the habits of and the precautions adopted by cohabitant smokers.

However, it should be noted that a small amount of cotinine is always present in human bodily fluids because of the consumption of foodstuffs containing nicotine (e.g., potatoes, tomatoes, eggplant, or beverages) (Domino et al. 1993). The exact

quantity of u-cotinine derived from dietary nicotine is not well-defined and very difficult to evaluate: a range from 0.6 to 6.2 μg/L of possible values for urinary cotinine concentrations was calculated based on estimated average and maximal consumption of food and beverages containing nicotine and cotinine (Davis et al. 1991). These results are in line with u-cotinine levels we found in the group of children not living with smoker(s).

Possible sources of nicotine for the study participants are listed in Table 4.

Table 4. The possible contributions of secondhand (SHS) and thirdhand smoke (THS) to children's urinary-cotinine levels (Latium region, Italy; winter season of the academic years 2007-08 and 2008-09)

		Possible contributors to nicotine uptake					
Group		Dietary intake of nicotine	Occasional passive smoking through exposure to noncohabiting smokers	SHS from cohabitants occurring outside the home	THS from contaminated hair and clothing	THS from household surfaces and dust	SHS from cohabitants smoking at home
Children not living with smoker(s)		+	+				
Children living with smoker(s)	Total home-smoking restriction	+	+	+	+		
	Partial home-smoking- restriction	+	+	+	+	+	
	No home-smoking-restriction	+	+	+	+	+	+

Taking nicotine ingested in food and resulting from occasional ETS exposure unrelated to cohabitant smokers as the sources of the base amount of u-cotinine excreted by both children not living and children living with smoker(s), we can assume that the nicotine intake of the latter group is also affected by SHS from cohabitants smoking outside the home, THS from contaminated hair and clothing and from household surfaces and dust, and SHS from cohabitants smoking at home. Which of these sources is relevant depends on the smoking policy adopted in the participants' homes.

Strengths and weaknesses of the study

To the best of our knowledge, the present study is the first to examine the impact of the home-smoking rules of cohabitant smokers on a large sample of Italian children, using a proven biomarker of exposure to tobacco smoke such as u-cotinine as an objective parameter.

There are several methodological limitations that should be considered in interpreting the findings of this study. First, urine samples were collected only once from each child (at the end of the sampling day), so possible changes in u-cotinine excretion over time could not be examined. However, previous studies have indicated that although multiple-occasion urine sampling does provide highly accurate estimates of an individual child's exposure to nicotine, cotinine measurements from single urine samples provide a very accurate estimation of a child's recent exposure (2-3 days) (Matt et al. 2007). Second, the findings of the present study should be confirmed by conducting similar studies in different seasons and with children living in other areas of Italy. In addition to the variables investigated in the present study, u-cotinine concentrations may also be affected by the size of each room in a child's house and the level of ventilation (Blackburn et al. 2003). However, we did not collect any data regarding these potentially variables. Besides, final adjusted R^2 was quite low for both models; in our opinion, residual variability in u-cotinine levels could be explained by other determinants of exposure, evidenced in previous studies but not considered in the present one (parental education, socio-economic status, genetic polymorphism, etc) (Mannino et al. 2001).

Implications for policy-makers

Our findings suggest that the u-cotinine levels of Italian children are correlated with the smoking habits of and home-smoking precautions adopted by their cohabitants. As there is a constant policy debate about possible strategies for limiting ETS exposure, especially among children, this is an issue with major public health significance.

For this reason, in addition to adopting smoking bans for workplaces and public places, educational interventions on parents are essential to increase their awareness of the negative impacts of ETS exposure in childhood and promoting behaviours that will better protect the children's health.

REFERENCES ARE PRESENTED IN THE GENERAL REFERENCE LIST

CHAPTER 7

THE NEW DANGER OF THIRDHAND SMOKE: WHY PASSIVE SMOKING DOES NOT STOP AT SECONDHAND SMOKE

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Environ Health Perspect 119:a422-a422

Passive smoking exposure is a topic of great concern for public health because of its well-known adverse effects on human health (IARC 2004). Two news articles on this topic were published in the February 2011 issue of Environmental Health Perspectives (Burton 2011; Lubick 2011). Lubick (2011) discussed the global health burden of secondhand smoke, and Burton (2011) emphasized a new and alarming consequence of smoking in indoor environments - "thirdhand smoke" - a term first coined in 2006 (Szabo 2006).

Secondhand smoke is defined as "the combination of smoke emitted from the burning end of a cigarette or other tobacco products and smoke exhaled by the smoker" (WHO 2007). Thus, secondhand smoke exposure consists of an unintentional inhalation of smoke that occurs close to people smoking and/or in indoor environments where tobacco was recently used.

Thirdhand smoke is a complex phenomenon resulting from residual tobacco smoke pollutants that adhere to the clothing and hair of smokers and to surfaces, furnishings, and dust in indoor environments. These pollutants persist long after the clearing of secondhand smoke. They are reemitted into the gas phase or react with oxidants or other compounds present in the environment to form secondary contaminants, some of which are carcinogenic or otherwise toxic for human health (Matt et al. 2011b). Thus, thirdhand smoke exposure consists of unintentional intake (mainly through inhalation but also via ingestion and dermal routes) of tobacco smoke and other related chemicals that occurs in the absence of concurrent smoking. Exposure can even take place long after smoking has ceased, through close contact with smokers and in indoor environments in which tobacco is regularly smoked.

Lubick (2011) considers secondhand smoke synonymous with passive smoking, as do the majority of the authors publishing on this topic. However, in light of new evidence about thirdhand smoke (Matt et al. 2011b), it is no longer appropriate to use the term "secondhand smoke" as a synonym for passive smoking or environmental tobacco smoke, because it represents a pars pro toto. In other words, using the term "secondhand smoke" mistakes one part of the problem for the whole. Instead, we propose that "passive smoking" or "environmental tobacco

smoke" be used as a more inclusive term to describe any tobacco smoke exposure outside of active smoking.

This question of terminology is of particular concern for researchers evaluating passive smoking exposure in indoor settings, especially in domestic environments. Since numerous countries have introduced smoking bans in enclosed public places, domestic environments have become the main sources of passive smoking exposure (WHO 2007). We believe researchers should determine the independent contributions of secondhand and thirdhand smoke when they assess the magnitude of pollutant intake due to passive smoking exposure.

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CHAPTER 8

CONCLUSIONS

The results of the present doctoral thesis allow to draw some final considerations:

- The main sources of benzene exposure of the investigated sample are the urbanization degree of residence area, indirect indicator of air pollution, and the exposure to passive smoking.

- The use of the biological indices of benzene exposure, already successfully used to assess benzene exposure in occupational setting and for adult groups of general population showed that:
    - u-SPMA was a good biomarker for assessing exposure to benzene derived from air pollution in childhood;
    - u-UB can be effectively used as a tobacco-related carcinogen biomarker for assessing the intake of benzene resulting from passive smoking exposure in childhood.

- The intake of benzene specifically related to exposure to passive smoking increases significantly in parallel to increased environmental tobacco smoke exposure at home, and it shows the following sequence among subject groups: unexposed to environmental tobacco smoke < cohabitant(s) smoker(s) not smoking inside the home < cohabitant(s) smoking inside the home only when children are out < cohabitant(s) smoking inside the home even when children are in. It is even more demonstrated that smoking is a health threat for smokers and for others with whom they share an environment, regardless of the 'precautions' taken by smokers.

- Exposure to passive smoking derives from the sum of exposures to secondhand and thirdhand smoke. This result is of fundamental importance for several reasons:

- Appropriateness of the terminology: the term "secondhand smoke", often used as synonymous with passive smoking, is only part of the phenomenon; thus, it would be more appropriate to use the term "passive smoking" or "environmental tobacco smoke" to describe any tobacco smoke exposure outside of active smoking.
- Appropriateness of the studies: researchers should determine the independent contributions of secondhand and thirdhand smoke when they assess the magnitude of pollutant intake due to passive smoking exposure.

– Implications for policy makers in health policies: Apart from smoking bans in public places, educational interventions on parents are essential to increase their awareness about the negative impact of environmental tobacco smoke exposure during childhood, and to teach correct behaviors to protect health of kids, especially in household environment.

In conclusion, we can affirm that benzene is an old and well known pollutant but it must still be considered of great concern for children health, despite its use has been progressively reduced and rigorously regulated worldwide by occupational exposure limits and air quality standards.

For these reasons, it is essential that Public Health authorities encourage and promote monitoring programs for the assessment of children exposure to benzene. The results should be use as objectifiable data for orienting the strategies aimed to control air pollution.

On the other hand, the same data should be included in community-level health promotion interventions. They could determine a higher awareness of the actual state of the living environments and, consequently, encourage the adoption of "health and ecological friendly" lifestyles.

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GIUDIZIO ESPRESSO DAL COLLEGIO DEI DOCENTI DEL DOTTORATO DI RICERCA IN

"SCIENZE DI SANITÀ PUBBLICA E MICROBIOLOGIA" - XXIV Ciclo

DOTTORATO DI RICERCA IN
SCIENZE DI SANITA' PUBBLICA E MICROBIOLOGIA
Sede amministrativa: Università di Roma "La Sapienza" Tel. 06.49914632 fax 06.4454845

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La Dott.ssa Carmela PROTANO presenta la tesi dal titolo:

"L'inquinamento dell'aria e l'età pediatrica: come un'indagine di igiene ambientale può supportare le scelte in Sanità Pubblica"

1. PREMESSA E OBIETTIVI DELLA RICERCA DI DOTTORATO

Lo studio oggetto della presente Tesi di Dottorato è stato effettuato allo scopo di proseguire e ampliare una ricerca iniziata nel 2006 come progetto finanziato dall'Istituto Superiore per la Prevenzione e la Sicurezza del Lavoro (ISPESL), al fine di valutare alcuni aspetti dell'inquinamento dell'aria in relazione ai possibili effetti avversi sulla salute umana.
La ricerca è stata condotta secondo differenti fasi:
Fase conoscitiva preliminare
Durante tale fase è stata effettuato lo studio della letteratura scientifica sull'argomento "esposizione non professionale al benzene" al fine di identificare la metodologia più adatta per la valutazione dell'esposizione a basse dosi di benzene ed individuare i principali fattori che incidono sull'esposizione della popolazione generale, in particolar modo nei bambini.
Elaborazione di un questionario "ad hoc" per indagare i fattori maggiormente correlati con l'esposizione a benzene in età pediatrica. Il questionario è stato realizzato per ottenere informazioni sulle attività del bambino durante la giornata di campionamento, sulla sua giornata "tipo", sulle caratteristiche dell'abitazione e alcune abitudini dei conviventi, con particolare riferimento alle abitudini al fumo di sigaretta inambiente domestico.
Arruolamento del campione e realizzazione delle campagne di monitoraggio.
Le aree geografiche in cui effettuare i campionamenti sono state scelte sulla base del grado di urbanizzazione (indice indiretto di inquinamento atmosferico) mediante alcuni indicatori specifici quali la popolazione residente, la densità di popolazione, la densità di aree verdi e il tasso di motorizzazione; grazie a questi indicatori sono state identificate tre aree nella regione Lazio, suddivise rispettivamente in area altamente urbanizzata, area mediamente urbanizzata e area rurale.
La strategia utilizzata per il reclutamento dei bambini è stata quella di coinvolgere alcune strutture scolastiche primarie, per un totale di 348 bambini nel'area altamente urbanizzata, 150 bambini nell'area mediamente urbanizzata e 166 nell'area rurale.
Il progetto di ricerca è stato illustrato a tutti gli alunni delle scuole, a cui è stata consegnata una lettera per i genitori, in cui si chiarivano gli scopi e le istruzioni per poter partecipare allo studio e le modalità di gestione per garantire l'assoluto anonimato nel trattamento dei campioni di urine e dei questionari.
Successivamente, all'inizio della settimana "scolastica", è stato consegnato a tutti i bambini il materiale necessario per partecipare al progetto ed è stato chiesto ai genitori di compilare il questionario e di raccogliere un campione di urine del bambino corrispondente all'ultima minzione prima di andare a dormire.
I campioni urinari raccolti sono stati conservati a +4°C e immediatamente trasportati in laboratorio, dove sono stati preparati i campioni analitici per le determinazioni di benzene immodificato, dei metaboliti, della cotinina (u-cotinina) e della creatinina. La cotinina è stata analizzata come marker

biologico di esposizione a fumo passivo e la creatinina come fattore di normalizzazione urinario per gli altri analiti.
I risultati ottenuti hanno permesso di trarre alcune conclusioni e spunti di riflessione:
– le principali sorgenti di esposizione a benzene nel campione di bambini indagati sono risultate l'urbanizzazione dell'area di residenza, indice indiretto di inquinamento atmosferico, e l'esposizione a fumo passivo;
– tra gli indici biologici di esposizione utilizzati con efficacia per la valutazione dell'esposizione a benzene in ambito occupazionale e per la popolazione generale:
– l'u-SPMA è risultato un buon *biomarker* di valutazione per l'esposizione a benzene derivante dall'inquinamento atmosferico nel campione di bambini indagato;
– l'u-UB può essere impiegato con successo come *tobacco-related carcinogen biomarker* nella valutazione dell'*intake* di benzene derivante dal fumo passivo;
– l'assunzione di benzene specificamente correlata con l'esposizione a fumo passivo varia secondo i comportamenti dei conviventi fumatori in ambiente domestico, con differenze nei livelli di escrezione urinaria di benzene già evidenziabili tra i bambini esposti ma con conviventi fumatori che dichiarano di fumare in casa quando il bambino non è presente o di non fumare in casa rispetto ai non esposti;
– i comportamenti e le *Home Smoking Policies* adottate dai fumatori in ambiente domestico incidono in maniera significativa sull'esposizione a fumo passivo la quale, globalmente, risulta derivare dalla sommatoria delle esposizioni a *secondhand smoke* e *thirdhand smoke*; tale risultato è di fondamentale importanza per diversi motivi:
– appropriatezza della terminologia: il termine *secondhand smoke*, spesso utilizzato come sinonimo di fumo passivo, rappresenta solo una parte del fenomeno; pertanto, sarebbe più appropriato utilizzare il termine "fumo passivo" oppure "*environmental tobacco smoke*" quando si tratta l'argomento;
– approfondimenti negli studi di valutazione all'esposizione a fumo passivo: nella stima dell'esposizione a fumo passivo andrebbe considerato il singolo contributo del *secondhand smoke* e *thirdhand smoke*;
– implicazioni per i *policy makers* nell'ambito delle politiche sanitarie: la riduzione dell'esposizione al fumo passivo rappresenta un obiettivo prioritario di politica sanitaria, come esplicitato dall'introduzione del divieto di fumo nei luoghi pubblici da parte di numerosi paesi. L'ambiente domestico rimane, comunque, il luogo a maggior rischio di esposizione a fumo passivo, anche quando sono adottati comportamenti ritenuti cautelativi, come fumare fuori casa o in casa solo in assenza dei bambini. Oltre ai divieti di fumo applicati per legge negli ambienti pubblici, sono essenziali interventi di promozione alla salute sui genitori, al fine di incrementare la loro consapevolezza sugli effetti negativi dovuti all'esposizione a fumo passivo durante l'infanzia e di aumentare le conoscenze in tema di *secondhand smoke* e *thirdhand smoke* e di idonei comportamenti cautelativi.

Il Consiglio di Dottorato ha espresso giudizio positivo, manifestando apprezzamento per il lavoro di tesi.