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Scuola di Dottorato in Scienze della Natura e Tecnologie Innovative

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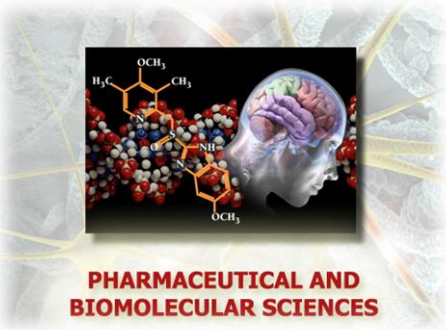

LIFE AND OCCUPATIONAL ENVIRONMENT, LIFESTYLE AND PHYSIOPATOLOGICAL CONDITIONS OF HUMAN POPULATIONS.

AN EPIDEMIOLOGICAL STUDY OF OXIDATIVE STRESS AND HEALTH EFFECT TO DESIGN THE BEST POLICIES OF PRIMARY PREVENTION.

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INTRODUCTION: GENERAL CONCEPTS

1. OXIDATIVE STRESS

The term oxidative stress indicates the whole of alterations that occur in the biological tissues, cells and macromolecules when they are exposed to an excess of oxidizing agents (1). In all aerobic organisms, the balance among the oxidizing substances, including the Reactive Oxygen Species (ROS), and the antioxidant defenses, is called oxidative-reductive balance. This last plays the role of preventing and/or repairing any damage produced. Thus, all life forms conserve, within their cells, a reducing environment protected by enzymes able to maintain the reduced state through a constant supply of metabolic energy. ROS and other reactive species are continuously produced through numerous biochemical processes (2). Therefore, a quantity of oxidizing substances are necessary for maintaining the correct cellular functioning and regulating the mechanisms of homeostasis (3). However, during the reactions of oxygen reduction, the reactive species generated can exceed the physiological threshold value; if these molecules are not neutralized by the antioxidant systems, can occur cellular damage able to conduct to apoptosis (4). Thus, if an imbalance between the ROS production and the effectiveness antioxidant system is generated, a condition of oxidative stress is created (5). Disorders of the normal redox state may cause toxic effects through the over-production of reactive chemical species that damage the cell components including proteins, lipids and nucleic acids (6).

1.1. Reactive chemical species

The reactive chemical species (SCR) are simple and complex ions which have tendency to react depending on their nature and/or the environment in which they are located. On the whole, they acts as oxidizing agents and this characteristic gives them the ability to induce oxidative damage if produced in excess (3). Oxygen has the ability to oxygenate other molecules and it can break chemical bonds generating new radical agents, by electron transfer, that can oxidize other molecules. The role of SCRs in biological systems is twofold: beneficial and harmful (7). Indeed, SCRs show a beneficial effect when, for example, they are used by the immune system as agents able to stop the pathogenic action of various microorganisms, or when they are used as cellular communicators by mediating the biochemical signals transmission between cells. On the contrary, if the oxidizing substances are present at high concentrations, and/or the antioxidant system is not able to neutralize them, they can react with all biological molecules, such as: proteins, lipids, nucleic acids, and carbohydrates (1). In general, we can distinguish two sources of SCR production: endogenous and exogenous. Endogenous sources include mitochondria, cytochrome-P450 metabolism, peroxisomes and the activation of inflammatory cells. Exogenous processes includes the environmental agents, which can directly or indirectly generate ROS (7). At this concern, stress induction and oxidative damage were observed after exposure to different types of xenobiotics such as metals (reduced and not reduced), ions, radiation (UV, gamma and X rays), drugs, environmental contaminants and carcinogens (3). Depending on atom responsible for their reactivity, SCR can be classified

as reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive carbon species (RCS). Moreover, these can be distinguished in radical (FR) and non-radical forms (RS), depending on the possession of unpaired electron in one of the outermost orbitals at least (3). FR are inorganic or organic chemical compounds with one or more unpaired electron(s), highly reactive and short lived. Among FR, ROS are of particular biological importance. They may act as a physiological intracellular agent that, in excess, is considered to be a risk factor for several diseases such as neurodegenerative, cardiovascular and respiratory disease (COPD, pulmonary fibrosis), arterial hypertension, cancer (8), diabetes and in general aging (9). However, oxidative stress is caused not only by an increased burden of oxidants but also by a decrease of the antioxidant potential. Many recent studies show that in inflammatory diseases such as COPD, antioxidant mechanisms are not sufficiently adapted as the increase of ROS expression is lacking, so that oxidants subsequently may take over the leading role (10).

1.2. Mechanism of oxidative stress

Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness or numerousness of antioxidant defenses, such as glutathione (11). The effects of oxidative stress depend on the magnitude of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis. Varieties of indices have been developed to assess FR-mediated injury or

FR generation in vivo. Most of these measure tissue changes that are consistent with an oxidative process but are not always specific for free radicals. Most long-term effects are caused by damage to DNA (12) such as mutations (13,14). Among substrates for attack by FRs, polyunsaturated fatty acids, particularly arachidonic acid and linoleic acid, are primary targets for free radical and singlet oxygen oxidations. The relationships between ROS and lipids have been the most extensively studied because of the ready accessibility (15). After synthesis, lipoproteins are susceptible to lipid peroxidation triggered by ROS and RNS and this peroxidation of lipoproteins might occur in vivo and play a role in the pathogenesis of several diseases, including neurodegenerative and cancer diseases (16,17). Lipid peroxidation is considered the main sources of damage induced by ROS. Lipid peroxidation is divided in 3 phases (initiation, propagation, termination) and starts with an initially destabilization of the phospholipid bilayer structures and consequentially the formation of peroxide radicals. Under normal conditions, this process is kept under control by antioxidant defenses (enzymes and other substances) minimizing the negative consequences and damages (18). When oxidative imbalance occurs, the antioxidant efficiency is greatly reduced. Several studies have demonstrated that lipid peroxidation of lipoproteins is involved in mechanisms of human disease associated with oxidative damage (19,20). These study of the molecular mechanisms involved in lipid peroxidation of lipoproteins has shown, for example, that oxygen species (ROS) produced by the enzymes NADPH-oxidase (NADPH-ox) and myeloperoxidase (MPO) in activated polymorphonucleate leucocytes (PMN), may be

potential candidates for generation of oxidized lipoproteins in vivo (21).

1.3. Sources of oxidative stress

The most important endogenous sources are constituted by internal processes of the cell under physiological condition such as cellular respiration, the inflammatory response, the catalytic cycle of cytochrome P450 and the reaction catalyzed by xanthine oxidase (23). Exogenous sources are external to the organism and frequently are determined by environmental pollutants such as air pollutants, some types of food, toxic substances, and UV radiation. All of these factors may be directly or indirectly responsible for production of reactive oxygen species (ROS) (24). Among exogenous sources, air pollutants and cigarette smoke cover an important role in the production of oxidative stress imbalance (24). The respiratory system is the first apparatus of the human organism able to come in contact with atmospheric pollution and the first that can manifest the consequent pathological effects. (25). Inhalation of air pollutants promotes the production of ROS and an antioxidant deficiencies together with appropriate genetic polymorphisms, may result in increased airway inflammation and hyperactivity (26). Nowadays, the oxidative imbalance is due not only to environmental exposure but also to different life style habits; thus, among exogenous sources also diet, physical activities and BMI seem cover an important role in the production and exposure to oxidant species. Therefore, some of the most important pollutants are Endocrine disrupting chemicals (EDCs), and among these must be counted the xenoestrogens bisphenol A (BPA) and its substitute bisphenol S (BPS)

having the greatest attention from the scientific community, due to their effects on the human health. According to EFSA, exposure to BPs can take place in 3 ways. 1) external (by diet, drinking water, inhalation, and dermal contact to cosmetics and thermal paper), 2) internal exposure (absorbed dose of BPs, sum of conjugated and unconjugated BPs), and 3) aggregated (from diet, dust, cosmetics and thermal paper), expressed as oral human equivalent dose (HED) referring to unconjugated BPs only (27). However, breast milk represents the main vehicle of human intake of BPs which implies that the youngest children show the highest urinary BPs levels. (28). Upon ingestion, bisphenol molecules are metabolized in the liver by the enzyme uridine diphosphonate glucuronosyl transferase (UGT), which allows the conjugation of these molecules with the glucuronic acid forming primarily BP - monoglucuronate (BPs-G) and, to a lesser extent, BP-sulfate (BPs-S) before being excreted with urine (29). On the contrary, the non-conjugated form with glucuronic acid or sulfate of these molecules, and so their free form , represent the active estrogenic form (30) able to occur endocrine alterations (31,32) and cytotoxic effects (33). Anyway, the proportion of free (active) form comprises less than 1% of the total BPA in blood and urine. Thus, when exposure to BPA occurs orally, human studies have shown that BPA can be metabolized to polar conjugates (>99%) and rapidly cleared through urine (half – life of < 6h) (29, 30). In adult rats, BPA is rapidly metabolized to BPA-glucuronide (BPA-GA) by UGT2B1, UDP-glucuronosyl transferase (UGT) (34). In humans, UGT2B7 mediates metabolism of BPA (35). BPA-GA is a hydrophilic molecule, lacking the estrogenic activity of BPA. Given the high activity of such hepatic UGTs in adults, BPA is rapidly excreted in urine and feces. Therefore,

the glucuronidation in the liver is a reaction of detoxification, but is limited in the newborn because the expression of UGT is very weak (36); so this impairment has been hypothesized to slow clearance of these contaminants and increase serum and urine concentrations of free BPs in neonates showing that fetuses are more susceptible to these contaminants than adults (37, 38).

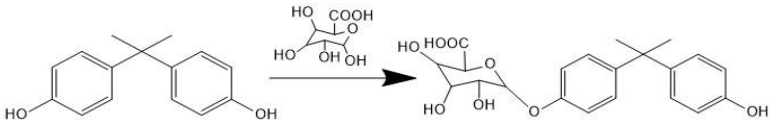


Figure 1. Reaction of conjugation of BPA with glucuronic acid

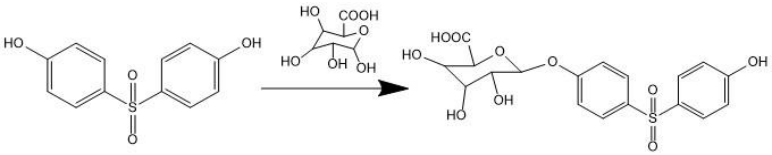


Figure 2. Reaction of conjugation of BPS with glucuronic acid

1.3.1. Bisphenol A (BPA)

Bisphenol A (4, 4'-isopropylidenediphenol, CASRN: 80-05-7), usually abbreviated as BPA, is a synthetic organic compound that consist of two phenolic rings joined by a bridging group formed by the reaction of phenol with acetone (39).

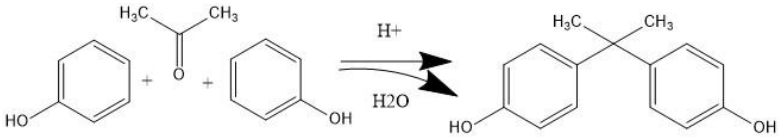


Figure 3. BPA synthesis

BPA is one of the most commonly produced and used chemical in the world. The global production of BPA is about 8 million tons per year and this means that BPA is almost everywhere found in everyday objects (40). At this concern, approximately 90% of the general population had detectable urinary BPA (i.e., > 0.2 or > 0.4 ng total BPA/mL) (41). BPA is mainly (in 95%) used in the production of synthetic polymers including epoxy resins and polycarbonates (42). BPA has many properties that are mechanically useful for many purposes: low adsorption of moisture and thermal stability of synthetic polymers made with BPA. These last are used in the production of various products including water pipes, bottles, toys, teats, medical and healthcare equipment, dental products, electronic devices and CD/DVD discs (43). BPA is also employed as a stabilizer in the production of vinyl chloride (44), and used in the production of thermal paper. In particular, thermal paper is produced in massive quantities because it is used in register receipts, books, faxes and labels and it is also utilized (after recycling) to produce

brochures, ticket, mailing envelopes, newspapers, kitchen rolls, toilet paper and food cartons(44,45). Moreover, BPA is widely used in the production of polyacrylates, polyester and lacquers coating for tins, which after degradation may be important sources of this compound in the environment and food (46). Diet is currently regarded as the major human exposure pathway to BPA (47–49), but the exposure to this contaminant can also occur breathing indoor and outdoor air (i.e. floor dust, paints) (45,50), as well as via transdermal route (i.e. body lotion or perfumes) (51). Usually, BPA is detectable in human urine, blood, breast milk, semen, cord blood, fetal serum, placental tissue and animal fat (46,52,53). Among all the matrix listed above, urine is considered one of the most appropriate body fluid to assess exposure to this contaminant (54); in fact in urine sampled from different countries and areas, the detection frequencies of this contaminant were in the range of 75-90% (55–57). BPA is able to bind to several kinds of receptors including estrogen and androgen receptors as well as aryl hydrocarbon receptor and peroxisome proliferator-activated receptor that are associated with hormones of the endocrine system and other systems of the body (58–60). Therefore, due to endocrine-disruption activity, oxidative and mutagenic potential as well as hypo-methylation ability, BPA is able to exhibit multidirectional toxic effects in animals and humans. BPA disrupts function of various hormones including sex hormones, leptin, insulin and thyroxin. It may lead to adverse effects, especially on reproductive abilities, such as female infertility (61), male low sperm quality (62), low sperm count (63), and sex hormone concentration changes (64). Moreover, its involvement is known in a wide variety of adverse health outcomes, such as: the increased susceptibility

to cancerous alterations (40); neurotoxic (65), cardiovascular and genotoxic effects (66); behavior change; metabolic disease; obesity (67–70); respiratory diseases, in particular asthma (71). Furthermore, prenatal and postnatal exposure to BPA is neurotoxic to mice and lead to altered behaviors, including depression, anxiety and hyperactivity because the neuron system exists within a crucial window during early development periods (72). The possible mechanisms of the effect of BPA on changes in behavior include BPA exposure disrupting the process of neurotransmissions (73), decreases in the gene expression levels of neurotransmitters (74), the influence of estrogen receptor β(ERβ) and GABA(A)-α2 receptors, as well as changes in the neuronal morphology in the hippocampus (75). In addition to the prenatal and neonatal periods, childhood is another important period for brain development (76). Moreover, the exposure to high doses of BPA (particularly during pregnancy and breast-feeding) produces effects on animal development, among which a reduction in survival (for concentration ≥500 mg / kg / day), a decrease in growth (≥300 mg / kg / day) and a delay on the onset of puberty (≥50 mg / kg / day) (77). Nevertheless, also low levels of BPA can evidence human health effects (78). However, the detailed mechanism of BPA influence remains to be investigated. Among the various toxic effects of BPA, I have to remember the contribution of this molecule provides to lipid peroxidation (LPO) and hence to oxidative stress. The intake of high levels of BPA results in increased generation of reactive oxygen species (ROS), culminating in oxidative imbalance (79). This is induced by a decrease of the activity of antioxidant enzymes and the increase of LPO (80). BPA induces oxidative stress and apoptosis in mice testes and, again through the formation of ROS, it can produces injury in

human tissues and organs such as liver, red blood cells, reproductive organs, brain, especially during the embryos phases (81–83).Currently, only few studies have examined the association between urinary BPA levels and oxidative stress (84,85). BPA intake is estimated to be highest in newborns and children because they eat, drink and breathe more per pound of their body weight and explore objects orally (86). Furthermore, these categories are thought to be sensitive and fragile to these contaminants because their metabolism system and organs are undeveloped (87). In this context, the assessment report surveyed from 2007 to 2009 in Canada also showed that the urinary levels of BPA in children were significantly higher than those in adults after creatinine adjustment (47). For pregnant women and the developing fetus, it is desirable to better understand the circulating maternal and fetal blood concentrations of BPA and its metabolites. Pregnancy represent a critical window of exposure due to developmental process in the fetus. Fetal biotransformation pathways are immature (25,41), and could lead to different BPA metabolite patterns in both the fetus and the mother. Moreover, animal studies have shown a slower clearance of conjugated BPA from the fetus relative to maternal serum (47,48). In fact BPA – glucuronide administers to fetal sheep was not only cleared more slowly but also interconverted to free BPA and led to a low but sustained free BPA exposure in the fetus (half –life of > 100 hours) (48). Finally, studies report that higher levels of total bisphenol in the urine are present in women and children of low social status, while lower levels have been measured in Hispanic women and children and this suggests that exposure to BPs probably varies with race-ethnicity, gender and age (88). Until now, BPA is authorized for use as a monomer in

plastic food contact materials, in accordance with Commission Regulation (EU) No 10/2011/EU on plastic materials and articles intended to get in touch with foodstuffs. Furthermore, based on the precautionary principle, in 2011 was introduced European Commission Implementing Regulation (EU) Nº 321/20118, which placed a restriction on the use of BPA in the manufacture of PC infant feeding bottles. In 2015 European Food Safety Authority (27) reduced the temporary Tolerable Daily Intake (t-TDI) of BPA from 50 to 4 µg/kg bw/day. Recently, Food and Drug Administration banned the use of BPA in baby formula packaging. Owing to increased scientific scrutiny of BPA, existing bans and/or restrictions/regulations to limit its applications in some consumer products and decreasing the human health risks, BPA is being replaced with a number of structural analogues speculatively considered safer (89). These alternatives have similar molecular sizes and structures compared to BPA and offer no obvious advantages in terms of their endocrine-disrupting activities (90,91), aquatic toxicity (29), persistence (30) or bioaccumulation potential (29). In particular, bisphenol S (BPS) has been employed as a major component of plastic substitutes for the productions of many articles (92).

1.3.2. Bisphenol S (BPS)

Bisphenol S, or BPS (bis-(4-hydroxyphenyl)sulfone (CASRN: 80-09-1), with a much similar chemical structure to that of BPA, contains a sulfone group with strong electron-absorbing ability and two hydroxyl groups, so it is stronger than other bisphenols in term of acidity, with more stability than BPA. For example, BPS is more resistant to heat and sunlight than BPA (93–95).

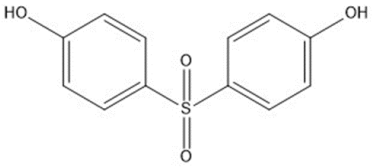


Figure 4. BPS molecule

BPS was first synthesized as a dye in 1869 and is actually used as the substitute of BPA introduced into consumer products in the 2000s (96). The annual manufactured or imported rate of BPS was as high as1000–10,000 tons in the European Economic Area reported by European Chemicals Agency (97). Therefore, humans are widely exposed to BPS, a chemical largely similar to BPA, also for toxicological reasons. Since the usage of BPS is not regulated, it is difficult to specify the products containing and leaching BPS. It is often used as an intermediate for the production of epoxy resins and polycarbonate plastics and it is exists in several consumer products. In particular, BPS is frequently found in

some daily consumer products, such as plastics, food cans linings and packaging, baby bottles and toys, dental materials, personal care products (PCPs) (46), various papers (98), to name a few. Moreover, BPS is the main substitute of BPA as a color developer into thermal papers. In addition, the recycling of paper and plastic, especially of the thermal paper, is a special source of BPS in related consumer products. As BPS is easy to phase-out in consumer products, all products mentioned above might be the potential sources for BPS in the environment. The "leached" BPS could enter and degrade in air, particle/dust, soil, water, sediment, biota, and foodstuffs, which causes the BPS pollution in the environment, and finally induces human exposure (99). In the same way of BPA, BPS can easily enter the human body by ingestion (food or dust), inhalation (air or particle), and dermal absorption (dust or PCPs). Therefore, humans are widely exposed also to BPS and this BPA-alternative has already been detected in 81% of urine samples from China, United States, and in six other Asian countries (100). The adsorption through thermal papers and clothes is relatively special routes of human exposure to BPS (46, 51, 101) even if, a very recent research indicates that food may be the principal source for human exposure to BPS. In this purpose, the study of Wang et al. (2015) emphasized that, for the population in China and U.S., the EDIs of total bisphenol analogues via diets accounted for 90% of the total EDIs when considering both dust and diet (102). Furthermore, a recent study found that textiles and baby clothing contained BPS, and newborns can be affected by skin absorption. Dermal BPS exposure doses from textiles ranged from 8.20 pg/kg_bw/d for 6– 12 months old infants to 10.1 pg/kg bw/d for newborns (<1 month) (103). Compared to numerous

studies on the toxicities of BPA, effects of BPS exposure are in a much smaller number and less conclusive about the biological mechanisms that govern the toxic effects. Although, in vitro and in vivo exposure studies indicate that BPS is an endocrine disruptor and may have potential health hazards, similar to that of BPA (104), no suggested daily exposure dose or oral maximum allow able dose of BPS was established for human exposure. However, considering the much similar chemical structure considering BPS and BPA, long-term exposure to BPS may have adverse effects on the development of reproductive and nervous system. In addition, as pregnancy is a particularly sensitive period in terms of exposure to BPS, special attention must be paid to avoid exposure to this dangerous chemical (e.g., reducing the use of plastic products, PCPs, canned food, and dermal contact with thermal receipt papers) (99). Several new studies show also that exposure to BPS may cause oxidative stress. In this purpose, a biomonitoring study conducted in Saudi Arabia found a significant positive association between concentrations of BPS and 8-OHdG in human urine. (85). Moreover, BPS may disrupt the function of the endocrine system, and then affect the reproductive system in mice (105). Finally, recent studies also indicated that exposure to BPS may induce obesity. In this purpose, Boucher incubated human preadipocytes at a BPS concentration ranging from 0.1 nM to 25 μM. They found that the 25 μM BPS treatment group increased the expression of several key adipogenic markers (e.g., lipoprotein lipase and adipocyte protein 2), both in mRNA and protein levels, and induced lipid accumulation. (106). Summarizing BPS, as a main substitute of BPA, is until now considered a little bit safer than BPA, partly for the lack of sufficient data to support risk

assessment, especially its toxicity at low dose exposure (104,105). Data are still limited and therefore it is necessary to continue the studies and deepen its toxic properties. BPS has the same qualitative effects on estrogen receptor and androgen receptor activities in the range of BPA, but BPS exhibit the greatest changes in efficacy on 17α-hydroxyprogesterone in all tested bisphenol analogues (107). Moreover, a Japanese study even indicated that BPS appear to be more resistant to environmental degradation if compared to BPA (94). Thus, these evidences suggest that the use of other chemicals to surrogate BPA may not solve this issue and, as long as a chemical structurally similar to BPA is adopted, this is not a "safe substitution" (99). Finally, the concentrations of BPS are generally lower than those of BPA, but with the prohibition/limitation in the use of BPA, the levels of BPS will constantly increase in the environment. This is an aspect just recorded when in U.S. and China where measured comparable concentrations of BPS and BPA in water and sludge samples (108). Consequently, considering the toxic potential of BPS, more studies are needed to investigate the occurrence, fate, and effects of this compound in the environment and in human populations.

1.3.3. Tobacco smoke

Oxidative stress could be influence also by tobacco smoke. In the last few years many hypothesis about this were explored and a lot of studies were focused on the biological response to exposure (109). Cigarette smoke is constituted by more than 4,500 components in its gaseous and particulate phases. These compounds include direct carcinogens (methylcholanthrene, FA, benzo-α-pyrenes, acrolein, etc.), toxins (carbon monoxide, nicotine, ammonia, acetone,

hydroquinone, etc.), reactive solids with chemically catalytic surfaces, and oxidants (superoxide and nitrogen oxides) (109). In addition, the immune system of a smoker is less efficient in combating bacterial infection compared with that of a non-smoker (110); also numerous studies have indicated greater levels of oxidative stress in cigarette smokers (111), which is most likely attributed to the high concentration of ROS in cigarette smoke (112). Besides, a significant increase of oxidative stress biomarkers level, such as 15F2t-IsoP, has been recently demonstrated comparing different levels of passive tobacco smoke in a population of adolescents (113,114). Overall, these findings strongly suggest that passive smoke, as well as the active one, cause oxidative stress and oxidative imbalance.

1.4. Oxidative stress and pregnancy

Pregnancy is a physiological condition with changed lipid profile parameters (115) and increased susceptibility to oxidative stress (116). This period is characterized by dynamic changes in body systems resulting by the increased in increased placental mitochondrial activity and a high maternal metabolism. This higher oxygen demand may stimulate the production of ROS. At the same time, the number of mitochondria tends to increase through gestation, leading to an oxidative environment too. However, antioxidants like superoxide dismutase or catalase also increase, probably to compensate the oxidative burden (117). Although antioxidant activity during physiological pregnancy is genetically determined, antioxidant concentration may be insufficient to balance high production of free radicals. Therefore, during pregnancy increased production of ROS exceeding the mother's antioxidant

potential leads to an oxidative imbalance, which can negatively affect the health of both the mother and the fetus and leading to complications in pregnancy (116). Numerous pregnancy-related complications (fetal death, intrauterine growth restriction, pre-eclampsia) are the result of insufficient maternal antioxidant protection to balance high production of free oxygen radicals and lipid peroxidation in the placenta (118). Moreover, numerous studies have shown a connection between the oxidative stress and different etiopathology in pregnancy (119). Gestational diabetes and hypothyroidism (120) are an example of a disease where oxidative stress plays a key causative role in metabolic disturbances and their consequences. Rajdl founds a statistically significant difference in the oxidative stress levels of diabetic mothers and their children in comparison to healthy mothers, suggesting an exceptional free radical activity expressed as increased lipid peroxidation (121). As pregnancy is a period of increased metabolic demands, insufficient supplies of essential vitamins and micronutrients can lead to a state of biological competition between the mother and fetus (122). In such a situation, an inadequate nutrition and a reduced intake of anti-oxidants or an increased exposure to pro-oxidants, may also disrupt the oxidative equilibrium and contribute to oxidative stress. For example, it is well known that smoking is followed by intense oxidative stress (123) and, in this purpose, pregnancy complications can arise due to the increased free radical damage caused by smoking (118).

1.4.1. Oxidative stress in pregnant

During pregnancy, increased production of ROS exceeding the mother's antioxidant potential leads to oxidative stress, which can negatively affect the health of both the mother and the fetus, leading to complications in pregnancy (116). Lipid peroxidation through the free radical pathway is generally described as unfavorable to mammalian health and often related to pathological consequences. However, it is also known that regulated free radical reactions in the body are beneficial, specifically for cell signaling, cell generation and degeneration, cellular homeostasis and defense against, for example, microorganisms. (124).

At this concern, numerous longitudinal study conducted in normal human pregnancies show that oxidative stress levels, measured by urinary F-2-isoprostanes, increase progressively throughout pregnancy. In particular, a significant increase in oxidative stress from week 9 until week 40 was recorded, while, thereafter, a significant decrease to basal levels at post-partum weeks 9, followed by a further increase from weeks 10 post-partum onwards, is observed (115,116). This suggests that free radical-mediated lipid peroxidation in mild form might have a role in pregnancy. Moreover, a longitudinal study has also shown that the antioxidative status decreased significantly in the first trimester and then increased throughout pregnancy to reach normal non-pregnant values post-partum (125). In a cross-sectional study, non-lipid-adjusted levels of vitamin E were found to be significantly higher in late normal pregnancy compared to non-pregnancy (126). These might be due to the higher levels of circulating serum lipids (cholesterol and triglycerides) found during pregnancy. Although the of oxidative stress

increase during pregnancy is considered a physiological process, the exposure to environmental pollution as well as wrong lifestyle habits, including an inadequate diet, because of the low in vitamins, and the exposure to tobacco smoke, may lead a worsening of the oxidative imbalance with serious consequences, mainly on the fetal development. In this concern, it is well known that tobacco smoke may invoke oxidative stress. In pregnant women, the nicotine and carbon monoxide components of cigarette smoke may cause damage to both the mother and the fetus, crossing the placental barrier (123). A relationship between maternal smoking and disturbances in postnatal growth and development (127) has been demonstrated, and not only intrauterine fetal growth retardation or low birth weight (118).

1.4.2. Oxidative stress in newborns

Newborns are particularly prone to oxidative damage induced by ROS. Oxidative stress affects various organs, mostly the lung epithelium and retinal and brain blood vessels. In particular, free oxygen radicals are produced in cell membranes and structures such as peroxisomes, mitochondria, endoplasmic reticulum, protein cell membranes and soluble enzymes (hemoglobin, xanthine oxygenase). The sensitivity of these cells and tissues results from their rapid growth and development, as well as weak antioxidant protection (128). The antioxidant status of newborns shows low concentrations of glutathione peroxidase, superoxide dismutase, ß-carotene, riboflavin, α-proteinase, vitamin E, selenium, copper, zinc, transferrin and

other plasmatic factors (129). Oxidative stress during the fetal period programs the metabolism of the neonate either directly, through gene expression modulation, or indirectly, through the influence of ROS on lipids and proteins in the critical window of development (130).Oxidative stress may last also after the birth (131). At this concern, numerous studies revealed that oxidative stress levels measured in various biological materials were significant higher in healthy, term newborn immediately after birth, and continued to grow and/or persist until the third day after birth (132). Delivery is associated to a considerable oxidative stress, even if the intensity of oxidative stress is independent of the mode of delivery and maternal infections during pregnancy (128). Defense against free radicals increases slightly before the delivery but in the last 15% of the pregnancy time, a 150% increase is observed (133). This defense depends on the activity of the enzymes glutathione, superoxide dismutase and catalases. Nevertheless, breast milk, especially after preterm delivery, contains high concentration of antioxidant defense factors, which might have a protective influence on the breastfed infant (134). It is a further argument in favor of breast milk for the nutrition of the infant.

2. MONITORING OXIDATIVE STRESS

Reactive oxygen species (ROS) are constantly produced in aerobic organisms by normal metabolic processes, such as cellular respiration and antibacterial defense. Furthermore, as previous mentioned, endogenous or exogenous exposures (such as ionizing radiation, smoking, and toxins) also induce production of ROS (135). Therefore, exposure to ROS is almost ubiquitous, and a certain level of oxidative damage is always present in any individual but, in order to contrast these damaging effects, aerobic organisms have developed multiple defense systems such as superoxide dismutase, catalase, glutathione peroxidases and endogenous antioxidants. Differences in both the intensity of ROS generation and the effectiveness of the antioxidant defense, produce variability in oxidative status between individuals (136). Variability in oxidative status within an individual between tissues as well as between individuals results from a complex interaction of multiple factors, including genetic and (137,138) epigenetic differences, endogenous promoters of ROS, chronic inflammation and (139) or other chronic conditions. On the whole, a "normal oxidative status" (non-stress range) in opposition to a pathological condition has not been yet defined. Therefore, it is not clear which levels should be considered "normal" (non-stress) and which represent a serious imbalance between ROS generation and antioxidant defense (stress). Because ROS have short lifetimes and

cannot be directly detected in humans (140), a very useful alternative is the measurement of oxidative stress biomarkers. Although the quantification of these oxidative biomarkers do not measure the ROS levels per se, they are assumed to be proportional to the ROS levels.

2.1. Biomarkers of oxidative stress

F2-isoprostanes: F2-isoprostanes are synthesized during non-enzymatic oxidation of arachidonic acid by different types of free radicals, including reactive oxygen species (141). Depending on the position where the oxygen molecule is added to arachidonic acid, four regioisomers are formed, giving each of the four F2-isoprostane series. Furthermore, each series comprises 16 stereoisomers (142). F_2-isoprostanes can be measured in detectable quantities in biological fluids and in human blood and urine (143). They have two important characteristics : 1) isoprostanes describe a lipid peroxidation not completely stopped by the antioxidant defenses that leads to an exponential increase in the isoprostane concentrations (142); 2) isoprostane quantification can be easily used as a sensitive expression of systemic oxidative stress status and inflammatory response (142,144). There are three main techniques used to assay F2-isoprostanes: gas chromatography with mass spectrometry detection (GC-MS), liquid chromatography with tandem mass spectrometry detection (LC-MS/MS), and enzyme-linked immunosorbent assay (ELISA). The advantages of GC/MS and LC-MS/MS techniques are numerous, the main of which is the great sensitivity of the method. The immuno-enzymatic ELISA-based tests are inherently inferior as compared to the chromatography-based techniques due to cross-reactivity but they allow to measure faster a large

number of samples, preserving sensitivity and sensibility (145). In the epidemiological studies, the 15-F2t-isoprostane is the most used biomarker of oxidative stress. Through ELISA quantification, it is able to express relationships between ROS and chronic diseases (asthma, BPCO, cancer, etc.)(136,146).

Cytokines: oxidative stress and ROS, especially those derived from mitochondria, stimulate the activation of mediator signaling molecules as the transcription factor nuclear factor kappa-B (NF-κB) (147), that up-regulates the production of inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and others mediators as tumor necrosis factor-α (TNF-α) or chemokines. In physiological circumstances, the deleterious effects caused by the highly reactive nature of ROS are balanced by the presence of antioxidants, including glutathione, carotenoids, and antioxidant enzymes such as catalase and glutathione peroxidase. Several chronic human diseases characterized by excessive ROS production (148), or more in general, uncontrolled inflammatory response results in extensive cell and tissue damage, giving rise to normal cell and tissue destruction (149). Therefore, the levels of multiple cytokines collectively might appropriately reflect subtle status of a deregulated immune response or a failure to contrast adequately the ROS production due to endogenous or exogenous exposures. Cytokines can be quantified at various levels: intracellular proteins can be measured by fluorescence-activated cell sorter staining of permeabilized cells, and secreted cytokines can be quantified with bioassays, enzyme-linked immune sorbent assays (ELISAs), radioactive immunosorbent assays, and microarrays.

Multiplex assays for detection of cytokines at the mRNA (150) and cellular levels are commonly used. However, these assays have one or more limitations, like the need for a large sample volume or detection of precursor proteins rather than native secreted proteins. In addition, these techniques are time-consuming and laborious. At the same time, these assays have proven to be very useful in the simultaneous detection of cytokines in many body fluids (151).

3. OXIDATIVE STRESS AND EPIDEMIOLOGICAL APPROACH

The environment is a vehicle of many biological, chemical, and physical risk factors capable to induce the onset of many human diseases. A recent report from the World Health Organization (WHO) showed that in 2012, an estimated 12.6 million global death (23% of all deaths) were attributable to the environment. The environmental impacts on health mostly affected children less than 5 years of age (152). Therefore, indoor and outdoor air pollution are factors possibly associated with environment-related health outcomes, including respiratory cardiovascular diseases, and cancer. Among all the environmental factors, hazardous chemicals ubiquity, food and water contaminants and tobacco smoke (153) have become a problem of growing international interest (154–156). In particular, for their endocrine disruptor's properties and their widespread presence in the human life environment, BPA and BPS are an important topic in terms of Public Health. Since bisphenols are often used as an intermediate for the production of epoxy resins and polycarbonate plastics, they exists in a very big number of consumer products, and for this reason humans are largely exposed to these contaminants (99). According to EFSA, exposure to BPs can take place in 3 ways. 1) external (by diet, drinking water, inhalation, and dermal contact to cosmetics and thermal paper), 2) internal exposure to total BPA (absorbed dose of BPA, sum of conjugated and unconjugated BPA), and 3) aggregated (from diet, dust, cosmetics and thermal paper), expressed as oral human equivalent dose (HED) referring to unconjugated BPA only (27). Mostly, BPA and BPS are considered among the Food Contact

Materials (FCMs), being key monomers used in the production of the resins employed to prevent the direct contact of food with metal cans and to ensure their thermal and mechanical stability (40). There is a tendency to think that plastics are stable compounds; instead, the chemical bond between bisphenol molecules is highly unstable and, therefore, there is a high risk that these substances will spread in the water, in the drinks or in the food in contact with the plastic materials in which they are contained. The main factors influencing the migration of BPs from the surface of cans or bottles, are the temperatures used in the manufacturing process and, particularly, the prolonged usage (44). Migration can also be induced by storage time and, consequently, the accumulation of these molecules can be observed in canned foods. Moreover, BPA migration ratio from PC bottles to other solutions varied dependently on their chemical properties. In ethanol-water mixture, faster release of BPA from PC (in comparison to water) was noted, whereas in oil from olives negligible release of BPA was observed (157). Children are considered as vulnerable populations when it comes to exposure of environmental chemicals for a number of reasons. Due to the children's continuing development and behavioral activities (e.g. hand-to-mouth touching, playing on the floor, etc.), exposure to environmental chemicals could lead to increased incidences of acute and/or chronic diseases in children such as asthma, attention-deficit/hyperactivity disorders, obesity and diabetes (158). Furthermore, given that dietary intake is the chief sources of exposure to bisphenols and phthalates, children and adolescents are uniquely vulnerable to these chemicals as they have greater food consumption per unit body weight and will have prolonged exposure over the course of the lifespan

(159). Moreover, children have smaller body storage for compounds due to their small body size and blood volume capacity as well as less mature metabolic pathways, which might lead to longer half-lives in the body (158,160). There are also specific critical programming periods during children's *in utero* development, postnatal development and puberty, when the body is particularly vulnerable to disrupting effects, and exposure to environmental chemicals might lead to persistent epigenetic changes, resulting in adverse effects (161–163). Lastly, exposure to environmental chemicals during early life results in longer life-times exposure and higher risk of developing chronic diseases (158). The resulting impact on human health may evolve with different characteristics and intensity levels (153,164,165) even if its mechanisms of action remains partially unclear (166,167). Consequently there is a need to monitor the exposure levels of environmental chemicals mostly in vulnerable populations to ensure the risk of developing adverse health effect or diseases attributable to environment can be minimized as much as possible (168). Thus, the human biomonitoring provides measures of internal exposure and/or effects, integrating all sources and routes of uptakes by direct measurements of biological specimens such as urine (169). The indoor environments, particularly the sites where people smoke or have smoked, and different life style habits are often characterized by the highest levels of pollutants that induce oxidative stress (170). Accordingly, oxidative stress plays a crucial role in the inflammatory response to tobacco smoke (171), frequently in co-exposure with airborne ultrafine particles (172) and in many other environmental factors.

OBJECTIVES

4. GENERAL OBJECTIVES

To maintain the population healthy and safety, molecular epidemiology represents a crucial tool for the impact analysis of environmental exposure to the different risk factors. Therefore, the deepening of the biological relationship between humans and environment is crucial to build a complete OXIDATIVE STRESS PROFILE of exposed subjects.
The research work of last three years has been driven by the idea of monitoring different environmental expositions through a complete OXIDATIVE STRESS PROFILE analysis. This intending to highlighting different conditions of environmental and life-style risk and, consequently, potential pre pathological conditions. The project, in its broader meaning, has been structured on the main purpose of investigating thoroughly and extensively some life-style habits and consequently the mechanisms that can increase oxidative stress imbalance in humans, in particular in children and newborns, in order to better understand responses and adaptations to this different risk conditions.

4.1. Oxidative stress in pediatric populations. The role of passive smoking and the living environment.

In the fully aware, that OS originates from many endogenous and exogenous factors, purpose of this first study-line was to investigate the presence of BPA in the urine of a group of adolescents, its role in the induction of OS, and to confirm the same role of the tobacco smoke. Therefore, the purpose

of this first study was to clarify the role that some independent environmental, individual, and physiological variables have on the oxidative stress status of a large population of healthy adolescents. To achieve this goal, a sample of urine provided by each of the 223 young healthy volunteers (7-19 years old) attending three different schools of a little town named Chivasso (close to Torino, Piedmont, North-Western Italy) was analyzed to quantify BPA, cotinine, and 15F2t-IsoP in urine. The first as the chemical playing the role of internal dose marker, the second as a nicotine metabolite to quantify exposure to smoking and the third as a marker of OS.

4.2. Bisphenols as endocrine disruptors and the onset of oxidative stress in newborns

The assessment of Endocrine Disrupting Chemicals (EDCs) exposure is particularly important in case of infants who, being in the early stages of development and therefore with still underdeveloped metabolic mechanisms and organs, appear to be more vulnerable and susceptible to the onset of potentially stress and pathological conditions. UGT in the liver of human fetuses is present at concentrations 5 times lower than that of an adult liver; therefore, newborns may be exposed to higher concentrations of free bisphenol if compared to adults. In this contest, studies show that the levels of unconjugated bisphenol in human adults' serum are typically low, while higher levels have been reported among newborns (173).
In addition, animal study demonstrate the passage of bisphenol through the placenta, with subsequent passage in

the umbilical cord and amniotic fluid of the fetus. In particular, the β-glucuronidase enzyme was found to be very active in human placental tissues, increasing fetal exposure to bisphenol in free form by the hydrolysis of the conjugated form (174). Due to the prolonged exposure of the fetus to the free form of bisphenol, the multiple negative effects of this molecule on the endocrine system is observed during the Moreover, the increasing concern about the late effects consecutive to early exposures identifies sensitive populations such as pregnant women and newborns as a priority for biomonitoring studies (175). Then, breast milk as mothers' and babies' urines appear as a relevant biological materials for conducting such studies, giving access both to an estimated internal exposure level of the mothers and their babies and to an estimated food exposure level of the breastfed newborn (176). Therefore, the first purpose of this second study was to clarify if during pregnancy and the first days of breast-feeding may occur oxidative alterations in newborns, also in relation to a possible exposure to bisphenols and in particular BPA and BPS. Furthermore, the second aim of this Ph.D project was to evaluate if and how, during pregnancy, the neo - mothers oxidative stress levels may change depending on their health status and on active and / or passive exposure to tobacco smoke, compared to the control population considered.

STUDY-LINE 1: OXIDATIVE STRESS IN PEDIATRIC POPULATIONS. THE ROLE OF PASSIVE SMOKING AND THE LIVING ENVIRONMENT.

5. EXTENDED METHOD

- Epidemiological sampling:

The epidemiological sample was selected with the aim to investigate the oxidative status in several range of age measuring biological markers in a large population of healthy children and adolescence, also in relation to the presence of BPA. Schools were recruited on October 2015 and the enrolment started on January 2016; the "Provveditore agli Studi della Regione Piemonte" have facilitated the relationship with the schools. Managers and teachers have been informed about the project and the students have been called up for the information meeting during school hours. All the 223 students involved in the study were volunteers who attended the same school district but in three different school of Chivasso; in particular, a primary school, a secondary school and a high school were selected. No other selection criteria was adopted. Since the subjects were underage, parents were informed on the objective of the study and a written informed consent was signed and delivered from any parent who allowed to his son to participate. Thus, the participation of all subjects did not occur until informed consent was obtained. Sampling was carried out from January to March 2016, involving one class per day, on Wednesday or Thursday, according to a pre-established timetable. For each student, a short version of the questionnaire "SIDRIA"

was prepared to acquire information on age, sex, place of residence, hobbies, diet, therapies, and parent's smoking habits (177). An interviewer administered the questionnaire during school hours, the same day the urine sampling, some anthropometrics parameters took place. The local Ethics Committee of "San Luigi" Turin Hospital (11th March 2015, practice n ° 27/2015) approved the study protocol.

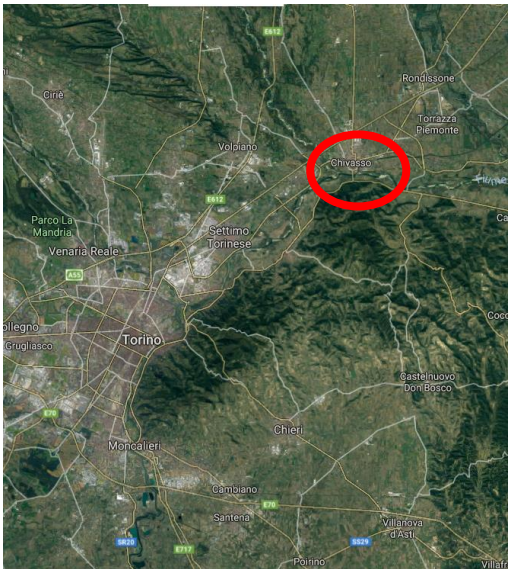


Figure 5: Map of the sampling site. Chivasso is a smaller and less urbanized city than Torino with about 26,000 inhabitants (Sources by Piedmont Region, 2011).

- Biological analysis:

Urinary cotinine: Cotinine measurement was carried out to quantify the passive exposure to tobacco smoke, which represents a possible factor of oxidative stress formation. A specimen of the first morning urine was collected from each volunteer and stored at −80 °C until analysis. Cotinine was measured by gas chromatography–mass spectrometry (GC/MS). A spot of fresh urine was collected in the morning and approximately at the same time from each volunteer and then stored at -80°C. 10 ml of urine was transferred into a glass tube and 4 g of NaCl, 500 µl of NaOH (5M) and 10 µl of cotinine-d^3 (internal standard) were added. Cotinine was than extracted from urine repeating the following procedure twice: addiction of 2 ml of $CHCl_3$, liquid-liquid extraction in a shaking wheel for 15 min., centrifugation for 10 min at 1000 g. The two phases were collected together and evaporated with nitrogen. The cotinine calibration curve was built with a blank non-smoking subject's urine pool extracted with the same procedure, in order to measure a concentration range from 10 to 100 ng/ml. The dry residue was dissolved in 200 µl of $CHCl_3$ and transferred in conical vial. In collaboration with Fondazione Salvatore Maugeri (FSM) of Pavia, GC/MS analysis was performed using an Agilent Technologies 689 GC, interfaced to a 5973 MSD Inert Agilent MS. The analytical procedure has been described in detail elsewhere (178). Cotinine concentrations were normalized to the urinary creatinine (CREA) levels, as usual for every urinary measurement.

Urinary Isoprostane: 15-F2t-IsoP was measured in urine by ELISA technique. A microplate kit specific for urinary 15-

F2t-IsoP (Oxford, MI, USA) was used following manufacturers' instructions. This kit is based on a competitive ELISA so microplates are directly coated with polyclonal antibody specific for F_2-IsoPs. Urine sample were mixed with an enhancing reagent to limit interferences due to specific binding. Both samples and standard compete with 15-F2t-IsoP conjugated to horseradish peroxidase (HRP) for binding a polyclonal antibody on the microplate surface. After the addition of the substrate, the HRP activity resulting was measurable in color development. The intensity of the color is inversely proportional to amount of unconjugated F_2IsoPs in the samples or standards. Sulfuric acid (3N) was then added to each well to stop the reaction and the microplate was read at 450 nm using a microplate reader (Tecan). The declared limit of detection is 0.2 ng/ml and the possible cross-reactivity of this method is fixed below 3%. To achieve better accuracy by the ELISA method, a dilution rate of 1:4 (v/v) was adopted. 15-F2t-IsoP concentrations were normalized to the CREA levels.

Urinary BPA – glucuronide: To exclude contamination from BPA, all urine samples were collected in BPA-free plastic vessels (polypropylene) and stored at - 80°C until analysis. All the laboratory glass material used were washed with methanol and then kept in methanol for 12 hours, which was subsequently analyzed to verify the possible contamination of BPA. Each thawed urine was vortexed and 700 µl of acetonitrile, 750 µl of ethyl acetate and 10 µl of BPA-d_{16} (1 ng/µl), used as internal standard, were added to 400 µl urine sample. To facilitate the liquid – liquid extraction (LLE), samples were vortexed for 3 minutes,

centrifuged at 4000 rpm for 15 min and the supernatants were evaporated to dryness by a gentle stream of nitrogen. The dried extract was dissolved with 125 µl of methanol/water (1:1 v/v) and analyzed by HPLC – MS/MS to quantify GlcA–BPA. GlcA–BPA was identified and quantified by liquid chromatography equipped with a low-pH resistant reverse phase column, Kinetex EVO C18 (2.6 µm, 150 x 3.0 mm). The binary solvent system was: a) acidified ultrapure water with formic acid 0.1% v/v and b) acetonitrile (HPLC ultrapure grade) acidified with formic acid 0.1% v/v. The chromatographic separation was carried out at constant flow rate (200 µl/min-1) and constant temperature (23°C ± 1°C) by a column thermostat. The solvent linear gradient was from 10% to 30% of B in 5 min, to 65% of B at 30 min, at 33 min 95% of B. The concentration of solvent B was maintained at 95% for 5 min. The initial mobile phase was re-established for 10 min before the next injection. The injection volume was 20 µl and quantification was performed by internal standard method (BPA-d_{16}). Quantitative analyses were carried out by tandem mass spectrometry with a 6330 Series Ion Trap LC-MS system equipped with an Electrospray Ionization Source (ESI). The analytes were detected in negative mode. The dry gas (Nitrogen) was at 325°C, 20.0 psi and 10 l min-1; capillary voltage was at 2000V. Data acquisition was made in Multiple Reaction Monitoring (MRM) mode by monitoring the transitions of quasi-molecular ions [M-H]: 227 for BPA, 242 for BPA-d_{16}, 307 for HO3S–BPA, 403 for GlcA-BPA and 419 for OH-GlcA-BPA. Procedural blank samples with ultrapure water in place of urine were collected, extracted and analyzed by HPLC-MS/MS with the same samples protocol. In the processed

blanks were not detected BPA contaminations above the limit of detection (LOD, 0.065 ng mL-1).

Urinary Creatinine: urinary creatinine was determined by the kinetic Jaffè procedure (179) to normalize the excretion rate of the all urinary markers described above.

6. RESULTS

The characteristics of the students enrolled in the project are reported in **Table 1**. Numerousness, mean, standard deviation (s.d.) and percentage (%) for gender, age (years), height (m), weight (kg) and smoking exposure (number of cigarettes per day) are reported for the subjects grouped for educational level. Among the 223 students, 18 reported being active smokers (8%), all from the age group 14-19, 52 passive (23.3%), and 153 non-smokers (68.7%). All these parameters were proved not to be statistically different among educational level (non-parametric test: **p>0.05**). Therefore, these individual characteristics could be analyze as homogeneous in the three different groups.

	PRIMARY SCHOOL (7-10 years)	**SECONDARY SCHOOL (11-14 years)**	**HIGH SCHOOL (15-19 years)**	**TOTAL**
N.	87	34	102	223
Gender N. (%)	Male 48 **(55.2%)** Female 38	Male 15 **(44.1%)** Female 19	Male 57 **(55.8%)** Female 45	Male 119 **(53.3%)** Female 104
Age (years) Mean ± s.d.	8.87 ± 1.0	11.7 ± 0.8	16.6 ± 1.71	12.8 ± 3.8
Height (m) Mean ± s.d.	1.39 ± 0.08	1.54 ± 0.1	1.71 ± 0.08	1.56 ± 0.17
Weight (kg) Mean ± s.d.	35.6 ± 9.8	45.0 ± 7.5	64.5 ± 12.4	50.2 ± 17.2
Smoking habits N. (%)	Active 0 Passive 26 (30%) No exp 61 (70%)	Active 0 Passive 5 (14.7%) No exp 29 (85.3%)	Active 18 (17.6%) Passive 21 (20.5%) No exp 63 (61.9%)	Active 18 (8%) Passive 52 (23.3%) No exp 153 (68.7%)

Table 1. Gender, age, height, weight and number of active and passive smokers in the whole population and in three groups sub grouped according to the three educational level considered**.**

In **Table 2**, cotinine, 15F2t-IsoP, and GlcA – BPA, all expressed as nanogram per 1 milligram of creatinine, are listed sub grouped *per* educational level as mean, standard deviation, and minimum and maximum.

	Cotinine [ng/mg CREA]	**15F2t-IsoP [ng/mg CREA]**	**Total BPA inactivated [ng/mg CREA]**
	Mean (±sd) Min - Max	Mean (±sd) Min - Max	Mean (±sd) Min - Max
PRIMARY SCHOOL (7-10)	73.7 (± 109) 1.06 - 382.9	5.07 (± 5.4) 0.6– 38.8	7.5 (± 7.8) 0.02 – 38.7
SECONDARY SCHOOL (11-14)	51.2 (± 111.0) 0.1 – 372.3	3.4 (± 2.9) 0.5 – 17.1	6.9 (± 5..3) 0.9- 34.4
HIGH SCHOOL (15-19)	179.0(±282.5) 0.1 – 1730.9	5.76 (± 5.4) 0.4– 23.2	10.8 (± 0.8) 0.3 –55.4
TOTAL Mean (± s.d.) Min - Max	107 (± 200) 0.03 –1730	4.8 (± 4.8) 0.41 –38.8	8.56 (± 7.8) 0.02 –55.4

Table 2. Urinary cotinine, 15F2t-IsoP and total BPA inactivated values in the three groups subgrouped according to the three educational level considered. Min = minimum value; Max = maximum value. Units of biological markers are nanograms of every 1 mg of urinary creatinine.

GlcA – BPA (inactivated BPA) show an increase of concentration proportional with increasing age, even if the intermediate age group (11-14 years) is slightly lower. The same thing is observed also for 15F2t-IsoP and the exposure to tobacco (mainly passively breathed) quantified by cotinine. According to the Box-Cox regression results, the values of urinary cotinine, 15F2t-IsoP, and GlcA – BPA were subjected to a logarithmic transformation before carrying out the subsequent analysis, in order to stabilize the variance and normalize the distribution. If inspecting the two way plot of log (ng 15F2t-IsoP/mg CREA) versus log (ng GlcA BPA/mg CREA) highlighted a non-linear relationship between these

variables, suggesting a spline function to estimate the relationship, we used piecewise linear or "hockey stick" robust regression point (180). It presupposes that the effect of predictive variables on dependents can be best fitted by two straight lines, with different slopes, and calculating the two slopes and the value of the dependent at which the slope changes (the breakpoint or spline point). Therefore, the result of piecewise linear robust regression shows a break point at 1.79 (**95%CI: 1.56 - 2.02; p < 0.001**) of the effect of log-BPA on log-15F2t-IsoP (**Figure 6 and Table 3**). Thus, concentration of 15F2t-IsoP increases exponentially (more than threefold each one log unit of BPA), when log-BPA concentration overcomes the break point identified at 1.79.

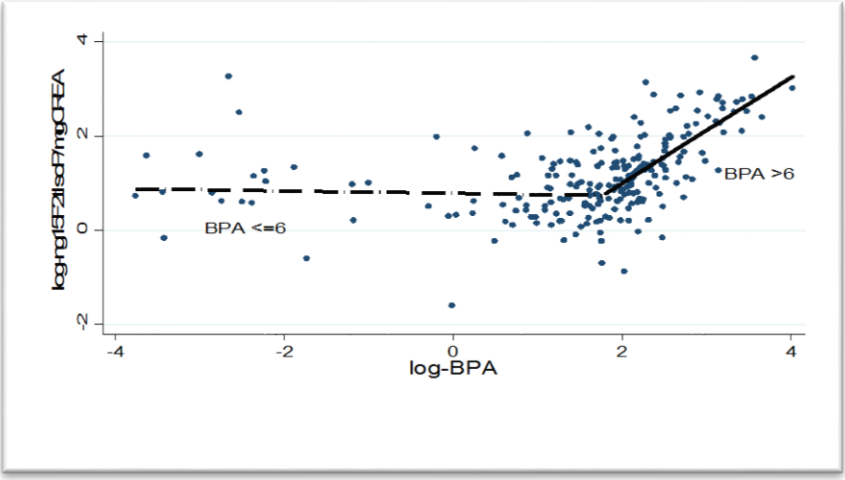


Figure 6: Piecewise linear robust regression of the relation of log (GlcA–BPA) on log (ng 15F2t-IsoP/mg CREA) – (break point at BPA = 6, 95% CI = 4.5 - 7.5). Exp (1.79) = 6

MLR analysis shows a positive effect also of log-cotinine concentration on log 15F2t-IsoP (**Table 3**). This last effect is evident also by observing **figure 7** where an increase of 12% of 15F2t-IsoP is observed for each one-unit-increment of log-cotinine.

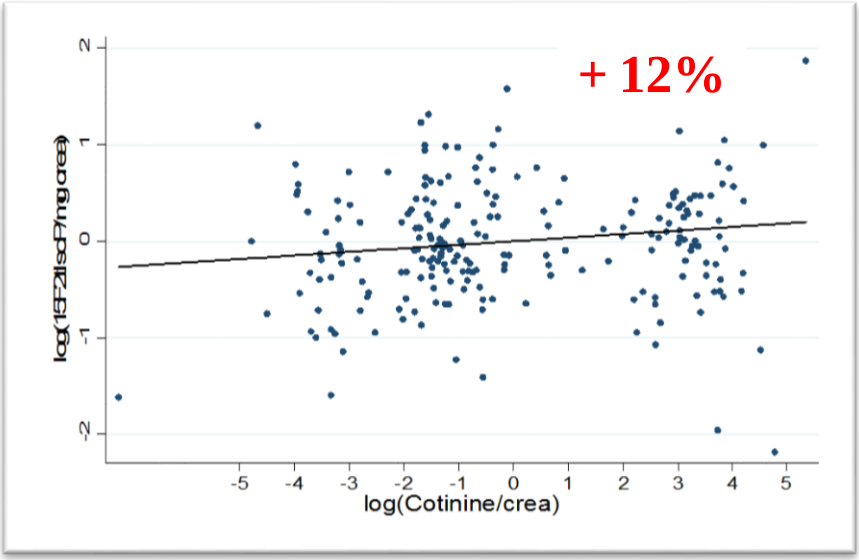


Figure 7: Added Variable Plot of the relation between log 15F2t-IsoP and log – cotinine given age.

Furthermore, the analysis of the relationship between log(ng15F2t-IsoP/mg CREA) and age shows a trend V-shaped (**figure 8**); with a significant decrease (**p = 0.026**) between infancy (7-10 year old) and the beginning of adolescence (11 – 15 years old) and then a new increase starting from 15 year of age (**Figure 8 and Table 3**).

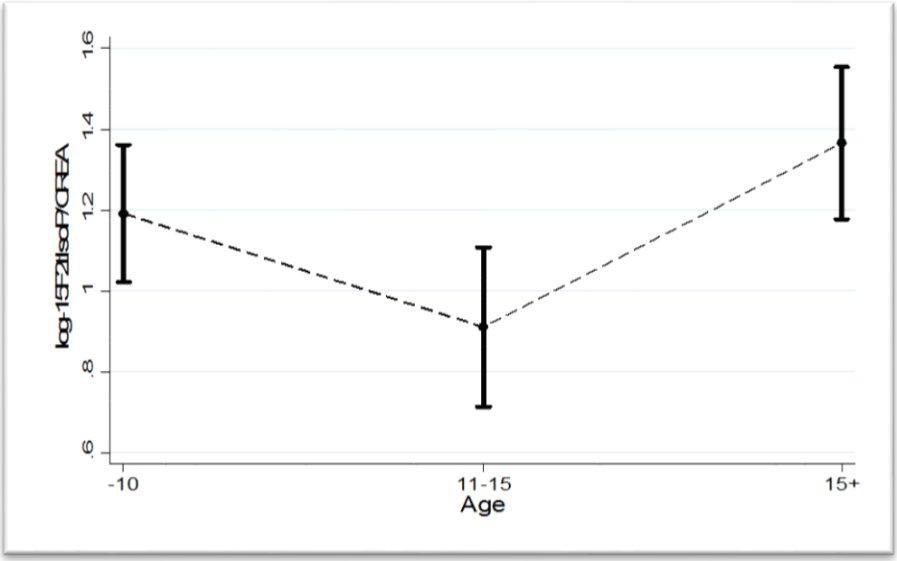


Figure 8: Margins-plot of the relation between log 15F2t –IsoP and age classes.

	Means	C.I. 95% Lower limit – Upper Limit		*p* <
Total inactive BPA (ng/mg CREA)	1.79	1.56	2.02	**<0.05**
Urinary Cotinine (ng/mg CREA)	0.03	0.00	0.06	**< 0.001**
Age (years old)**: < 10**	1.19	**1.02**	**1.36**	NS
11–14	0.91	0.71	1.11	**<0.05**
≥ 15	1.37	1.37	1.18	NS

Table 3: Multiple linear regression parameters, with means and 95% confidence interval (C.I.), of log 15F2t-IsoP as dependent variable and log (total inactive BPA), log cotinine, age as predictors.

7. DISCUSSION AND CONCLUSION STUDY –LINE 1

The main objective of this work was to evaluate the environmental diffusion and the consequent absorption of BPA in a population of children and adolescents attending primary, secondary and high school in a city located in Piedmont region, in the northwestern part of Italy. At the same time, the aim was to observe the role of this environmental pollutant in the induction of oxidative stress, taking into account as confounders, the role of passive and active exposure to tobacco smoke and age, other predictors of the same effect. These youth were enrolled as a population suitable to investigate as accurately as possible, some environmental conditions as predictors of oxidative status development. This because their life habits leads them to be more in contact with the outside environment and because their lower body weight makes them more sensitive and vulnerable. At this concern, young people are still in a phase of development of the body and of their metabolic system and therefore still fragile and hypersensitivity to environmental stimuli. The oxidative stress level was monitored through the quantification of urinary 15-F2t-IsoP concentration, a biomarker unaffected by diet potentially confounding the relationship we have investigated (141,181,182). Diet is very similar among all the students. This information originates from the answers to the questionnaire outlining a homogeneous domestic diet and because they benefit from the same school lunch prepared by the same company according to the requirements imposed by nutritionists working at the local health authority to minimize oxidant food. Since the exposure to BPA can influence the oxidative stress level, urinary GlcA – BPA was measured to know the

role of this contaminant in the onset of 15-F2t-IsoP values. Findings show that the effect of GlcA – BPA on 15F2t-IsoP has a threshold value around a break point of 1.79; this suggest that values of GlcA – BPA lower than 4.5 ng/mg of creatinine (exponential value of lower confidential limit) have no measurable effect on isoprostane; conversely, above the break point the 15F2t-IsoP grows linearly (**p < 0.005**). To explain this log-linear relationship characterized by a threshold value, we must remember the higher commitment of liver to contrast the higher concentrations of this contaminant, or an insufficient sensitivity of analytical technique to detect BPA at lower concentrations. Nevertheless, this last hypothesis seems to be contradicted by the log - linear relationship without threshold of the 15F2t-IsoP versus cotinine. Indeed, the induction of oxidative stress by passive and/or active smoking is confirmed in adolescent subject independently from age also in our previous paper (113). The age of the subject proved to be another factor that can significantly influence the 15-F2t-IsoP concentration. The 15-F2t-IsoP levels in the 11-15 age group has been studied in a previous work (178) where a slight decrease (6%) was recorded passing from 11 to 15 years. In the present study, the analysis of 15-F2t-IsoP levels according to age (7-19 years old) highlighted the V-shape previously illustrated. This seems to show that the oxidative stress experiences a lowering of intensity in the first years considered, and then return to grow regularly. This may result in the establishment and growth of a condition of chronical inflammation until senescence (182–184). Finally, urine GlcA – BPA concentrations were positively associated with BMI but not in significant way. Due to its rapid metabolism (half – life less than 6 h), BPA exposure estimates from first morning

urine may just represent the exposure at the prior meal (dinner), not daily or average exposure level. Given the food indigestion as the main exposure route to BPA, should be collect more urine samples throughout the day preceding the sampling to avoid underestimation of exposure to this contaminant. To conclude, the adolescent studied show an increase in oxidative stress dependent from tobacco smoke passively and/or actively breathed, and from GlcA – BPA higher than 4.8 ng/mg of crea. This last theme, not analyzed in depth yet by the International Scientific Community, must be taken care of by the Public Health Authorities in a careful manner and without forgetting the other Bisphenols, now present in the living environment. Thus, the evidence of these risky conditions for public health may represent a platform for designing new preventive strategies addressed to promoting adolescent health in a sensitive period of growth, sexual differentiation, and brain development. Thus it is crucial to continue the study of new and safer materials having the least impact on environment and human health. The two main results obtained in this work are: BPA causes an increase in oxidative stress in the adolescents selected for the study, but only from 6 ng / mg of CREA up. In addition, the tobacco smoke passively breathed is able to induce an increase of the oxidative stress. The promotion of health must therefore also consist of the preventive contrast to BPA still present in the living environment.

STUDY-LINE 2: BISPHENOLS AS ENDOCRINE DISRUPTORS AND THE ONSET OF OXIDATIVE STRESS IN NEWBORNS.

8. EXTENDED METHOD

- Epidemiological sampling:

The epidemiological sample was selected in order to represent the population of mothers and their newborns potentially and variously exposed to bisphenols and to evaluate if their exposure to BPA and BPS, during pregnancy and already the first 24 hours of breast-feeding may cause oxidative alterations. The subjects were recruited by consulting the register of subjects born during 2015-2017 at the University Unit of Neonatology of the Sant'Anna Gynecological Hospital of Turin. In particular, the enrollment started on March 2016. First, the doctors and nurses staff have been informed about the project in order to help in the recruitment of hospitalized mothers and babies. Only term pregnancies (> 37 EG-W) were included in the study; babies were excluded from the project if they had an Apgar score of less than 5 at 5 minutes of life, if they were admitted to the neonatal intensive care unit or, more in general, if they had a certain specific diagnosis certifying the danger of life. Every recruited mom was informed on the objective of this study and a written informed consent was signed and returned, while for the participation of the baby was requested the authorization of both parents. Thus, the participation of all the subjects did not occur until informed consents were obtained. Then the sensitive data of the women recruited, as well as those of the newborns, have been replaced by an

identification code to ensure full respect for privacy during all subsequent stages of sampling and analysis of data. At the end of the sampling, 100 mothers in physiological conditions with their babies and 50 mothers in pre-delivery circumstances potentially at risk (gestational diabetes, hypertension, and hypothyroidism) with their babies were enrolled. During the hospitalization mothers were asked to fill in a short questionnaire in order to acquire information on individual, clinical, smoking, diet and working mother's habits and on the baby's health status at birth time. Moreover, before hospital discharge few milliliters of milk with manual BPA/BPS-free breast pump and a pool of urine were collected. Furthermore, a specific polypropylene bag (Urinocol® Pediatric, BRAUN) was positioned inside the diaper of each newborn enrolled to collect a few milliliters of urine. Urine and milk samples were transported to the laboratory within 3 hours of sample collection and were stored at -80°C until laboratory analysis. In both cases, the biological samples were gathered in special glass little jars pretreated in methanol to reduce the risk of environmental contamination. Moreover, to better evaluate the oxidative trend, a control population constituted of 50 women, aged between 25 and 35 years, healthy and far, at least of 2 years, from any pregnancy was selected. To this sample has been proposed the same protocol adopted for the neo-mothers, with the aim to highlight if pregnancy may have any influences on the oxidative status. The local Ethics Committee of "A.O.U. Città della Salute e della Scienza" of Turin (22nd October 2015, practice n ° CS/709) approved the study protocol.

- *Biological analysis*

Urine

Total (Free + Conjugate) BPA and BPS: 4 mL of fresh urine was thawed and vortexed; 2 mL was used for the determination of free bisphenols, while the other 2 were used for the determination of bisphenol-glucuronides after 12 hours of incubation with 20 units of β-glucuronidase /arylsulfatase. Briefly, for the determination of free BPA and BPS: 2 ml of urine were transferred in a specially tube pretreated with methanol, vortexed and acidified with HCl at Ph 1. Subsequently NaCl was added (Salting Out process) together with 30 µL of a standard solution containing BPS^{d8} and BPA-d_{16} with a concentration of 0.10 and 0.05 mg/L respectively. At this point 750 µL of chloroform and 500 µL of acetone for the liquid-liquid extraction (LLE) were added. Each sample was vortexed and then sonicated for 1 minute. Finally, the sample was centrifuged at 4000 rpm for 20 min, and then the supernatant was collected and transferred to a new pretreated vial. LLE was repeated adding only 800 µl of chloroform and finally all the extracted supernatant was brought to dryness under nitrogen and then was suspended in 100 µl of a solution composed of ammonium acetate 5 mM in ultrapure water and acetonitrile 5mM. Concerning the determination of conjugate BPA and BPS: after 12 hours of incubation with the enzyme β-glucuronidase /arylsulfatase, the same extraction procedure described in detail above was performed.

Isoprostane: 15-F_{2t}IsoP was measured in urine by ELISA technique. All the details have been previously described.

Cotinine: Urinary cotinine was measured aiming to consider the possible role played by tobacco smoke in the onset of an oxidative stress status. Cotinine was measured in urine sample with a solid phase competitive ELISA kit, according to manufacturer's instructions. The samples and Cotinine enzyme conjugate were added to the wells coated with anti-Cotinine antibody. Cotinine in the samples competed with a Cotinine enzyme (HRP) conjugate for binding sites. Unbound Cotinine and Cotinine enzyme conjugate was washed off by washing step. Upon the addition of the substrate, the intensity of color was inversely proportional to the concentration of Cotinine in the samples. A standard curve was prepared relating color intensity to the concentration of the Cotinine.

Cytokine: the analysis of these parameters were carried out in collaboration with prof.ssa Tiziana Musso of the Microbiology Unit of the Dept. of Public Health and Pediatrics of University of Turin. Briefly, urine samples were aliquoted and immediately stored at −20°C until assayed. The urinary concentration of IL-1β and IL-6 was determined by an enzyme-linked immunosorbent assay human Duo-Set Kit (ELISA) (R & D Systems, Minneapolis, MN, USA). This method involves covering the bottom of the well with a specific antibody for the antigen to be measured. A wash is performed and the antigen is then introduced, which will bind to the antibody. A further wash is performed to remove the excess antigens. Finally, a specific antibody is introduced, which will bind the antibody and antigen complex forming a triple layer. At the last added antibody, a specific enzyme was bound, and by adding its substrate a colored product is

formed, which will highlight the well. The development of color is indicative of the presence of the antigen researched and the intensity of the color, measurable thanks to the spectrophotometer, provides a quantitative indication. The range of the Il-6 and IL-1β standard curves was 9.4 pg/ml and 3.9 pg/ml, respectively. All samples were measured in duplicate. The intra- and inter-assay coefficients of variation for 2 different proteins were < 10% and < 15%, respectively.

Creatinine: urinary creatinine was determined by the kinetic Jaffè procedure (Bartels and Cikes, 1969) to normalize the excretion rate of the all urinary markers described above.

Milk

Free BPA and BPS: 1 mL of breast milk was thawed, vortexed and subsequently diluted with 9 ml of a mixture of Milli-Q water and methanol (8 ml of water + 1 ml of methanol); later the sample was positivized with 30 uL of a standard solution containing BPS – d_8 and BPA - d_{16} with a concentration of 0,10 and 0,05 mg/L respectively. In parallel, a SPE C-18 cartridge (Strata C18-E 55 um, 70 A) was conditioned with 5 ml of methanol and immediately rebalanced with 5 ml of Milli-Q water. After loading the sample in the C-18 column, a wash with 8 ml of Milli-Q water and another one with 2 ml of a solution of methanol and water (9:1) were carried out. Finally, the sample was eluted with 5 ml of methanol, brought to dryness under a gentle stream of nitrogen and then re-suspended in 100 µl of a solution composed of ammonium acetate 5 mM in ultrapure water 60%and acetonitrile 5 mM 40%.

- Set up of the instrumental method in urine and milk for BPA/BPS

The accurate determination of BPs at ultra-low concentrations is frequently hindered by many factors. Organic solvents used in the laboratory may release BPs from plastic labware, leading to contamination, and pure water has also been found to contain detectable levels of BPs (185). Therefore, in the present project, the urine and milk collection tubes, other equipment used to handle and store samples, and each step in the chemical analysis procedure were screened to confirm the absence of BPA and BPS. All the solvents used for sample preparation were proven to BPs free. Only glassware was used to avoid BPs contamination by plastic

goods; moreover, the glassware was baked for 4 h at 400°C in a muffle furnace and then rinsed with methanol before use. Procedural blanks, containing water in place of urine or milk, were subjected to the same extraction protocol as the actual samples and analyzed to evaluate the level of contamination from the extraction procedure. No BPA and BPS were detected in the procedural blanks above the limit of detection for the biological matrices. As illustrated in the figure 9 below, BPA and BPS molecules contain in their structure hydroxyl groups (-OH) and, therefore, is preferred to operate in mass spectrometry by evaluating negative ions. The m/z ratios for bisphenol A and S are respectively 227 and 249, while for BPA-d_{16} and BPA-d8 are 241 and 257 respectively.

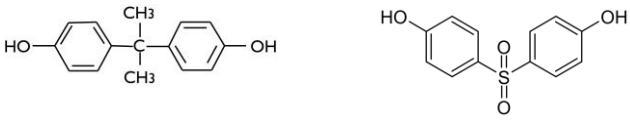


Figure 9. BPA and BPS chemical structures

The instrumental analysis was carried out with a UPLC Shimadzu Nexera X2 System interfaced through an ESI source (Turbo V™ Ion) to a mass spectrometer Sciex QTrap 5500 system. The transitions considered during this research are shown in the **table 5** below. The bold transitions are those used to quantify the analytes because they are the most sensitive.

	Molecular ion (m/z)	*Fragmented ion (m/z)*	*Reitention Time (min.)*
BPA	**227**	**212**	2,6
BPA	227	133	2,6
BPS	**249**	**108**	1,0
BPS	249	92	1,0
BPA-d_{16}	241	223	2,6
BPA-d_{16}	**241**	**142**	2,6
BPS-d_8	**257**	**112**	1,0
BPS-d_8	257	96	1,0

Table 5. The m / z ratios of the analytes molecular ions and those of their fragments

Once set–up the spectrometric part and therefore find the various m/z ratios to follow and optimize the values of the various gas and collision energy, a standard mix was prepared to set –up the chromatographic method and thus determine the times of retention for each analyte. The standards were dissolved in a 60% Acetic Acid (AA) 5mM / H2O 40% AA 5mM/ Acetonitrile (ACN) solution corrected at pH 7.5 with NH_3. The chromatographic column used is a Phenomenex Luna Omega C18 (1.6 μm, 100 mm × 2.1 mm). From the chromatographic run, it can be observed that deuterated and non-deuterated BPA is released around 2 minutes (**figure 11**), while the BPS deuterated and not deuterated around the minute 1.08 (**figure 12**). The best instrumental conditions to ensure maximum analytical sensitivity in the separation and determination of urine matrix analytes are reported in **Table 6**. Concerning to the milk samples, the chromatographic run has been prolonged because this matrix is more complex than urine. So the following instrumental conditions was optimized to ensure the maximum analytical sensitivity during the separation and determination of the matrix analytes (**Table 7**).

INJECTION	***Volume***: 20µl		
	SOURCE TEMPERATURE: 300 C°		
FLOW	350 µl/min		
CROMATOGRAPHIC RUN	***Time (min)***	***AA 5mM/H_2O %***	***AA 5mM/acetonitrile %***
	0	60	40
	5	0	100
	5,10	60	40
	11,.00	60	40

Table. 6: The optimal chromatographic conditions applied to urine samples.

INJECTION	***Volume***: 20µl		
	SOURCE TEMPERATURE: 300 C°		
FLOW	350 µl/min		
CROMATOGRAPHIC RUN	***Time (min)***	***AA 5mM/H_2O %***	***AA 5mM/acetonitrile %***
	0	70	30
	2,00	70	30
	12,00	0	100
	15,00	0	100
	15,10	70	30
	20,00	70	30

Table.7: The optimal chromatographic conditions applied to milk samples.

In this study the use of the mobile phase solvents (60% AA 5mM / H2O 40% AA 5mM / ACN) at pH = 7.5 / 8 was decisive to ensure a greater presence of bisphenol A and S molecules in deprotonate form. The optimal pH to obtain this

type of situation has been selected at the pH value of 7.5/8 instead of 10, because at higher pH values the stationary phase of the chromatographic column degrades. From the graph (**Figure 10**), it can be observed that the species of protonated bisphenol (blue line) decrease by increasing the pH values. At pH = 10, deprotonation of all bisphenol molecules occurs. Since the chromatographic column used during this PhD project would degrade at this values, we try to push the pH as close as possible to this value.

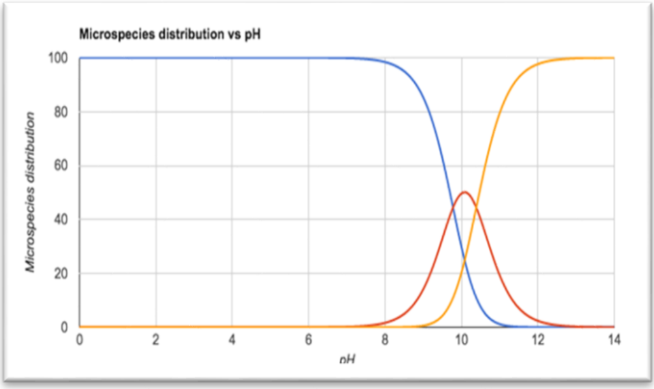


Figure 10: Graph showing the trend of protonated and deprotonated species at pH variation

Below are reported all together the chromatograms of the analytes: BPA, BPS and their respective deuterated internal standards (**Figure 11**); following are also reported the MRM chromatogram for BPA and its deuterated form (**Figure 12**) and the MRM chromatogram for BPS and its deuterated form (**Figure 13**).

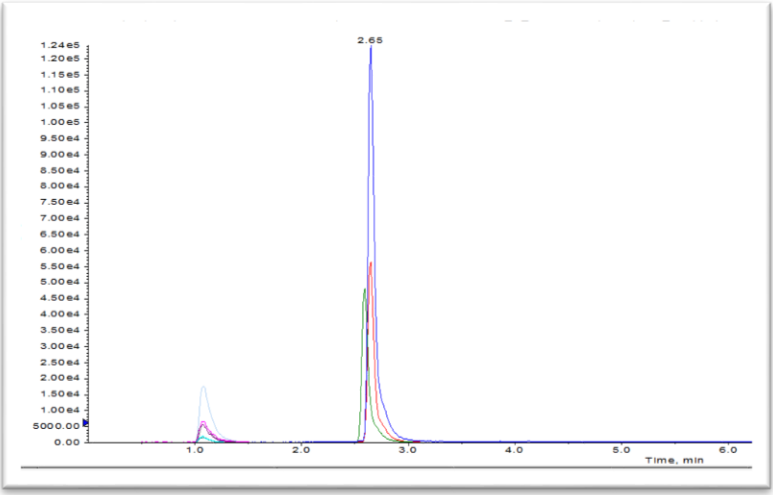


Figure.11: MRM chromatogram of the analytes.

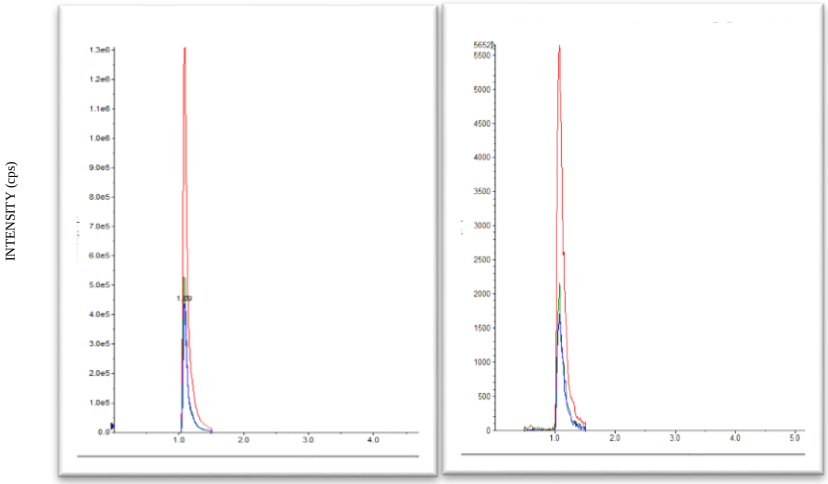


Figure 12: MRM chromatogram of the two BPA and BPA-d16 transitions.

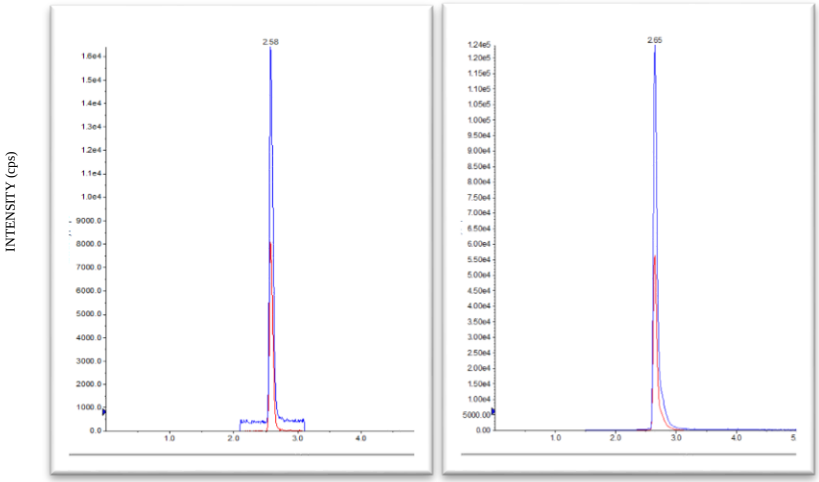
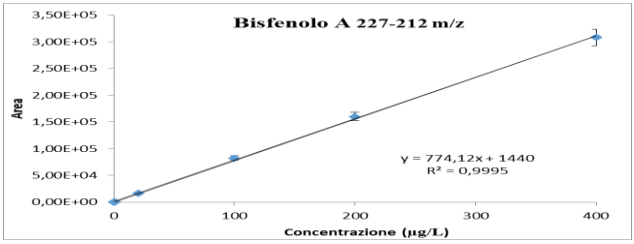
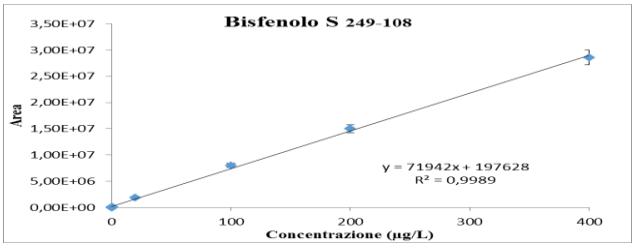


Figure 13: MRM chromatogram of the two BPS and BPS –d8 transitions.

- ***Validation of the instrumental method in urine and milk for BPA/BPS***

The methods were evaluated in terms of linearity, accuracy, precision and sensitivity. Isotopic internal standards were employed to compensate for the matrices effects and the loss during the analysis. Linearity was verified by injecting the standards for the two analytes, which exhibited satisfactory linearity, with correlation coefficients ($\mathbf{R^2}$) greater than 0.99 (**figure 14**). Accuracy was evaluated by measuring the recoveries of BPA and BPS in spiked, blank urine samples The LOQ and LOD values are listed in **table 8** and are defined as the lowest spiked concentration in blank urine and milk that can yield a signal to–noise (S/N).





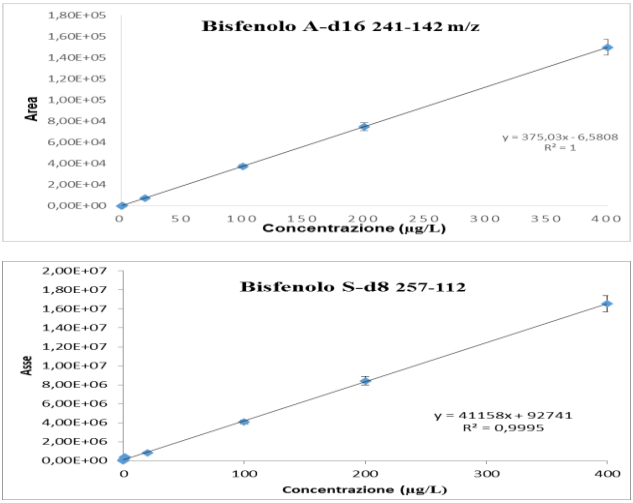


Figure 14: Calibration curves for each analyte considered and used for the quantification of the sample

Analyte	LOD (µg/L)	LOQ (µg/L)	LLOQ (µg/L)
BPA	9.16	30.52	0.5
BPA-d_{16}	0.63	2.10	0.5
BPS	15.44	51.46	0.1
BPS-d_8	10.13	33.77	0.1

Table 8: LOD, LOQ and LLOQ expressed as µg/L for each analytes considered and their corresponding internal standard.

9. RESULTS

The characteristics of the all mothers enrolled in this project are reported in **Table 9**.

	MOTHERS	*p*
N.	**172**	**N.S.**
Age (years) Mean ± s.d.	**33.6 ± 4.93**	
Height (m) Mean ± s.d.	**1.64 ± 0.07**	
Weight pre-pregnancy (kg) Mean ± s.d.	**63.8 ± 13.6**	
BMI Mean ± s.d	**23.7 ± 4.8**	
Scholarization level N. (%)	First level: **23 (13.4%)**	
	Second level: **69 (40.1%)**	
	Third level: **80 (46.5%)**	
Ethnic Group N. (%)	Caucasian **157 (91.3%)**	
	Non Caucasian **15 (8.7%)**	
Smoking habits N. (%)	Active **13 (7.5%)**	
	Passive **26 (15.1%)**	
	No Exp **133 (77.4%)**	

Table 9. Age, height, weight pre-pregnancy, BMI, scholarization level, ethnicity and number of active and passive smokers in the whole population considered.

Numerousness, mean and standard deviation (s.d.) for age, height, weight at the beginning of the pregnancy and BMI are reported. Moreover, in the bottom of the table are showed the numerousness and the percentage of the sample depending on ethnicity, scholarization level and exposure to tobacco smoke. Among the 172 mothers, 13 reported being active smokers (7.5 %), 26 passive (15.1%), and 133 non-smokers (77.4%). All these parameters are proved not to be statistically different (non-parametric test: **p>0.05**) and consequently the sample should be considered as a

homogeneous population. In the following table, the urinary levels of BPA and BPS, respectively free and conjugated form, 15-F2t Isop, cotinine, IL-6 and IL-1β, normalized for creatinine levels, are listed as mean and standard deviation (**table 10**).

N.	MOTHERS							
	BPA (ng/mg CREA) **Mean (± s. d.)**		**BPS** (ng/mg CREA) **Mean (± s. d.)**		**15-F2t Isop** (ng/mg CREA) **Mean ± s.d.**	**Cotinine** (ng/mg CREA) **Mean ± s.d.**	**IL – 1β** (pg/ml) **Mean ± s.d.**	**IL – 6** (pg/ml) **Mean ± s.d.**
	free	**con**	**free**	**con**				
172	0.23 (± 0.42)	0.19 (± 0.35)	0.04 (± 0.15)	0.023 (± 0.14)	3.28 ± 4.18	9.32 ± 33.4	34.4 ± 92.3	82.5 ±213.5

Table 10. Urinary free and conjugate BPA/ BPS, 15F2t-IsoP, cotinine, IL– 1β and IL – 6 values in the whole population considered.

Subsequently, in order to better understand the exposure level to bisphenols, mothers sample was sub-grouped according to the presence or absence of some specific diseases. In particular, we decided to include only those subjected affected by diabetes (pre or during pregnancy), hypertension and hypothyroidism. In fact, those pathologies probably could be influenced by the exposure to high levels of bisphenols. Therefore, **table 11** shows the same characteristics for the two categories previously mentioned as reported in **table 9**. The analysis shows differences statistically significant between the two groups for weight – pre pregnancy, BMI and educational levels ($p<0.005$), while the other parameters are proved not to be statistically different among the two groups. The biological markers, previously described, have been re-considered according to these two sub-groups. Thus, in **table 12**, no statistically

differences have been highlighted, even if, as showed in the bar-graphs reported below, the tendency among pathological mothers with higher oxidative-stress and inflammatory levels are evident if compared to the healthy mothers (**figure 14**).

	MOTHERS		***p***
	Pathological	**Non Pathological**	
N	**60**	**112**	N.S.
Age (years) **Mean ± s.d.**	**33.9 ± 5**	**33.4 ± 4.9**	N.S.
Height (m) **Mean ± s.d.**	**1.65 ± 0.06**	**1.63 ± 0.07**	N.S.
Weight pre-pregnancy(kg) **Mean ± s.d.**	**70.9 ± 17.1**	**60.1 ± 9.4**	**< 0.05**
BMI **Mean ± s.d.**	**25.9 ± 6.1**	**22.5 ± 3.3**	**< 0.05**
Scholarization level **N. (%)**	First level: **9 (15%)**	First level: **14 (12.6%)**	**< 0.05**
	Second level: **34 (56.6%)**	Second level: **35 (31.5 %)**	
	Third level: **17 (28.4%)**	Third level: 62 (**55.9%**)	
Ethnic Group **N. (%)**	Caucasian: **53 (88.3%)**	Caucasian **104 (92.8%)**	N.S.
	Non Caucasian **7 (11.7%**)	Non Caucasian **8 (7.2%)**	
Smoking habits **N. (%)**	Active **7 (11.6%)**	Active **6 (5.35)**	N.S.
	Passive **9 (15%)**	Passive **17 (14.2%)**	
	No Exp **44 (73.3%)**	No Exp **89 (79.5%)**	

Table 11. The two groups sub grouped according to the healthy condition considered. As pathological were considered only mothers with pregnancy or not diabetes, hypertension and hypothyroidism because these are the 3 diseases more probably associated with a high exposure to bisphenols.

	MOTHERS							
	BPA (ng/mg CREA) **Mean (± s.d.)**		**BPS** (ng/mg CREA) **Mean (± s.d.)**		**15-F2t Isop** (ng/mg CREA) **Mean ± s.d.**	**Cotinine** (ng/mg CREA) **Mean ± s.d.**	**IL – 1β** (pg/ml) **Mean ± s.d.**	**IL – 6** (pg/ml) **Mean ± s.d.**
	free	**con**	**free**	**con**				
Pat.	0.24 (± 0.42)	0.2 (± 0.41)	0.04 (± 0.18)	0.033 (± 0.19)	3.84 ± 4.31	5.9 ±9.2	43.9 ±91.05	92.9 ±154.6
Non Pat.	0.23 (± 0.43)	0.19 (± 0.31)	0.036 (± 0.12)	0.017 (± 0.11)	2.98 ± 4.10	11.15 ±40.8	29.34 ±92.99	76.9 ±239.6
p.	**N.S.**							

Table 12. Urinary free and conjugate BPA/ BPS, 15F2t-IsoP, cotinine, IL– 1β and IL – 6 values in the in the two groups sub-grouped according to the healthy condition considered.
Path: Pathological women;
Non Path: Non pathological women;

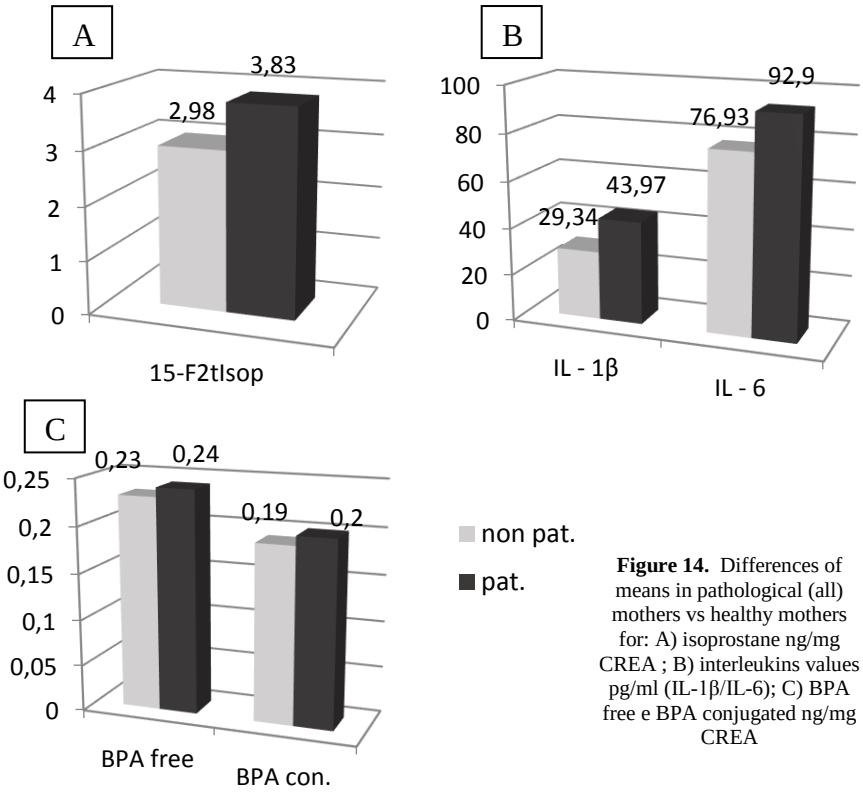


Figure 14. Differences of means in pathological (all) mothers vs healthy mothers for: A) isoprostane ng/mg CREA ; B) interleukins values pg/ml (IL-1β/IL-6); C) BPA free e BPA conjugated ng/mg CREA

For the second topic of this project and in order to better understand the relation between the level of oxidative stress and the healthy condition of the sample considered, mothers were sub-grouped again according to the presence of diseases. Unlike the division previously described, we decided to include in the pathological category all the mothers who referred a general disease during pregnancy. In particular, we selected also allergic, autoimmune or respiratory disease, such as asthma. Therefore, **table 13** shows the same characteristics already considered in Table 8, but sub-grouped into two different categories.

	MOTHERS		***p***
	Pathological	**Non Pathological**	
N	75	97	N.S.
Age (years) **Mean ± s.d.**	**33.7 ± 4.9**	**33.5 ± 4.9**	N.S.
Height (m) **Mean ± s.d.**	**1.65 ± 0.06**	**1.63 ± 0.07**	N.S.
Weight pre-pregnancy(kg) **Mean ± s.d.**	**68.3 ± 16.5**	**60.5 ± 9.6**	**< 0.005**
BMI Mean ± s.d	**25.1 ± 5.8**	**22.6 ± 3.4**	**< 0.005**
Scholarization level N. (%)	First level: **11 (14.6%)**	First level: **12 (12.3%)**	**< 0.005**
	Second level: **39 (52%)**	Second level: **30 (31%)**	
	Third level: **25 (33.4%)**	Third level: **54 (56.7%)**	
Ethnic Group N. (%)	Caucasian **67 (89.3%)**	Caucasian **90 (93%)**	N.S.
	Non Caucasian **8 (10.7%)**	Non Caucasian **7 (7%)**	
Smoking habits N. (%)	Active **10 (13.3%)**	Active **9 (9.27%)**	N.S.
	Passive **16 (21.3%)**	Passive **22 (22.7%)**	
	No Exp **49 (65.3%)**	No Exp **66 (68%)**	

Table 13. The two groups according to the healthy condition considered. In particular. As pathological were considered all mothers referred a pathological condition during pregnancy.

As reported in the **table 13**, all the parameters considered are proved not to be statistically different among the two groups, except for weight – pre pregnancy, BMI and educational levels. In **table 14** the urinary levels of 15-F2t-Isop, cotinine, IL-6 and IL-1β, normalized for creatinine, are listed as mean and standard deviation. Thus, it can be observed that no statistically difference has been highlighted, even if, as showed in the bar-graphs reported below, the tendency among pathological mothers with higher oxidative-stress levels are again evident if compared to the healthy (**figure 15-A**). Otherwise, the comparison between pathological mothers pathological mothers with higher inflammatory level and the control group show difference statistically significant ($p<0,005$) (**figure 15-B**)

	MOTHERS			
	15-F2t Isop (ng/mg CREA) **Mean ± s.d.**	**Cotinine** (ng/mg CREA) **Mean ± s.d.**	**IL – 1β** (pg/ml) **Mean ± s.d.**	**IL – 6** (pg/ml) **Mean ± s.d.**
Pat.	**3.9 ± 5.8**	**7.3 ± 13.8**	**48.4 ± 118.5**	**119.3 ± 295.9**
Non Pat.	**2.73 ± 1.93**	**10.8 ± 42.8**	**23.6 ± 63.8**	**54.0 ± 108.8**
p.	**N.S.**			

Table 14. Urinary free and conjugate BPA/ BPS, 15F2t-IsoP, cotinine, IL– 1β and IL – 6 values in the in the two groups sub-grouped according to the healthy condition considered

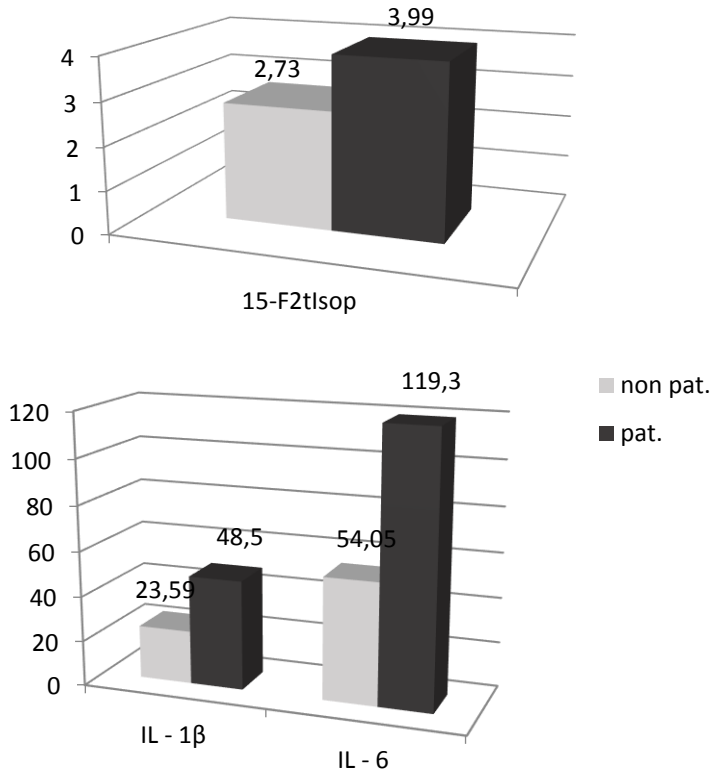


Figure 15. (**A)**: the bar-graph show that no statistically difference has been highlighted, even if, there is the tendency among pathological mothers with higher oxidative-stress levels are again evident if compared to the healthy group. (**B)**: the comparison between pathological mothers pathological mothers with higher with higher inflammatory level and the control group show difference statistically significant ($p<0,005$)

15-F2t Isoprostane		Regression coefficient (95% C.I.)	$p <$
BPA conjugated		**-2.9 (0.8 - 0.9)**	**0.004**
Oxidative stress pathologies correlated		**+ 3.49 (2.9 - 48.4)**	**0.000**
Ethnic group		**+0.39 (0.5 - 3.1)**	**> 0.05**
Tobacco smoke:	**Passive smokers**	**+5.31 (4.1 - 21.2)**	**0.000**
	Active smokers	**+6.34 (4.9 - 20.6)**	
Age		**- 5.94 (0.8 - 0.9)**	**> 0.05**
Scholarization level : High School		**+1.39 (0.8 - 3.9)**	**> 0.05**
	Degree, Ph.D, others	**-1.70 (0.1 - 1.2)**	

Table 15. GLM model with oxidative stress as dependent variable and BPA conjugated, pathology during pregnancy, ethnic group, tobacco smoke exposure, age and scholarization level as independent variables.

Proceeding with the statistical analysis, we analyzed a more complex model (for mothers) that took into account all the independent variables analyzed in relation to the dependent variable of oxidative stress. Therefore, the GLM has highlighted as statistically significant the relationships between the 15-F2t -Isop dependent variable and the following independent variables: conjugated BPA, pathologies during pregnancy and tobacco smoke exposure. In particular, the GLM showed an inversely proportional correlation between the 15-F2t-Isop and the BPA- conjugated values (p = 0.004), while the levels of oxidative stress are directly related to the presence of pathologies during pregnancy (p = 0.000) and with the increase of exposure to tobacco smoke (passive and active p = 0.000) (**table 15**). The other independent variables considered, such as ethnicity, age

and scholarization level, have proved to be not statistically significant, although the educational level shows an inversely proportional trend with isoprostane levels.
Finally, we decided to evaluate and compare this results with a control group of 50 healthy and not closed to pregnancy (almost to 2 years) women. The characteristics of the control group enrolled are reported in **table 16**.

	CONTROL SAMPLE	*p*
N.	**50**	
Age (years) Mean ± s.d.	**28.8 ± 4.83**	
Height (m) Mean ± s.d.	**1.65 ± 0.07**	**N.S.**
Weight (kg) Mean ± s.d.	**59 ± 9.7**	
BMI Mean ± s.d.	**21.5 ± 3.24**	
Smoking habits N. (%)	Active: **6 (12%)**	
	Passive: **12 (24%)**	
	No Exp: **32 (64%)**	

Table 16. Age, height, weight, BMI, and number of active and passive smokers in the control group.

Moreover, in the bottom of the **table 16** are showed the numerousness and the percentage of the sample depending on ethnicity, scholarization level and exposure to tobacco smoke. All these parameters were not statistically different (non-parametric test: $p > 0.05$) and consequently the sample should be considered as homogeneous. In the **table 17**, the urinary levels of BPA and BPS, respectively free and conjugated form, 15-F2t-Isop, cotinine, IL-6 and IL-1β, normalized for creatinine levels, are listed as mean and standard deviation.

N.	CONTROL SAMPLE							
	BPA (ng/mg CREA) **Mean (± s.d.)**		**BPS** (ng/mg CREA) **Mean (± s.d.)**		**15-F2t Isop** (ng/mg CREA) **Mean ± s.d.**	**Cotinine** (ng/mg CREA) **Mean ± s.d.**	**IL – 1β** (pg/ml) **Mean ± s.d.**	**IL – 6** (pg/ml) **Mean ± s.d.**
	free	**con**	**free**	**con**				
50	1.02 (± 1.38)	1.00 (± 1.37	0.07 (± 0.14)	0.07 (± 0.14)	11.7 ± 15.6	27.24 ± 68.3	3.8 ± 0	9.3 ± 0

Table 17. Urinary free and conjugate BPA/ BPS, 15F2t-IsoP, cotinine, IL– 1β and IL – 6 values in the in the two groups sub-grouped according to the healthy condition considered.

The comparison between the urinary mothers BPA-free, oxidative and inflammatory levels and those of the control group highlight differences statistically significant ($p < 0.005$). As illustrated in **figure 16,** this relationship is more evident when the model correlate isoprostane and BPA free levels. Moreover, subjects of the control show higher exposure if compared to mothers (**figure 17**)

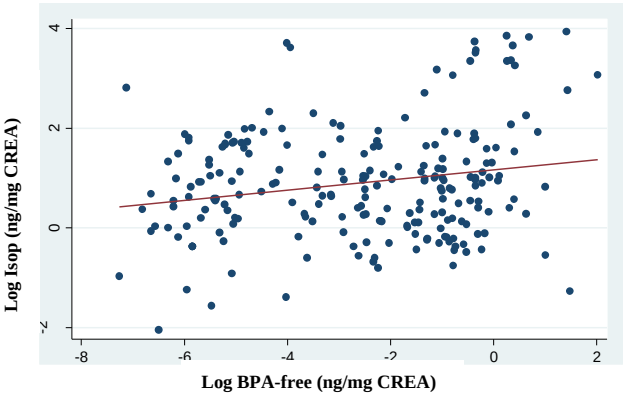


Figure 16. Correlation between Log Isop (ng/mg crea) and Log BPA free

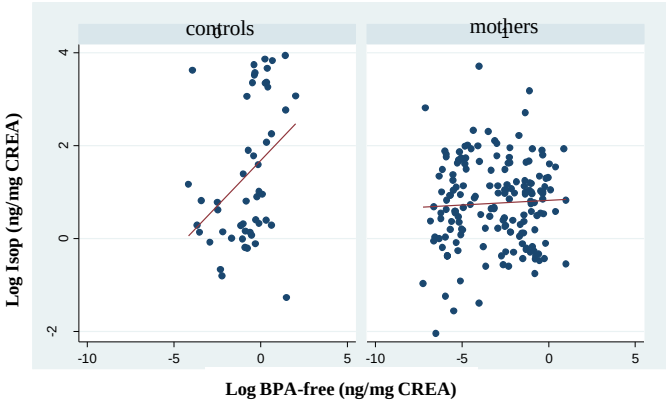


Figure 17. Correlation between isoprostane and BPA free level, subg-rouped for controls and mothers.

Another topic of this second Ph.D. project was to clarify if during pregnancy and, in particular at the moment of delivery, may occur oxidative alterations in newborns, also in relation to a possible exposure to BPA and BPS. Therefore in **table 18** are reported the characteristics of the 172 babies enrolled in the project. Numerousness, mean, standard deviation (s.d.) and percentage (%) for babies exclusively breast feed, breast and formula (as integration) feed and presence/absence of pathologies at birth are reported for the 172 subjects grouped for sex. All the parameters considered are proved not to be statistically different (non-parametric test: $\boldsymbol{p > 0.05}$) and consequently also the babies sample should be considered as a homogeneous population. As mothers and sample group, in the **table 19**, the urinary levels of BPA and BPS, respectively free and conjugated form and 15-F2t-Isop, normalized for creatinine levels, are listed as mean and standard deviation.

	BABIES		*p*
	BOYS	**GIRLS**	
N	**99**	**73**	**N.S.**
Length at birth (cm) **Mean ± s.d.**	**50.06 ± 1.7**	**49.3 ± 1.65**	
Cranial Circumference at birth (cm) **Mean ± s.d.**	**34.4 ± 1.10**	**33.9 ± 1.10**	
Weight at birth (kg) **Mean ± s.d.**	**3.34 ± 0.39**	**3.24 ± 0.41**	
Breast feeding N. (%)	YES: **81 (81.8%)**	YES: **60 (82.2%)**	
	NO: **18 (18.2%)**	NO: **13 (17.8%)**	
Breast feeding + formula N. (%)	YES: **69 (69.7%)**	YES: **54 (74%)**	
	NO: **30 (30.3)**	NO: **19 (26%)**	
Pathologies at birth N. (%)	YES: **19 (19.2%)**	YES: **21 (28.7%)**	
	NO: **80 (80.8%)**	NO: **52 (71.3%)**	

Table 18. Length, cranial circumference and weight at birth as mean and standard deviation in the babies groups sub grouped according to the sex. Moreover, for each category considered, the type of breastfeeding and the presence/absence of pathologies at birth is reported as numerousness and percentage.

	BABIES				
	BPA (ng/mg CREA) **Mean (± s.d.)**		**BPS** (ng/mg CREA) **Mean (± s.d.)**		**15-F2t Isop** (ng/mg CREA) **Mean ± s.d.**
	free	**con**	**free**	**con**	
BOYS	**0.24 (± 0.69)**	**0.063 (± 1.25)**	0.017 (± 0.66)	0.15 (± 0.52)	**5.21 ± 4.25**
GIRLS	**0.26 (± 0.52)**	**0.031 (± 0.92)**	0.024 (± 0.07)	0.15 (± 0.44)	**5.83 ± 9.8**
TOT.	**0.25 (± 0.63)**	**0.49 (± 1.12)**	0.020 (± 0.70)	0.15 (± 0.48)	**5.47 ± 7.1**
p.	**N.S.**				

Table 19. Urinary free and conjugate BPA/ BPS and 15F2t-IsoP in the in the babies group sub-grouped according to the sex.

The comparison between mothers BPA free levels and those of their babies does not highlight a statistically significant difference, but a little tendency among children with higher levels of BPA free appears. Differently, the urinary BPA-conjugate levels of the mothers show statistically differences ($p < 0.005$), as illustrated in **figure 19**, if compared with those of their respective newborns. Finally, the analysis performed between the 15-f2t-Isop levels of the mothers and their newborns show a positive correlation between increased maternal stress levels and the corrispective higher levels of oxidative stress in their newborn ($p < 0.005$) (**Figure 20**).

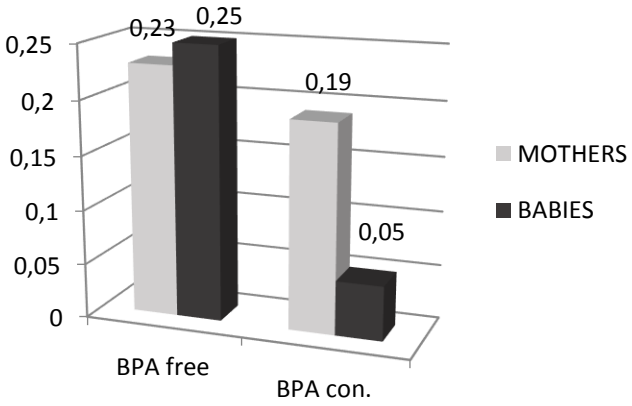


Figure 19. In the left side of the bar-graph the comparison between mothers and their babies BPA free level (ng/mg CREA) highlight only a tendency among newborns to have higher level; in the right side the urinary BPA-conjugate levels (ng/mg CREA) of the mothers if compared with those of their newborn statistically significant higher.

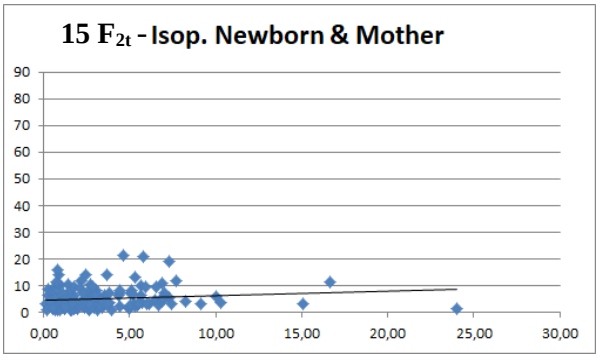


Figure 20. Correlation between 15 F2t -Isop (ng/mg CREA) mothers and babies levels.

To conclude, further analysis were carried out in order to assess if other characteristics of the population considered are somehow able to influence the bisphenols metabolization. The analysis showed an increase of 25% in the metabolic efficiency in BPA conjugation, and therefore detoxification, in the group of non-Caucasian (Asian, African and Afro-American) mothers, although due to the low numerosity of this group (5%) no statistical significance emerged.

10. DISCUSSION AND CONCLUSION STUDY – LINE 2

One of the main objectives of this Ph.D. project was to evaluate possible changes of oxidative stress during or in the immediately *post-partum*. For this reason, a population of 172 neo-mothers hospitalized at the neonatology department of the gynecological hospital S. Anna (Torino) were enrolled in order to investigate their status health, eating habits, life-style, eventual passive and/or active exposure to tobacco smoke related to oxidative stress and inflammatory status. Besides, the second aim of the project was to investigate the possible exposition to some endocrine disruptors (BPA and BPS), during pregnancy and the possible relationship with the oxidative stress trend both in mothers and in their babies. To best achieve these goals, the mothers sample was also related to a control sample consisting of 50 young women healthy and not closed to pregnancy almost to 2 years. A further objective was to evaluate the level of BPA and BPS in relation to diet, different lifestyles, and exposure to tobacco smoke, especially in conjunction with a so delicate period of life such as pregnancy.

The sample enrolled was proved to be homogenous for all the anthropometric and life-style characteristics analyzed. Among the 172 mothers, 97 were considered healthy while 75 had diseases such as asthma, allergies or autoimmune pathologies; among these 75 pathological mothers, 60 presented pregnancy diseases potentially at higher risk and influenced by an elevated exposure to endocrine disruptors: gestational diabetes, hypertension and hypothyroidism. Statistical analysis showed that women who started pregnancy in condition of overweight or even obesity, especially those with a lower educational level, are more

likely to develop diseases potentially dangerous for themselves and their fetus, such as diabetes or hypertension. This result leads to the hypothesis that overweight or obesity as a pathological condition preceding pregnancy, together with a lower educational level, seems to predispose to a situation of greater risk, especially because they do not effectively apply or more often underestimate the importance of the suggested the preventive strategies concerning the restraint and/or decrease of the body weight. The oxidative stress and inflammatory levels were measured respectively through urinary quantification of 15-F2t-Isop, IL-1β and IL-6. The general oxidative stress trend in the mothers group had significantly lower values, compared to the control population. However, the oxidative stress levels were higher in the pathological mothers group than those considered healthy. As a possible explanation, numerous longitudinal studies in normal human pregnancy show that oxidative stress levels, measured by urinary F-2-isoprostanes increase progressively throughout pregnancy. In particular, they found a significant increasing in oxidative stress from week 9 until week 40 while, thereafter, a significant decrease to basal levels at post-partum weeks 9, followed by a further increase at post-partum weeks 10 onwards, was observed (124,186). We can suggest that free radical-mediated lipid peroxidation in mild form might have a protective role in human right term-labor and delivery. Unlike the level of oxidative stress, the inflammation degree assessed through the urinary quantification of IL-1β and IL-6, is significantly higher in neo mothers if compared with the control population. In this purpose, interleukins, and in this case IL-1β and IL-6, are key molecules for triggering and promoting the cascade reaction of inflammation and mediators of other inflammatory cells,

but at the same time they also play a role in triggering term labor and so in encouraging contractions which then lead to the expulsion of the fetus (187). Therefore, as a possible explanation of the results obtained, the 172 mothers if compared with the control group showed physiologically and significantly higher inflammatory levels as indication of a recent birth and not of inflammation status due to a pathological conditions and/or a higher exposition to environmental pollutant.

Regarding the exposure to endocrine disruptors, the urinary quantification of BPA and BPS in their free (active) and conjugated (inactive) forms as well as the answers given to the questionnaire investigating the life-style habits, did not put in evidence significant differences. The exposure is homogeneous among the mothers who have been hospitalized for around 3 days. The hospitalization, and so the impossibility of mothers to keep their personal and usual life habits, as well as food, make-up and environmental exposition, together with the short half-life (about 6h) of the considered analytes, allowed to "photograph" only the exposure of the last hours before sampling. That condition most likely does not coincide with their usual exposure because the hospitalization interrupted and changed for some days the usual life-habits and, probably also the exposure to bisphenols. On the contrary, in the control group a typically situation closer to a common lifestyle was recorded, even if related to the last pre-sampling hours. The exposure appears a little higher although characterized by an efficient hepatic clearance since the levels of bisphenol (especially BPA) conjugated and then metabolized exceeds the share of the free and still active. GLM results highlight important relationships between oxidative stress levels and the most relevant

independent variables considered in this project. This type of model allows to analyze these relationships in their complex, evaluating all their contributions. In fact, GLM shows an inversely proportional relationship between oxidative stress levels and efficiency of metabolize and excrete BPA. This suggests that at higher levels of oxidative status corresponds a decreased capacity of hepatic clearance. Furthermore, GLM highlighted directly proportional relationships between higher isoprostane values and the presence of a pathological state and/or the active or passive exposure to tobacco smoke during pregnancy. This confirms how tobacco smoke and a pathological condition, although controlled by pharmacological therapies, are highly predisposing factors to an increase in the oxidative status of the subjects. Finally, a trend between isoprostane levels and the scholarization level was seen, as already confirmed by preliminary univariate analyzes, although GLM has not shown significant correlation. This suggests that a lower educational level seems to predispose to incorrect lifestyles and therefore to a possible redox balance alteration. In this study, newborns proved not to be mostly and significantly exposed to free BPA if compared to their mothers although the maternal uterus, but especially the placenta, constitute a predisposing environment to greater exposure given the passage, although partially, through the umbilical cord (173). This predisposition is also favored by the high and active presence of the placental glucuronidase enzyme which determines the continuous transition of BPA between its free (active) form, and the conjugated one (174). The result obtained on this newborns sample analyzed, can be explained considering the environmental exposure to which fetus were subjected, definitely lower than the values reported in literature and does

not allow to highlight statistically significant differences. However, the difference between the urinary BPA-conjugate levels in mother compared to their babies is significant. In fact, newborns, given their still underdeveloped enzymatic system, are not able to metabolize toxic substances with the same speed and efficiency of adults (175). To strengthen this data, the oxidative stress levels in newborns, also assessed through the urinary quantification of 15-F2t Isop, are significantly higher than those of mothers, as confirmed by several authors, and physiologically tent to decrease in the following months (132). This result suggests and strengthens the concept of a protective strategy for breastfeeding infants: in fact, breast milk is very rich in antioxidants molecules that help newborns to fight and to dispose toxic substances accumulated during the intrauterine growth period (134). Moreover, a greater BPA metabolic efficiency has been highlighted in non-Caucasian mothers (188). Despite this, due to the low numerosity of these women compared to the total population enrolled, there were no highlighted statistically significant differences.

Finally, I must remember that BPS levels were also measured in all the sampled subjects, but it was not possible to carry out a subsequent data analysis since the values obtained proved to be too close to the limit of detectability and therefore potentially misleading and negligible. Currently, we are proceeding with the quantification of BPA/BPS levels in breast milk. As previously and extensively described, the analytical method is definitely set up; in fact, we have reached a good level of sensitivity, accuracy and precision that, as for urine samples, will allow us to highlight even the lowest levels of exposure. However, the temporization is due to the need to improve the purification of the sample at the

time of its resuspension and loading in UPLC, in order to minimize the clogging of the chromatographic columns and thus facilitate the analysis of all 172 samples.

FINAL CONCLUSION

The biomolecular epidemiology and the biological monitoring can provide new information both for the evaluation of individual and population risk. A completely new optic is the connection between environmental measurements of pollutants and the possible biological alterations of the human body, in order to study not only the exposition but also the mechanisms of adaptations. The final purpose is to reduce and prevent the risk besides the evidence of the biological damage.

Thus, the evidence of this risky condition for individual and even more for public health, both for living and working conditions may represent a platform for designing new preventive strategies.

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Lung function changes from childhood to adolescence: a seven-year follow-up study

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Abstract

Background: As part of an investigation into the respiratory health in children conducted in Torino, northwestern Italy, our aim was to assess development in lung function from childhood to adolescence, and to assess changes or persistence of asthma symptoms on the change of lung function parameters. Furthermore, the observed lung function data were compared with the Global Lung Function Initiative (GLI) reference values.

Methods: We conducted a longitudinal study, which lasted 7 years, composed by first survey of 4–5 year-old children in 2003 and a follow-up in 2010. Both surveys consisted in collecting information on health by standardized SIDRIA questionnaire and spirometry testing with FVC, FEV_1, FEV_1/FVC% and FEF_{25-75} measurements.

Results: 242 subjects successfully completed both surveys. In terms of asthma symptoms (AS – asthma attacks or wheezing in the previous 12 months), 191/242 were asymptomatic, 13 reported AS only in the first survey (early transient), 23 had AS only in the second survey (late onset), and 15 had AS in both surveys (persistent). Comparing the lung function parameters observed with the predicted by GLI only small differences were detected, except for FVC and FEF_{25-75} for which more than 5% of subjects had Z-score values beyond the Z-score normal limits. Furthermore, as well as did not significantly affect developmental changes in FVC and FEV_1, the decrease in FEV_1/FVC ratio was significantly higher in subjects with AS at the time of follow-up (late onset and persistent phenotypes) while the increase in FEF_{25-75} was significantly smaller in subjects with persistent AS ($p < 0.05$).

Conclusions: The GLI equations are valid in evaluating lung function during development, at least in terms of lung volume measurements. Findings also suggest that the FEF_{25-75} may be a useful tool for clinical and epidemiological studies of childhood asthma.

Keywords: Longitudinal study, Lung function, Adolescents, Air pollution, Environmental tobacco smoke

Background

The prevalence of childhood pulmonary diseases, especially bronchial asthma, is increasing worldwide [Global Initiative for Asthma (GINA) 2012, http://www.ginasthma.org/]: lung disorders in children [1,2] are most frequently of obstructive type and usually limited to the intrathoracic-intrapulmonary airways [3].

Reliable information of lung function would greatly benefit clinical assessment and patient follow-up. Studies on respiratory function tests in children and adolescents have been published [4], and specific criteria have been proposed for acceptable maximal expiratory flow volume (MEFV) and other reference values [5,6]. Findings arising from many of these studies have been assembled to generate the reference lung function equations proposed by the Global Lung Function Initiative (GLI) [7-9] of the European Respiratory Society. The Global Lung Project in 2012 (http://www.lungfunction.org/tools/90-equations-and-toolsml. The) published multi-ethnic reference equations for spirometry that span all-ages. However, these equations are until today not enough tested on the field, especially in children.

While lung development is a continuous process during childhood, lung function is dependent on age, gender, height, and ethnicity [10]. Both lung volume and forced expiratory volumes increase during this period, but not at the same rate [11]. Forced vital capacity (FVC) is affected by changes in muscular strength, by the shape and stiffness of the thorax, and by the number

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and size of alveoli in the lungs. Airflow, as measured for example by the FEV_1, is also affected by the caliber of the airways, and by lung and airways elasticity. In childhood, the FVC increases more rapidly than the FEV_1, leading to falls in the FEV_1/FVC ratio, but these trends are temporarily reversed in adolescence [11] before continuing to decrease during adult life. Measurement of lung function is important for the evaluation of physical development and the presence of disease but few studies evaluated the effects of respiratory symptoms and their possible changes over time on transformation of lung function parameters.

In 2003, we studied a group of children, age 3–6 years, attending kindergarten in Torino, Italy [5]. We collected data on respiratory function (by spirometry) and respiratory health (by standardized questionnaire) [12]. In 2010, we carried out a follow-up study of the same group of children.

The aims of the present study were: 1) to establish if our data, drawn from a Northern Italian population, conforms with the GLI reference values; 2) to assess changes in lung function and to evaluate the effects of asthma symptoms (AS) on changes of lung function parameters in children during the transition to adolescence.

Methods

Study population

As part of a research project funded by the Piedmont Regional Council (northwest Italy) focusing on the effects of environmental pollution in preschoolers, in 2003 we studied 960 children, aged 3–6 years, drawn from 18 kindergarten schools located in Torino (6.700 inhabitants/km^2, 240 m a.s.l.), an urbanized Italian city with almost 900.000 inhabitants. This initial cohort was whittled down to 766 children as some declined to participate or spirometry testing was deemed invalid. To update the database in 2010, we again contacted the individuals studied in 2003. The demographic information provided by the parents, together with the information within the 2003 consent forms, allowed us to reconstruct a database of 573 children, now drawn from 20 secondary schools located in Torino. Each child was given the same the identification code in the 2003 and 2010 surveys to facilitate data comparison between surveys. Since the subjects were underage, during a public meeting in both the two occasions, parents and teachers were informed on the objective of this study. A written informed consent was signed and delivered by each the participants' parents. Thus, the participation of all the subjects did not occur until after informed consent was obtained. However, the local Ethics Committee "San Luigi Gonzaga Hospital" (previously named "ASL TO2") has expressed a favorable opinion with practice number 826/13/08.

Questionnaire

Child health information was collected in the 2003 and 2010 surveys using the same standardized SIDRIA (Italian Studies on Respiratory Disorders in Children and the Environment) questionnaire [12,13] compiled by the parents. The questionnaire was administered aiming to check the presence of respiratory symptoms and related risk factors.

Spirometry

Written informed consent was obtained from the parents prior to both 2003 and 2010 measurements. Spirometry was carried out in the morning during school activities. Height (measured with a stadiometer), weight and body mass index (BMI) (computed as weight/height^2) of each child were also recorded. Pulmonary function was measured using a turbine-based Masterscope Rotary Jaeger spirometer with subjects standing and wearing a nose-clip. In 2003, we organized the children into small groups and, with playful communication, we explained how to carry out the test. All the tests were performed using special incentive spirometry software ("blowing out candles" software). Spirometry testing was performed in a similar manner in 2010, without the use of "blowing out candles" software. In both surveys, each child recorded 3–6 MEFV curves within a 10–15 min interval. Subjects with only one acceptable measurement were excluded from the analysis. The exclusion criteria adopted in 2003 are specified elsewhere. In particular we have considered not acceptable the manoeuvres with: a) a sub-maximal expiratory effort in which a peak expiratory flow (PEF) was not clearly determined (i.e. in presence of flat or rounded curves), or with slow rise of PEF (top of the curve to the right) [14,15]; b) evidence of cough or glottis closure [6]; c) an expiration time lesser than 0.5 seconds [15]; d) an abrupt end of expiration effort (presence of a sharp drop or cessation in flow from a point in which the flows where >25% of PEF) [14,15]. Furthermore, children with reported skeletal anomalies or lung diseases, other than asthma, were excluded [5]. In 2010, the exclusion criteria adopted were in accordance with the current guidelines [10]. For subjects who reported actual acute symptoms or taking drugs for respiratory disorders, the examination was rescheduled after adequate washout period (at least 24 hours) or symptom remission.

Statistical methods

Lung function test results were used only if valid in both surveys. Descriptive statistics on both occasions were performed and reported for all subjects. All analysis were performed by means of STATA® 12 statistical package (StataCorp College Station, Texas 77845 USA). In the analyses, asthma symptoms (AS) where defined as

referred presence of asthma attacks or episodes of wheezing in the previous 12 months.

The subjects were categorized as following: *asymptomatic* if AS was absent in both surveys, *early transient* with AS in the first but not in the second survey, *late onset* with AS in the second survey only and *persistent* if AS was presents in both surveys. Following the results of Box-Cox regression, Linear transformations were applied when indicated to correct for heteroscedasticity and deviation from normal distribution.

To assess how our data for FEV_1, FVC, FEV_1/FVC% and FEF_{25-75} conformed with the GLI reference values, the GLI reference values were computed for each subject by means of the GLI-2012 Desktop Software for Data Sets vs 1.3.4, available on the web site http://www.lung-function.org/tools/90-equations-and-tools/196-obtain-software.html.

The means of observed values in asymptomatic as measured in each survey and values predicted by GLI were compared by means of *t*-test for paired data. The Z-score were also computed and the 5th and 50th percentiles were reported.

To measure the effects of time, anthropometric variables and symptoms on changes in lung function parameters over the time, annual changes were computed as the difference between the parameters from the second and the first survey, divided by years of follow-up. Annual changes were used in a set of multiple regressions as dependent variable, and gender and time varying covariates (anthropometric values: height, weight, BMI) and AS in both occasions as predictors, using asymptomatic subjects group as reference. Marginal means with confidence intervals (C.I.) at 95% were calculated from predictions at mean values of covariates and averaging over symptoms at the follow-up.

Results

In 2010, we traced and contacted 573 of the 766 children studied in 2003; of these, 174 declined to participate in the second survey. Consequently, the follow-up survey had 399 participants, mean age 11.8 years, drawn from 20 primary and secondary schools in Torino. Of these 399 subjects, 242 (60.3% males) adequately performed lung function tests in both surveys, and only these subjects were included in this analysis. The decision to participate in the surveys was not influenced by anthropometric variables or symptoms. Table 1 reports the main anthropometric characteristics and results of lung function tests in the two surveys.

Among the 242 subjects, 28 (11.6%) had AS in the first survey, 38 (17%) had AS in the second survey whereas 191 subjects did not have AS in either survey. Otherwise, from a longitudinal point of view, 13 children reported AS in the first but not in the second survey (early transient), 23 reported AS only in the second survey

Table 1 Characteristics and findings of lung function test of the 242 subjects at the first (2003) and second (2010) survey

	FEMALE			MALE			TOTAL		
	2003 Mean (sd)	2010 Mean (sd)	Δ/year Mean (sd)	2003 Mean (sd)	2010 Mean (sd)	Δ/year Mean (sd)	2003 Mean (sd)	2010 Mean (sd)	Δ/year Mean (sd)
AGE	4.5 (0.7)	11.7 (0.6)	7.34 (0.49)	4.5 (0.6)	11.8 (0.7)	7.23 (0.47)	4.5 (0.7)	11.8 (0.7)	7.30 (0.46)
HEIGHT (cm)	110.3 (6.2)	150.1 (8.5)	5.3 (0.7)	111.7 (6.3)	150.7 (8.8)	5.5 (0.7)	111.1 (6.3)	150.4 (8.7)	5.5 (0.7)
WEIGHT (Kg)	18.8 (3.2)	42.6 (9.3)	3.4 (1.2)	20.0 (3.8)	44.8 (11.3)	3.3 (1.8)	19.5 (3.6)	43.9 (10.5)	3.3 (1.6)
BMI%	15.4 (1.8)	18.6 (3.7)	0.41 (0.59)	15.9 (2.0)	19.0 (4.9)	0.44 (0.46)	15.7 (1.9)	18.8 (4.4)	0.43 (0.54)
FVC(ml)	1054 (239)	2647 (508)	221 (55)	1144 (226)	2842 (533)	231 (55)	1105 (236)	2756 (530)	227 (55)
FEV_1(ml)	1048 (224)	2293 (470)	146 (44)	1127 (211)	2426 (460)	147 (57)	1093 (220)	2367 (467)	147 (48)
FEV1%/FVC	96.6 (4.4)	85.3 (7.4)	−1.7 (1.1)	96.3 (4.1)	83.7 (6.0)	−1.8 (0.9)	96.4 (4.3)	84.3 (6.5)	−1.8 (1.0)
FEF_{25-75}(ml/s)	1561 (385)	2718 (720)	159 (88)	1735 (349)	2706 (731)	131 (167)	1660 (361)	2711 (720)	143 (136)

Table 2 Distribution of asthma symptoms (AS = Asthma or wheezing in the previous 12 months) on two observations

	No symptoms	Early transient	Late onset	Persistent
N	191	13	23	15
%	78.91	5.37	9.5	6.2

(late onset), and 15 had AS at the time of both surveys (persistent) (Table 2).

The Table 3 shows the results of comparison of observed with the GLI predicted values for FEV_1, FVC, FEV_1/FVC % and FEF_{25-75} in asymptomatic subjects. The FEV_1, FVC observed values were lower than predicted in both surveys while FEV_1/FVC% and FEF_{25-75} were lower than predicted in the second occasion only; the differences were significant at 5% level. However, the

Table 3 Means and changes by year of lung function parameter observed and predicted from GLI and their differences for asymptomatic subjects, compared with *t*-test for paired data

		2003		2010		Δ/year	
		Mean	p	Mean	p		p
FEV_1 (ml)	Observed	1107.4		2435.9		180.4	
	Predicted	1123.2		2497.5		188.3	
	δ	−15.8	NS	−61.6	*	−7.9	*
	95% CI of δ	(−127.8 – +5.6)		(−101.4 – −19.8)			
	Z-score SD_{50}	−0.138		−0.203			
	Z-score S_{LMS}	−1.583		−1.548			
	ln	879.5		2017.8			
FVC (ml)	Observed	1120.7		2804.1		213.4	
	Predicted	1216.0		2876.4		227.8	
	δ	−95.2	**	−52.3	**	5.6	**
	95% CI of δ	(−116.2 – −74.3)		(−91.9 – −12.7)			
	Z-score SD_{50}	−0.645		−0.113			
	Z-score S_{LMS}	**−1.954**		−1.468			
	ln	941.9		2139.9			
FEV_1/FVC (%)	Observed	96.6		85.5		−1.57	
	Predicted	92.9		87.1		−0.8	
	δ	3.7	**	−2.4	**	−0.77	**
	95% CI of δ	(+3.0 – +4.3)		(−2.4 – −0.8)			
	Z-score SD_{50}	0.961		−0.207			
	Z-score S_{LMS}	−0.905		−1.664			
	ln	81.6		76.3			
FEF_{25-75} (ml/s)	Observed	1621.3		2766.9		152.8	
	Predicted	1602.6		2955.8		185.2	
	δ	18.7	NS	−269.6	**	−32.1	**
	95% CI of δ	(−25.0 – +62.3)		(−269.6 – −108.2)			
	Z-score SD_{50}	0.045		−0.306			
	Z-score S_{LMS}	−1.357		−1.912			
	ln	1617.0		1994.3			

Legend:
δ: Observed. Predicted by GLI.
Δ: (Differences between the 2 occasions/Years of follow up).
* $p < 0.05$ ** $p < 0.01$.
CI = Confidence Interval.
Z Score = (standard deviation scores from regression Equation).
SD_{50} = Median S_{LMS} = fifth percentile.

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%	78.91	5.37	9.5	6.2

(late onset), and 15 had AS at the time of both surveys (persistent) (Table 2).

The Table 3 shows the results of comparison of observed with the GLI predicted values for FEV_1, FVC, FEV_1/FVC % and FEF_{25-75} in asymptomatic subjects. The FEV_1, FVC observed values were lower than predicted in both surveys while FEV_1/FVC% and FEF_{25-75} were lower than predicted in the second occasion only; the differences were significant at 5% level. However, the

Table 3 Means and changes by year of lung function parameter observed and predicted from GLI and their differences for asymptomatic subjects, compared with t-test for paired data

		2003		2010		Δ/year	
		Mean	p	Mean	p		p
FEV_1 (ml)	Observed	1107.4		2435.9		180.4	
	Predicted	1123.2		2497.5		188.3	
	δ	−15.8	NS	−61.6	*	−7.9	*
	95% CI of δ	(−32.8 ÷ +5.6)		(−101.4 ÷ −19.8)			
	Z-score 50_{Me}	−0.138		−0.203			
	Z-score S_{Me}	−1.583		−1.548			
	Δn	89.5		201.8			
FVC (ml)	Observed	1120.7		2824.1		231.4	
	Predicted	1216.0		2876.4		227.8	
	δ	−95.2	**	−52.3	**	5.6	**
	95% CI of δ	(−116.2 ÷ −74.3)		(−91.9 ÷ −12.7)			
	Z-score 50_{Me}	−0.645		−0.113			
	Z-score S_{Me}	**−1.954**		−1.468			
	Δn	94.9		210.9			
FEV_1/FVC (%)	Observed	96.6		85.5		−1.57	
	Predicted	92.9		87.1		0.8	
	δ	3.7	**	−2.4	**	−0.77	**
	95% CI of δ	(+3.0 ÷ +4.3)		(−2.4 ÷ −0.8)			
	Z-score 50_{Me}	0.961		−0.207			
	Z-score S_{Me}	−0.905		−1.664			
	Δn	81.6		76.3			
FEF_{25-75} (ml/s)	Observed	1621.3		2766.9		152.8	
	Predicted	1602.6		2955.8		185.2	
	δ	18.7	NS	−269.6	**	−12.1	**
	95% CI of δ	(−25.0 ÷ +62.3)		(−269.6 ÷ −108.2)			
	Z-score 50_{Me}	0.045		−0.306			
	Z-score S_{Me}	−1.357		−1.912			
	Δn	95.7.0		1954.3			

Legend:
δ: Observed, Predicted by GLI.
Δ: (Differences between the 2 occasions/Years of follow up).
* $p < 0.05$ ** $p < 0.01$.
CI = Confidence interval.
Z Score = Standard deviation scores from regression Equation).
50_{Me} = Median S_{Me} = 50th percentile.

during the first survey (adjusted for sex and height) were not significantly different among symptoms status groups. However, annual changes seemed to be affected by AS. When volumes were taken into account, AS of symptomatic subjects did not induced significant changes in FVC, FEV_1 if compared to asymptomatic subjects. Comparing the symptomatic subjects to asymptomatic ones, the decrease in the FEV_1/FVC% was significantly higher in subjects with AS in the second survey (late onset and persistent), but not in transient. Finally, FEF_{25-75} showed a significantly higher decrease in subjects with persistent AS ($p < 0.05$).

Discussion

The interpretation of functional parameters in the transition from childhood to adolescence was highlighted by the multiplicity of reference values and the discontinuity between age groups. The Global Lung Initiative (GLI) equations are the first global multiethnic reference equations for spirometry that span all ages and which seem to have solved this problem. A major limitation of any reference equation is that it is based on a cross-sectional snapshot of a population composed of individuals at different ages, but the age differences do not necessarily reflect the individual changes over time [16-18]. However, our results suggest that, at least for the age and time range examined in this study, the predictions of the GLI reference equations are a good approximation of the changes in lung function observed over time and adequately describe pulmonary function in growing subjects.

When we compared the values observed in asymptomatic subjects with the predicted by the GLI, the means were consistently different only for FVC and FEF_{25-75}. Although, for the most part of subjects these differences were within the range of normal variation of the GLI references values, they may indicate that some regional differences are possible, even among the same ethnic group. Thus, at least for FEF_{25-75} we recommend the use of regional data; at least until a proper longitudinal equation including individuals who have completed their lung growth.

Factors having a negative impact on the age-related growth of pulmonary function in children and adolescents may result in a lower maximally attained level of pulmonary function and perhaps in an earlier onset in decline of pulmonary function. Therefore, these factors potentially increase the risk of subsequently developing both reversible and irreversible obstructive pulmonary diseases [17-19].

Numerous follow-up studies in children with asthma have consistently shown that more severe respiratory symptoms in childhood predict reduced lung function in early adulthood [20,21]. In the mean time, epidemiological studies of childhood asthma are highlighting the causality between asthma and deficits in lung function, although the characteristics of growth in pulmonary function from childhood to adulthood are not completely

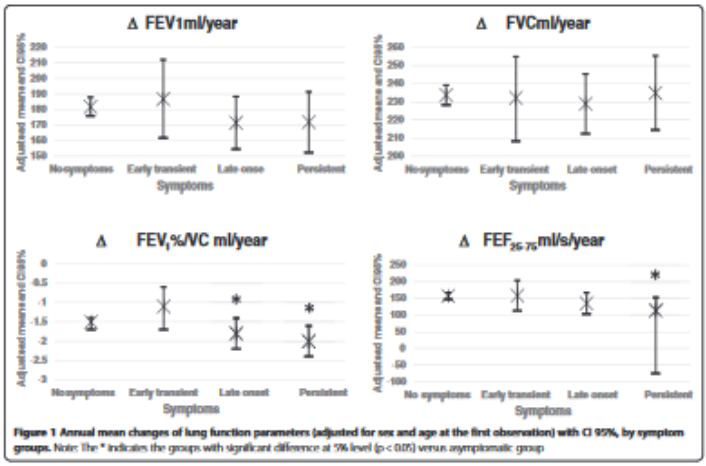

Figure 1 Annual mean changes of lung function parameters (adjusted for sex and age at the first observation) with CI 95%, by symptom groups. Note: The * indicates the groups with significant difference at 5% level ($p < 0.05$) versus asymptomatic group

clarified yet [22-26]. Determining whether the loss of lung function and asthma are, respectively, the cause or the effect of one another is crucial for the prevention of asthma and for our understanding of the origins of this important disease. The contradictory results can be due to differences in the frequency of assessment, cohort retention rates, and the use of quantitative measurements [27,28].

In the present study, the longitudinal analysis of a cohort of children examined in 2003 and 2010 showed that the presence of wheezing disorders (wheezing or asthma attack) at the time of both measurements (persistent phenotype) was associated with a smaller increase in forced flows (particularly FEF_{25-75}). In contrast, changes in the FEV_1/FVC% (and FEV_1 and $FEV_{0.5}$, although not statistically significant at 5% level) was affected by the presence of symptoms in the second measurement.

The FEV_1 (and the FEV_1/FVC%), which is considered to be reproducible and to represent an appropriate measure of airway obstruction, often shows normal values even in children with symptoms of uncontrolled asthma. In this regard, asthmatic patients may have ventilatory defects in the presence of normal FEV_1 [29-32].

The FEF_{25-75} is a more sensitive marker of symptomatic asthma than the FEV_1 in children [31] and in adults [32]. The middle volume flow rates, measured by the FEF_{25-75}, is theoretically less effort-dependent than the FEV_1, also because FEF_{25-75} is a measurement of the "small airways" patency [32,33] and does not include high flows in the lung volume. On the contrary, the guidelines of the American Thoracic Society and the GLI do not suggest that the assessment of FEF_{25-75} could play a significant role in the measurement of airflow obstruction [10,34]. The coefficient of variation of instantaneous flow is quite large, which partly explains their unsatisfactory performance in clinical decision-making. Moreover, the bronchodilators can affect the natural transformations of flows and, consequently, the values of FVC, making them no longer comparable with one another.

On the other hand, the FEF_{25-75} measured in children provides, compared to the FEV_1, additional information about clinical status and airway inflammation. Furthermore, the FEF_{25-75} is well correlated with bronchodilator responsiveness in asthmatic children with normal FEV_1, and the FEF_{25-75} is associated with increased childhood asthma severity and morbidity [35].

Middle volumes flows rates, particularly the FEF_{25-75}, seem to be a more sensitive indicator of small airway disease. Despite concerns of test-to-test variability and the lack of a clearly defined normal range, our findings suggest that the FEF_{25-75} is clinically relevant in children with asthma and could distinguish between subjects with symptoms that persist over time from subjects with transient or late-onset symptoms. However, in large and statistically powerful studies, that can compensate for the variability, the FEF_{25-75} can be a useful tool to detect mean differences between groups with different clinical characteristics. In these circumstances, the benefits of the increased physiological sensitivity of the FEF_{25-75} remain.

Study potential limitations

A possible limitation of this study could either be the relevant loss of subjects to follow-up because we were not able to trace them or because the subjects declined to repeat lung function testing or were not able to perform it. A further limitation can be due to impossibility to measure FEV1 in some children 3–4 years [5] aged because their expiratory time was less than 1 second: this produced a loss of subjects for longitudinal comparison.

Due to the initial estimate, the sample size in the symptomatic strata could be too small to show as statistically significant effects. However, although still inaccurate the estimation and the relative confidence intervals are quite informative to assess the direction of the effects.

Conclusions

Our results confirm the validity of the GLI reference equations in evaluating lung function during growth, at least as regards the dynamic volumes. Furthermore, our results highlight the usefulness of FEF_{25-75} measurement as a tool for clinical and epidemiological studies of asthma in children.

Abbreviations

GLI: Global Lung function Initiative; SIDRIA: Italian Studies on Respiratory Disorders in Children and the Environment; ALS: Asthma Like Symptoms; FVC: Forced Vital Capacity; FEV_1: Forced Expiratory Volume in the 1st second; FEV_1/FVC: Forced Expiratory Volume related to some portion of the FVC; FEF_{25-75}: Forced Expiratory Flow 25–75% of FVC.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PP contributed to ideation and design the study, to data acquisition and their interpretation. BT has contributed to the design of the study and interpretation of data, written the paper and collected the larger part of data. AC and CC have written the paper, contributed to the interpretation of data and revised the paper critically for important intellectual content. MB performed statistical analysis and contributed to the interpretation of data. BB contributed substantially to the design of the study, to writing and critically revise the manuscript, and to contributing with important intellectual content. All authors read and approved the final manuscript.

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RESEARCH ARTICLE Open Access

Air pollution, aeroallergens and admissions to pediatric emergency room for respiratory reasons in Turin, northwestern Italy

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Abstract

Background: Air pollution can cause respiratory symptoms or exacerbate pre-existing respiratory diseases, especially in children. This study looked at the short-term association of air pollution concentrations with Emergency Room (ER) admissions for respiratory reasons in pediatric age (0–18 years).

Methods: Daily number of ER admissions in a children's Hospital, concentrations of urban-background PM_{10}, NO_2, O_3 and total aeroallergens (Corylaceae, Cupressaceae, Gramineae, Urticaceae, Ambrosia, Betula) were collected in Turin, northwestern Italy, for the period 1/08/2008 to 31/12/2010 (883 days). The associations between exposures and ER admissions were estimated, at time lags between 0 and 5 days, using generalized linear Poisson regression models, adjusted for non-meteorological potential confounders.

Results: In the study period, 21,793 ER admissions were observed, mainly (81 %) for upper respiratory tract infections. Median air pollution concentrations were 22.0, 42.5, 34.1 μg/m³ for urban-background PM_{10}, NO_2, and O_3, respectively, and 2.9 grains/m³ for aeroallergens. We found that ER admissions increased by 1.3 % (95 % CI: 0.3-2.2 %) five days after a 10 μg/m³ increase in NO_2, and by 0.7 % (95 % CI: 0.1-1.2 %) one day after a 10 grains/m³ increase in aeroallergens, while they were not associated with $PM_{2.5}$ concentrations. ER admissions were negatively associated with O_3 and aeroallergen concentrations at some time lags, but these association shifted to the null when meteorological confounders were adjusted for in the models.

Conclusions: Overall, these findings confirm adverse short-term health effects of air pollution on the risk of ER admission in children and encourage a careful management of the urban environment to health protection.

Keywords: Airborne pollutants, Pollens, Time-series analysis, Pediatric emergency room, Short-term respiratory effects

Background

Over the last decades, the prevalence of respiratory diseases, and in particular of asthma and allergies, has increased considerably, especially in industrialized countries [1, 2]. The etiology of respiratory diseases is multifactorial and includes, among others, interactions between genetic predisposition and environmental factors [3]. The environmental dynamics, characterised by climate change, qualitative and quantitative aspects of chemical air pollution and airborne pollens, may partially explain the increased incidence of respiratory symptoms and respiratory diseases during the last years [4]. The short-term respiratory effects of air pollution include decreases in pulmonary function [5], increases in inflammatory biomarkers [6] and respiratory symptoms [7, 8], exacerbations of chronic obstructive pulmonary disease (COPD), infections [9, 10], school absenteeism [11] and respiratory mortality [12, 13].

The respiratory system is a primary target of air pollution. In children, the small airway caliber allows for a higher chance of being affected by inflammation resulting from air pollution [14, 15]. Due to their respiratory rates, children breathe a proportionately greater volume of air than adults and their oxygen demand is significantly

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higher, as well as their respiration rates. Young people also spend more time engaged in intense activities than adults, often outdoors and during midday when air pollution levels tend to be higher. As a result, children inhale more pollutants per kilogram of body weight. Irritation caused by air pollutants that would produce only a slight response in an adult can result in potentially significant obstruction in the airways of a young child [16].

The environmental risk factors that may have an impact on children's respiratory health, especially in urban areas, include chemical outdoor pollution, aeroallergens, indoor air pollution including environmental tobacco smoke, microorganisms such as virus and bacteria that infect the airways. The latter can exacerbate or re-exacerbate their manifestations in presence of other risk factors.

Several epidemiological studies have documented a positive association between exposure to particulate air pollution and respiratory symptoms of cough and wheeze, especially among children [17, 18]. In this regard, the findings from two Swiss studies showed that the reduction of exposure to particulate matter (PM) <10 μm in aerodynamic diameter (PM_{10}) contributes to improved respiratory health, observed through fewer cases of chronic cough in children [19] and through fewer cough, wheezing and breathlessness in adults [20]. Exposure to ozone (O_3) at environmental concentrations is associated with lung function decrease and respiratory symptoms including cough, shortness of breath and pain on deep inspiration [21]. Nitrogen dioxide (NO_2) concentrations have also been associated with cough, wheeze and breath shortness in children. Residential traffic-related air pollution exposure is associated with reduced expiratory flows in schoolchildren [7, 22]. Variations in lung function that mirror changes in PM exposure have been reported in children who move to areas with different air pollution levels [23].

Pollen is a well know trigger of allergies and asthma aggravation, and actually has a changing profile; in fact new pollen types have emerged following the cultivation and spread of exotic ornamental plants in public and private places [24]. Moreover, global climate change has been linked to an earlier onset and an extended duration of the pollens season, to an increase in pollen production, and a stronger allergenicity for some pollen types [25]. Thunderstorm asthma epidemics may be triggered by pollen grains rupture in the atmosphere and the entrapment of respirable-size particles in the outflows of air masses at ground level [24, 25]. Increasing pollution is responsible for an increase in pollen-induced respiratory allergy, including asthma, because of airway inflammatory reaction and the passage of pollen grains into the lower respiratory tract [24].

The aim of this study was to analyze the short-term relationships between hospital emergency room (ER) admissions for respiratory diseases in children and concentrations of NO_2, $PM_{2.5}$, O_3, and aeroallergens, in Turin, Italy, between 2008 and 2010.

Methods

Turin, the capital of Piedmont region (North-Western Italy) has 900,000 inhabitants, is located at 200 m above sea level and it is one of the most polluted Italian cities [26–29] Additional file 1: Figure S1. Daily data for the period 01/08/2008 to 31/12/2010 (883 days) for the city of Turin were collected or derived as described below. The locations of the data sources are shown in Additional file 1: Figure S1.

ER admissions for respiratory diseases

Daily data on ER admissions (date of admission, primary diagnosis and diagnostic code) for 19 respiratory diseases to "Regina Margherita" Pediatric Hospital of Turin were collected (age range 0–18 years). Diagnoses were coded according to the International Classification of Disease (ICD) 9th edition (Table 1).

Meteorological data

Meteorological data derived from the station placed on the roof of the Department of Physics of the University of Turin, located at about 1 km from the city center. The station is permanently active since 1989 in order to collect and display in real time the weather data in the urban surface layer of the city. The station is equipped with the instruments reported in Additional file 1: Table S1. Data are collected every 5 s by the acquisition system, and subsequently averaged every 5 min and stored in an electronic archive. Data acquired in this way were aggregated in a daily form for the subsequent analysis.

Chemical air pollution data

Daily concentrations of NO_2, $PM_{2.5}$, and O_3 were derived from hourly data collected at the urban background monitoring station "Lingotto" located in Turin (viale Augusto Monti, 21) by the Local Environmental Protection Agency (ARPA Piemonte), coordinated by the regional air pollution service of Piedmont Region, according to the current European legislation (DIR 2008/50/ECK).

Aeroallergen data

Among the pollen taxa usually considered in aerobiological monitoring for being allergenic, Corylaceae, Cupressaceae, Gramineae, Urticaceae, Ambrosia, and Betula were quantified in this study. Daily data were derived from a station located 12 m above the ground, as required by the standard [30], on the flat roof of a building located in a semi-central area of the city of Turin. In this site, atmospheric circulation is local and not affected by surrounding obstacles such as walls or other types of protection. The station is equipped with

Table 1 Distribution of daily ER admissions, for the diagnoses of the respiratory diseases considered in the analysis, during the study period (883 days)

Respiratory disease diagnoses			total n. of admissions	daily n. of admissions		
Group	Description	ICD-IX-CM code	count	mean ± SD	median	min-max
Upper respiratory tract infections	Acute rhino pharyngitis	460	17684	18.1 ± 9.0	17	0-45
	Acute pharyngitis	462				
	Acute tonsillitis	463				
	Acute laryngitis without obstruction	46400				
	Acute laryngitis with obstruction	46401				
	Acute upper respiratory infections	4658				
Lower respiratory tract infections	Acute bronchitis	4660	1989	3.0 ± 3.2	2	0-20
	Acute bronchiolitis other infectious agents	46619				
	Flu with respiratory manifestations	4871				
	Bronchitis	490				
Deep lung infections	Viral pneumonia	4809	839	2.1 ± 2.1	2	0-8
	Bacterial pneumonia	4859				
	Bronchopneumonia	485				
	Pneumonia	486				
Asthma	Asthma, without status asthmaticus	49390	1.881	1.5 ± 1.6	1	0-7
	Asthma, with status asthmaticus	49391				
Total			21793	24.7 ± 11.7	23	0-80

International classification of diseases, 9th edition, clinical modification (ICD-IX-CM) codes

a HIRST sampler, which consists of three main parts: a swivel head, a suction pump and a deposition drum (the sampling part), which rotates at 2 mm / h with 7 days of power reserve. Weekly, a specific adhesive tape is fixed on the drum. This tape captures the aeroallergens avoiding any loss for rebound or natural detachment. The air pump provides a constant airflow of 10 L / min inside the sampler, equivalent to 14.4 m^3 each 24 h. Daily aeroallergen counts were carried out at the Department of Life Science and System Biology, University of Turin, and expressed as concentrations (grains/m^3). For the statistical analysis, daily total aeroallergen concentrations were obtained as the sum of the concentrations of the single aeroallergen types.

Statistical Analysis

Quantitative variables were summarized with means ± SD, medians with interquartile ranges (IQR), and minimum and maximum values. The interquartile ratio (IQR/median ratio) was also computed in order to compare variability across different air pollutants. Linear correlations among exposure variables were evaluated using Pearson's r coefficients.

The association between daily ER admission counts for all diagnoses in Table 1 combined (dependent variable) and air pollution exposure variables were analyzed using Generalized Linear Models (GLMs) fitting a non-stationary Poisson process [30, 31]. We used the following model:

$$\log(\lambda_t) = \alpha + \sum_{i=1}^{k} \beta X_i + NS(Z)$$

Where λ_t denotes the count of daily ER admissions at day t, α is a constant, β is the vector of estimated parameters, X_i is the matrix of k independent variables (exposure and adjustment variables), and $NS(Z_df)$ is a natural spline smoothing function of calendar day Z with df of freedom (df), [31] which was included to take the medium/long term trend into account [30, 31]. The number of df of the smoothing function was chosen by minimizing the sum of the absolute values of the partial autocorrelation function (PACF) of the residuals [11, 30, 31]. Day of the week was included when estimating the smoothing function to remove the 7-day positive correlation across PACF residuals. To avoid overfitting, the maximum number of df allowed was 15, which corresponds to about 6 df per calendar year (60-day windows) [32].

The adjustment variables considered were: a) day of the week, b) influenza outbreaks, defined as days when influenza incidence was greater than 2‰ [33], which were

computed by the Regional Reference Service of Epidemiology for the Surveillance, Prevention and Control of Infectious Diseases, ASL Alessandria, Italy Reference Service Regional Epidemiology and Infectious Disease (SeREMI), e) holidays (4-level variable coded as: Christmas and Easter; 3 days around Christmas and Easter; other holidays; other days), d) summer population decrease (from Saturday before Mid-August to the next Sunday for a total of 16 days/year; from 16 July to 31 August, except for the aforementioned period; all other days) [34], e) average daily temperature, f) average relative humidity, and g) cumulative daily precipitations. The following models were fit to the data:

A) One exposure variable + medium/long trend function + non meteorological variables (day of the week, influenza outbreaks, holidays and summer population decrease) (*single pollutant models*);
B) Model A + meteorological variables (daily temperature, daily relative humidity, cumulative daily precipitations). Temperature and relative humidity were modeled using natural splines with 3 and 2° of freedom, respectively. The number of df was chosen using the PACF criterion as above. Daily precipitations were binary coded (present if ≥1 mm; absent otherwise);
C) One chemical pollutant ($PM_{2.5}$, NO_2 or O_3) + aeroallergens + medium/long trend function + non meteorological variables (*two-pollutant models*). To avoid multicollinearity, two-pollutant models only combined one chemical pollutant per time and aeroallergens, because correlations across chemical pollutants were very strong (Pearson's coefficients of correlation in absolute value |r| >0.50).

Exposure variables were included in the models at single time lags, from the same day when ER admissions were evaluated (Lag 0) to 5 days before (Lag 5). Associations between exposure variables (10 $\mu g/m^3$ increase in $PM_{2.5}$, NO_2, O_3 concentrations; 10 grains/m^3 increase in aeroallergen concentrations) and ER admissions were reported with rate ratios (RR) with 95 % confidence intervals (CI).

Results

In the study period, 21,793 pediatric ER admissions for respiratory diseases were observed, mainly (81 %) for infections of the upper airways (Table 1). Figure 1 shows average daily ER admissions by month of the year. The cold months showed the highest frequency of ER admissions, probably due to the more frequent outbreaks of colds. Table 2 shows a general description of the daily concentrations of airborne pollutants during the study period. NO_2 concentration showed the highest mean absolute levels and, overall, the air pollution concentrations observed underline the poor air condition in Turin as compared to the rest of Europe [26, 35].

Aeroallergen concentrations showed larger variability than chemical air pollution concentrations: the interquartile ratio for aeroallergens was 4 (O_3) to 8-fold (NO_2) the interquartile ratio of the chemical pollutants. Both $PM_{2.5}$ and NO_2 (Fig. 2a and b) showed a prevailing maximum level during the coldest months, which is a typical behavior of primary pollutants. An opposite trend

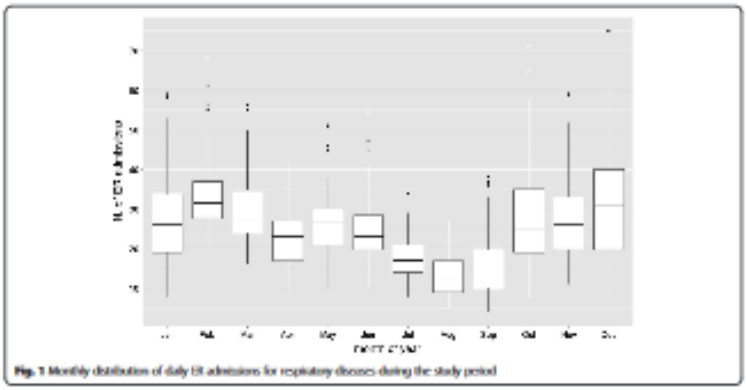

Fig. 1 Monthly distribution of daily ER admissions for respiratory diseases during the study period

Table 2 Distribution of daily concentrations of air pollution and aeroallergens at the Lingotto urban background monitoring station during the study period (883 days)

Exposure variable	available data (days)	median (IQR)	interquartile ratio	min-max	mean ± SD	EU annual reference value[b]
$PM_{2.5}$ (μg/m^3)	833	23.0 (18.0)	1.4	4–157	32.0 ± 26.2	25
NO_2 (μg/m^3)	851	42.5 (32.0)	0.8	7.4–192.9	48.3 ± 25.0	40
O_3 (μg/m^3)	858	34.1 (53.6)	1.6	1.8–127.3	36.6 ± 29.1	
aeroallergens (grains/m^3)[a]	836	2.9 (9.6)	6.7	0–221.9	16.1 ± 29.6	

[a]Includes Cupressaceae, Cupressaceae, Taxaceae, Urticaceae, Ambrosia, Betula
[b]European Union (EU) Directive 2008/50/CE® (http://ec.europa.eu/environment/air/quality/legislation/directive.htm)

was shown by O_3, with higher concentrations in summertime (Fig. 2c). The concentrations of aeroallergens were high in the warm season, and virtually absent in winter (Fig. 2d).

There was a strong positive linear correlation between $PM_{2.5}$ and NO_2 ($r = 0.762$, $p < 0.001$). The negative correlations of O_3 with $PM_{2.5}$ and NO_2 were also strong ($r = -0.591$ and -0.695, respectively, all $p < 0.001$) (Fig. 3). The correlations between chemical pollutants and aeroallergens were weaker ($|r|$ between 0.257 and 0.459).

Table 3 shows the mean number of daily ER admissions, air pollution and aeroallergen concentrations according to the potential confounders considered. ER admissions were more frequent during weekends, holidays, and during influenza outbreaks than during the other days. They were also more likely in days with lower temperatures and more extreme (low and high) relative humidity levels. $PM_{2.5}$ and NO_2 were positively associated with temperature, whereas the opposite was true for O_3 and aeroallergens. As expected, airborne pollution was lower during rainy days.

The associations between exposure variables and ER admissions for respiratory diseases, adjusted for non-meteorological potential confounders (model A), is described in Fig. 4. There was no statistically significant association of $PM_{2.5}$ and ER admissions at any time lag (Fig. 4a). Instead, an increase of 10 μg/m^3 of NO_2 concentrations (Fig. 4b) was associated with a significant 1.3 % (95 % CI: 0.3–2.2 %) increase of ER admissions after 5 days (lag 5). O_3 concentrations were significantly negatively associated with ER admissions for respiratory diseases, starting from lag 4 (Fig. 4c). Finally, a 0.7 % (95 % CI: 0.1–1.2 %) increase of ER admissions was observed 1 day after (lag 1) an increase of 10 grains/m^3 of aeroallergens (Fig. 4d). When meteorological variables were also included as adjustment variables in the analyses, the results were consistent, with the exception that the negative associations between O_3 (lags 4–5) and aeroallergen (lag 4) concentrations and ER admissions shifted to the null (Fig. 5). Joint models including individual chemical air pollutants and aeroallergens confirmed the main models results completely (Additional file 1: Figure S2), suggesting that the

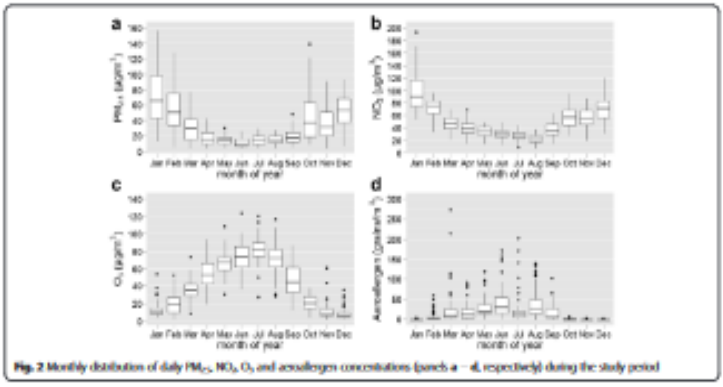

Fig. 2 Monthly distribution of daily $PM_{2.5}$, NO_2, O_3 and aeroallergen concentrations (panels a – d, respectively) during the study period

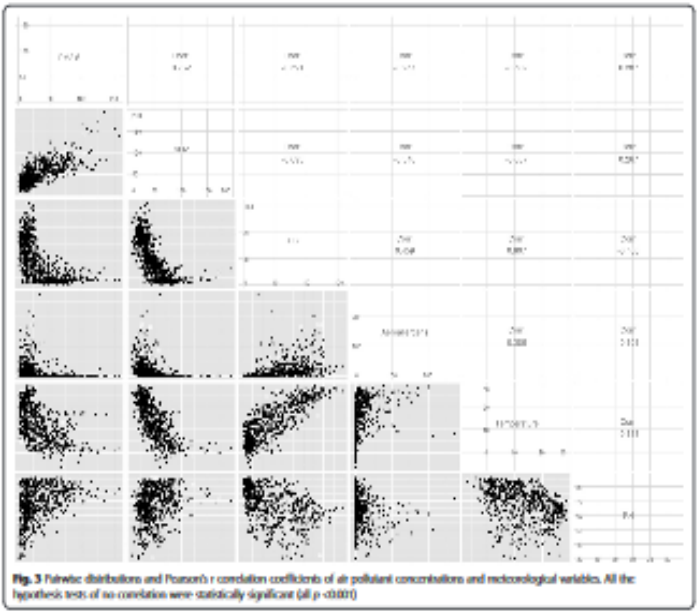

Fig. 3 Pairwise distributions and Pearson's r correlation coefficients of air pollutant concentrations and meteorological variables. All the hypothesis tests of no correlation were statistically significant (all $p < 0.001$)

observed associations of chemical pollutants and aeroallergens with ER admissions were independent.

Discussion and conclusion

The main purpose of this study was to analyze in the selected period the trend of ER admissions for respiratory reasons in a Children's Hospital in Turin and the relationships of ER admissions with urban background chemical air pollutants and aeroallergens.

To achieve this objective, we considered the monitoring site of chemical air pollution where the data were the most complete for the study period and which was the closest to the pediatric emergency room (Additional file 1: Figure S1).

Among the 19 diseases diagnosed at the moment of the access to pediatric emergency room, as expected the upper respiratory tract infections was the most frequent reason for access to the emergency room. These infectious diseases of viral etiology are very common in pediatric age also because children generally attend kindergarten, nurseries and schools and they are therefore more exposed both to the etiological agents and to environmental risk factors such as poor air quality. Air pollution in fact, as well as tobacco smoke, can inhibit defensive mechanisms against oxidative stress [7, 15] and inflammation of the upper respiratory tract, which can favor the development of respiratory virus infections.

Despite the fact that the concentration of $PM_{2.5}$ appears quite high when compared to European levels [29], this pollutant did not reveal any significant short-term association with ER pediatric admission, as documented by other authors [36]. Instead, NO_2 showed a positive significant association with ER admissions, but only after 5 days (lag 5). Also Li et al. have shown a positive association between NO_2 air pollution at lag 5 and ER admission in children of Detroit (MI, U.S.A.) in 2011 [37]. However, in contrast to what we observed, they have also shown a positive and significant association between $PM_{2.5}$ concentrations and ER admissions. This does not seem to depend primarily on the average concentrations of $PM_{2.5}$, which were much lower than in our study area, and it may be due to a different

Table 3 Distribution of the daily number of ER admissions for respiratory diseases and daily concentrations of chemical air pollution and aeroallergens during the study period, according to the potential confounders considered in the analysis[a]

	ER admissions (counts)	PM_{10} (μg/m³)	NO_2 (μg/m³)	O_3 (μg/m³)	Aeroallergens (grains/m³)
Day of the week					
Monday	22.7 ± 10.2	30.4 ± 26.5	47.2 ± 25.1	58.2 ± 27.7	12.7 ± 28.9
Tuesday	21.4 ± 9.8	30.8 ± 25.4	50.3 ± 24.5	57.8 ± 28.6	14.9 ± 28.8
Wednesday	20.4 ± 9.6	32.5 ± 25.4	51.0 ± 23.8	58.9 ± 29.2	18.8 ± 38.1
Thursday	21.6 ± 9.7	35.1 ± 27.8	52.9 ± 25.9	58.9 ± 30.2	15.1 ± 23.0
Friday	21.4 ± 11.1	33.3 ± 25.0	50.9 ± 24.7	59.9 ± 29.7	17.2 ± 32.1
Saturday	21.1 ± 12.4	31.5 ± 26.0	45.0 ± 25.1	47.2 ± 29.8	18.2 ± 33.4
Sunday	22.2 ± 12.8	30.4 ± 27.4	40.9 ± 24.0	41.3 ± 28.5	15.8 ± 27.4
p	<0.001	0.79	0.000	0.87	0.73
Holidays					
Christmas and Easter	47.6 ± 17.5	25.4 ± 15.2	45.1 ± 27.5	52.0 ± 29.9	1.0 ± 1.9
3 days before/after Christmas and Easter	34.9 ± 16.3	37.9 ± 21.7	58.6 ± 25.8	25.7 ± 21.7	6.4 ± 15.8
Other holidays	15.6 ± 15.6	37.4 ± 27.7	49.3 ± 22.7	37.2 ± 30.4	12.6 ± 1.9
Other days	23.8 ± 10.9	31.6 ± 26.4	48.0 ± 25.0	48.4 ± 29.1	16.6 ± 30.2
p	<0.001	0.39	0.23	0.62	0.20
Influenza outbreaks					
No	19.9 ± 9.0	22.8 ± 21.0	36.8 ± 16.9	55.2 ± 27.4	23.8 ± 31.9
Yes	31.0 ± 12.0	43.9 ± 27.5	61.0 ± 25.8	19.9 ± 16.4	6.3 ± 23.0
p	<0.001	<0.001	<0.001	<0.001	<0.001
Summer population decrease					
2 weeks around 15 August	13.4 ± 4.71	15.8 ± 5.6	20.7 ± 6.3	66.1 ± 15.4	34.7 ± 28.5
From 16/7 to 31/8 (except 2 weeks around 15 August)	14.4 ± 5.8	14.5 ± 6.4	26.1 ± 8.5	28.0 ± 19.3	29.1 ± 31.6
Other days	26.4 ± 11.6	34.5 ± 27.1	52.5 ± 24.5	33.7 ± 26.3	13.4 ± 28.7
p	<0.001	<0.001	<0.001	<0.001	<0.001
Temperature[b] (°C)					
-6.4, 6.4	29.1 ± 11.6	57.3 ± 28.3	78.9 ± 27.6	12.1 ± 8.8	0.8 ± 2.5
6.5, 12.8	28.1 ± 11.3	31.7 ± 21.5	51.7 ± 15.2	21.3 ± 12.3	10.1 ± 31.9
12.9, 20.3	24.8 ± 11.5	27.5 ± 23.9	43.4 ± 17.1	41.2 ± 20.2	14.9 ± 21.6
20.4, 28.5	19.5 ± 8.2	14.9 ± 6.8	29.4 ± 9.5	71.7 ± 18.4	32.5 ± 33.7
p	<0.001	<0.001	<0.001	<0.001	<0.001
Cumulative precipitation					
<1 mm	25.4 ± 11.4	35.5 ± 28.0	51.5 ± 26.2	38.0 ± 33.7	16.0 ± 29.9
≥1 mm	25.2 ± 11.5	25.5 ± 19.2	45.0 ± 20.1	33.7 ± 26.0	9.5 ± 27.3
p	0.87	<0.001	0.01	0.10	0.02
Relative humidity[b] (%)					
30.4, 60.4	26.4 ± 10.3	17.8 ± 13.1	41.6 ± 17.9	52.1 ± 24.6	25.1 ± 34.3
60.5, 68.6	23.1 ± 11.6	25.5 ± 18.9	43.2 ± 22.2	50.9 ± 31.3	21.8 ± 32.5
68.7, 76.4	25.3 ± 11.3	44.7 ± 30.5	56.9 ± 32.1	28.3 ± 23.0	9.6 ± 25.4
76.5, 85.5	26.7 ± 12.1	42.5 ± 28.8	58.4 ± 22.1	16.7 ± 15.9	1.9 ± 6.0
p	0.01	<0.001	<0.001	<0.001	<0.001

[a] mean ± SD reported; overall *p*-values were calculated using non-parametric Kruskall-Wallis tests, under the null hypothesis that the distribution of a variables is homogeneous among the strata of a potential confounder
[b] coded in groups according to the quantiles of their frequency distribution

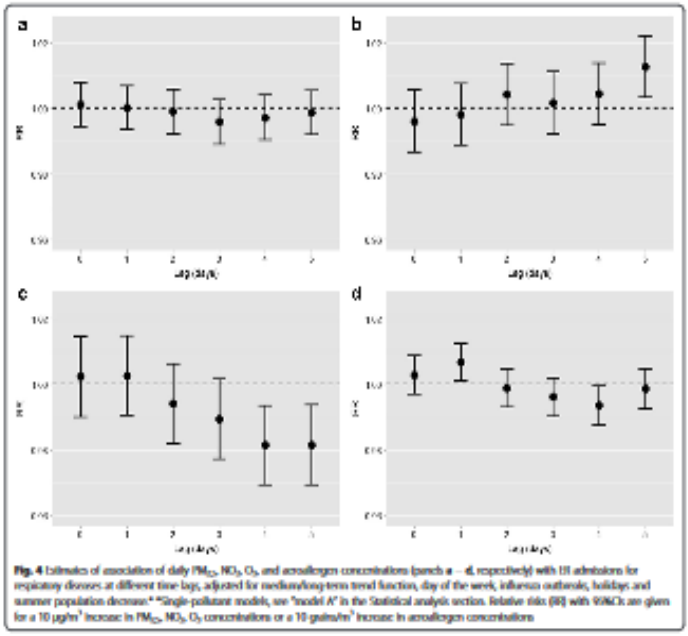

Fig. 4 Estimates of association of daily PM_{10}, NO_2, O_3, and aeroallergen concentrations (panels **a** – **d**, respectively) with ER admissions for respiratory diseases at different time lags, adjusted for medium/long-term trend function, day of the week, influenza outbreaks, holidays and summer population decrease.* *Single-pollutant models, see "model A" in the Statistical analysis section. Relative risks (RR) with 95%CIs are given for a 10 $\mu g/m^3$ increase in PM_{10}, NO_2, O_3 concentrations or a 10 grains/m^3 increase in aeroallergen concentrations

composition of particulate, perhaps more toxic in the City of Detroit than in Turin.

The adjusted estimates of relative risk for the effect of O_3 were significantly less than one, seemingly suggesting a little protective effect. In 2009, Jerrett et al. also showed how relative risk for the effect of ozone on the risk of death from cardiovascular causes were significantly less than 1.0 [38].

Such beneficial influence of ozone, however, is currently completely to exclude from toxicological point of view. In experimental studies, O_3 can increase airway inflammation [39] and can worsen pulmonary function and gas exchange [40]. In addition, exposure to elevated concentrations of tropospheric O_3 has been associated with numerous adverse health effects, including the induction [26] and exacerbation [27, 28] of asthma, pulmonary dysfunction [33, 34] and hospitalization for respiratory reasons [31]. In our study, the apparent protective effect of O_3 seems to be due to a confounding by meteorology, or to the fact that O_3 acts as a mediator of the effect of temperature. In fact, when temperature, relative humidity and precipitations were included in the models as adjustment factors, the associations between O_3 and ER admissions shifted to the null. Measurements of $PM_{2.5}$ and NOx obtained using background monitoring stations are probably more representative of population's exposure than measurements of O_3. In fact, O_3 concentrations tend to vary within cities more than $PM_{2.5}$, because of the scavenging of O_3 by NO near roadways and principally, for its photochemical origin [37]. Thus, in the presence of a high density of local traffic, the measurement error is probably higher for exposure to O_3 than for exposure to $PM_{2.5}$. The effects of O_3 could therefore be confounded by the presence of $PM_{2.5}$ because of collinearity between the measurements of the two pollutants and the higher precision of measurements of $PM_{2.5}$ [38].

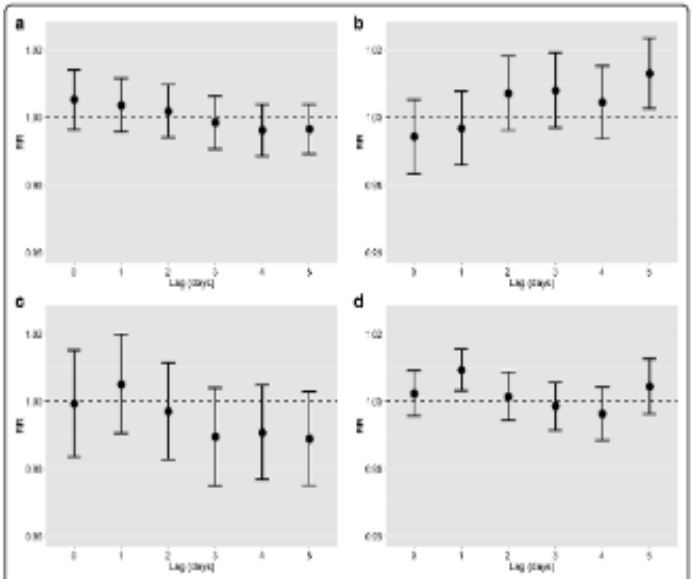

Fig. 5 Estimates of association of daily PM_{10}, NO_2, O_3, and aeroallergen concentrations (panels **a** – **d**, respectively) with ER admissions for respiratory diseases at different time-lags, adjusted for medium/long-term trend function, day of the week, influenza outbreak, holidays, summer population decrease, and for the meteorological variables (daily temperature, daily relative humidity, cumulative daily precipitations)[*]. * 'Single-pollutant models, see "model II" in the Statistical analysis section of the article. Relative risks (RR) with 95%CIs are given for a 10 μg/m^3 increase in PM_{10}, NO_2, O_3 concentrations or a 10 grains/m^3 increase in aeroallergen concentrations

Finally, a 0.7 % (95 % CI: 0.1–1.2 %) increase of ER admissions for every 10 grains/m^3 increase of aeroallergens was observed at lag 1. This indicates a lower latency between the stimulus and the effect, compared to chemical pollutants. An apparent protective effect of aeroallergens at lag 4 shifted to the null when the models included meteorological adjustment variables. Adverse short-term effects of aeroallergens are supported by other studies [41, 42], although the time lags when excesses of ER admissions are observed vary according to a number of reasons, including differences in study populations, air pollutant mixtures, as well as exposure assessment and statistical methodologies applied.

A limitation in our study is that we used only one monitoring site to estimate air pollution concentrations. However, both chemical pollution and aeroallergen monitoring stations were located close to the children's hospital. It is likely that children are referred to the closest hospital, especially in the case of acute health events that are captured by ER admissions, and we can therefore hypothesise that children lived at relative close distance to the monitoring area. Any measurement error in exposures due to spatial heterogeneity in airborne air pollution concentrations is more likely to bias risk estimates toward the null than in the opposite direction [43].

In conclusion, we observed consistent and positive associations of background NO_2 and aeroallergen concentrations with ER admissions in children in a populated and heavily polluted city in western Italy. Our findings add to the existing evidence and call for urgent public health policies especially in the Po valley in northern Italy, one of the most polluted areas in Europe because

of high emissions but also poor ventilation and precipitation especially in winter. Moreover, replacement of non-allergenic cultivated plant species and their management (for example frequent grassland mowing which limit the production of flowers and consequently of pollens) can reduce the concentrations of allergenic pollens in the air [44]. Air pollution reduction policies are also recommended in the protection and promotion of public health, especially in children.

Additional file

Additional file 1: Table S1. Characteristics of the instruments of the meteorological station. **Figure S1.** Map of the city of Turin (grey line). The dots indicate the locations of the "Regina Margherita" Children's Hospital (red), the aeroallergen sampling station (green), and the chemical air pollution monitoring and weather station (blue) (© 2015 Google maps). **Figure S2.** Estimates of the association of daily PM2.5 + aeroallergens (panel A), NO2 + aeroallergens (panel B), O3 + aeroallergens (panel C) with ER admissions for respiratory diseases at different time lags, adjusted for medium/long trend function, day of the week, influenza outbreaks, holidays and summer population decrease* (DOCX 951 kb)

Abbreviations
COPD, chronic obstructive pulmonary disease; ER, emergency room; GAMs, generalized additive models; ICD, international classification of diseases; IQR, interquartile ranges; PACF, partial autocorrelation function of the residuals; PM, particulate matter

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Authors' contributions
RB has conceived, designed, and coordinated the study, performed the analysis and wrote the manuscript; VR and VB have made substantial contributions to acquisition of data and to their analysis and interpretation; RT and GT have made substantial intellectual contribution and have contributed to the design of the study and to organization and quality control of database; AU, CC, and CS have made substantial intellectual contribution in the execution of the work and have collected and processed data related to their profession (admission data, aeroallergens, and meteorological data); PM and AM have contributed to the design of the study, revised the manuscript, performed statistical analysis and contributed to the interpretation and discussion of data.
All authors have made substantive intellectual contributions in the study, read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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RESPIRATORY RESEARCH

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Diverging trends of chronic bronchitis and smoking habits between 1998 and 2010

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Abstract

Background: No study has been carried out on the time trend in the prevalence of chronic bronchitis (CB) in recent years, despite its clinical and epidemiological relevance. We evaluated the trend in CB prevalence during the past decade among young Italian adults.

Methods: A screening questionnaire was mailed to general population samples of 20–44 year-old subjects in two cross-sectional surveys: the Italian Study on Asthma in Young Adults (ISAYA) (1998/2000; n = 18,873, 9 centres) and the screening stage of the Gene Environment Interactions in Respiratory Diseases (GEIRD) study (2007/2010; n = 10,494, 7 centres). CB was defined as having cough and phlegm on most days for a minimum of 3 months a year and for at least 2 successive years. The prevalence rates and the risk ratios (RRs) for the association between CB and each potential predictor were adjusted for gender, age, season of response, type of contact, cumulative response rate, and centre.

Results: CB prevalence was 12.5% (95% CI: 12.1–12.9%) in 1998/2000 and 12.6% (95% CI: 11.7–13.7%) in 2007/2010; it increased among never smokers (from 7.6 to 9.1%, p = 0.003), current light smokers (<15 pack-years; from 15.1 to 18.6%, p < 0.001), and unemployed/retired subjects (from 14.3 to 19.1%, p = 0.001). In this decade, the prevalence of current smoking decreased (from 33.6 to 26.9%, p < 0.001), whereas the prevalence of unemployment/premature retirement (from 5.3 to 6.0%, p = 0.005), asthma (from 5.0 to 6.2%, p = 0.003), and allergic rhinitis (from 19.5 to 24.5%, p < 0.001) increased. In both 1998/2000 and 2007/2010, the likelihood of having CB was significantly higher for women, current smokers, asthmatic patients, and subjects with allergic rhinitis. During this period, the strength of the association between CB and current heavy smoking (≥15 pack-years) decreased (RR: from 4.82 to 3.57, p = 0.018), whereas it increased for unemployment/premature retirement (from 1.11 to 1.53, p = 0.019); no change was observed for gender, asthma, and allergic rhinitis.

Conclusions: Despite the significant reduction in current smoking, CB prevalence did not vary among young Italian adults. The temporal pattern of CB prevalence can only be partly explained by the increase of unemployment/premature retirement, asthma and allergic rhinitis, and suggests that other factors could have played a role.

Keywords: Allergic rhinitis, Asthma, Chronic bronchitis, Cigarette smoking, Epidemiology

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Background

Chronic bronchitis (CB) (i.e. a mucus-producing cough on most days for a minimum of 3 months a year and for at least 2 consecutive years) is a highly prevalent respiratory disorder, usually associated with cigarette smoking [1,2]. CB can precede the development of chronic obstructive pulmonary disease (COPD) [3,4], and it increases the likelihood of exacerbations, hospitalization, and mortality in COPD patients [5-7]. Passive smoking, air pollution, and occupational exposures to dust, gas, and fumes can also increase the risk of CB [8-10]. Furthermore, CB may be associated with asthma and may be a marker of both asthma severity [11] and poor control of symptoms [12], which in turn are associated with a heavy socio-economic burden [13-15]. CB in asthmatic subjects may also indicate the presence of the asthma-COPD overlap syndrome, which is a difficult condition to treat [16]. In addition, CB can be related to allergic rhinitis and gastroesophageal reflux disease (GORD) [17,18].

While the prevalence of asthma and allergic rhinitis increased during the last decade in Italy [19], so far no study has been carried out on the time trend in the prevalence of CB in recent years, despite its clinical and epidemiological relevance. Accordingly, the present study is aimed at evaluating the ten-year trend in the prevalence of CB among young adults (20–44 years) in Italy, and at evaluating whether the pattern of the association with potential predictors changed during the last decade. To fulfil these objectives, we used the data from two cross-sectional surveys, the Italian Study on Asthma in Young Adults (ISAYA) [20] and the screening stage of the Gene Environment Interactions in Respiratory Diseases (GEIRD) study [21].

Methods

Design of the study and definitions

ISAYA and GEIRD-screening stage were two cross-sectional surveys on respiratory health in the general adult population, which were carried out in Italy between 1998–2000 and between 2007–2010, respectively. ISAYA involved 25,969 subjects from 9 centres (Ferrara, Pavia, Pisa, Sassari, Sassuolo, Siracusa, Turin, Udine, Verona) with an overall response rate of 72.7% [20], whereas GEIRD involved 18,357 subjects from 7 centres (Ancona, Pavia, Salerno, Sassari, Terni, Turin, Verona) with an overall response rate of 57.2% [19]. In both the studies, random samples of 3,000 subjects aged 20–44 (men/women ratio = 1) were selected in each centre from the general population, using the local health authority registers. A screening questionnaire [21,22] was mailed to each subject up to three times and then administered by telephone in case of non response. Ethics approval was obtained in each centre involved in GEIRD from the appropriate ethics committee (Comitato Etico dell'Azienda Ospedaliero-Universitaria Ospedali Riuniti di Ancona; Comitato di Bioetica della Fondazione IRCCS Policlinico San Matteo di Pavia; Comitato Etico dell'Azienda Sanitaria Locale SA/2 di Salerno; Comitato di Bioetica dell'Azienda Sanitaria Locale di Sassari; Comitato Etico delle Aziende Sanitarie dell'Umbria di Perugia; Comitato Etico dell'Azienda Sanitaria Locale TO/2 di Torino; Comitato Etico per la Sperimentazione dell'Azienda Ospedaliera Istituti Ospitalieri di Verona). All participants were fully informed about all aspects of the research project and consented to complete and return the questionnaire.

The screening questionnaires used in ISAYA and GEIRD shared a set of core questions (mainly taken from the European Community Respiratory Health Survey [ECRHS] questionnaires [23]) on the presence of asthma (self-report of the disease during lifetime with or without a physician diagnosis, frequency of asthma attacks and use of anti-asthmatic drugs in the last 12 months), asthma-like symptoms (wheezing, nocturnal tightness in the chest, and attacks of shortness of breath at night time in the last 12 months), allergic rhinitis (any nasal allergies, including hay fever), and CB (cough and phlegm on most days for a minimum of 3 months a year and for at least 2 successive years), as well as questions on smoking habits (the age of smoking commencement, the amount of tobacco smoked, and the age at which smokers stopped smoking). The screening questionnaire used in GEIRD also contained questions on the frequency of vehicular traffic (cars and trucks) near home.

Smoking habits were classified as current light/heavy smoking, past light/heavy smoking, or never smoking according to lifetime pack-years, considering 15 pack-years as the cut-off. Ever smoking was defined as having smoked at least one cigarette per day or one cigar a week for one year. Asthma was considered present if a subject had reported asthma confirmed by a doctor and at least one respiratory symptom (wheezing, nocturnal tightness in the chest, attacks of shortness of breath at night time) or at least one attack of asthma or use of medicines because of breathing problems in the last 12 months.

Statistical analysis

The data from all the centres that participated in ISAYA and/or GEIRD were used in the analysis. Gender, age, season of response, type of contact, and cumulative response rate were considered as potential confounders because of differences in their distribution between the two surveys (Table 1). In particular, the cumulative response percentile rank was used: each subject was ordered by study and centre according to the date of response to the questionnaire, and then he/she was

Table 1 Distribution of the design variables

	ISAYA (1998/2000) N = 25,969	GEIRD (2007/2010) N = 18,357	p-value*
N° of responders (response rate, %)	18,873 (72.7)	10,494 (57.2)	<0.001
Season at time of the interview, %			<0.001
spring	18.5	41.0	
summer	16.4	22.1	
autumn	40.4	25.7	
winter	24.7	11.2	
Phone contact, %	27.9	11.8	<0.001
Females, %	50.7	52.1	0.008
Age (years), mean (sd)	33.1 (8.9)	34.7 (7.1)	<0.001

* p value obtained by Pearson's chi squared test and two sample t test with unequal variances.

attributed the ratio between his/her rank and the number of eligible subjects [19].

Adjusted prevalence rates of CB were computed by logistic regression models. The models had an indicator of the presence of CB as the dependent variable and the study (GEIRD vs ISAYA), the potential confounders reported above, and centre as covariates. Centre was included in the models in order to partly control for the potential confounding effect of ecological-level variables, such as environmental factors. Since the subjects were nested into groups (the study -ISAYA or GEIRD- in which they participated, crossed by centre), clustered sandwich estimators of the variance were used. The prevalence rates in each survey were estimated by setting the distribution of gender, season of response, type of contact, and centre equal to the average distribution, and by setting age and the cumulative response rate equal to the overall mean [24], in order to make the ISAYA and GEIRD estimates comparable.

The association between gender, smoking habits, occupational status, asthma, allergic rhinitis, and the presence of CB was estimated by the risk ratios (RRs), separately for each survey. The RRs were computed by Poisson regression models with robust standard errors (obtained by the Huber/White/sandwich estimator of the variance) and no offset [25], and were adjusted for the potential confounding effect of the same variables as the prevalence rates. The RRs were also computed by including a dummy variable for the high frequency of vehicular traffic (i.e. a continuous passage of cars and/or trucks) near home in the GEIRD model.

The statistical analysis was performed using STATA software, release 12 (StataCorp, College Station, TX).

Results

Ten year trend in the prevalence of CB

The prevalence of CB was 12.5% (95%CI: 12.1-12.9%) in 1998/2000 and 12.6% (95%CI: 11.7-13.7%) ten years later, and it did not significantly vary according to gender and age (Table 2). A statistically significant trend in the prevalence of CB was observed among never smokers (from 7.6 to 9.1%, p = 0.003) and current light smokers (<15 pack-years: from 15.1 to 18.6%, p < 0.001), but not among current heavy smokers (≥15 pack-years) and past smokers (Figure 1). According to occupational status, the prevalence of CB significantly increased among unemployed/retired subjects (from 14.3 to 19.1%, p = 0.001), but not among employed subjects/house-persons/students (from 12.3 to 12.1%, p = 0.764).

Ten year trend in the distribution of smoking habits, occupational status, asthma, and allergic rhinitis

During this period, there was a statistically significant decrease in the percentage of current smokers (from 33.6 to 26.9%, p < 0.001) and in the percentage of current smokers according to the amount of exposure (current light smokers: from 22.5 to 19.8%, p < 0.001; current heavy smokers: from 7.0 to 4.2%, p < 0.001) (Table 3). Moreover, a statistically significant change was observed in the percentage of past heavy smokers (from 1.2 to 0.8%, p < 0.001), but not in the percentage of past light smokers. In addition, a statistically significant increase was observed in the percentage of unemployed/retired individuals (from 5.3 to 6.0%, p = 0.005), asthmatic patients (from 5.0 to 6.2%, p = 0.003), and subjects with allergic rhinitis (from 19.5 to 24.5%, p < 0.001). In the same decade, there was no statistically significant change in the percentage of asthmatic patients who also reported CB, and in the percentage of subjects with allergic rhinitis and coexisting CB (Table 4).

Changes in the pattern of the association between the presence of CB and the potential predictors

In both 1998/2000 and 2007/2010, the likelihood of having CB was significantly higher for women, current smokers and past heavy smokers (as compared to never smokers), asthmatic patients, and subjects with allergic rhinitis (Table 5). During this ten-year period, the

Table 2 Adjusted prevalence of CB according to gender and age

		Adjusted prevalence (%) (95% CI)		
		ISAYA (1998/2000)	GEIRD (2007/2010)	p-value[†]
Gender[*]	Males	12.5 (12.0 - 12.6)	12.6 (11.8 - 13.4)	0.575
	Females	12.6 (11.9 - 13.3)	12.7 (11.3 - 14.3)	0.920
Age[‡]	<30 years	11.8 (11.2 - 12.5)	12.2 (10.9 - 13.6)	0.577
	≥30 years	12.7 (12.2 - 13.2)	12.9 (12.0 - 13.8)	0.692
ICOA[§]		12.5 (12.1 - 12.9)	12.6 (11.7 - 13.7)	0.981

[*] prevalence obtained by stratifying on gender and adjusted for age, season of response, type of contact, cumulative response rate, and center.
[‡] prevalence obtained by stratifying on age and adjusted for gender, season of response, type of contact, cumulative response rate, and center.
[§] prevalence adjusted for gender, age, season of response, type of contact, cumulative response rate, and center.
[†] p-value obtained by testing the null hypothesis that the regression coefficient of the dummy indicator of the study (GEIRD vs ISAYA) was zero

strength of the association between smoking habits and the presence of CB decreased (with a dissimilar change in the RRs according to pack-years for both past and current smoking), whereas the strength of the association increased for unemployment/premature retirement. No change in the RRs was observed for gender, asthma, and allergic rhinitis. When the high frequency of vehicular traffic near home was also considered in the GEIRD model (2007/2010), traffic-related air pollution was significantly associated with the presence of CB (RR = 1.27; 95%CI: 1.14-1.41).

Sensitivity analysis

These results were confirmed when only the data from the four centres (Pavia, Sassari, Turin, Verona) that had participated in both the surveys were considered in the analysis. In particular, the prevalence of CB was 12.4% in 1998/2000 and 12.5% in 2007/2010, and the estimates of the RRs were comparable to those obtained in the main analysis (an additional table shows this in more detail [see Additional file 1]).

Discussion

The main results of the present study are the following:

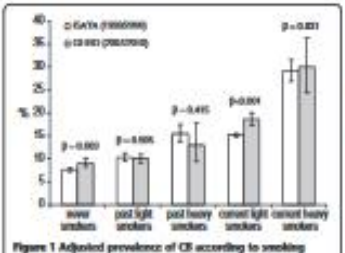

Figure 1 Adjusted prevalence of CB according to smoking habits. Bars represent 95% confidence intervals.

- the prevalence of CB did not vary during the past decade among young adults in Italy, despite a 7% reduction in the percentage of current smokers;
- the increase in the prevalence of CB among never smokers and the decrease in the strength of the association between current smoking and CB observed in the last ten years, suggest that factors other than smoking could have played a major role in determining the trend in the prevalence of CB;
- CB is common in asthmatic patients and in subjects with allergic rhinitis, and its association with these respiratory diseases has not changed with time.

The prevalence of CB did not change in the past decade, despite the striking reduction in the percentage of current smokers

The prevalence rates of CB among 20–44 year-old subjects remained stable during the past ten years in Italy and were similar to that reported in the three Italian centres (Pavia, Turin, Verona) that had participated in the ECRHS in 1991/1993 (median prevalence: 11.3%) [17]. Moreover, the estimates from ISAYA and GEIRD are comparable to that obtained for the same age class in 16 industrialized countries in the ECRHS (10.2%) [17]. The impressive prevalence rates of CB estimated from the ISAYA and GEIRD studies enlighten the fact that CB is a substantial health problem even in young adults. Although CB may not lead to COPD in many of these subjects, our estimates suggest that the base of the COPD iceberg was wide and did not decrease during the past decade in Italy [2].

It could be surprising that there was no significant decrease in the prevalence of CB during the past decades in view of the fact that the percentage of current smokers decreased in Italy during the last twenty years [26], as also documented in our study. In fact, the relationship between smoking and CB is well known, and the amount of tobacco smoke determines the frequency and severity of symptoms [2,27]. Accordingly, we found that smoking habits is the strongest predictor of the presence of CB, since the RRs were the highest for current smoking. In

Table 3 Adjusted percentage[a] of past/current smokers, unemployed/retired subjects, subjects with asthma, and subjects with allergic rhinitis

	Adjusted % [95% CI]		
	ISAYA (1998/2000)	GERD (2007/2010)	p value[b]
Past light smokers	11.2 [10.9–11.6]	11.7 [11.1–12.2]	0.219
Past heavy smokers	1.2 [1.1–1.3]	0.8 [0.7–1.0]	<0.001
Current light smokers	22.5 [22.0–23.1]	19.8 [18.9–20.7]	<0.001
Current heavy smokers	7.0 [6.7–7.4]	4.2 [3.8–4.6]	<0.001
Unemployed/retired subjects	5.3 [5.1–5.6]	6.0 [5.7–6.3]	0.005
Subjects with asthma	5.0 [4.7–5.3]	6.2 [5.6–6.8]	0.003
Subjects with allergic rhinitis	19.5 [19.2–19.8]	24.5 [23.8–25.2]	<0.001

[a] adjusted for gender, age, season of response, type of contact, cumulative response rate, and centre.
[b] p value obtained by testing the null hypothesis that the regression coefficient of the dummy indicator of the study (GERD vs ISAYA) was zero.

addition, the strength of the association increased according to the amount of past and current active exposure. However, the prevalence of CB significantly increased among never smokers during the past decade, resulting in a reduction of the strength of the association between CB and smoking. Hence, our findings add to the emerging evidence that factors other than smoking play a major role in respiratory diseases [28].

Temporal variation in the association between the presence of CB and other factors

The risk of having CB among unemployed/retired subjects significantly increased during the past decade. Unemployment/premature retirement can be considered a proxy measure for socio-economic status. Socio-economically disadvantaged subjects might be more susceptible to respiratory infections, such as pertussis, repeated viral infections, and tuberculosis. Moreover, the observed association could be due to factors, such as residential and workplace pollutant exposures [29], which affect respiratory health and which are more frequent in lower social classes, or could reflect a cumulative life course disadvantage [30]. The high prevalence of CB among unemployed/retired subjects in 2007/2010 supports the need for public health programs focused on vulnerable populations. In fact, the relationship between good health and sustaining employment strengthened during the last decades due to decreasing employment rates and increasing economic inactivity rates among subjects with poor health [31].

The strength of the association between the presence of CB and gender has not significantly changed during the past decade in Italy, and females showed a higher risk of having CB as compared to males, regardless of their smoking habits and socio-economic status, and the coexistence of asthma and allergic rhinitis. A possible explanation is that females may have more sensitive cough receptors than males [32]. Moreover, sex hormones may have an effect on airway reactivity [33].

The time trend in the prevalence of CB could have been influenced by factors other than smoking habits or socio-economic status, which were not measured in the present study. In fact, many causes may account for CB, especially in never smokers. In particular, we were not able to investigate the association with: i) passive smoking exposure, which is still common and it has been found to be significantly related to all types of respiratory symptoms [34]; ii) occupational exposures to dust, gas, and fumes over a long period of time, which has been documented to be a cause of CB [10]; iii) GORD [17,18], which is due to the fact that the acid reflux consumes the airways or the bronchi and triggers the body to create increased levels of mucus.

Finally, the exposure to outdoor air pollution was only investigated in 2007/2010 using a proxy variable of traffic-related air pollution near home. However, the

Table 4 Adjusted prevalence of CB according to the presence of asthma and allergic rhinitis

		Adjusted prevalence (%) [95% CI]		
		ISAYA (1998/2000)	GERD (2007/2010)	p value[b]
Asthma[a]	Absent	11.0 [10.5–11.5]	10.7 [9.7–11.8]	0.665
	Present	31.2 [29.9–32.5]	33.3 [31.5–35.1]	0.144
Allergic rhinitis[a]	Absent	10.5 [10.2–10.9]	9.7 [8.8–10.6]	0.124
	Present	19.9 [19.0–20.8]	21.2 [20.0–22.5]	0.365

[a] prevalence obtained by stratifying on asthma/allergic rhinitis and adjusted for gender, age, season of response, type of contact, cumulative response rate, and centre.
[b] p value obtained by testing the null hypothesis that the regression coefficient of the dummy indicator of the study (GERD vs ISAYA) was zero.

Table 5 Mutually adjusted risk ratios (RRs)[a] for the association between CB and each potential predictor

	RR (95% CI)		
	ISAYA (1998/2000)	GEIRD (2007/2010)	p-value for heterogeneity[b]
Gender (female vs male)	1.37 (1.09–1.27)	1.31 (1.00–1.23)	0.721
Smoking habits (vs never smoking):			
Past light smoking	1.40 (1.21–1.62)	1.05 (0.86–1.25)	0.015
Past heavy smoking	2.47 (1.96–3.13)	1.66 (1.19–2.31)	0.151
Current light smoking	2.15 (1.95–2.37)	1.95 (1.73–2.19)	0.144
Current heavy smoking	4.82 (4.31–5.39)	3.57 (3.11–4.11)	0.018
Occupational status (unemployed/retired subject vs employed subject/house person/student)	1.31 (0.97–1.28)	1.53 (1.31–1.79)	0.079
Asthma (present vs absent)	2.08 (1.84–2.34)	2.01 (1.77–2.30)	0.635
Allergic rhinitis (present vs absent)	1.76 (1.60–1.93)	1.81 (1.61–2.02)	0.680

[a] RRs also adjusted for age, season of response, type of contact, cumulative response rate, and centre.
[b] p-value obtained by considering the data from both the studies in the model and by testing the null hypothesis that the regression coefficient of the interaction term between the covariate and the dummy indicator of the study (GEIRD vs ISAYA) was zero.

observed association between vehicular traffic and the presence of CB supports the evidence that long-term exposure to air pollution is causally associated with respiratory illness [35,36]. In particular, living close to traffic has been documented to be associated with the prevalence of CB, which indicates that traffic-related air pollution has long-term effects on chronic respiratory disease [9,37]. Thus, in addition to the relatively rare episodes of incidental, heavy air pollution, the common levels of exposure to air pollutants may increase the prevalence of CB and other respiratory diseases.

CB, asthma, and allergic rhinitis

The prevalence of asthma and allergic rhinitis increased in the last ten years in Italy, as already reported in a previous analysis of the ISAYA and GEIRD data [19]. Proposed contributing factors to the increase in the prevalence of asthma are the exposure to air pollution, infections, microbial substances in the environment, and obesity [38], whereas the upward trend in the prevalence of allergic rhinitis could be due to increasing air pollution, indoor environmental factors, improved hygiene practices, geo-climatic factors, or all of the above [19].

Despite the positive trend in their prevalence, the strength of the association with the presence of CB did not change for both asthma and allergic rhinitis, and our estimates are comparable to the results obtained in 16 industrialized countries in the ECRHS [17]. As already reported, the association between asthma and CB identifies a subgroup of patients characterized by frequent exacerbations, inadequate treatment, or poor disease control [11,12], which result in a heavy socio-economic burden [13-15]. Alternatively, it may be a result of the coexistence of asthma with COPD [16].

In agreement with previous studies [17,39], CB was highly prevalent among subjects with allergic rhinitis. This association confirms that post-nasal drip is a frequent cause of cough, which could also contribute to the volume of sputum expectoration. In particular, allergic rhinitis is a risk factor for sinusitis [40], which is highly prevalent in the general Italian population [41] and is a cause of post-nasal drip. However, especially in this case, CB may be multifactorial because many patients with asthma have rhinitis and, in turn, many patients with rhinitis have asthma or are at risk of asthma [42,43].

Strengths and weaknesses of the study

The temporal change in CB is likely to reflect the trend in the prevalence rather than differences in the study design or changes in health care practice. In fact, our results were obtained by analyzing the data from two large surveys, which were carried out in the general population by using the same design, sampling frame, and protocol. Moreover, our estimates are based on self-reported symptoms, which were collected throughout international validated questionnaires [23] and are less influenced by diagnostic procedures.

A few caveats should be taken into account when interpreting our results. The Italian centres participating in ISAYA or GEIRD were not chosen randomly, but on the availability of research teams that were able to carry out the survey, and only a sub-sample of the Italian centres (Pavia, Sassari, Turin, Verona) was involved in both the surveys. However, despite this non-random selection, the study centres are located all over Italy and are representative of the geographical and climatic features of the country. Moreover, the sensitivity analysis carried out by considering only these four centres gave the same results as that obtained in the main analysis.

The participation rate declined from 72.7% in 1998/2000 to 57.2% in 2007/2010, as observed in other epidemiological studies over the past decades [44], and this decrease may have biased our estimates. In fact, symptom prevalence may be overestimated in GEIRD if the willingness to respond to the questionnaire is related to respiratory health [45]. However, it has been suggested that declines in participation rates are not likely to have a substantial influence on the exposure-disease associations or on the point estimates of measures of interest [44].

Conclusions

Despite the significant reduction in current smoking, the prevalence of CB did not vary during the past decade among young adults in Italy. The temporal pattern in the prevalence of CB can only be partly explained by the increase in the prevalence of unemployment/premature retirement, asthma, and allergic rhinitis in the same period, and suggests that other factors could have played a role.

Additional file

Additional file 1: Table E1. Mutually adjusted RRs for the association between CB and each potential predictor. The RRs were obtained by considering only the data from the four centres (Pavia, Sassari, Turin, Verona) that had participated in both the surveys.

Abbreviations

CB: Chronic bronchitis; COPD: Chronic obstructive pulmonary disease; GORD: Gastroesophageal reflux disease; ISAYA: Italian study on asthma in young adults; GEIRD: Gene environment interactions in respiratory diseases; ECRHS: European community respiratory health survey; RR: Risk ratio; 95% CI: 95% confidence interval.

Competing interests

RdM, AGC, LC, MF, and PP have received a grant for the clinical stage of the GEIRD study from Chiesi Pharmaceuticals. LC has received a reimbursement for attending a symposium from Sigma-Tau Pharmaceuticals and a fee for organizing education from Chiesi Pharmaceuticals. All remaining authors declare that they have no competing interests.

Authors' contributions

SA and RdM designed the study. SA, RdM, and AGC prepared the draft of the manuscript. SA did the statistical analysis. All the authors collected the data in their own centre and contributed to the interpretation of results and to the revision of the manuscript. All the authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Adult eczema in Italy: prevalence and associations with environmental factors

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Abstract

Background Studies on the prevalence of eczema and atopic dermatitis (AD), and on the factors associated with these diseases, have been mostly performed in children, whereas studies on adult populations are lacking.

Objectives To determine the prevalence of eczema and AD in the Italian adult population, and to investigate risk factors associated with the disease.

Methods A postal screening questionnaire was administered to 18 357 randomly selected subjects aged 20–44 years in the Gene–Environment Interaction in Respiratory Diseases study, which involved seven centres distributed across northern, central and southern Italy. The questionnaire included items on the occurrence of doctor-diagnosed eczema, asthma and hay fever, socio-demographic characteristics and environmental exposures.

Results In all, 10 464 (57.0%) subjects responded to the questionnaire. The prevalence of current eczema was 8.1% (95% CI: 7.6–8.7%), while the prevalence of eczema with asthma and/or hay fever (EAH), which was adopted as proxy of AD, was 3.4% (95% CI: 3.1–3.8%). About 60% of the subjects with current eczema reported the onset of the disease in adulthood. In multi-variable models, the prevalence of eczema was significantly associated with female sex, older age, living close to industrial plants, high levels of heavy traffic near home and living in central–southern Italy.

Conclusions Eczema and EAH are highly prevalent in Italian young adults, especially in women. Our results suggest that adult onset is not unusual, and that environmental factors may influence the occurrence of eczema and EAH.

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Conflict of interest

The authors declare that they have no conflicts of interest

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Introduction

Atopic dermatitis (AD), a.k.a. atopic eczema, is an inflammatory disease, characterized by epidermal dryness and itchiness, that causes typical relapsing lesions. AD is often considered a disease typical of childhood, and it is regarded as infrequent in adulthood.[1] While most published research on AD has been performed in children, very few studies investigated the epidemiology of the disease in adults, showing that AD is not uncommon in grown-up subjects.[2–5]

AD considerably impacts the quality of life of patients. The irritating, uncomfortable and unlikeable plaques can cause severe distress and sleepless nights,[6,7] influencing the personal,

social and occupational life.[8–11] Despite the high prevalence and socioeconomic costs,[12] AD still has a uncertain aetiology. The increasing trend in the prevalence of AD both in children[13] and adults[14] suggests that genetic and immunologic predisposition can be triggered by environmental factors, which seem to play a critical role in the manifestation of the disease.

This report from a multi-centre study aimed at determining the prevalence adult eczema and AD in Italy, and at studying the socio-demographic and environmental factors associated with the disease.

Methods

Study design and participants

The Gene–Environment Interactions in Respiratory Diseases (GEIRD) project is an ongoing two-stage multi-centre study, aiming at investigating allergic and respiratory conditions in the Italian adult population.[15] As part of GEIRD stage-I, a cross-sectional screening questionnaire was administered between 2005 and 2010 to 18 357 subjects aged 20–44, which were randomly selected from 7 Italian centres: three from northern Italy (Verona, Turin and Pavia), two from central Italy (Ancona and Terni) and two from southern Italy (Sassari and Salerno) (Fig. 1). The GEIRD screening questionnaire (available on www.geird.org) is a modified version of the European Community Respiratory Health Survey (ECRHS) questionnaire,[16] and includes questions on the occurrence of allergic diseases, socio-demographic characteristics and environmental exposures. The questionnaire was mailed up to three times in the case of non-response, and a final phone interview was carried out to reach the remaining non responders. All participants were informed about all aspects of the research project and consented to complete and return the questionnaire. Approval to conduct the study was granted from the local ethical committee in each participating centre.

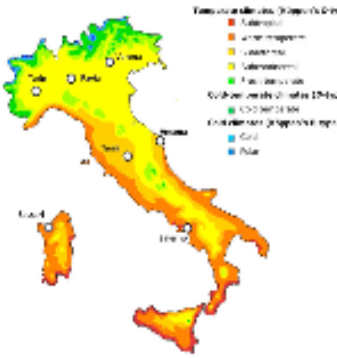

Figure 1 Centres participating in Gene–Environment Interaction in Respiratory Diseases survey, according to climatic regions.

History of eczema, asthma and hay fever

A subject was considered to have:

1. *Current asthma*: in case of affirmative answer to the questions 'Have you ever had asthma?' and 'Was this confirmed by a doctor?', plus he/she reported asthma attacks or use of medicines for asthma in the previous 12 months;
2. *Current hay fever*: in case of affirmative answer to both the questions: 'Do you have nasal allergies including hay fever?', and 'Do you still have these nose problems?'.
3. *Current eczema*: in case of affirmative answer to the questions 'Have you ever had atopic dermatitis or eczema, confirmed by a doctor?', and 'Do you still have these problems?'

A composite binary variable for eczema with concurrent asthma and/or hay fever (EAH) was also created and considered as proxy of AD.[3] Childhood and adulthood onset eczema were defined according to reported age at disease onset <18 and ≥18 years, respectively.[17]

Covariates and potential confounders

The following covariates were collected from the questionnaire and considered:

1. Socio-demographic factors: sex, age (20–29; 30–39; 40–44), school education level (university degree; high school diploma; or lower education otherwise) as a proxy of socio-economical status, and occupation (clerk; manager; unemployed; housewife; self-employed; workman; student).
2. Environmental determinants: current smoking habits (non-smoker; smoker; ex smoker), self-reported level of heavy traffic near home ('high' if trucks constantly passed near home; 'moderate' if trucks frequently passed near home; 'low' if trucks never or seldom passed near home), proximity to industrial plants, residential area (inner city; suburb of a city; village or rural area) and geo-climatic area (Sub-continental in the three northern centres; Mediterranean, in the 4 centres from central and southern Italy, Table 1).[18]

The season when the questionnaire was completed and the type of contact (mail/phone) were considered as potential confounders. Moreover, as the centres had different final response rates, the centre-specific cumulative percentile rank of response was also included as potential confounder.[19]

Statistical analyses

All the statistical analyses were performed with STATA 13.1 (Stata Corp LP, College Station, TX, USA), and a 5% significance level was adopted. Multi-variable logistic regression models were used to evaluate the adjusted associations of the above-

Table 1 Geo-climatic characteristics of the GEIRD centres

GEIRD centre	Latitude North	Mean temperature (°C)	Temperature range (°C)	Degree day (HDD)	Climatic classification
Sub-continental area					
Verona	45°26'	12.7	21.7	2617	E
Pavia	45°11'	12.6	22.5	2623	E
Torino	45°04'	12.0	20.4	2868	F
Mediterranean area					
Ancona	43°37'	13.6	17.3	1888	D
Terni	42°34'	15.5	19.2	1680	D
Sassari	40°43'	16.1	16.2	1165	C
Salerno	40°41'	18.2	15.7	994	C

The meteorological data from each centre were obtained using the data collected in the period 1981–2010 from the reference weather stations of the Italian Air Force Meteorological Service. The annual mean temperature is defined as the mean of the daily temperature mean values, and the temperature range is defined as the difference between the average temperature of the hottest and the coldest month. The centre-specific values of HDD and their climatic classification were obtained by the Italian National Agency for Energy (ENEA) (http://efficienzaenergetica.acs.enea.it/doc/dpr412-93_all4_tabdl-legradigiorno.pdf). According to the Italian law n.412/1993, HDD is defined as the annual summation of the difference between the conventional base indoor temperature (i.e. 20°C) and daily outdoor temperature measured in the days with mean temperature lower than 12°C.
HDD, heating degree day.

Table 2 Response rate and prevalence of Eczema and EAH in the centres participating in the GEIRD study

GEIRD centre	Eligible subjects n	Response rate %	Subjects included* n	Eczema % (95% CI)	EAH % (95% CI)
Sub-continental area	7393	53.0	3918	7.4 (6.6 8.2)	2.8 (2.3 3.3)
Verona	3579	62.7	1716	8.3 (7.0 9.6)	2.7 (2.0 3.5)
Pavia	2605	37.1	944	8.4 (6.6 10.1)	3.9 (2.7 5.2)
Torino	2208	64.8	1168	5.4 (4.1 6.7)	2.0 (1.2 3.8)
Mediterranean area	10 965	58.7	6965	8.5 (7.8 9.2)	3.8 (3.4 4.3)
Ancona	2811	58.1	1744	8.4 (7.1 9.7)	3.8 (2.9 4.7)
Terni	3018	61.9	1818	8.1 (6.7 9.4)	2.8 (2.0 3.7)
Sassari	3347	53.0	1705	9.7 (8.0 11.4)	4.8 (3.6 6.0)
Salerno	2792	63.6	1700	6.3 (7.0 9.6)	4.1 (3.1 5.0)
Total	18 357	57.0	10 883	8.1 (7.6 8.7)	3.4 (3.1 3.8)

EAH: current eczema with overlapping asthma and/or rhinitis. *Subjects with missing data on the occurrence of eczema, asthma or hay fever were excluded from the analyses.

mentioned variables with eczema and EAH, adjusting for potential confounders. Because the questionnaire administered from centre of Ancona missed the information on 'proximity to industrial plants' and 'residential area', the subjects recruited in this centre (n = 1744) were excluded from the multi-variable analyses.

Results

Response rates and prevalence across centres

Of 18 357 eligible subjects, 10 464 subjects responded to the GEIRD screening questionnaire (response rate: 57%) (Table 2). In all, 10 883 subjects with complete information on eczema, asthma and hay fever occurrence were included in the study. A total of 1492 subjects (14.8%, 95% CI: 14.1–15.5) reported that they had suffered from eczema at some time in the past, and 819 (8.1%, 95% CI: 7.6–8.7) reported that they were currently suffering from the disease. In particular, 347 subjects (3.4%, 95% CI: 3.1–3.8) had EAH, defined as current eczema with concurrent asthma and/or hay fever (Fig. 2). The prevalence of subjects with current eczema and EAH was minimum in Turin (5.4% and 2.0%, respectively) and maximum in Sassari (9.7% and 4.8%, respectively).

Age at the onset in subjects with current disease

Figure 3 represents the age at the onset of the disease in the subjects with current eczema and EAH, stratified by age group. Nearly 60% of the subjects in their twenties reported that they had symptoms for the first time when they were children (<18 years). This percentage decreased to 35% and 25% in

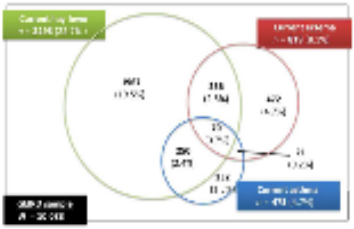

Figure 2 Distribution of current eczema, hay fever and asthma in the GEIRD sample.

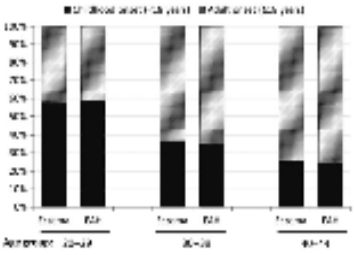

Figure 3 Proportion of childhood and adult onset eczema and EAH in subjects with current disease, stratified by age group.

subjects in their thirties and forties, respectively. Overall, only 40% of adults with eczema or EAH reported that they had their first symptom before the age of 18. There was no significant difference in the age at onset between subjects with eczema and subjects with EAH.

Associations of socio-demographic factors and environmental determinants with adult eczema

The prevalence of eczema and EAH across strata of socio-demographic and environmental variables is shown in Table 3. The prevalence of both eczema and EAH was lower in men and in subjects in their twenties compared to women and to older subjects. Unemployed subjects had the highest prevalence of eczema and EAH (9.9% and 4.1%, respectively), while students the lowest (6.9% and 2.2%, respectively).

Subjects who lived in proximity to roads trafficked by trucks or to industrial plants had a higher prevalence of eczema and EAH compared to subjects living farther away. Finally, centres from central/southern Italy had a higher prevalence of eczema and EAH with respect to centres from northern Italy.

After adjusting for all covariates and potential confounders (Table 4), the risk of having eczema and EAH was 57% and 86%, respectively, significantly higher in women than men ($P < 0.001$). Compared to older subjects, adults in their twenties had nearly 30% lower risk of having eczema ($P < 0.01$). Occupation was no longer associated with eczema in multi-variate analysis. Subjects living in proximity of industrial plants had about 40% higher risk of having eczema or EAH ($P = 0.001$ and $P = 0.03$, respectively), and living close to heavy trafficked roads was significantly associated with 30% higher risk of having eczema ($P = 0.02$). The risks of having eczema and EAH were, respectively, 24% ($P = 0.01$) and 56% ($P = 0.001$) higher in subjects living in cities with Mediterranean climate than in those living in cities with sub-continental climates in northern Italy.

Discussion

Eczema represents a group of diseases with similar clinical characteristics, and there is not a clinical standard for the definition of the disease.[1] Similarly to the approach used by Silverberg and Hanifin,[3] two definitions were used in this study, which captured different subsets of the eczema/AD disease spectrum. Our definition of eczema, which was based on self-reported doctor diagnosed 'eczema or atopic dermatitis', might have resulted in an overestimation of the true prevalence of AD by including other eczematous skin disorders, such as irritant and/or allergic contact dermatitis. In contrast, the composite definition of *EAH*, based on the concurrent presence of eczema and asthma or hay fever, is likely be more specific in identifying the clinical cases of extrinsic AD, but might underestimate the real prevalence of AD since many patients develop AD without having other allergic disorders.[3] Both of these definitions are accepted and have been used in previous epidemiological studies.[20] In the present study, we assumed the definition of EAH as proxy indicator of AD.[3]

The main findings of the study were as follows:

1 In Italian subjects aged 20–44 years, the prevalence rates of current eczema and current EAH were 8.1% and 3.4%, respectively, and most of the subjects reported an adult onset of disease;
2 The prevalence of eczema and EAH is significantly higher in centres of central and southern Italy compared to those in northern Italy;
3 Living in air polluted areas, such as in proximity of heavy trafficked roads or industrial plants, is associated with significantly increased risk of eczema and EAH.

Eczema and AD are common in adults

In this national multi-centre population-based study, we found a 8.1% prevalence of eczema and 3.4% prevalence of EAH in Italian young adults. These results are consistent with recent

Table 3 Prevalence (with 95% confidence interval) of eczema and EAH across strata of socio-demographic and environmental variables

Variable	n	Eczema		EAH	
		Prevalence	95% CI	Prevalence	95% CI
Socio-demographic factors					
Gender					
Male	4788	6.5	5.8 7.2	2.7	2.2 3.2
Female	5290	9.6	8.8 10.4	4.1	3.6 4.7
Age					
20 29	3850	6.7	5.7 7.6	2.9	2.3 3.5
30 39	4399	8.8	8.0 9.6	3.6	3.1 4.2
40+	7829	8.5	7.5 9.5	3.7	3.0 4.4
Schooling					
Lower education	1920	8.3	7.0 9.5	3.3	2.5 4.1
High school	5409	7.9	7.1 8.8	3.4	3.0 3.9
University	3700	8.6	7.6 9.7	3.6	2.9 4.3
Occupation					
Clerk	4092	8.4	7.5 9.2	3.9	3.3 4.5
Manager	537	7.8	5.5 10.1	3.0	1.5 4.4
Unemployed	677	9.9	7.6 12.2	4.1	2.6 5.6
Housewife	709	9.4	7.3 11.6	2.7	1.4 3.9
Self-employed	1597	8.6	7.2 10.0	3.7	2.7 4.6
Workman	1426	6.4	5.2 7.7	3.0	2.1 3.9
Student	1108	6.9	5.4 8.4	2.2	1.3 3.0
Environmental exposures					
Smoking habits					
Non-smoker	5616	7.7	7.0 8.4	3.3	2.9 3.7
Smoker	3732	8.3	7.3 9.4	3.6	2.9 4.2
Past smoker	1903	9.4	8.0 10.9	3.7	2.8 4.7
Traffic level near home					
Low	5893	7.4	6.7 8.1	3.1	2.6 3.6
Moderate	3615	8.4	7.3 9.4	3.4	2.7 4.1
High	1698	10.0	8.6 11.4	4.5	3.5 5.5
Proximity to industrial plants					
No	6479	7.6	7.0 8.2	3.1	2.7 3.5
Yes	1647	10.0	8.6 11.5	4.4	3.4 5.4
Residential area					
City centre	3217	8.3	7.3 9.2	3.5	2.8 4.1
Suburbs	3999	8.0	7.2 8.8	3.3	2.7 3.9
Countryside	1040	7.5	5.9 9.1	3.4	2.3 4.5
Geo-climatic area					
Sub-continental	3816	7.4	6.6 8.3	2.8	2.3 3.3
Mediterranean	6265	8.5	7.8 9.2	3.8	3.4 4.3

EAH: current eczema with overlapping asthma and/or rhinitis.

reports from other large population-based surveys conducted in Europe and in the United States.[3–5] A US study, which used the 2010 National Health Interview Survey (NHIS) data, reported a 12-month prevalence of adult eczema of 10.2% and 3.2% prevalence of EAH.[3] Another study from the ICRHS reported that the 12-month prevalence of eczema and AD in 11 European countries was approximately 7.1% and 2.4%, respectively, in the 37–56 years age group, with great variation between and within countries.[5]

There are no Italian population-based studies in young adults to directly compare our study with. We are aware of only a prior survey that assessed the prevalence of skin disorders in a representative sample of the Italian adult population, aged 45 and older, which reported a lifetime prevalence of AD of 4.7%.[23]

Table 4 Adjusted estimates of association of socio-demographic and environmental factors with prevalence of eczema and EAH

Variable, n (%)	Eczema		EAH	
	Adjusted OR† (95% CI)	*P*-value	Adjusted OR† (95% CI)	*P*-value
Socio-demographic factors				
Gender				
Male	1		1	
Female	1.57 (1.31 1.88)	<0.001	1.88 (1.40 2.49)	<0.001
Age				
20 29	0.71 (0.56 0.90)	0.007	0.79 (0.55 1.13)	0.192
30 39	1		1	
40 44	0.97 (0.80 1.18)	0.740	0.98 (0.73 1.31)	0.873
Schooling				
Lower education	1		1	
High school	0.91 (0.72 1.14)	0.407	1.00 (0.70 1.43)	0.998
University	0.86 (0.66 1.13)	0.283	0.84 (0.56 1.26)	0.406
Occupation				
Clerk	1		1	
Manager	1.12 (0.76 1.65)	0.558	1.01 (0.56 1.82)	0.985
Unemployed	1.31 (0.95 1.81)	0.097	1.20 (0.75 1.92)	0.453
Housewife	1.03 (0.76 1.41)	0.850	0.63 (0.37 1.07)	0.087
Self-employed	1.22 (0.95 1.57)	0.114	1.13 (0.78 1.64)	0.533
Workman	0.83 (0.61 1.13)	0.233	0.90 (0.58 1.41)	0.653
Student	1.31 (0.94 1.83)	0.115	0.76 (0.43 1.29)	0.298
Environmental exposures				
Smoking habits				
Non-smoker	1		1	
Smoker	1.18 (0.98 1.41)	0.126	1.10 (0.82 1.47)	0.537
Past smoker	1.25 (0.99 1.58)	0.057	1.02 (0.71 1.45)	0.934
Traffic level near home				
Low	1		1	
Moderate	1.08 (0.89 1.32)	0.427	0.97 (0.71 1.31)	0.817
High	1.29 (1.04 1.60)	0.021	1.24 (0.89 1.73)	0.195
Proximity to industrial plants				
No	1		1	
Yes	1.38 (1.13 1.69)	0.001	1.41 (1.05 1.90)	0.023
Residential area				
City centre	1	0.807	1	
Suburbs	0.96 (0.81 1.16)	0.696	0.99 (0.74 1.31)	0.917
Countryside	0.93 (0.70 1.24)	0.126	1.05 (0.69 1.60)	0.833
Geo-climatic area				
Sub-continental	1		1	
Mediterranean	1.24 (1.05 1.48)	0.013	1.56 (1.20 2.03)	0.001

†Adjusted for all the variables included in the table plus season of response, centre-specific cumulative percentile rank of response and type of survey (postal waves/telephone).
OR, odds ratio.
Statistically significant associations are shown in bold.

Subjects with adult eczema report prevalently an adult onset of the disease

Eczema is conventionally regarded as a disease of childhood, developing in the first years of life and commonly remitting by the adolescence. If this was true, it would be reasonable to expect a large difference in the prevalence of current eczema between adults and children. However, a recent survey reported a 1-year prevalence of eczema of 10.1% and 7.7% in Italian children aged

6–7 and 13–14, respectively,[13] which is similar to the prevalence that we found in adults (8.1%). This result is quite in agreement with the literature. When comparing the results of large epidemiological surveys carried out in children and in adults within the same country, the 12-months prevalence of eczema is similar in the adolescent and adult populations. In the United States, for example, the reported prevalence of current eczema was 10.7% in children and 10.2% in adults.[18] The same parallel can be done in Europe by comparing the results of the ISAAC study in children and the ECRHS study in adults.[3,13]

Another finding is that more than half of the subjects with eczema and EAH reported an adult onset (≥18 years) of the disease. The prevalence of subjects with adult onset is about 40% in the 20–29 years age group, and it raises to over 70% in the subjects aged 40–44. These data are consistent with a prior US epidemiologic study report,[17] and suggest that eczema in adults is mostly represented by new adult onset disease rather than the comeback or the persistence of childhood disease. However, given the self-reported design of the study, which makes it potentially prone to recall bias, these results might be influenced by the fact that the memory of childhood eczema might vanish with increasing age. Prospective cohort studies examining the age at onset are needed to understand the connection between childhood and adult eczema.

Geo-climatic and environmental factors are associated with adult eczema

Previous research showed that climatic factors influence the prevalence of childhood eczema in US.[21] Our analyses highlighted a regional variability in the prevalence of eczema within Italy. In fact, the risk of having eczema and EAH was, respectively, 24% and 50% higher in central/southern Italy, which has a milder, Mediterranean climate, with respect to northern Italy, which has sub-continental climate.[19] These differences in the prevalence of eczema could be ascribed by variations of climate itself and of its intrinsic components (e.g. mean annual temperature, atmospheric temperature range, UV light exposure, outdoor humidity, precipitations, characteristics and length of the pollen season), as well as to other differences that are closely correlated with geo-climatic variations, such as different lifestyle, time spent outdoors and diet.

Given the boot shape of the Italian peninsula, most of the principal towns in southern Italy lie on the coast. Accordingly, 3 out of 7 centres that participated to the study, all in central/southern Italy, are coastal cities. The proximity to sea not only directly contributes to the climate (e.g. humidity, precipitations, outdoor temperature range) but it also modifies population's life-styles and behaviours, which might in turn influence the risk of developing eczema.

Living close to heavy traffic was another independent risk factor for eczema and EAH. Some earlier studies have shown a similar positive association in children[20,22] and in adults.[4,26] In experimental studies, diesel exhaust particles have been reported to modulate the immune response towards IgE production and to concur in the onset of allergic inflammation by activating macrophages and other mucosal cell types and by affecting the production of cytokines and chemokines.[27] Moreover, exposure to NO_2 has been shown to damage the skin barrier function and increase trans-epidermal water loss.[28] Heavy traffic exposure may therefore worsen the skin and immunological function, facilitating the development and the relapse of eczema.

Further emphasizing the role of pollution in the occurrence of eczema, our results showed that living in proximity to industrial plants significantly increases the risk of eczema and EAH of about 40%. This is consistent with the results from two studies carried out in Turkey and Sweden, which recently reported that occupational exposure to chemical fumes is associated with an increased risk of eczema.[5,29] Taken together, these data suggest that environmental air pollution may affect the susceptibility to eczema, and agree with many epidemiological studies that showed an increasing trend in the prevalence of eczema and AD, especially in developing countries that are undergoing rapid urbanization and industrialization.[30]

Strengths and limits of the study

The strength of this study includes being a large-scale, population-based study, and controlling for confounding socio-demographic and environmental factors. The use of a standardized methodology enabled the direct comparison of disease prevalence across the centres. We acknowledge that the moderate response rate of 57% is a limitation of this study. However, it is consistent with those obtained by other recent epidemiological surveys in randomly selected subjects from the general population.[3] In the analysis, we have tried to account for differential response rates in participating centres by including percentile rank of response in multi-variable models.

Since eczema is commonly thought as a disease of childhood, most of the questionnaires are specifically designed to identify the skin lesions in children. In adults, however, eczema has a more assorted distribution, for example with frequent hand and head-neck involvement.[3] To partially overcome these problems, our definitions of eczema and AD were based on self-reported doctor diagnosis rather than on symptoms. These definitions, however, have not been validated in previous studies and they might be prone to recall and to diagnostic bias.

The study also suffers of the limits of the cross-sectional design and, consequently, the associations of the variables with eczema must be interpreted cautiously. Finally, this study was based on a short screening questionnaire developed to investigate mainly respiratory diseases. As a consequence, it lacks of more detailed information on the anatomical location of skin lesions, which would have been useful to differentiate among possible eczema phenotypes.

Conclusions

Eczema and EAH are highly prevalent in Italian adults, especially in women, and most of the investigated subjects reported an adult onset. The associations of eczema with proximity to heavy-trafficked roads and industrial plants suggest a link between environmental air pollution and the disease. Geo-climatic variations seem also to influence the occurrence of eczema in adults.

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GPe and AM conceived the study and planned the statistical analyses. GPe performed the statistical analyses, wrote the first draft of the manuscript and contributed to the survey design and data collection. AM, AC, LA, LC, MF, AGF, PM, MO, PP, GPo, RT, GV, SV, RdM critically reviewed the manuscript for important intellectual content.

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Urban air and tobacco smoke as conditions that increase the risk of oxidative stress and respiratory response in youth

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ABSTRACT

Background: Air pollution and tobacco smoke can induce negative effects on the human health and often leads to the formation of oxidative stress.

Objective: The purpose of this study was to clarify the role of the urbanization degree and of passive exposure to tobacco smoke in the formation of oxidative stress. Thus, a group of non smoking adolescents was recruited among those who live and attend school in areas with three different population densities. To each subject a spot of urine was collected to quantify 15-F_{2t} isoprostane as a marker of oxidative stress and cotinine as a marker of passive exposure to tobacco smoke. Furthermore, respiratory functionality was also measured.

Results: Multiple linear regression analysis results showed a direct correlation ($p<0.0001$) of 15-F_{2t} isoprostane with both the urbanization and passive smoke. Lung function parameters proved significantly lower for the subjects living in the most populous city of Torino.

Conclusion: This remarks the negative effect that urbanization has on the respiratory conditions. Lastly, lung functionality presented a low inverse correlation with 15-F_{2t} isoprostane, suggesting an independent mechanism than that of the urban factor.

1. Introduction

The airborne particulate matter (PM) has several origins, is formed in different places where its precursors may be different; thus it possesses various physico-chemical and toxicological properties (Gieschi et al., 2005; Hazenkamp-Von Arx et al., 2004; Traversi et al., 2008). Depending on type and quantity, the presence of airborne PM can determine deleterious effects on the global environment, cultural heritage, human activities and health (Fang et al., 2010; Hemchel et al., 2012; Katsouyanni et al., 2009; Levy et al., 2012; Porchi, 2005; Ranchou-Nielsen et al., 2013; Strak et al., 2012). To contain the problem, the European Union established air quality guidelines for PM as well as for other risky air pollutants (European Union, 2008). At the same time, the research activities of the scientific community were focused on the urban air pollution and its potential risk for health (Bono et al., 2001, 2014; Cohen et al., 2005; Fraser et al., 2003; Tzivian, 2011), in search of the best preventive techniques against the onset of diseases related to air pollution.

Exposure to urban air pollutants, whose concentration is partly dependent on proximity and intensity of traffic, is connected with the onset of asthma, development of respiratory allergies (Badyda et al., 2013; Chio et al., 2012; Lauenbach and Klpen, 2012), lung dysfunction (Kelly and Fussell, 2011; Wright and Brauer, 2013), inflammation, and exacerbation of other respiratory and cardiovascular problems (Mills et al., 2009). Numerous among these pathological conditions can be preceded or highlighted by the presence of internal dose markers, by biosynthesis of biological effects markers or, in some cases, by the formation of oxidative stress (Castro-Giner et al., 2009; Patel et al., 2013). An imbalance of the oxidative status is often a condition that precedes the onset of these respiratory diseases, and it is due to the exposure to airborne

Abbreviations: PM, particulate matter; 15-F_{2t} IsoP, 15-F_{2t}-isoprostane; SIDRIA, Italian Studies on Respiratory Disorders of Childhood and the Environment; CIBA, creatinine; ELISA, Enzyme-Linked Immuno Sorbent Assay; FVC, forced vital capacity; FEV1, forced expiratory volume in one second; PEF, maximal expiratory flows at peak of FVC; FEF50, maximal expiratory flows at 50% of FVC; FEF25, maximal expiratory flows at 25% of FVC; FEF25–75, maximal expiratory flows among 25–75% of FVC; MLR, multiple linear regression; CI, confidence interval

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oxidants (Saxa and Carlsten, 2012) and a decreasing biosynthesis of endogenous antioxidant molecules (Yang and Omaye, 2009). To date, the mechanisms by which oxidants interact with molecules, cells, and tissue remain largely unclear. Remarkably, oxidative stress is also related to the inflammatory response due to tobacco smoke (Doruk et al., 2011; Howard et al., 1998), which contains a complex mixture of mutagenic chemicals (Granella et al., 1996) able to promote lipid peroxidation (Kalra et al., 1991), protein and DNA oxidation (Vadhanam et al., 2012; van Rijt et al., 2012).

F2-isoprostanes, specific and stable products of lipid peroxidation (Basu et al., 2009), are non-invasive biomarkers for in vivo investigations of oxidative stress status (Roberts and Morrow, 2000; Romanazzi et al., 2013), airways inflammation (Basu, 2008) and asthma (Wedes et al., 2000). They can also be implicated in a larger number of human diseases, even if a clear correlation between many of these pathological conditions and oxidative stress is far from being proven (Giustarini et al., 2008). The determination of F2-isoprostanes levels in selected populations may help understanding the role that some environmental factors play in the expression of oxidative stress. In particular, the 15-F_{2t}-isoprostane (15-F_{2t} IsoP) can be monitored, since it has been proven capable to highlight different biological responses to environmental stimuli, particularly those concerning airborne chemicals (Bono et al., 2014).

Quantification of oxidative stress by means of F2-IsoPs has several advantages if compared to other biomarkers, including the one that its levels are unaffected by diet (Gopaul et al., 2000; Jacob et al., 2013). At this concern, Roberts and Morrow reported that urinary F2-IsoPs, in subjects consuming a normal diet, does not decrease after a four days diet consisting only of glucose (Roberts and Morrow, 2000), and Richelle refers that the lipid content of the diet does not affect the level of urinary F2-IsoPs (Richelle et al., 1999). This aspect of F2-IsoPs is particularly useful when, as in this case, the role of the diet is not object of interest, although it is very important in the manifestation of oxidative stress.

Finally, the relationship between biosynthesis of 15-F_{2t} IsoP and levels of respiratory functionality, in relation to the environmental conditions of life, are still largely to deepen.

That is, the purpose of this study was to clarify the role that some independent environmental, individual, and physiological variables have on the oxidative stress status of a large population of healthy non-smoking adolescents, living in three different areas of the Piedmont region (northwestern Italy).

2. Materials and methods

15-F_{2t} IsoP levels were studied in relation to the urbanization degree of the selected areas where the adolescents live and attend school, in order to understand the role that urbanization might play on oxidative stress formation. Any additional information, essential for the study, was collected through a questionnaire filled out by all the adolescents, after their parents or legal tutors had signed an informed consent. In detail:

2.1. Sampling sites

As shown in Fig. 1, three geographic areas with different levels of urbanization and anthropization were chosen in the Piedmont region (northwestern Italy, 25,401.56 km^2): Torino, capital of Piedmont, a urbanized city with almost 900,000 inhabitants (6,700 inhabitants/km^2, 130.2 km^2, 240 m above sea level); Chivasso, a smaller and less urbanized city with about 26,000 inhabitants (507 inhabitants/km^2, 51.3 km^2 183 m above sea level); and Casalborgone, a rural site with 1880 inhabitants (93.3 inhabitants/km^2, 20.2 km^2, 205 m above sea level). Due to the relative proximity with one another, the three locations do not have significant differences in climate, geography, altitude or social habits.

2.2. Epidemiological sample

The epidemiological sample was prepared with the aim to represent the young population of the three locations of the Piedmont region. All subjects were volunteers recruited in lower secondary schools. In more detail, three schools were located in residential and commercial areas of the city of Torino and 214 subjects were recruited from there; one school was in Chivasso, where 119 subjects were recruited; one school was in the rural area of Casalborgone and 57 subjects were enrolled from there. Since all the students were minors, parents or legal tutors were asked to sign an informed consent. Sampling was carried out over the period from March to May 2012. Each adolescent was asked to fill out a questionnaire, perform a spirometry test to evaluate their respiratory functionality, and provide a urine sample for the determination of 15-F_{2t} IsoP and cotinine.

2.2.1. Questionnaire

For each student, a short version of the questionnaire "SIDRIA" was prepared to acquire information on age, sex, place of residence, hobbies, therapies, and parent's smoking habits (SIDRIA, 1997). An interviewer administered the questionnaire during school hours, the same day the urine sampling and the spirometry took place.

2.3. Biological samples and statistical analysis

2.3.1. Urinary cotinine

Cotinine measurement was carried out to quantify the passive exposure to tobacco smoke, which represents a possible factor of oxidative stress formation. A specimen of the first morning urine was collected from each volunteer and stored at −80 °C until analysis. Cotinine was measured by gas chromatography–mass spectrometry. The analytical procedure has been described in detail elsewhere (Bono et al., 2014). Cotinine concentrations were normalized to the urinary creatinine (CREA) levels, as usual for every urinary measurement.

2.3.2. Urinary 8-isoprostane

15-F_{2t} IsoP was measured in urine by ELISA, as previously described (Romanazzi et al., 2013). A microplate kit specific for urinary 15-F_{2t} IsoP (Oxford, MI, USA) was used following manufacturers' instructions. The declared limit of detection is 0.2 ng/ml and the possible cross-reactivity of this method is fixed below 3%. To achieve better accuracy by the ELISA method, a dilution rate of 1:4 (v/v) was adopted (Romanazzi et al., 2013). 15-F_{2t} IsoP concentrations were normalized to the CREA levels.

2.3.3. Spirometry

According to the current standards (ATS/ERS, 2005), maximal expiratory flow-volume curves were obtained while the subjects were in a standing position, wearing a nose clip and breathing into a pneumotachograph (Medicalgraphics). The instrument was calibrated with a 3-l syringe. The measurements were repeated until the volume variability did not exceed 150 ml for at least 2 times. Forced vital capacity (FVC), forced expiratory volume in one second (FEV1) and maximal expiratory flows at peak 50%, 25% and among 25–75% of FVC (PEF, FEF50, FEF25, FEF 25-75) were recorded (Bono et al., 1998; Miller et al., 2005).

2.3.4. Statistical analysis

Statistical analysis was carried out with the statistical package

Fig. 1. Map of the sampling sites. Torino, the capital of Piedmont region in Italy is a urbanized city with almost 900,000 inhabitants. Chivasso is a smaller and less urbanized city with about 26,000 inhabitants; Casalborgone is a rural site with 1900 inhabitants.

"Stata" (version 12 SE for MS Windows® 64 bit). In Table 1 descriptive statistics was reported *per* each location of sampling. A Box–Cox regression (Box and Cox, 1964) was performed to find the power transformation that stabilize the variance and normalize the distribution.

A multiple linear regression (MLR) was carried out to assess the effect on covariates on 15-F_2 IsoP and lung function parameters respectively, using 15-F_2 IsoP or lung function parameters as dependent variable, and age, height, weight, gender (female as reference value), urinary cotinine, and sampling location as independent variables. A significant level (a two tailed *p*-Value) of 0.05 (CI—95%) was chosen for the statistical tests. For the final regression model, only variables that proved to be significant were selected.

Table 2
15-F_2 IsoP values in the three sampling locations. The three concentrations prove a direct relationship to population density: Turin, the most populated city, display the highest mean value, Casalborgone, the rural site, the lowest.

	15-F_2IsoP (ng/mg)		
	Mean ± sd	min	max
Torino	7.2 ± 4.0	<LOD*	37.0
Chivasso	6.4 ± 5.4	<LOD*	39.8
Casalborgone	4.8 ± 3.0	1.5	14.7
Total	6.5 ± 4.4	<LOD*	39.8

min—minimum value; max—maximum value; sd—standard deviation. Units of 15-F_2 IsoP is nanograms of 15-F_2 IsoP every 1 mg of urinary creatinine.
* LOD—limit of detection. 15-NT IsoP fixed to limit of detection (0.2 ng/ml) if < of LOD.

3. Results

The characteristics of the population enrolled in the study are described in Table 1. Cohort numerousness, mean, and standard deviation (sd) and percentage (%) for gender, height and weight, age, and passive smoking exposure are reported per each investigated location where the adolescents live and attend school. All these parameters proved not to be statistically different among locations. Therefore, we could consider these individual characteristics as homogeneous in the three different sampling sites.

In Table 2, means and sd, minimum and maximum values of 15-F_2 IsoP concentrations, normalized to the urinary creatinine values (ng/mg), are listed *per* sampling location. Torino is the area with highest mean value of 15-F_2 IsoP in comparison to Chivasso e Casalborgone ($p < 0.001$). Since Torino is the most densely populated site, this result suggests the presence in the city of a possible "urban factor": the greater the urbanization level, the higher the 15-F_2 IsoP concentrations. According to the Box–Cox regression results, the values of 15-F_2 IsoP and urinary cotinine were subjected to a logarithmic transformation prior to execute the multiple linear regression (MLR) analysis.

Running the MLR test allowed us to observe that sex, height and weight are not statistically significant in the model ($p > 0.050$)

Table 1
Gender, age, height, weight, and number of passive smokers in the whole population and in three groups divided according to the three sites where the adolescents live and attend school.

	Torino (urban site)		Chivasso (intermediate site)		Casalborgone (rural site)		Total	
N	214		119		57		390	
Gender *N* (%)	Male n. (%)	122 (57%)	Male n. (%)	64 (53.8%)	Male n. (%)	25 (43.9%)	Male n. (%)	211 (54.1%)
	Female n. (%)	92 (43%)	Female n. (%)	55 (46.2%)	Female n. (%)	32 (55.1%)	Female n. (%)	179 (45.9%)
Height cm ± sd	149.6 ± 9.3		154.0 ± 8.9		153.3 ± 9.8		151.7 ± 9.5	
Weight kg ± sd	43.93 ± 11.0		47.1 ± 12.3		47.7 ± 13.2		45.6 ± 11.7	
Age years ± sd	11.5 ± 0.8		12.7 ± 0.8		12.5 ± 0.6		12.0 ± 1.0	
Smoking habits *N* (%)	Passive	70 (32.7)	Passive	52 (43.7%)	Passive	24 (42.1%)	Passive	146 (37.4%)
	Not exposed	144 (67.3%)	Not exposed	67 (56.3%)	Not exposed	33 (57.9%)	Not exposed	244 (62.6%)

Table 3
Multiple linear regression parameters, with 95% confidence interval (C.I.), of log $Ti\text{-}F_{Bi}$ IsoP as dependent variable and log (cotinine); age and sampling site as predictors.

		Regression coefficient (95% C.I.)	p<
Urinary cotinine		−0.158 (−0.199 to −0.117)	0.0001
Sampling sites	Chivasso vs Casalborgone	+0.308 (0.152–0.465)	0.0001
	Torino vs Casalborgone	+0.434 (0.268–0.601)	0.0001
	Torino vs Chivasso+Casalborgone	+0.234 (0.108–0.138)	0.0001
Age		−0.058 (−0.076 to −0.039)	0.050

Note: gender, height, weight, and diet indicators, not significant at 5% level, were excluded from the model. 15-F_{2t} IsoP fixed to limit of detection (0.2 ng/ml) if ≤ of LOD (limit of detection).

and, therefore, were excluded from the computation of the final regression model. On the contrary, urinary cotinine, sampling location and age had a significant relationship with log 15-F_{2t} IsoP (r^2=0.17; $p<0.001$) and, thus used to compute the model (Table 3). In particular, log 15-F_{2t} IsoP adjusted for age and sampling site, proved to be positively correlated to cotinine, as shown in Fig. 2, with an estimated increase of 17% for every increasing unit of cotinine concentration in a log scale.

The mean value of log transformed 15-F_{2t} IsoP concentrations, referring to the entire population, and adjusted for log cotinine and sampling site, significantly decreases of 6% for every year of age (Fig. 3).

As stated above, the mean value of 15-F_{2t} IsoP concentrations was significantly higher in the adolescents of Torino when compared to those who live in Chivasso (+12%) and Casalborgone (+51%). The adolescents living in Chivasso also presented higher concentrations in respect to those who live in Casalborgone (+34%). All the effects are orthogonal like (Fig. 4).

Table 4 shows the marginal geometric means, adjusted for the covariates (age, gender, BMI), and the lower and upper limits at a 95% confidence interval (C.I.) of the lung function parameters *per* sampling location. All lung function parameters (volumes and flows), were significantly lower for the adolescents of Torino when compared to the other locations. Adjusting the concentrations for age, gender, BMI, and sampling location, the middle volume flow rates (FEF50 and FEF 25-75) and FEV1/FVC% proved to be negatively correlated with the population density of the three sampling sites (Fig. 5); while the volumes FVC (mean −0.002, CI 95% −0.022

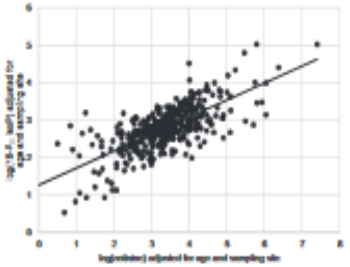

Fig. 2. Plot of the relation between log $Ti\text{-}F_{2t}$ IsoP and log cotinine, given age and sampling site.

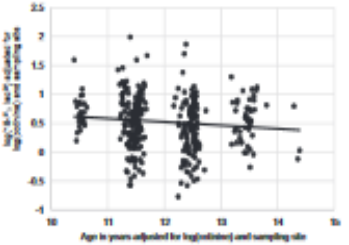

Fig. 3. Plot of the relation between log $Ti\text{-}F_{2t}$ IsoP and age, given log (cotinine) and sampling site.

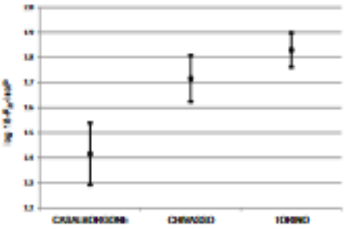

Fig. 4. Marginal means and confidence intervals of 15-F_{2t} IsoP levels measured in the three sampling sites, adjusted for log (cotinine) and age by means of multiple regression model (with log link). Casalborgone is the rural site, Chivasso the medium size city, Torino the big city.

Table 4
Marginal geometric means with 95% confidence intervals (C.I.) of the lung function parameters by sampling site, as estimated by multiple regression analysis adjusted by sex, age, height, BMI index and log (cotinine).

		Means	C.I. 95%		p <
			Lower limit	Upper limit	
FVC (l)	Casalborgone	2.91	2.82	2.99	
	Chivasso	2.86	2.82	2.95	0.22
	Torino	2.77	2.73	2.82	0.01
FEV1 (l)	Casalborgone	2.62	2.53	2.71	
	Chivasso	2.58	2.51	2.65	0.52
	Torino	2.39	2.34	2.44	0.01
FEV1/FVC (%)	Casalborgone	89.68	87.97	90.99	
	Chivasso	88.54	86.31	90.77	0.17
	Torino	86.45	85.45	87.44	0.04
MEF50 (l/s)	Casalborgone	3.53	3.30	3.75	
	Chivasso	3.66	3.51	3.81	0.16
	Torino	3.19	3.07	3.32	0.02
FEF25-75 (l/s)	Casalborgone	3.35	2.96	3.74	
	Chivasso	3.25	3.11	3.38	0.24
	Torino	2.80	2.68	2.91	0.02

+0.026) and FEV1 (CI 95% −0.019 −0.048 +0.009) did not. This evidence can be accepted considering that, compared to the volumes, the flows are more sensitive, especially in the pediatric age.

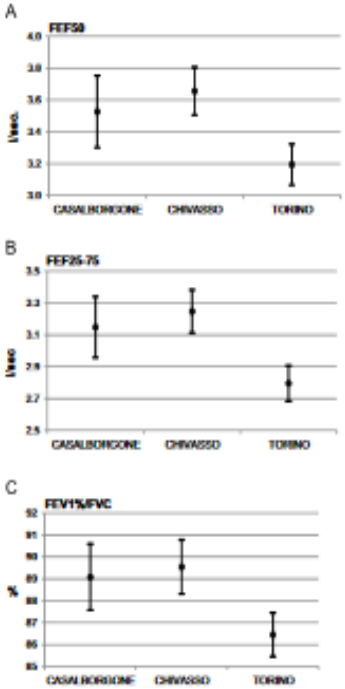

Fig. 3. Marginal means and confidence intervals of lung function parameters by the three sampling sites, adjusted for age, height, gender, BMI and log (cotinine) by means of multiple regression model. A) FEF 50=Forced Expiratory Flow at 50% of Forced Vital Capacity (FVC), B) FEF 25–75 mean forced expiratory flows at 25/75% of FVC, C) FEV1/FVC%=Forced expiratory volume in 1 s as % of FVC.

4. Discussion

The main goal of this work was to highlight the role that the urbanization level of the location where people inhabit may have in the oxidative stress formation. Healthy non-smoking adolescents were chosen as target population. Three areas of the Piedmont region with different demographic and road-traffic intensity, though not very far from each other, were investigated: Torino (a big city), Chivasso (a small town), and Casalborgone (a small rural village). The oxidative stress level was monitored through the quantification of 15-F_{2t} IsoP urinary concentration. Levels of this biomarker are unaffected by diet, an antioxidant factor, potentially confounding the relationship we have investigated (Gopaul et al., 2000; Roberts and Morrow, 2000; Jacob et al. 2013; Richelle et al., 1999). In particular, the diet is very similar among all the students because: a) they benefit from the same school lunch prepared by the same company according to the requirements imposed by nutritionists working at the local health authority to minimize oxidant food, b) all the students are white and of Caucasian ethnicity. This may mean that the diet consumed the previous evening at home is likely to be similar, c) although the three groups of students are different for population density, the distance between them does not exceed 50 km and the altitude above sea level is the same.

Since the passive exposure to tobacco smoke can also influence the oxidative stress level, urinary cotinine was measured to know the role of the tobacco in the onset of 15-F_{2t} IsoP values and used it to adjust the relationship between 15-F2t IsoP and the urban factor. Finally, the respiratory function has also been taken into account as physiological factor potentially able to be altered by the two environmental aspects regarded in the present study. The results showed the presence of a direct correlation between 15-F_{2t} IsoP and the degree of urbanization of the areas where the adolescents live and attend school. This suggests that an "urban factor" plays a direct role in the synthesis of 15-F_{2t} IsoP inducing its increase up to about 50%. Thus, the level of urbanization highlights a role of risk factor able to increase oxidative stress in adolescents, which proved to be a population particularly sensitive to even small environmental differences.

Passive exposure to tobacco smoke and age of the subject proved to be other factors that can significantly influence the 15-F_{2t} IsoP concentrations but while the exposure to passive smoke increases 15-F_{2t} IsoP levels, the age leads to the opposed result. The latter effect has been recently observed in an independent population of a similar age (Bono et al., 2014) but opposes to the general trend observed in adults. Indeed, recent scientific studies showed an increase in the intensity of oxidative stress with aging, and with the onset of many age-related diseases, including Alzheimer (Bouzid et al., 2014; Jacob et al., 2013; Montine et al., 2011).

Another finding of this study is the significant lower level of respiratory fluxes in the adolescents living in Torino, in comparison to those living in less urbanized locations. This finding shows the responsibility of the higher level of urbanization of Turin in the reduction of respiratory flows. This aspect highlights, at the same time, an increase of respiratory risk.

Furthermore, the three measures of fluxes (FEF 50, FEF 25–75, and FEV1/FVC), adjusted for age, gender, and BMI were negatively correlated to 15-F_{2t} IsoP ($p<0.047$, $p<0.013$, $p<0.005$ respectively) when compared per sampling location (Fig. 5). This allows us to consider the low values of flux intensity as a respiratory condition inversely correlated with the onset of oxidative stress. This is true even after removing the effect of 15-F_{2t} IsoP, which highlights the relationship of the respiratory effects from urbanization independent from inflammation and oxidative stress. Finally, we can conclude that the adolescents studied show an increase in oxidative stress and a decrease in respiratory flow dependent from the urbanization and the tobacco smoke passively breathed. Thus, the evidence of this risky condition for public health may represent a platform for designing new preventive strategies against tobacco smoke exposure and urban pollution.

Ethical considerations

Since the subjects were underage, during a public meeting, parents and teachers were informed on the objective of this study. A written informed consent was signed and delivered by each the participants' parents. Thus, the participation of all the human subjects did not occur until after informed consent was obtained. However, the local ethics committee (ASL TO2, Turin Italy) has expressed a favorable opinion with practice number 826/13/08.

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RESEARCH ARTICLE Open Access

The impact of asthma, chronic bronchitis and allergic rhinitis on all-cause hospitalizations and limitations in daily activities: a population-based observational study

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Abstract

Background: Chronic respiratory diseases are a significant cause of morbidity and mortality worldwide. We sought to evaluate the impact of asthma, chronic bronchitis and allergic rhinitis on all-cause hospitalizations and limitations in daily activities in adults.

Methods: In the Gene Environment Interactions in Respiratory Diseases study (2007//2010), a screening questionnaire was mailed to 9,739 subjects aged 20–44 (response rate: 53.0%) and to 3,480 subjects aged 45–64 (response rate: 67.3%), who were randomly selected from the general population in Italy. The questionnaire was used to: identify the responders who had asthma, chronic bronchitis, allergic rhinitis or asthma-like symptoms/dyspnoea/other nasal problems; evaluate the total burden [use of hospital services (at least one ED visit and/or one hospital admission) and number of days with reduced activities (lost working days and days with limited, not work related activities) due to any health problems (apart from accidents and injuries) in the past three months]; evaluate the contribution of breathing problems to the total burden (hospitalizations and number of days with reduced activities specifically due to breathing problems).

Results: At any age, the all-cause hospitalization risk was about 6% among the subjects without any respiratory conditions, it increased to about 9-12% among the individuals with allergic rhinitis or with asthma-like symptoms/dyspnoea/other nasal problems, and it peaked at about 15-18% among the asthmatics with chronic bronchitis aged 20–44 and 45–64, respectively. The expected number of days with reduced activities due to any health problems increased from 1.5 among the subjects with no respiratory conditions in both the age classes, to 6.3 and 4.6 among the asthmatics with chronic bronchitis aged 20–44 and 45–64, respectively. The contribution of breathing problems to the total burden was the highest among the asthmatics with chronic bronchitis (23-29% of the hospitalization risk and 39-50% of the days with reduced activities, according to age).

Conclusions: The impact of asthma, chronic bronchitis and allergic rhinitis on all-cause hospitalizations and limitations in daily activities is substantial, and it is markedly different among adults from the general population in Italy. The contribution of breathing problems to the total burden also varies according to the respiratory condition.

Keywords: Burden of allergic rhinitis, Burden of asthma, Burden of chronic bronchitis, Determinants

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Background

Asthma, chronic bronchitis and allergic rhinitis are common health problems in industrialized countries [1-3] and they often coexist [4-6]. Furthermore, chronic bronchitis is associated with both a more severe form of the disease and poor control of symptoms in subjects with asthma [7,8], and it may indicate the presence of the asthma-chronic obstructive pulmonary disease (COPD) overlap syndrome [9,10].

Asthma generates a high socio-economic burden among European adults, which significantly increases when chronic bronchitis is also present [11,12]. Moreover, asthma and allergic rhinitis together account for most of the allergy-related morbidity associated with the respiratory system [13]. Therefore, real-world evaluations of the impact of these diseases in the general population are fundamental to meet the widespread need for updated estimates, which should be used as a valid support to public health decisions.

In recent years, the socio-economic impact of asthma, chronic bronchitis and allergic rhinitis has been largely investigated [11,12,14-16]. However, in many studies, the total burden (i.e. the burden due to any health problems) in patients with these respiratory illnesses has not been estimated because only disease-related costs have been considered. Moreover, the total burden should be compared between patients with a certain disease and unaffected subjects from the general population. Indeed, a better understanding of the burden of these respiratory diseases requires the assessment of their total impact on the health and social systems.

The aim of the present paper is to compare all-cause hospitalizations and limitations in daily activities among adult subjects with asthma, chronic bronchitis or allergic rhinitis, and unaffected individuals from the general population, and to assess the contribution of breathing problems to the total burden. To fulfil these purposes, the data from the screening stage of the Gene Environment Interactions in Respiratory Diseases (GEIRD) study [17] were used.

Methods

Design of the study

The screening stage of GEIRD was a cross-sectional survey on respiratory health, which was carried out between 2007 and 2010 in Italy [17]. A total sample of 9,739 subjects aged 20–44 and of 3,480 subjects aged 45–64 (men/women ratio = 1) was randomly selected from the general population in four centres (Pavia, Sassari, Turin and Verona) by using the local health authority registers. A screening questionnaire was mailed to each individual up to three times and then administered by telephone in case of nonresponse. Overall, the responders were 5,162 (53.0%) in the 20–44 age group and 2,167 (62.3%) in the 45–64 age group (Table 1).

Table 1 Distribution of the design variables

		20-44 yrs	45-64 yrs	p-value
N° of eligible subjects*		9739	3480	-
N° of responders (response rate, %)		5162 (53.0)	2167 (62.3)	<0.001
Season at the time of response, %	Spring	46.7	51.5	<0.001
	Summer	15.7	8.6	
	Autumn	32.0	30.9	
	Winter	6.6	9.0	
Telephone interview, %		11.6	9.6	0.012
Females, %		53.6	52.1	0.267

*Eligible = initial sample – (dead or moved away from the target area).

Ethics statement

Ethics approval was obtained in each centre from the appropriate ethics committee (*"Comitato di Bioetica della Fondazione IRCCS Policlinico San Matteo di Pavia"*; *"Comitato di Bioetica dell'Azienda Sanitaria Locale di Sassari"*; *"Comitato Etico dell'Azienda Sanitaria Locale TO/2 di Torino"*; *"Comitato Etico per la Sperimentazione dell'Azienda Ospedaliera Istituti Ospitalieri di Verona"*). All participants were fully informed about all aspects of the research project and consented to complete and return the questionnaire.

Screening questionnaire and definitions

The screening questionnaire (available at www.geird.org) is a modified version of the questionnaire used in the Italian Study on Asthma in Young Adults [18]. It contains validated questions (mainly taken from the European Community Respiratory Health Survey questionnaires [19]) on asthma (self-report of the disease during lifetime with or without a physician's diagnosis, frequency of asthma attacks, and use of anti-asthmatic drugs in the past 12 months), asthma-like symptoms (wheezing, nocturnal tightness in the chest, and attacks of shortness of breath at night time in the past 12 months), chronic cough and phlegm, and nasal problems, as well as questions on occupational status, smoking habits and outdoor air pollution (frequency of heavy traffic near home and living near industrial plants).

On the basis of their answers to the questions described above, the responders to the screening questionnaire were classified as subjects with:

- *asthma* (i.e. self-report of the disease during lifetime with or without a physician's diagnosis or at least one asthma attack in the past 12 months or use of anti-asthmatic drugs in the past 12 months) *and chronic bronchitis* (i.e. cough and phlegm on most

days for a minimum of three months a year and for at least two successive years);
- *chronic bronchitis without asthma*;
- *asthma without chronic bronchitis*;
- *allergic rhinitis* (i.e. any nasal allergies, including hay fever) *without asthma and chronic bronchitis*;
- *asthma-like symptoms or dyspnoea or other nasal problems without asthma, chronic bronchitis and allergic rhinitis*;
- *no respiratory conditions* (i.e. none of the conditions described above).

In addition, the responders were considered exposed to a high level of outdoor air pollution if they had reported a continuous passage of trucks near home and/or living near industrial plants.

The screening questionnaire also includes questions on the use of hospital services [at least one Emergency Department (ED) visit and/or one hospital admission], the number of lost working days and the number of days with limited, not work related activities (such as looking after children, housework or studying) due to any health problems (apart from accidents and injuries) in the past three months. If hospital services have been used, the responders should specify if this has been due to breathing problems. If lost working days and/or days with limited, not work related activities have been reported, the subjects should specify the number of the impaired days because of breathing problems.

The total burden was evaluated at an individual level on the basis of the use of hospital services and the number of days with reduced activities (i.e. the number of lost working days plus the number of days with limited, not work related activities) due to any health problems (apart from accidents and injuries) in the past three months. The days with limited, not work related activities reported by full-time housepersons were considered as lost working days. The hospital services utilization and the number of days with reduced activities specifically due to respiratory disorders in the past three months were used to evaluate the contribution of breathing problems to the total burden.

Statistical analysis

The cumulative response rate, the season at the time of response to the questionnaire, the type of contact (postal waves vs telephone interview), and gender were considered as potential confounders because of differences in their distribution between the two age classes (20–44 and 45–64) (Table 1). In particular, the cumulative response percentile rank, which is an indicator of the individual propensity to respond to the questionnaire, was used. Each subject was ordered by centre and age class according to the date of response to the questionnaire, and then he/she was attributed the ratio between his/her rank and the number of eligible subjects [20].

The prevalence of each respiratory condition was estimated by using a logistic model, which included age class, centre and the potential confounders reported above, as covariates. The all-cause hospitalization risk [i.e. the proportion of subjects with at least one ED visit and/or one hospital admission due to any health problems (apart from accidents and injuries) in the past three months] and the expected number of days with reduced activities due to any health problems in the same period were estimated by using logistic and negative binomial models [21], respectively, which included the respiratory condition crossed by the age class, centre and the potential confounders reported above, as covariates. Since the subjects were nested into centres, clustered sandwich estimators of the variance were used. The estimates were computed in each age class and for each respiratory condition by setting the distribution of gender, season at the time of response to the questionnaire, type of contact and centre equal to the average distribution, and by setting the cumulative response rate equal to the overall mean [22].

A multivariable analysis was carried out to evaluate the association of each component of the total burden (i.e. all-cause hospitalizations and limitations in daily activities) with the respiratory condition, gender, occupational status, smoking habits and a high level of outdoor air pollution. Logistic and negative binomial models were computed separately in each age class and the estimates were adjusted for centre and the potential confounders reported above. Clustered sandwich estimators of the variance were used. The results of the negative binomial models were summarized as mutually adjusted Ratios of Expected Number of Days with reduced activities (RENDs).

In each age class and for each respiratory condition, the percentage contribution of breathing problems to all-cause hospitalizations and limitations in daily activities in the past three months was obtained by dividing the hospitalization risk and the expected number of days with reduced activities specifically due to respiratory disorders, by the corresponding component of the total burden. All the estimates of the hospitalization risk and of the expected number of days with reduced activities were adjusted for centre and the potential confounders reported above. Ninety-five percent confidence intervals (95%CIs) were computed by using the bias-corrected bootstrap method [23] with 2,000 replications.

The statistical analysis was performed by using STATA software, release 13 (StataCorp, College Station, TX).

Results

All-cause hospitalizations and limitations in daily activities according to the respiratory condition

The percentage of subjects who reported any respiratory condition was 57.7% (95%CI: 57.4 to 58.0%) in the 20–44 age class and 54.8% (95%CI: 54.0 to 55.6%) in the 45–64

age class, with differences in the disease distribution according to age (Figure 1).

The risk of having at least one ED visit and/or one hospital admission due to any health problems (apart from accidents and injuries) in the past three months was 8.8% (95%CI: 8.3 to 9.3%) and 9.5% (95%CI: 8.1 to 11.0%) among the subjects aged 20–44 and 45–64, respectively. At any age, the risk of all-cause hospitalization was about 6% among the subjects with no respiratory conditions, it increased to about 9-12% among the individuals reporting allergic rhinitis or asthma-like symptoms/dyspnoea/other nasal problems, and it peaked at about 15-18% among the asthmatics with chronic bronchitis aged 20–44 and 45–64, respectively (Figure 2). The same trend was observed after adjusting for the effect of the other potential determinants: the odds ratio (OR; reference category: no respiratory conditions) ranged from 1.62 to 2.36 in the 20–44 age class and from 2.09 to 4.24 in the 45–64 year-old group, respectively (Table 2).

The expected number of days with reduced activities due to any health problems (apart from accidents and injuries) in the past three months was 2.4 (95%CI: 2.3 to 2.5) and 2.3 (95%CI: 2.1 to 2.5) among the subjects aged 20–44 and 45–64, respectively. This estimate increased from 1.5 days among the individuals with no respiratory conditions in both the age classes, to 6.3 and 4.6 days among the asthmatics with chronic bronchitis aged 20–44 and 45–64, respectively (Figure 2). The subjects aged 45–64 with asthma-like symptoms/dyspnoea/other nasal problems were characterised by a particularly high figure (3.2 days). The increasing trend in the number of days with reduced activities was confirmed after adjusting for the effect of the other potential determinants: the REND

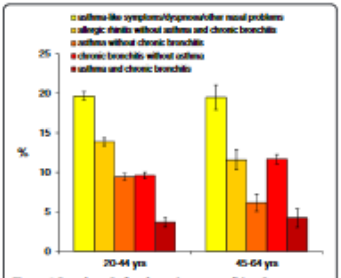

Figure 1 Prevalence* of each respiratory condition. Bars represent 95% confidence intervals. *The estimates were adjusted for the cumulative response rate, the season at the time of response to the questionnaire, the type of contact, gender and centre.

(reference category: no respiratory conditions) ranged from 1.64 to 3.87 in the 20–44 age class and from 2.05 to 4.33 in the 45–64 year-old group, respectively (Table 3).

Contribution of breathing problems to all-cause hospitalizations and limitations in daily activities

Breathing problems accounted for less than 50% of all-cause hospitalizations and limitations in daily activities, and the percentage contribution of breathing problems to the total burden varied according to the respiratory condition in both the age classes (Figure 3). In particular, the highest contribution of breathing problems to the total burden was observed among the asthmatics with chronic bronchitis (23-29% of the hospitalization risk and 39-50% of the days with reduced activities, according to age), whereas the lowest figure was observed among the individuals with asthma-like symptoms/dyspnoea/other nasal problems (4-5% of the hospitalization risk and 10-11% of the days with reduced activities, in the two age groups).

Other determinants of all-cause hospitalizations and limitations in daily activities

According to the multivariable analysis, females showed an increased hospitalization risk (OR: 1.46) and an increased number of days with reduced activities (REND: 1.73) in the population aged 20–44, whereas they had less hospitalizations (OR: 0.70) in the population aged 45–64, as compared to males in the same age class (Tables 2 and 3). As compared to white collars/housepersons/students of the same age, unemployed/retired subjects had an increased hospitalization risk in both the age classes (OR: 1.60 and 1.39, respectively), as found for blue collars (OR: 1.31) in the 20–44 age class; moreover, blue collars showed heavier limitations in daily activities in both the age groups (REND: 1.73 and 2.17, respectively). As compared to never smokers aged 45–64, past smokers had an increased hospitalization risk (OR: 1.20) and an increased number of days with reduced activities (REND: 1.80), whereas current smokers showed lesser limitations in daily activities (REND: 0.66). In the same age class, the individuals reporting a high level of outdoor air pollution showed an increased hospitalization risk (OR: 1.47), as compared to the subjects reporting a low exposure.

Sensitivity analyses

The stability of the results was evaluated by repeating the analysis also adjusting for smoking habits or without adjusting for the cumulative response rate, the season at the time of response to the questionnaire and the type of contact. The estimates obtained under these two conditions were comparable with the figures computed in the main analysis (data not shown).

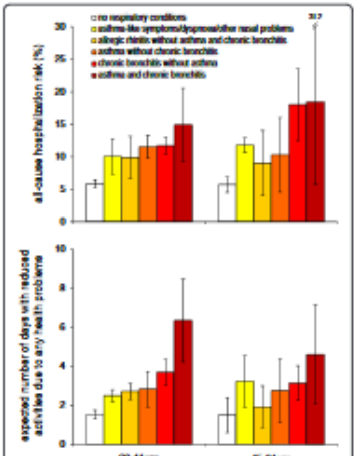

Figure 2 All-cause hospitalizations and limitations in daily activities in the past three months*. Bars represent 95% confidence intervals. *The all-cause hospitalization risk is the proportion of subjects with at least one ED visit and/or one hospital admission due to any health problems (apart from accidents and injuries) in the past three months; the estimates were adjusted for the cumulative response rate, the season at the time of response to the questionnaire, the type of contact, gender and centre.

Discussion

The main results of the present study are the following:

- the subjects with asthma, chronic bronchitis or allergic rhinitis have a two- to four-fold increased risk of all-cause hospitalizations and limitations in daily activities, as compared to unaffected individuals from the general population;
- among the subjects with asthma, chronic bronchitis or allergic rhinitis, breathing problems account for less than 50% of all-cause hospitalizations and limitations in daily activities, and the contribution of breathing problems to the total burden varies according to the respiratory condition;
- female gender, a low occupational status and a high level of outdoor air pollution contribute to the total burden.

The presence of asthma, chronic bronchitis or allergic rhinitis is associated with an increase in all-cause hospitalizations and limitations in daily activities

Our data show that the risk of hospitalization and the number of days with impaired activities due to any health problems (apart from accidents and injuries) are impressively higher among adults with asthma, chronic bronchitis or allergic rhinitis, as compared to unaffected individuals from the general population in Italy. This result is in agreement with the findings in other studies, which show that respiratory symptoms are among the major causes of consultation at primary health care centres in different countries [24], and that these diseases impair work performance, social life and physical quality of life [25-27].

In each age class, the heaviest burden is associated with the coexistence of asthma and chronic bronchitis. In particular, the risk of all-cause hospitalization peaks among the 45–64 year-old subjects with both these conditions, whereas this risk is not so much higher than the figures observed among the individuals with the other respiratory conditions in the 20–44 age class. A possible explanation for this could be that the presence of chronic bronchitis may be an early expression of COPD in the older age group [28], whereas it may be an expression of post nasal drip among the individuals aged 20–44 [29].

Among the 45–64 year-old subjects, those reporting asthma-like symptoms/dyspnoea/other nasal problems show a heavy burden. This result could be explained by the age-related increase in the incidence of pneumonia, lung cancer, COPD or other diseases that cause dyspnoea or respiratory symptoms, which often require ED visits or hospital admissions.

The contribution of breathing problems to all-cause hospitalizations and limitations in daily activities varies according to the respiratory condition

In our study, breathing problems account for less than 50% of all-cause hospitalizations and limitations in daily activities. This is not surprising because Druss and colleagues [30] reported that about one fourth of the costs due to five chronic conditions (mood disorders, diabetes, heart disease, asthma and hypertension) in the United States were incurred in treating the conditions themselves, whereas the remaining costs were due to coexisting illnesses.

The contribution of breathing problems to the total burden varies according to the respiratory condition, even if the results pertaining to the 45–64 age class should be interpreted with caution because of the large confidence intervals, which is due to the smaller sample size of that group, as compared with the dimension of the 20–44 age class. In particular, the highest weight of breathing problems is found among the subjects with coexisting asthma and chronic bronchitis, which suggests that the burden for

Table 2 Potential determinants of all-cause hospitalizations in the past three months

		20-44 yrs		45-64 yrs	
		OR* [95% CI]	p-value	OR* [95% CI]	p-value
Respiratory condition	No respiratory conditions	1.00	-	1.00	-
	Asthma-like symptoms/dyspnoea/other nasal problems	1.62 [1.15 to 2.28]	0.005	2.09 [1.65 to 2.65]	<0.001
	Allergic rhinitis without asthma and chronic bronchitis	1.71 [1.09 to 2.71]	0.019	1.79 [1.07 to 2.98]	0.027
	Asthma without chronic bronchitis	2.08 [1.84 to 2.35]	<0.001	2.09 [1.32 to 3.32]	0.002
	Chronic bronchitis without asthma	1.85 [1.44 to 2.39]	<0.001	3.38 [1.95 to 5.88]	<0.001
	Asthma and chronic bronchitis	2.36 [1.45 to 3.84]	0.001	4.24 [2.06 to 8.70]	<0.001
Gender	Male	1.00	-	1.00	-
	Female	1.46 [1.04 to 2.07]	0.030	0.70 [0.53 to 0.91]	0.008
Occupational status	White collar/house-person/student	1.00	-	1.00	-
	Blue collar	1.31 [1.05 to 1.63]	0.016	0.74 [0.43 to 1.28]	0.278
	Unemployed/retired subject	1.60 [1.03 to 2.50]	0.038	1.39 [1.00 to 1.93]	0.049
Smoking habits	Never smoking	1.00	-	1.00	-
	Past smoking	1.22 [0.87 to 1.70]	0.252	1.20 [1.08 to 1.33]	0.001
	Current smoking	1.45 [0.96 to 2.18]	0.076	0.97 [0.62 to 1.53]	0.892
High level of outdoor air pollution	Absent	1.00	-	1.00	-
	Present	1.05 [0.81 to 1.37]	0.708	1.47 [1.18 to 1.84]	0.001

*Mutually adjusted odds ratios (ORs) of hospital services utilization (at least one ED visit and/or one hospital admission) due to any health problems (apart from accidents and injuries) in the past three months; the ORs were also adjusted for the cumulative response rate, the season at the time of response to the questionnaire, the type of contact and centre.

Table 3 Potential determinants of all-cause limitations in daily activities in the past three months

		20-44 yrs		45-64 yrs	
		REND* [95% CI]	p-value	REND* [95% CI]	p-value
Respiratory condition	No respiratory conditions	1.00	-	1.00	-
	Asthma-like symptoms/dyspnoea/other nasal problems	1.64 [1.21 to 2.38]	0.001	2.05 [0.85 to 4.97]	0.112
	Allergic rhinitis without asthma and chronic bronchitis	1.72 [1.37 to 2.17]	<0.001	1.46 [0.64 to 3.31]	0.366
	Asthma without chronic bronchitis	1.76 [1.33 to 2.32]	<0.001	2.15 [1.43 to 3.22]	<0.001
	Chronic bronchitis without asthma	2.42 [1.90 to 3.08]	<0.001	3.19 [2.22 to 4.59]	<0.001
	Asthma and chronic bronchitis	3.87 [2.99 to 5.01]	<0.001	4.33 [2.10 to 8.89]	<0.001
Gender	Male	1.00	-	1.00	-
	Female	1.73 [1.37 to 2.18]	<0.001	0.95 [0.84 to 1.08]	0.436
Occupational status	White collar/house-person/student	1.00	-	1.00	-
	Blue collar	1.73 [1.57 to 1.91]	<0.001	2.17 [1.06 to 4.47]	0.035
	Unemployed/retired subject†	-	-	-	-
Smoking habits	Never smoking	1.00	-	1.00	-
	Past smoking	1.17 [0.97 to 1.40]	0.093	1.80 [1.22 to 2.66]	0.003
	Current smoking	1.07 [0.93 to 1.23]	0.324	0.66 [0.53 to 0.82]	<0.001
High level of outdoor air pollution	Absent	1.00	-	1.00	-
	Present	1.17 [0.88 to 1.55]	0.288	1.54 [0.94 to 2.54]	0.087

*Mutually adjusted ratios of expected number of days with reduced activities (RENDs) due to any health problems (apart from accidents and injuries) in the past three months; the RENDs were also adjusted for the cumulative response rate, the season at the time of response to the questionnaire, the type of contact and centre. †The RENDs are not reported because unemployed/retired subjects have no lost working days.

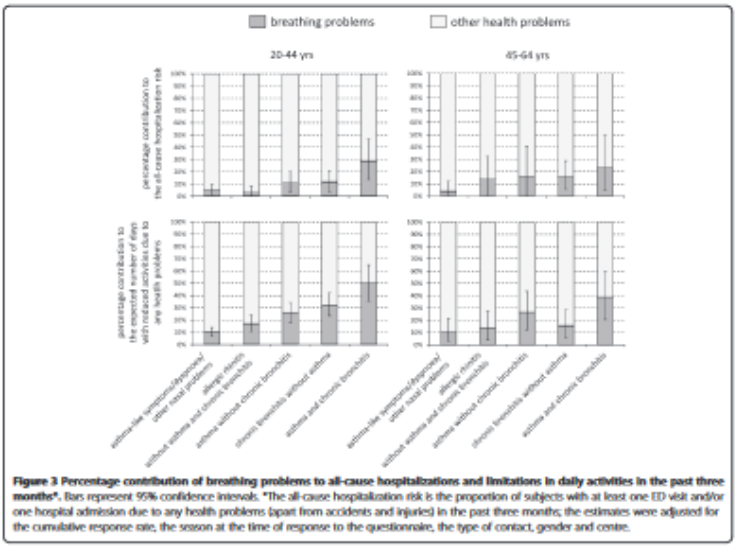

Figure 3 Percentage contribution of breathing problems to all-cause hospitalizations and limitations in daily activities in the past three months*. Bars represent 95% confidence intervals. *The all-cause hospitalization risk is the proportion of subjects with at least one ED visit and/or one hospital admission due to any health problems (apart from accidents and injuries) in the past three months; the estimates were adjusted for the cumulative response rate, the season at the time of response to the questionnaire, the type of contact, gender and centre.

these patients is mainly caused by the disease. This result indirectly confirms that chronic bronchitis is associated with both a more severe form of the disease and poor control of symptoms in asthmatic subjects [7,8], or it may be an expression of the coexistence of asthma and COPD [9,10]. Accordingly, poor control of symptoms and coexisting chronic bronchitis were both associated with increased disease-related costs in European adults with asthma [12]. On the contrary, the contribution of breathing problems is particularly low among the subjects with allergic rhinitis only, whereas it is at an intermediate level among those with asthma or chronic bronchitis only, which suggests a progressive increase in the level of impairment due to these respiratory diseases.

Breathing problems have the lowest weight in determining all-cause hospitalizations and limitations in daily activities for the subjects with asthma-like symptoms/dyspnoea/other nasal problems. Different factors may contribute to explain this finding. Smoking is associated with respiratory symptoms and it is strongly associated with cardiac and cerebrovascular diseases, and with diabetic complications. Dyspnoea and non-specific respiratory symptoms may be attributable to other illnesses, such as heart failure, essential hypertension, anaemia, electrolyte disorders or gastroesophageal reflux. Moreover, in the case of comorbidities such as diabetes, anaemia, hypertension or tachycardia, the decision to admit a patient can be based not only on factors that are directly related to the respiratory disease, but also on the difficulty in managing an outpatient. In fact, these patients are likely to require more health services and more complex health management strategies.

Other determinants of all-cause hospitalizations and limitations in daily activities

Females aged 20–44 report a heavier burden as compared to males in the same age class, regardless of their disease status. In fact, females are probably more concerned about their health than males [31], as previously found among young adults with asthma in Italy [32]. On the contrary, the risk of all-cause hospitalization is lower among females aged 45–64, in accordance with official Italian statistics for the same period [33]. A heavier burden is reported by subjects with a low occupational status (i.e. blue collars and unemployed/retired individuals), in accordance with previous results for adult asthma in Italy [32]. This could be

due to factors that affect health and are more frequent in lower social classes, such as residential and workplace pollutant exposures [34], or it could reflect a cumulative life course disadvantage [35]. Self-reported high levels of outdoor air pollution are associated with an increased risk of all-cause hospitalization among the subjects aged 45–64, which suggests that the exposure to air pollution has a long-term effect on health [36,37]. Finally, the pattern of association between smoking habits and the total burden could be due to the "healthy smoker effect" bias, since the subjects with the most severe underlying disease are the least likely to smoke [38].

Strengths and weaknesses of the study

The main strength of the present analysis is that the data were collected through a highly standardized protocol [17], which ensures the comparability of the information among the centres. Moreover, the prevalence rates are based on self-reported symptoms, which were measured through an international validated questionnaire [18], and are less influenced by diagnostic procedures. In addition, the data were collected from patients who had been identified in the general population, rather than from clinically selected groups, which should guarantee that the studied sample encompasses a wide spectrum of respiratory conditions. Finally, a potential measurement error [39] could have influenced our estimates of the burden only to a minor extent, because our study did not involve elderly patients and the recall period considered in the questionnaire (three months) was short.

A few caveats should be taken into account when interpreting our results. Only four centres participating in GEIRD collected the information on the subjects aged 45–64 and so there could be a potential limitation in the generalizability of the results. Moreover, the participation rate was quite low (53.0%) among young adults, as observed in other epidemiological studies over the past decades [40], and this may have biased our estimates for this age class. However, it has been suggested that decreasing participation rates are not likely to have a substantial influence on the point estimates of measures of interest [40]. Finally, it was not possible to directly evaluate the impact of comorbidities on the total burden because questions on coexisting diseases had not been included in the screening questionnaire, in order to minimize its length and therefore increase the response rate [18]. In addition, the screening questionnaire does not allow to compute separate estimates of the risk of having at least one ED visit and of the risk of having at least one hospital admission in the past three months. Therefore, the possible overestimation of hospital services utilization cannot be detected through the comparison with ED visit rates and hospital in-patient admission rates from other sources. However, participation bias could have inflated our estimates of the hospitalization risk to some extent.

Conclusions

The impact of asthma, chronic bronchitis and allergic rhinitis on all-cause hospitalizations and limitations in daily activities is substantial, and it is markedly different among adult subjects from the general population in Italy. The contribution of breathing problems to the total burden also varies according to the respiratory condition. Gender, occupational status and outdoor air pollution contribute to the total burden.

Abbreviations

COPD: Chronic obstructive pulmonary disease; GEIRD: Gene Environment Interactions in Respiratory Diseases; ED: Emergency Department; IRNDs: Ratios of Expected Number of Days with reduced activities; 95%CI: 95% confidence interval; OR: Odds ratio.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SA and RdM conceived and designed the study. SA, AGC, RB, IC, AF, PP, RT, GV and RdM contributed to the data collection. SA performed the statistical analysis. SA, AGC and RdM draft the manuscript. All authors revised the manuscript critically for important intellectual content and gave final approval of the version to be published.

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RESEARCH ARTICLE Open Access

The impact of asthma, chronic bronchitis and allergic rhinitis on all-cause hospitalizations and limitations in daily activities: a population-based observational study

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Abstract

Background: Chronic respiratory diseases are a significant cause of morbidity and mortality worldwide. We sought to evaluate the impact of asthma, chronic bronchitis and allergic rhinitis on all-cause hospitalizations and limitations in daily activities in adults.

Methods: In the Gene Environment Interactions in Respiratory Diseases study (2007/2010), a screening questionnaire was mailed to 9,739 subjects aged 20–44 (response rate: 53.0%) and to 3,480 subjects aged 45–64 (response rate: 62.3%), who were randomly selected from the general population in Italy. The questionnaire was used to: identify the responders who had asthma, chronic bronchitis, allergic rhinitis or asthma-like symptoms/dyspnoea/other nasal problems; evaluate the total burden [use of hospital services (at least one ED visit and/or one hospital admission) and number of days with reduced activities (lost working days and days with limited, not work related activities) due to any health problems (apart from accidents and injuries) in the past three months]; evaluate the contribution of breathing problems to the total burden (hospitalizations and number of days with reduced activities specifically due to breathing problems).

Results: At any age, the all-cause hospitalization risk was about 6% among the subjects without any respiratory conditions, it increased to about 9-12% among the individuals with allergic rhinitis or with asthma-like symptoms/dyspnoea/other nasal problems, and it peaked at about 15-18% among the asthmatics with chronic bronchitis aged 20–44 and 45–64, respectively. The expected number of days with reduced activities due to any health problems increased from 1.5 among the subjects with no respiratory conditions in both the age classes, to 6.3 and 4.6 among the asthmatics with chronic bronchitis aged 20–44 and 45–64, respectively. The contribution of breathing problems to the total burden was the highest among the asthmatics with chronic bronchitis (23-29% of the hospitalization risk and 39-50% of the days with reduced activities, according to age).

Conclusions: The impact of asthma, chronic bronchitis and allergic rhinitis on all-cause hospitalizations and limitations in daily activities is substantial, and it is markedly different among adults from the general population in Italy. The contribution of breathing problems to the total burden also varies according to the respiratory condition.

Keywords: Burden of allergic rhinitis, Burden of asthma, Burden of chronic bronchitis, Determinants

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Background

Asthma, chronic bronchitis and allergic rhinitis are common health problems in industrialized countries [1-3] and they often coexist [4-6]. Furthermore, chronic bronchitis is associated with both a more severe form of the disease and poor control of symptoms in subjects with asthma [7,8], and it may indicate the presence of the asthma-chronic obstructive pulmonary disease (COPD) overlap syndrome [9,10].

Asthma generates a high socio-economic burden among European adults, which significantly increases when chronic bronchitis is also present [11,12]. Moreover, asthma and allergic rhinitis together account for most of the allergy-related morbidity associated with the respiratory system [13]. Therefore, real-world evaluations of the impact of these diseases in the general population are fundamental to meet the widespread need for updated estimates, which should be used as a valid support to public health decisions.

In recent years, the socio-economic impact of asthma, chronic bronchitis and allergic rhinitis has been largely investigated [11,12,14-16]. However, in many studies, the total burden (i.e. the burden due to any health problems) in patients with these respiratory illnesses has not been estimated because only disease-related costs have been considered. Moreover, the total burden should be compared between patients with a certain disease and unaffected subjects from the general population. Indeed, a better understanding of the burden of these respiratory diseases requires the assessment of their total impact on the health and social systems.

The aim of the present paper is to compare all-cause hospitalizations and limitations in daily activities among adult subjects with asthma, chronic bronchitis or allergic rhinitis, and unaffected individuals from the general population, and to assess the contribution of breathing problems to the total burden. To fulfil these purposes, the data from the screening stage of the Gene Environment Interactions in Respiratory Diseases (GEIRD) study [17] were used.

Methods

Design of the study

The screening stage of GEIRD was a cross-sectional survey on respiratory health, which was carried out between 2007 and 2010 in Italy [17]. A total sample of 9,739 subjects aged 20–44 and of 3,480 subjects aged 45–64 (men/women ratio = 1) was randomly selected from the general population in four centres (Pavia, Sassari, Turin and Verona) by using the local health authority registers. A screening questionnaire was mailed to each individual up to three times and then administered by telephone in case of nonresponse. Overall, the responders were 5,162 (53.0%) in the 20–44 age group and 2,167 (62.3%) in the 45–64 age group (Table 1).

Table 1 Distribution of the design variables

		20-44 yrs	45-64 yrs	p-value
N° of eligible subjects*		9739	3480	-
N° of responders (response rate, %)		5162 (53.0)	2167 (62.3)	<0.001
Season at the time of response, %	Spring	46.7	51.5	<0.001
	Summer	15.7	8.6	
	Autumn	32.0	30.9	
	Winter	6.6	9.0	
Telephone interview, %		11.6	9.6	0.012
Females, %		53.6	52.1	0.267

*Eligible = initial sample – (dead or moved away from the target area).

Ethics statement

Ethics approval was obtained in each centre from the appropriate ethics committee (*"Comitato di Bioetica della Fondazione IRCCS Policlinico San Matteo di Pavia"*; *"Comitato di Bioetica dell'Azienda Sanitaria Locale di Sassari"*; *"Comitato Etico dell'Azienda Sanitaria Locale TO/2 di Torino"*; *"Comitato Etico per la Sperimentazione dell'Azienda Ospedaliera Istituti Ospitalieri di Verona"*). All participants were fully informed about all aspects of the research project and consented to complete and return the questionnaire.

Screening questionnaire and definitions

The screening questionnaire (available at www.geird.org) is a modified version of the questionnaire used in the Italian Study on Asthma in Young Adults [18]. It contains validated questions (mainly taken from the European Community Respiratory Health Survey questionnaires [19]) on asthma (self-report of the disease during lifetime with or without a physician's diagnosis, frequency of asthma attacks, and use of anti-asthmatic drugs in the past 12 months), asthma-like symptoms (wheezing, nocturnal tightness in the chest, and attacks of shortness of breath at night time in the past 12 months), chronic cough and phlegm, and nasal problems, as well as questions on occupational status, smoking habits and outdoor air pollution (frequency of heavy traffic near home and living near industrial plants).

On the basis of their answers to the questions described above, the responders to the screening questionnaire were classified as subjects with:

- *asthma* (i.e. self-report of the disease during lifetime with or without a physician's diagnosis or at least one asthma attack in the past 12 months or use of anti-asthmatic drugs in the past 12 months) *and chronic bronchitis* (i.e. cough and phlegm on most

days for a minimum of three months a year and for at least two successive years);
- *chronic bronchitis without asthma*;
- *asthma without chronic bronchitis*;
- *allergic rhinitis* (i.e. any nasal allergies, including hay fever) *without asthma and chronic bronchitis*;
- *asthma-like symptoms or dyspnoea or other nasal problems without asthma, chronic bronchitis and allergic rhinitis*;
- *no respiratory conditions* (i.e. none of the conditions described above).

In addition, the responders were considered exposed to a high level of outdoor air pollution if they had reported a continuous passage of trucks near home and/or living near industrial plants.

The screening questionnaire also includes questions on the use of hospital services [at least one Emergency Department (ED) visit and/or one hospital admission], the number of lost working days and the number of days with limited, not work related activities (such as looking after children, housework or studying) due to any health problems (apart from accidents and injuries) in the past three months. If hospital services have been used, the responders should specify if this has been due to breathing problems. If lost working days and/or days with limited, not work related activities have been reported, the subjects should specify the number of the impaired days because of breathing problems.

The total burden was evaluated at an individual level on the basis of the use of hospital services and the number of days with reduced activities (i.e. the number of lost working days plus the number of days with limited, not work related activities) due to any health problems (apart from accidents and injuries) in the past three months. The days with limited, not work related activities reported by full-time housepersons were considered as lost working days. The hospital services utilization and the number of days with reduced activities specifically due to respiratory disorders in the past three months were used to evaluate the contribution of breathing problems to the total burden.

Statistical analysis

The cumulative response rate, the season at the time of response to the questionnaire, the type of contact (postal waves vs telephone interview), and gender were considered as potential confounders because of differences in their distribution between the two age classes (20–44 and 45–64) (Table 1). In particular, the cumulative response percentile rank, which is an indicator of the individual propensity to respond to the questionnaire, was used. Each subject was ordered by centre and age class according to the date of response to the questionnaire, and then he/she was attributed the ratio between his/her rank and the number of eligible subjects [20].

The prevalence of each respiratory condition was estimated by using a logistic model, which included age class, centre and the potential confounders reported above, as covariates. The all-cause hospitalization risk [i.e. the proportion of subjects with at least one ED visit and/or one hospital admission due to any health problems (apart from accidents and injuries) in the past three months] and the expected number of days with reduced activities due to any health problems in the same period were estimated by using logistic and negative binomial models [21], respectively, which included the respiratory condition crossed by the age class, centre and the potential confounders reported above, as covariates. Since the subjects were nested into centres, clustered sandwich estimators of the variance were used. The estimates were computed in each age class and for each respiratory condition by setting the distribution of gender, season at the time of response to the questionnaire, type of contact and centre equal to the average distribution, and by setting the cumulative response rate equal to the overall mean [22].

A multivariable analysis was carried out to evaluate the association of each component of the total burden (i.e. all-cause hospitalizations and limitations in daily activities) with the respiratory condition, gender, occupational status, smoking habits and a high level of outdoor air pollution. Logistic and negative binomial models were computed separately in each age class and the estimates were adjusted for centre and the potential confounders reported above. Clustered sandwich estimators of the variance were used. The results of the negative binomial models were summarized as mutually adjusted Ratios of Expected Number of Days with reduced activities (RENDs).

In each age class and for each respiratory condition, the percentage contribution of breathing problems to all-cause hospitalizations and limitations in daily activities in the past three months was obtained by dividing the hospitalization risk and the expected number of days with reduced activities specifically due to respiratory disorders, by the corresponding component of the total burden. All the estimates of the hospitalization risk and of the expected number of days with reduced activities were adjusted for centre and the potential confounders reported above. Ninety-five percent confidence intervals (95%CIs) were computed by using the bias-corrected bootstrap method [23] with 2,000 replications.

The statistical analysis was performed by using STATA software, release 13 (StataCorp, College Station, TX).

Results

All-cause hospitalizations and limitations in daily activities according to the respiratory condition

The percentage of subjects who reported any respiratory condition was 57.7% (95%CI: 57.4 to 58.0%) in the 20–44 age class and 54.8% (95%CI: 54.0 to 55.6%) in the 45–64

age class, with differences in the disease distribution according to age (Figure 1).

The risk of having at least one ED visit and/or one hospital admission due to any health problems (apart from accidents and injuries) in the past three months was 8.8% (95%CI: 8.3 to 9.3%) and 9.5% (95%CI: 8.1 to 11.0%) among the subjects aged 20–44 and 45–64, respectively. At any age, the risk of all-cause hospitalization was about 6% among the subjects with no respiratory conditions, it increased to about 9-12% among the individuals reporting allergic rhinitis or asthma-like symptoms/dyspnoea/other nasal problems, and it peaked at about 15-18% among the asthmatics with chronic bronchitis aged 20–44 and 45–64, respectively (Figure 2). The same trend was observed after adjusting for the effect of the other potential determinants: the odds ratio (OR; reference category: no respiratory conditions) ranged from 1.62 to 2.36 in the 20–44 age class and from 2.09 to 4.24 in the 45–64 year-old group, respectively (Table 2).

The expected number of days with reduced activities due to any health problems (apart from accidents and injuries) in the past three months was 2.4 (95%CI: 2.3 to 2.5) and 2.3 (95%CI: 2.1 to 2.5) among the subjects aged 20–44 and 45–64, respectively. This estimate increased from 1.5 days among the individuals with no respiratory conditions in both the age classes, to 6.3 and 4.6 days among the asthmatics with chronic bronchitis aged 20–44 and 45–64, respectively (Figure 2). The subjects aged 45–64 with asthma-like symptoms/dyspnoea/other nasal problems were characterised by a particularly high figure (3.2 days). The increasing trend in the number of days with reduced activities was confirmed after adjusting for the effect of the other potential determinants: the REND (reference category: no respiratory conditions) ranged from 1.64 to 3.87 in the 20–44 age class and from 2.05 to 4.33 in the 45–64 year-old group, respectively (Table 3).

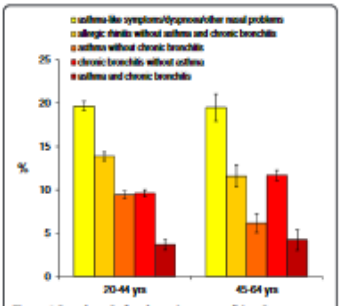

Figure 1 Prevalence* of each respiratory condition. Bars represent 95% confidence intervals. *The estimates were adjusted for the cumulative response rate, the season at the time of response to the questionnaire, the type of contact, gender and centre.

Contribution of breathing problems to all-cause hospitalizations and limitations in daily activities

Breathing problems accounted for less than 50% of all-cause hospitalizations and limitations in daily activities, and the percentage contribution of breathing problems to the total burden varied according to the respiratory condition in both the age classes (Figure 3). In particular, the highest contribution of breathing problems to the total burden was observed among the asthmatics with chronic bronchitis (23-29% of the hospitalization risk and 39-50% of the days with reduced activities, according to age), whereas the lowest figure was observed among the individuals with asthma-like symptoms/dyspnoea/other nasal problems (4-5% of the hospitalization risk and 10-11% of the days with reduced activities, in the two age groups).

Other determinants of all-cause hospitalizations and limitations in daily activities

According to the multivariable analysis, females showed an increased hospitalization risk (OR: 1.46) and an increased number of days with reduced activities (REND: 1.73) in the population aged 20–44, whereas they had less hospitalizations (OR: 0.70) in the population aged 45–64, as compared to males in the same age class (Tables 2 and 3). As compared to white collars/housepersons/students of the same age, unemployed/retired subjects had an increased hospitalization risk in both the age classes (OR: 1.60 and 1.39, respectively), as found for blue collars (OR: 1.31) in the 20–44 age class; moreover, blue collars showed heavier limitations in daily activities in both the age groups (REND: 1.73 and 2.17, respectively). As compared to never smokers aged 45–64, past smokers had an increased hospitalization risk (OR: 1.20) and an increased number of days with reduced activities (REND: 1.80), whereas current smokers showed lesser limitations in daily activities (REND: 0.66). In the same age class, the individuals reporting a high level of outdoor air pollution showed an increased hospitalization risk (OR: 1.47), as compared to the subjects reporting a low exposure.

Sensitivity analyses

The stability of the results was evaluated by repeating the analysis also adjusting for smoking habits or without adjusting for the cumulative response rate, the season at the time of response to the questionnaire and the type of contact. The estimates obtained under these two conditions were comparable with the figures computed in the main analysis (data not shown).

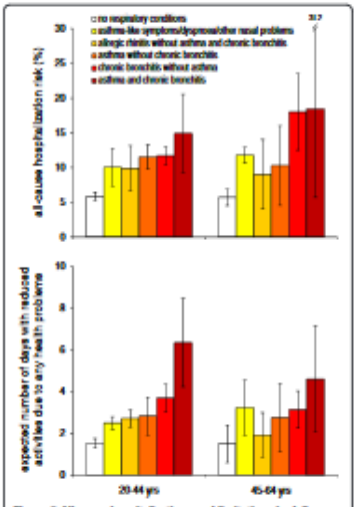

Figure 2 All-cause hospitalizations and limitations in daily activities in the past three months*. Bars represent 95% confidence intervals. *The all-cause hospitalization risk is the proportion of subjects with at least one ED visit and/or one hospital admission due to any health problems (apart from accidents and injuries) in the past three months; the estimates were adjusted for the cumulative response rate, the season at the time of response to the questionnaire, the type of contact, gender and centre.

Discussion

The main results of the present study are the following:

- the subjects with asthma, chronic bronchitis or allergic rhinitis have a two- to four-fold increased risk of all-cause hospitalizations and limitations in daily activities, as compared to unaffected individuals from the general population;
- among the subjects with asthma, chronic bronchitis or allergic rhinitis, breathing problems account for less than 50% of all-cause hospitalizations and limitations in daily activities, and the contribution of breathing problems to the total burden varies according to the respiratory condition;
- female gender, a low occupational status and a high level of outdoor air pollution contribute to the total burden.

The presence of asthma, chronic bronchitis or allergic rhinitis is associated with an increase in all-cause hospitalizations and limitations in daily activities

Our data show that the risk of hospitalization and the number of days with impaired activities due to any health problems (apart from accidents and injuries) are impressively higher among adults with asthma, chronic bronchitis or allergic rhinitis, as compared to unaffected individuals from the general population in Italy. This result is in agreement with the findings in other studies, which show that respiratory symptoms are among the major causes of consultation at primary health care centres in different countries [24], and that these diseases impair work performance, social life and physical quality of life [25-27].

In each age class, the heaviest burden is associated with the coexistence of asthma and chronic bronchitis. In particular, the risk of all-cause hospitalization peaks among the 45–64 year-old subjects with both these conditions, whereas this risk is not so much higher than the figures observed among the individuals with the other respiratory conditions in the 20–44 age class. A possible explanation for this could be that the presence of chronic bronchitis may be an early expression of COPD in the older age group [28], whereas it may be an expression of post nasal drip among the individuals aged 20–44 [29].

Among the 45–64 year-old subjects, those reporting asthma-like symptoms/dyspnoea/other nasal problems show a heavy burden. This result could be explained by the age-related increase in the incidence of pneumonia, lung cancer, COPD or other diseases that cause dyspnoea or respiratory symptoms, which often require ED visits or hospital admissions.

The contribution of breathing problems to all-cause hospitalizations and limitations in daily activities varies according to the respiratory condition

In our study, breathing problems account for less than 50% of all-cause hospitalizations and limitations in daily activities. This is not surprising because Druss and colleagues [30] reported that about one fourth of the costs due to five chronic conditions (mood disorders, diabetes, heart disease, asthma and hypertension) in the United States were incurred in treating the conditions themselves, whereas the remaining costs were due to coexisting illnesses.

The contribution of breathing problems to the total burden varies according to the respiratory condition, even if the results pertaining to the 45–64 age class should be interpreted with caution because of the large confidence intervals, which is due to the smaller sample size of that group, as compared with the dimension of the 20–44 age class. In particular, the highest weight of breathing problems is found among the subjects with coexisting asthma and chronic bronchitis, which suggests that the burden for

Table 2 Potential determinants of all-cause hospitalizations in the past three months

		20-44 yrs		45-64 yrs	
		OR* [95% CI]	p-value	OR* [95% CI]	p-value
Respiratory condition	No respiratory conditions	1.00	-	1.00	-
	Asthma-like symptoms/dyspnoea/other nasal problems	1.62 [1.15 to 2.28]	0.005	2.09 [1.65 to 2.65]	<0.001
	Allergic rhinitis without asthma and chronic bronchitis	1.71 [1.09 to 2.71]	0.019	1.79 [1.07 to 2.98]	0.027
	Asthma without chronic bronchitis	2.08 [1.84 to 2.35]	<0.001	2.09 [1.32 to 3.32]	0.002
	Chronic bronchitis without asthma	1.85 [1.44 to 2.39]	<0.001	3.38 [1.95 to 5.88]	<0.001
	Asthma and chronic bronchitis	2.36 [1.45 to 3.84]	0.001	4.24 [2.06 to 8.70]	<0.001
Gender	Male	1.00	-	1.00	-
	Female	1.46 [1.04 to 2.07]	0.030	0.70 [0.53 to 0.91]	0.008
Occupational status	White collar/house-person/student	1.00	-	1.00	-
	Blue collar	1.31 [1.05 to 1.63]	0.016	0.74 [0.43 to 1.28]	0.278
	Unemployed/retired subject‡	1.60 [1.03 to 2.50]	0.038	1.39 [1.00 to 1.93]	0.049
Smoking habits	Never smoking	1.00	-	1.00	-
	Past smoking	1.22 [0.87 to 1.70]	0.252	1.20 [1.08 to 1.33]	0.001
	Current smoking	1.45 [0.96 to 2.18]	0.076	0.97 [0.62 to 1.53]	0.892
High level of outdoor air pollution	Absent	1.00	-	1.00	-
	Present	1.05 [0.81 to 1.37]	0.708	1.47 [1.18 to 1.84]	0.001

*Mutually adjusted odds ratios (ORs) of hospital services utilization (at least one ED visit and/or one hospital admission) due to any health problems (apart from accidents and injuries) in the past three months; the ORs were also adjusted for the cumulative response rate, the season at the time of response to the questionnaire, the type of contact and centre.

Table 3 Potential determinants of all-cause limitations in daily activities in the past three months

		20-44 yrs		45-64 yrs	
		REND* [95% CI]	p-value	REND* [95% CI]	p-value
Respiratory condition	No respiratory conditions	1.00	-	1.00	-
	Asthma-like symptoms/dyspnoea/other nasal problems	1.64 [1.21 to 2.18]	0.001	2.05 [0.85 to 4.97]	0.112
	Allergic rhinitis without asthma and chronic bronchitis	1.72 [1.37 to 2.17]	<0.001	1.46 [0.64 to 3.31]	0.366
	Asthma without chronic bronchitis	1.76 [1.33 to 2.32]	<0.001	2.15 [1.43 to 3.22]	<0.001
	Chronic bronchitis without asthma	2.42 [1.90 to 3.08]	<0.001	3.19 [2.22 to 4.59]	<0.001
	Asthma and chronic bronchitis	3.87 [2.99 to 5.01]	<0.001	4.33 [2.10 to 8.89]	<0.001
Gender	Male	1.00	-	1.00	-
	Female	1.73 [1.37 to 2.18]	<0.001	0.95 [0.84 to 1.08]	0.436
Occupational status	White collar/house-person/student	1.00	-	1.00	-
	Blue collar	1.73 [1.57 to 1.91]	<0.001	2.17 [1.06 to 4.47]	0.035
	Unemployed/retired subject†	-	-	-	-
Smoking habits	Never smoking	1.00	-	1.00	-
	Past smoking	1.17 [0.97 to 1.40]	0.093	1.80 [1.22 to 2.66]	0.003
	Current smoking	1.07 [0.93 to 1.23]	0.324	0.66 [0.53 to 0.82]	<0.001
High level of outdoor air pollution	Absent	1.00	-	1.00	-
	Present	1.17 [1.01 to 1.35]	0.388	1.54 [1.14 to 2.54]	0.010

*Mutually adjusted ratios of expected number of days with reduced activities (RENDs) due to any health problems (apart from accidents and injuries) in the past three months; the RENDs were also adjusted for the cumulative response rate, the season at the time of response to the questionnaire, the type of contact and centre. †The RENDs are not reported because unemployed/retired subjects have no lost working days.

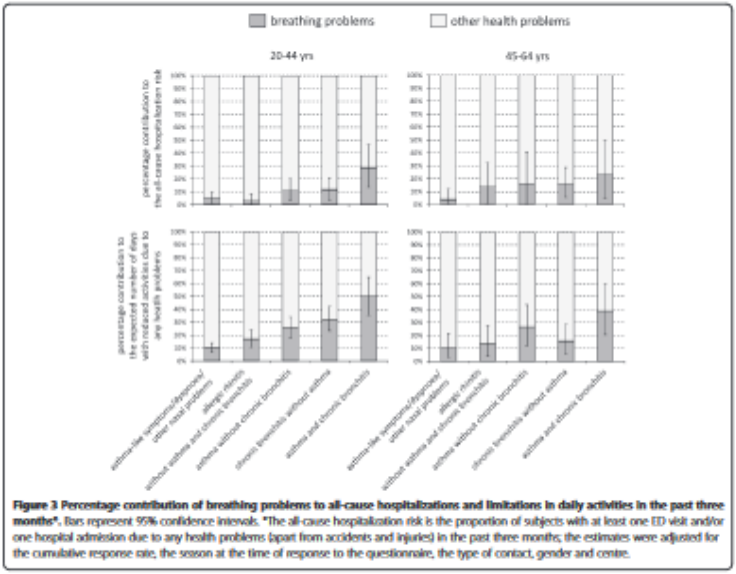

Figure 3 Percentage contribution of breathing problems to all-cause hospitalizations and limitations in daily activities in the past three months*. Bars represent 95% confidence intervals. *The all-cause hospitalization risk is the proportion of subjects with at least one ED visit and/or one hospital admission due to any health problems (apart from accidents and injuries) in the past three months; the estimates were adjusted for the cumulative response rate, the season at the time of response to the questionnaire, the type of contact, gender and centre.

these patients is mainly caused by the disease. This result indirectly confirms that chronic bronchitis is associated with both a more severe form of the disease and poor control of symptoms in asthmatic subjects [7,8], or it may be an expression of the coexistence of asthma and COPD [9,10]. Accordingly, poor control of symptoms and coexisting chronic bronchitis were both associated with increased disease-related costs in European adults with asthma [12]. On the contrary, the contribution of breathing problems is particularly low among the subjects with allergic rhinitis only, whereas it is at an intermediate level among those with asthma or chronic bronchitis only, which suggests a progressive increase in the level of impairment due to these respiratory diseases.

Breathing problems have the lowest weight in determining all-cause hospitalizations and limitations in daily activities for the subjects with asthma-like symptoms/dyspnoea/other nasal problems. Different factors may contribute to explain this finding. Smoking is associated with respiratory symptoms and it is strongly associated with cardiac and cerebrovascular diseases, and with diabetic complications. Dyspnoea and non-specific respiratory symptoms may be attributable to other illnesses, such as heart failure, essential hypertension, anaemia, electrolyte disorders or gastroesophageal reflux. Moreover, in the case of comorbidities such as diabetes, anaemia, hypertension or tachycardia, the decision to admit a patient can be based not only on factors that are directly related to the respiratory disease, but also on the difficulty in managing an outpatient. In fact, these patients are likely to require more health services and more complex health management strategies.

Other determinants of all-cause hospitalizations and limitations in daily activities

Females aged 20–44 report a heavier burden as compared to males in the same age class, regardless of their disease status. In fact, females are probably more concerned about their health than males [31], as previously found among young adults with asthma in Italy [32]. On the contrary, the risk of all-cause hospitalization is lower among females aged 45–64, in accordance with official Italian statistics for the same period [33]. A heavier burden is reported by subjects with a low occupational status (i.e. blue collars and unemployed/retired individuals), in accordance with previous results for adult asthma in Italy [32]. This could be

due to factors that affect health and are more frequent in lower social classes, such as residential and workplace pollutant exposures [34], or it could reflect a cumulative life course disadvantage [35]. Self-reported high levels of outdoor air pollution are associated with an increased risk of all-cause hospitalization among the subjects aged 45–64, which suggests that the exposure to air pollution has a long-term effect on health [36,37]. Finally, the pattern of association between smoking habits and the total burden could be due to the "healthy smoker effect" bias, since the subjects with the most severe underlying disease are the least likely to smoke [38].

Strengths and weaknesses of the study

The main strength of the present analysis is that the data were collected through a highly standardized protocol [17], which ensures the comparability of the information among the centres. Moreover, the prevalence rates are based on self-reported symptoms, which were measured through an international validated questionnaire [18], and are less influenced by diagnostic procedures. In addition, the data were collected from patients who had been identified in the general population, rather than from clinically selected groups, which should guarantee that the studied sample encompasses a wide spectrum of respiratory conditions. Finally, a potential measurement error [39] could have influenced our estimates of the burden only to a minor extent, because our study did not involve elderly patients and the recall period considered in the questionnaire (three months) was short.

A few caveats should be taken into account when interpreting our results. Only four centres participating in GEIRD collected the information on the subjects aged 45–64 and so there could be a potential limitation in the generalizability of the results. Moreover, the participation rate was quite low (53.0%) among young adults, as observed in other epidemiological studies over the past decades [40], and this may have biased our estimates for this age class. However, it has been suggested that decreasing participation rates are not likely to have a substantial influence on the point estimates of measures of interest [40]. Finally, it was not possible to directly evaluate the impact of comorbidities on the total burden because questions on coexisting diseases had not been included in the screening questionnaire, in order to minimize its length and therefore increase the response rate [18]. In addition, the screening questionnaire does not allow to compute separate estimates of the risk of having at least one ED visit and of the risk of having at least one hospital admission in the past three months. Therefore, the possible overestimation of hospital services utilization cannot be detected through the comparison with ED visit rates and hospital in-patient admission rates from other sources. However, participation bias could have inflated our estimates of the hospitalization risk to some extent.

Conclusions

The impact of asthma, chronic bronchitis and allergic rhinitis on all-cause hospitalizations and limitations in daily activities is substantial, and it is markedly different among adult subjects from the general population in Italy. The contribution of breathing problems to the total burden also varies according to the respiratory condition. Gender, occupational status and outdoor air pollution contribute to the total burden.

Abbreviations

COPD: Chronic obstructive pulmonary disease; GEIRD: Gene Environment Interactions in Respiratory Diseases; ED: Emergency Department; IRNDs: Ratios of Expected Number of Days with reduced activities; 95%CI: 95% confidence interval; OR: Odds ratio.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SA and RdM conceived and designed the study. SA, AGC, RB, IC, AF, PP, RT, GV and RdM contributed to the data collection. SA performed the statistical analysis. SA, AGC and RdM draft the manuscript. All authors revised the manuscript critically for important intellectual content and gave final approval of the version to be published.

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ARTICLE UNDER REVIEW

TITLE PAGE

Bisphenol A, tobacco smoke, ad age as predictor of oxidative stress in children and adolescents

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Thanks are due to Dr. Andrea Occhipinti for his precious help in the laboratory and to all the students who participated in the study.

Implications and contribution:
BPA cause an increase in OS in the adolescents, but only starting from 6 ng / mg of CREA up; as well as tobacco smoke passively breathed. All bisphenols still present in the living environment have to be preventively contrasted, as well as carefully regulated and supervised.

List of abbreviations:

Bisphenol A, tobacco smoke, ad age as predictor of oxidative stress in children and adolescents

Implications and contribution:
BPA cause an increase in OS in the adolescents, but only starting from 6 ng / mg of CREA up; as well as tobacco smoke passively breathed. All bisphenols still present in the living environment have to be preventively contrasted, as well as carefully regulated and supervised.

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Abstract

Purpose of this study was to investigate the presence of bisphenol A (BPA) and its role in the induction of oxidative stress (OS) in a group of adolescents and to confirm the same role of the tobacco smoke.

Methods. 223 young healthy volunteers (7-19 years old) were recruited among students attending three different schools of Chivasso, close to Torino, Piedmont, North-Western Italy. A spot of urine provided by each subject was analyzed to quantify BPA, cotinine, and 15F2t-isoprostane (15F2t-IsoP).

Results. BPA shows a slight increase of concentration proportional with increasing age, even if the intermediate age group (11-14 years) is slightly lower, inducing a v-shape. The same trend and v-shaped appearance were observed for 15F2t-IsoP and cotinine. After a logarithmic transformation, the result of piecewise linear robust regression shows a break point of the effect of log-BPA on log-15F2t-IsoP at 1.79 (6 ng/mg CREA) ($p < 0.001$). At higher levels than this, 15F2t-IsoP underlines an increases exponentially more than threefold each one-log unit of BPA.

Conclusions. The two main results obtained in this work consist of an increase of OS due to BPA in the adolescents selected for the study, but only from 6 ng / mg of CREA up. Secondly, the passively breathed tobacco smoke is able to induce an increase of the O.S. The preventive action against BPA still present in the living environment represents an important tool to promote highest health standard in the living environment in general, and in urban one in particular.

Keywords:
Oxidative stress, adolescents, passive tobacco smoke, age, bisphenol A, public health

For its endocrine disruptor's properties and its widespread presence in the human life environment, bisphenol "A" (BPA) is an important topic in terms of Public Health. BPA, whose IUPAC name is 2, 2-bis (4-hydroxyphenyl) propane (CASRN: 80-05-7), is a synthetic organic compound with a relatively short life. It consists of two phenolic rings joined by a bridging group formed by the reaction of phenol with acetone [1]. Over 3.6 billion kilograms of BPA are produced annually as a component of polycarbonate plastic and epoxy resins. BPA is authorized for use as a monomer in plastic food contact materials, in accordance with Commission Regulation (EU) No 10/2011/EU on plastic materials and articles intended to get in touch with foodstuffs. Furthermore, based on the precautionary principle, in 2011 was introduced European Commission Implementing Regulation (EU) No 321/20118, which placed a restriction on the use of BPA in the manufacture of PC infant feeding bottles.

The general population can be exposed to BPA via food, dermal contact, drinking water, by swimming, and/or breathing indoor and outdoor air. According to EFSA, exposure to BPA can take place in 3 ways. 1) external (by diet, drinking water, inhalation, and dermal contact to cosmetics and thermal paper), 2) internal exposure to total BPA (absorbed dose of BPA, sum of conjugated and unconjugated BPA), and 3) aggregated (from diet, dust, cosmetics and thermal paper), expressed as oral human equivalent dose (HED) referring to unconjugated BPA only [2]. However, breast milk represents the main vehicle of human intake of BPA emphasizing that the youngest children have the highest urinary BPA levels [3].

Although BPA is not dangerous in polymeric form, it is unstable in acidic or basic solutions and when exposed to UV light. These conditions can convert BPA from polymeric to monomeric form and, due to its long-term release from food containers to food, beverages or environment, its presence can represent a health risk for a large part of humans [4]. In particular, the negative effects of BPA can be evident for children and adolescents,

pregnant women and their embryos, as confirmed by numerous tests on animal *in vivo* and *in vitro* [5,6]. Nevertheless, only free (unconjugated) BPA is a weak estrogen [7] and its presence in the different biological matrices is substantially negligible. This is due to an efficient metabolization of BPA together with a biological half – life in humans of less than 6 hours [8,9].

BPA and other bisphenols, in addition to the role of endocrine disruptor, are also able to contribute or induce to several others negative effects: obesity, Attention-Deficit/Hyperactivity Disorder (ADHD), and diabetes, related to its possible interaction with the estrogen receptor. Possible adverse effects following the assumption of low dose of BPA may be subsequent to endocrine disruption and to the possible presence of a non-monotonic dose-response relationship (NMDR) [10,11].

Usually, BPA is detectable in human urine, blood, breast milk, semen, cord blood, fetal serum, placental tissue and animal fat [12,13], but in urine its detection frequency is about 75-90% [14,15]. Glucuronic acid of BPA (GlcA–BPA) is a metabolite of BPA, also urinary, with a half-life of 6 hours. It is currently considered the major residue of BPA, both *in vitro* and *in vivo* [16].

Among the various toxic effects of BPA, we have to remember the contribution that this molecule provides to lipid peroxidation (LPO) and hence to oxidative stress (O.S.). OS is the expression of a biological imbalance occurring when endogenous and/or exogenous oxidants overtakes the level of antioxidant defenses indicating a risky condition for health [17,18]. The intake of high levels of BPA results in increased generation of reactive oxygen species (ROS), culminating in oxidative stress [19]. This is due to a decrease of the activity of antioxidant enzymes and the increase of LPO [20]. BPA induces OS and apoptosis in testes of mice and, again for the formation of ROS, it can produces injury in human tissues and organs such as liver, red blood cells, reproductive organs, brain, especially during the embryos phases [21–23]. The urinary BPA in children is significantly more concentrated

than in adults because they eat, drink, and breathe in greater quantities per kilogram of body weight [13,24]. Furthermore, children are more sensitive and fragile because their metabolism system and organs are not yet fully developed [25]. In particular, infants up to 2 or 3 months of age might have higher free-BPA levels in urine since detoxifying enzymes, such as UDP-glucuronosyltransferase are not yet fully developed [26,27]. As a result of the widespread exposure to BPA and consequent potential health risk to humans, restrictions and dedicated regulations for the use of this toxic chemical have been suggested world widely. In 2015 European Food Safety Authority [2] reduced the temporary Tolerable Daily Intake (t-TDI) of BPA from 50 to 4 μg/kg bw/day. Consequently, BPA is being replaced with a number of alternatives, such as Bisphenol S, which however presents similar genotoxic and estrogenic activity to that of BPA [28] but which, to date, does not presents regulatory limits.

Currently, only few studies on human have explored the exposure to BPA in relation to the induction of inflammation, LPO and OS [29,30], despite these processes to be very important as mediators of numerous health effects, including atherosclerosis, cardiovascular disease, cancer and pregnancy outcomes [31]. In the fully aware, that OS originates from many endogenous and exogenous factors, many of which not here considered; purpose of the present study was been to investigate the presence of BPA in the urine of a group of adolescents, its role in the induction of OS, and to confirm the same role of the tobacco smoke. To achieve this goal, a sample of urine provided by every one of the 223 young healthy volunteers (7-19 years old) attending three different schools of Chivasso (close to Torino, Piedmont, North-Western Italy) was analyzed to quantify BPA, cotinine, and 15F2t-IsoP. The first as a compound directly detectable in urine, the second as a nicotine metabolite to quantify exposure to smoking and the third as a marker of O.S.

METHODS

All 223 students who voluntarily participated to this study attended three different schools at Chivasso, a medium urbanized town with about 27,000 inhabitants (522 inhabitants/km^2) located at 180 m a.s.l. close to Torino (the Metropolitan City of the Piedmont Region, Italy - 890,500 inhabitants). No other selection criteria have been adopted to recruit volunteers. Because the subjects were underage, parents and teachers were informed during a public meeting on the objective of this study and, consequently, a written informed consent was signed and delivered by each participants' parents. Moreover, participation of all subjects took place only after obtaining the assent of the local Ethics Committee of "San Luigi" Turin hospital (session on 11th March 2015 aut. number 27/2015). Samplings were carried out from January to March 2016, involving one class per day, on Wednesday or Thursday, according to a pre-established timetable. A questionnaire was administered and a urine sample was collected from each student. In particular:

a) To each subject, one interviewer administered a questionnaire during the school time. The answers provided information on individual and clinical features, such as age, weight and height, gender, residence, diet (dinner the day before), hobbies, therapies and health conditions. The questionnaire used was mainly a synthesis of the most extensive questionnaire "SIDRIA", described in detail elsewhere [32].

b) A spot of urine was collected to each volunteer during the morning sampling to measure the following parameters:

b1) BPA. To exclude contamination from BPA, all urine samples were collected in BPA-free plastic vessels (polypropylene) and stored at - 80°C until analysis. All the laboratory glass material used were washed with methanol and then kept in methanol for 12 hours, which was subsequently analyzed to verify the possible contamination of BPA.

Each thawed urine was vortexed and 700 µl of acetonitrile, 750 µl of ethyl acetate and 10 µl of BPA-d_{16} (1 ng/µl), used as internal standard, were added to 400 µl urine sample. To facilitate the liquid – liquid extraction (LLE), samples were vortexed for 3 minutes, centrifuged at 4000 rpm for 15 min and the supernatants were evaporated to dryness by a gentle stream of nitrogen. The dried extract was dissolved with 125 µl of methanol/water (1:1 v/v) and analyzed by HPLC – MS/MS to quantify GlcA–BPA. GlcA–BPA was identified and quantified by liquid chromatography equipped with a low-pH resistant reverse phase column, Kinetex EVO C18 (2.6 µm, 150 x 3.0 mm). The binary solvent system was: a) acidified ultrapure water with formic acid 0.1% v/v and b) acetonitrile (HPLC ultrapure grade) acidified with formic acid 0.1% v/v. The chromatographic separation was carried out at constant flow rate (200 µl/min^{-1}) and constant temperature (23°C ± 1°C) by a column thermostat. The solvent linear gradient was from 10% to 30% of B in 5 min, to 65% of B at 30 min, at 33 min 95% of B. The concentration of solvent B was maintained at 95% for 5 min. The initial mobile phase was re-established for 10 min before the next injection. The injection volume was 20 µl and quantification was performed by internal standard method (BPA-d_{16}). Quantitative analyses were carried out by tandem mass spectrometry with a 6330 Series Ion Trap LC-MS system equipped with an Electrospray Ionization Source (ESI). The analytes were detected in negative mode. The dry gas (Nitrogen) was at 325°C, 20.0 psi and 10 l min^{-1}; capillary voltage was at 2000V. Data acquisition was made in Multiple Reaction Monitoring (MRM) mode by monitoring the transitions of quasi-molecular ions [M-H]: 227 for BPA, 242 for BPA-d_{16}, 307 for HO_3S–BPA, 403 for GlcA-BPA and 419 for OH-GlcA-BPA.

Procedural blank samples with ultrapure water in place of urine were collected, extracted and analyzed by HPLC-MS/MS with the same samples protocol. In the processed blanks were not detected BPA contaminations above the limit of detection (LOD, 0.065 ng mL^{-1}).

b2) Cotinine. Urine samples were prepared for analysis as follows: 10 ml of urine were fortified with 10 µl of cotinine-d_3 as internal standard, 4 g NaCl and 500 µl NaOH (5 M). Then, 2 ml $CHCl_3$ were added two times to extract the cotinine by means of LLE for 15 min. Each sample was then centrifuged for 10 min at 1000 x g and the resulting organic phase was collected in a glass tube and evaporated to dryness in a rotary evaporator at room temperature. The dry residue was reconstituted in 200 µl of $CHCl_3$ and transferred into a conical vial for GC-MS determination [33].

b3) $15F_{2t}$-Isoprostane ($15F_{2t}$-IsoP). $15F_{2t}$-IsoP was measured to quantify OS by ELISA technique carried out with a specific microplate kit (Oxford, MI, USA) and accordingly to manufacturer instructions. To achieve the better accuracy in the competitive ELISA method each sample was diluted 1:4. Our previous paper reports all the details of this procedure [34].

b4) Creatinine. In order to normalize the excretion rate of cotinine, $15F_{2t}$-IsoP and GlcA–BPA, and an aliquot of fresh urine was used to quantify the concentration of creatinine (CREA) by the kinetic Jaffè procedure.

Statistical analysis. The analysis was performed by means of Stata 12 Statistical Package (Stata Corp LP, Lakeway Drive, TX, USA). Appropriate linear transformation was applied on data whenever suggested by distributional diagnostic plots (symmetry plot, quantile plot) and descriptive statistic inspection (looking at variance stability among categories). To alleviate multivariate heteroscedasticity, a Box-Cox power transformation on the dependent variable was applied to the data by means of maximum likelihood estimates of the parameters. If inspecting the two way plot of log (ng $15F_{2t}$-IsoP/mg CREA) versus log (GlcA–BPA) highlighted a non-linear relationship between these variables, suggesting a spline function to estimate the relationship, we used piecewise linear or "hockey stick" robust regression point [35]. It presupposes that the effect of predictive variables on dependents can be best fitted by two straight lines, with different slopes, and calculating

the two slopes and the value of the dependent at which the slope changes (the breakpoint or spline point). In the model log (ng cotinine/mg CREA), BMI linear effect, gender and effect of age classes were also tested and used as covariate if reached the 5% significance effect or changed significantly the estimates. A multiple linear regression (MLR) was carried out to assess the effect on covariates on $15F_{2t}$-IsoP and BPA exposure respectively, using log (ng $15F_{2t}$-IsoP/mg CREA), log (GlcA–BPA) as dependent variable, and age, height, weight, gender (female as reference value) and urinary cotinine as independent variables. A p value of ≤ 0.05 (two–tailed) was considered significant for all tests. All the variables that proved non–significant at 5% were excluded with a stepwise backward removal procedure.

RESULTS

In **table 1**, characteristics of students enrolled for the study are reported. Numerousness, mean, standard deviation (s.d.) and percentage (%) for gender, age (years), height (m), weight (kg) and smoking exposure (number of cigarettes *per* day) are reported for the subjects grouped for educational level. Among the 223 students, 18 reported being active smokers (8%), all from the age group 14-19, 52 passive (23.3%), and 153 non-smokers (68.7%). In **table 2**, cotinine, $15F_{2t}$-IsoP, and GlcA–BPA, all expressed as nanogram *per* 1 milligram of creatinine, are listed subgrouped *per* educational level as mean, standard deviation, and minimum and maximum.

GlcA–BPA shows an increase of concentration proportional with increasing age, even if the intermediate age group (11-14 years) is slightly lower. The same thing is observed also for $15F_{2t}$-IsoP and the exposure to tobacco (mainly passively breathed) quantified by cotinine. According to the Box–Cox regression results, the values of biological markers analyzed were subjected to a logarithmic transformation before carrying out the subsequent analysis. The result of piecewise linear robust regression shows a break point

at 1.79 (95%CI: 1.56 - 2.02; $p < 0.001$) of the effect of log- GlcA–BPA on log-15F_{2t}-IsoP (**figure 1 and table 3**). Thus, concentration of 15F_{2t}-IsoP increases exponentially (more than threefold each one log unit of GlcA–BPA), when log-GlcA–BPA concentration overcomes the break point identified at 1.79. MLR analysis shows a positive effect also of log-cotinine concentration on log 15F_{2t}-IsoP (**table 3**). This last effect is evident even considering that a 12% increase of 15F2t-IsoP is observed for each increment of a log-cotinine unit. Furthermore, the analysis of the relationship between log(ng15F_{2t}-IsoP/mg CREA) and age shows a trend V-shaped (**figure 2**); with a significant decrease ($p = 0.026$) between infancy (7 -10 year old) and the beginning of adolescence (11 – 15 years old) and then a new increase starting from 15 year of age (**figure 2** and **table 3**).

DISCUSSION AND CONCLUSION

The main objective of this work was to evaluate the environmental diffusion and the consequent absorption of BPA in a population of children and adolescents attending primary, secondary and high school in a city located in Piedmont region, in the Northwestern part of the Italy. At the same time, we wanted to observe the role of this pollutant in the induction of OS, taking into account as confounders, the role of passive and active exposure to tobacco smoke and age, other predictors of the same effect. These youth were enrolled as a population useful to investigate as accurately as possible, some environmental conditions as predictors of OS status development. This because their life habits leads them to be more in contact with the outside environment and because their lower body weight makes them more sensitive and vulnerable. At this concern, it is also known that young people are still in a phase of development of the body and of their metabolic system and therefore still fragile and hypersensitivity to environmental stimuli.

The OS level was monitored through the quantification of urinary 15F_{2t}-IsoP concentration, a biomarker unaffected by diet potentially confounding the relationship we have

investigated [36,37]. Furthermore, diet is very similar among all the students. This was known from the replies to the questionnaire they outlined a homogeneous domestic diet and because they benefit from the same school lunch prepared by the same company according to the requirements imposed by nutritionists working at the local health authority to minimize oxidant food.

Since the exposure to BPA can influence the OS level, urinary GlcA–BPA was measured to know the role of this contaminant in the onset of $15F_{2t}$-IsoP values. Findings show that the effect of log GlcA–BPA on $15F_{2t}$-IsoP has a threshold value around a break point of 1.79. This suggest that values of GlcA–BPA lower than 4.5 ng/mg of creatinine (exponential value of lower confidential limit) have no measurable effect on isoprostane; conversely, above the break point (6 ng/mg crea) the $15F_{2t}$-IsoP grows linearly ($p < 0.005$). To explain this log-linear relationship characterized by a threshold value, we have to remember the higher commitment of liver to contrast the higher concentrations of this contaminant, or an insufficient sensitivity of analytical technique to detect BPA at lower concentrations. Nevertheless, this last hypothesis seems to be contradicted by the log - linear relationship without threshold of the $15F_{2t}$-IsoP versus cotinine. Indeed, the induction of oxidative stress by passive and/or active smoking is confirmed in adolescent subject independently from age also in our previous paper [38].

The age of the subject proved to be another factor that can significantly influence the $15F_{2t}$-IsoP concentration. In a previous work [38] the $15F_{2t}$-IsoP levels has been studied in the 11-15 age group. A slight decrease (6%) was recorded passing from 11 to 15 years. In the present study, the analysis of $15F_{2t}$-IsoP levels according to age (7-19 years old) highlighted the V-shape previously illustrated. This seems to confirm that the O.S. experiences a lowering of intensity in the first years considered, and then return to grow regularly. This may result in the establishment and growth of a condition of chronic inflammation until senescence [37,39,40].

Finally, we found that urine GlcA–BPA concentrations were positively but not significantly associated with BMI. Due to its rapid metabolism (half – life less than 6 h), BPA exposure estimates from first morning urine may just represent the exposure at the prior meal (dinner), not daily or average exposure level. Given the food indigestion as the main exposure route to BPA, perhaps should be collect more urine samples throughout the day preceding the sampling to avoid underestimation of exposure to this contaminant.

We can conclude that the adolescent studied show an increase in OS dependent from GlcA–BPA higher than 4.8 ng/mg crea and from tobacco smoke passively and/or actively breathed. The induction of oxidative stress by GlcA–BPA is a theme not yet analyzed in depth by the International Scientific Community. It must be taken into consideration by the Public Health Authorities in a careful manner and without forgetting the other Bisphenols, now present in the living environment. Thus, the evidence of these risky conditions for public health may represent a platform for designing new preventive strategies addressed to promoting adolescent health in a sensitive period of growth, sexual differentiation, and brain development. Further studies on new and safer materials, on the least impact on environment and human health are therefore crucial.

The main results obtained in this work are: GlcA–BPA causes an increase in OS in the adolescents selected for the study, but only starting from 6 ng / mg of CREA. In addition, the passively breathed tobacco smoke is able to induce an increase of the S.O. The promotion of health must therefore also consist of the preventive contrast to BPA and all the bisphenols still present in the living environment.

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	PRIMARY SCHOOL (7-10 years)	SECONDARY SCHOOL (11-14 years)	HIGH SCHOOL (15-19 years)	TOTAL
N.	87	34	102	223
Gender N. (%)	Male 48 (55.2%) Female 38	Male 15 (44.1%) Female 19	Male 57 (55.8%) Female 45	Male 119 (53.3%) Female 104
Age (years) Mean ± s.d.	8.87 ± 1.0	11.7 ± 0.8	16.6 ± 1.71	12.8 ± 3.6
Height (m) Mean ± s.d.	1.39 ± 0.06	1.54 ± 0.1	1.71 ± 0.06	1.56 ± 0.17
Weight (kg) Mean ± s.d.	35.6 ± 9.8	45.0 ± 7.5	64.5 ± 12.4	50.2 ± 17.2
Smoking habits N. (%)	Active 0 Passive 26 (30%) No exp 61 (70%)	Active 0 Passive 5 (14.7%) No exp 29 (85.3%)	Active 18 (17.6%) Passive 21 (20.5%) No exp 63 (61.9%)	Active 18 (8%) Passive 52 (23.3%) No exp 153 (68.7%)

Table 1. Gender, age, height, weight and number of active and passive smokers in the whole population and in three groups subgrouped according to the three educational level considered.

	Cotinine [ng/mg CREA]	15F$_{2t}$-IsoP [ng/mg CREA]	Total BPA Inactivated [ng/mg CREA]
	Mean (±sd) Min - Max	Mean (±sd) Min - Max	Mean (±sd) Min - Max
PRIMARY SCHOOL (7-10)	73.7 (± 109) 1.06 - 382.9	5.07 (± 5.4) 0.6– 38.8	7.5 (± 7.8) 0.02 – 38.7
SECONDARY SCHOOL (11-14)	51.2 (± 111.0) 0.1 – 372.3	3.4 (± 2.9) 0.5 – 17.1	6.9 (± 5..3) 0.9- 34.4
HIGH SCHOOL (15-19)	179.0 (± 282.5) 0.1 – 1730.9	5.76 (± 5.4) 0.4– 23.2	10.8 (± 0.8) 0.3 –55.4
TOTAL Mean (± s.d.) Min - Max	107 (± 200) 0.03 –1730	4.8 (± 4.8) 0.41 –38.8	8.56 (± 7.8) 0.02 –55.4

Table 2. Urinary cotinine, 15F$_{2t}$-IsoP and total BPA inactivated values in the three groups subgrouped according to the three educational level considered. Min = minimum value; Max = maximum value. Units of biological markers are nanograms of every 1 mg of urinary creatinine.

		Means	C.I. 95% Lower limit – Upper Limit		$p <$
Total Inactive BPA (ng/mg CREA)		1.79	1.56	2.02	**<0.05**
Urinary Cotinine (ng/mg CREA)		0.03	0.00	0.06	**< 0.001**
Age (years old):	< 10	1.19	1.02	1.36	NS
	11–14	0.91	0.71	1.11	**<0.05**
	≥ 15	1.37	1.37	1.18	NS

Table 3. Multiple linear regression parameters, with means and 95% confidence interval (C.I.), of log 15F_{2t}-IsoP as dependent variable and log (total inactive BPA), log cotinine, age as predictors.

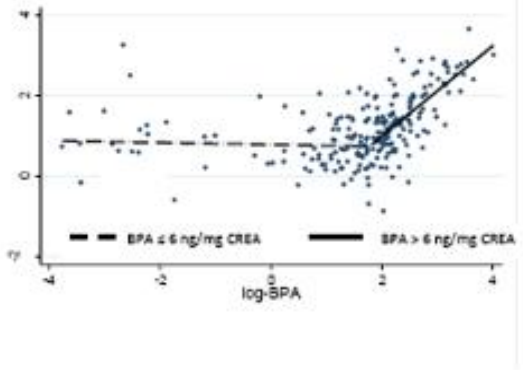

Figure 1. Piecewise linear robust regression of the relation of log (GlcA–BPA) on log (ng 15F_{2t}-IsoP/mg CREA) – (break point at BPA = 6 ng/mg CREA, 95% CI: 4.5 - 7.5). Exp (1.79) = 6

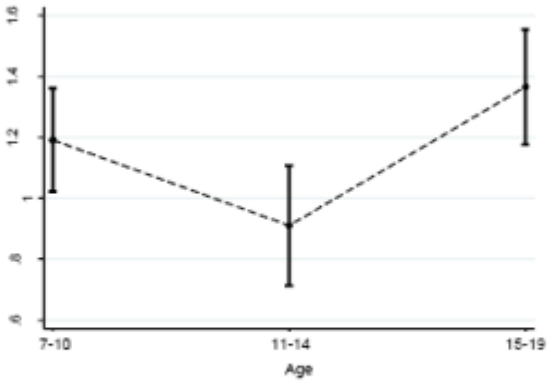

Figure 2. Margins-plot of the relation between log 15F2t –isoP and age classes.