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UNIVERSITÀ DEGLI STUDI DI TORINO

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

Roberto Bono^{1*}, Roberta Tassinari¹, Valeria Bellisario¹, Giorgio Gilli¹, Marco Pazzi²,
Valentina Pirro², Giulio Mengozzi³, Massimiliano Bugiani⁴, Pavilio Piccioni⁴

¹ Department of Public Health and Pediatrics. University of Torino, Italy

² Department of Chemistry, University of Torino, Italy

³ Clinical Chemistry Laboratory, San Giovanni Battista Hospital, Torino, Italy

⁴ Unit of Respiratory Medicine, National Health Service (ASL TO2), Torino, Italy

* Corresponding author: prof. Roberto Bono. University of Turin, ITALY. Department of Public Health and Pediatrics. Via Santena 5 bis. 10126 Turin, Italy. Phone: (+39) 011-670-5818
Fax: (+39) 011-236-5818. e-mail: roberto.bono@unito.it

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.

KEY WORDS: tobacco smoke, urban pollution, adolescents, oxidative stress, respiratory fluxes.

ABBREVIATIONS

PM = particulate matter

15-F_{2t} IsoP = 15-F_{2t}-isoprostane

SIDRIA = Italian Studies on Respiratory Disorders of Childhood and the Environment

CREA = creatinine

E.L.I.S.A. = 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
C.I. = confidence interval

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.

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 (Götschi T. 2005; Hazenkamp-Von Arx, 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; Henschel et al., 2012; Katsouyanni et al., 2009; Levy et al., 2012; Poschl, 2005; Raaschou-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 (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., 2010; Bono R., 2001; Bono R., 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; Ghio et al., 2012; Laumbach and Kipen, 2012), lung dysfunction (Kelly and Fussell, 2011; Wright and Brunst, 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 oxidants (Kelly et al., 2011; Sava and Carlsten, 2012) and a decreasing biosynthesis of endogenous antioxidant molecules (Yang W., 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 (Granello 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, 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., 2009). 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., 2009). 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 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 2000, Jacob 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 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 **Figure 1**, three geographic areas with different levels of urbanization and anthropization were chosen in the Piedmont region (northwestern Italy, 25.401,56 km²): Torino, capital of Piedmont, a urbanized city with almost 900.000 inhabitants (6.700 inhabitants/km², 130,2 km², 240 m. above sea level); Chivasso, a smaller and less urbanized city with about 26.000 inhabitants (507 inhabitants/km², 51,3 km² 183 m above sea level); and Casalborgone, a rural site with 1880 inhabitants (93,3 inhabitants/km², 20,2 km², 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.

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 R., 2014). Cotinine concentrations were normalized to the urinary creatinine (CREA) levels, as usual for every urinary measurement.

2.3.2. Urinary 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-liter 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 M.R. and McKay R., 2005).

2.3.4. Statistical analysis. Statistical analysis was carried out with the statistical package “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 GEP, 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_{2t} IsoP and lung function parameters respectively, using 15-F_{2t} 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.

3. RESULTS

The characteristics of the population enrolled in the study are described in **table 1**. Cohort numerosness, 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_{2t} 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_{2t} 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_{2t} IsoP concentrations. According to the Box-Cox regression results, the values of 15-F_{2t} 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 ($p>0.050$) 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 ($r^2=0.37$; $p<0,001$) 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 **figure 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 (**figure 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 (**figure 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 (**figure 5**); while the volumes FVC (mean

+0.002, CI 95% -0022 +0026) and FEV1 (CI 95% -0019 -0048 +0009) did not. This evidence can be accepted considering that, compared to the volumes, the flows are more sensitive, especially in the pediatric age.

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 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 kilometers 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-F_{2t} 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 R., 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 (Bouزيد MA, 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 (**figure 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.

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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		153.1 ± 9.8		151.7 ± 9.5	
Weight kg ± sd	43.53 ± 11.0		47.3 ± 12.3		47.7 ± 12.2		45.4 ± 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 2. 15-F_{2t} IsoP values in the three sampling locations. The three concentrations prove a direct relationship to population density: Turin, the most populated city, displays the highest mean value, Casalborgone, the rural site, the lowest.

	15-F _{2t} IsoP [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

* LOD = limit of detection. 15-F_{2t} IsoP fixed to limit of detection (0.2 ng/ml) if ≤ of LOD.

min = minimum value, max = maximum value, sd = standard deviation. Units of 15-F_{2t} IsoP is nanograms of 15-F_{2t} IsoP every 1 mg of urinary creatinine.

Table 3. Multiple linear regression parameters, with 95% confidence interval (C.I.), of log 15-F2t IsoP as dependent variable and log (cotinine); age and sampling site as predictors. Note: gender, height, weight, and diet indicators, not significant at 5% level, were excluded from the model. 15-F2t IsoP fixed to limit of detection (0.2 ng/ml) if \leq of LOD (limit of detection).

	Regression coefficient (95% C.I.)	<i>p</i> <
Urinary cotinine	+0.158 (0.119-0.197)	0.0001
Sampling sites	Chivasso vs Casalborgone	+0.301 (0.152 -0.561)
	Torino vs Casalborgone	+0.414 (0.268 - 0.561)
	Torino vs Chivasso+Casalborgone	+0.224 (0.108 - 0.338)
Age	-0.058 (-0.115 - -0.001)	0.050

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 (liters)	Casalborgone	2.91	2.82	2.99	
	Chivasso	2.88	2.82	2.95	0.22
	Torino	2.77	2.73	2.82	0.01
FEV1 (liters)	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	89.08	87.57	90.59	
	Chivasso	89.54	88.31	90.77	0.37
	Torino	86.45	85.45	87.44	0.04
MEF50 (liters/sec.)	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 (liters/sec.)	Casalborgone	3.15	2.96	3.34	
	Chivasso	3.25	3.11	3.38	0.24
	Torino	2.80	2.68	2.91	0.02

Figure 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 1880 inhabitants.

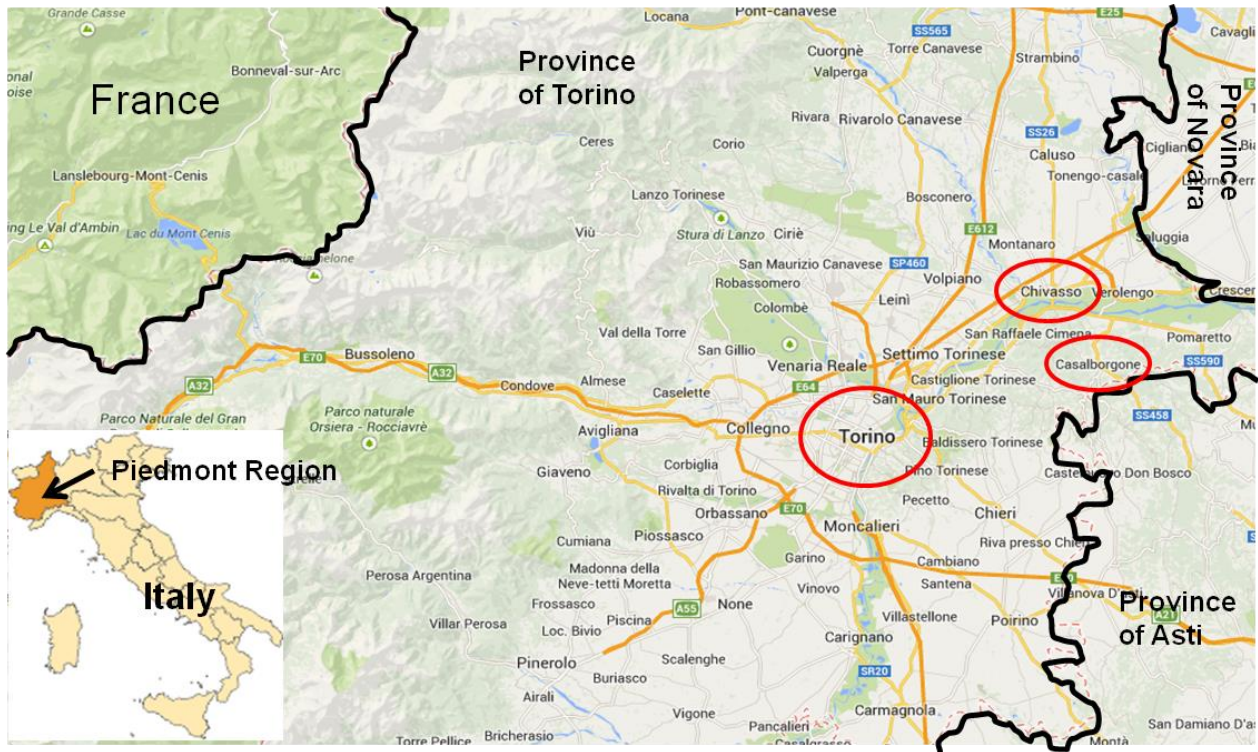


Figure 2. Plot of the relation between log 15-F_{2t} IsoP and log cotinine, given age and sampling site.

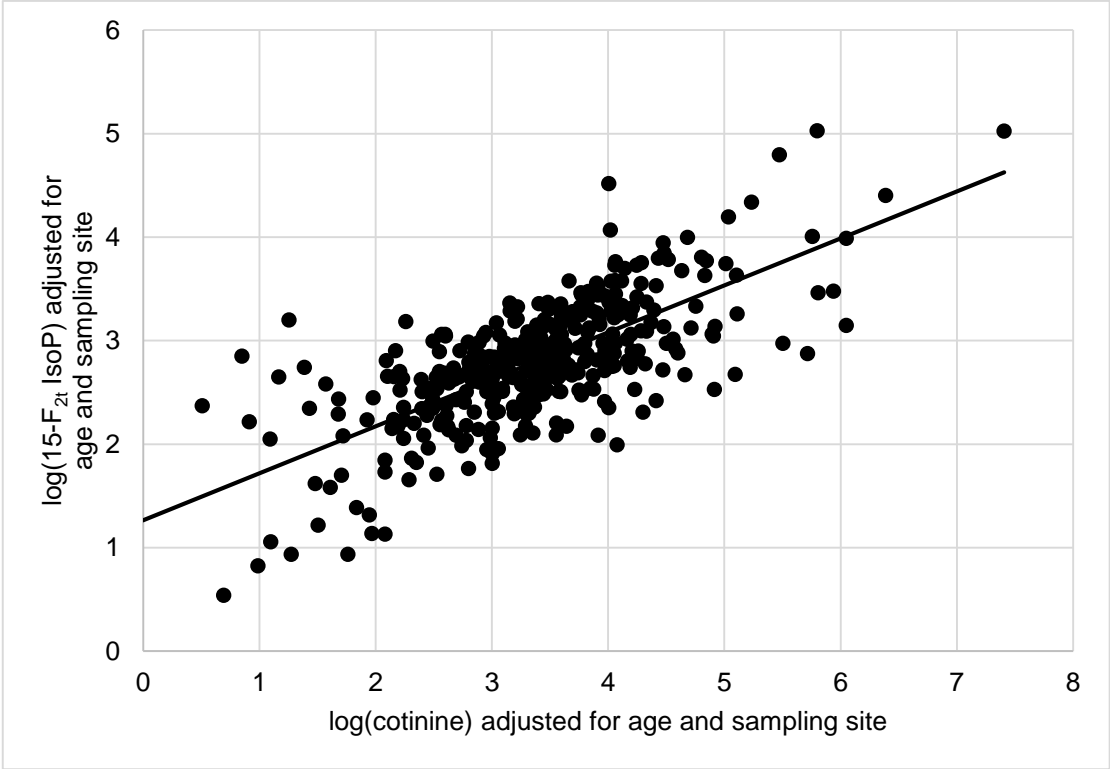


Figure 3. Plot of the relation between log 15 F2t IsoP and age, given log (cotinine) and sampling site.

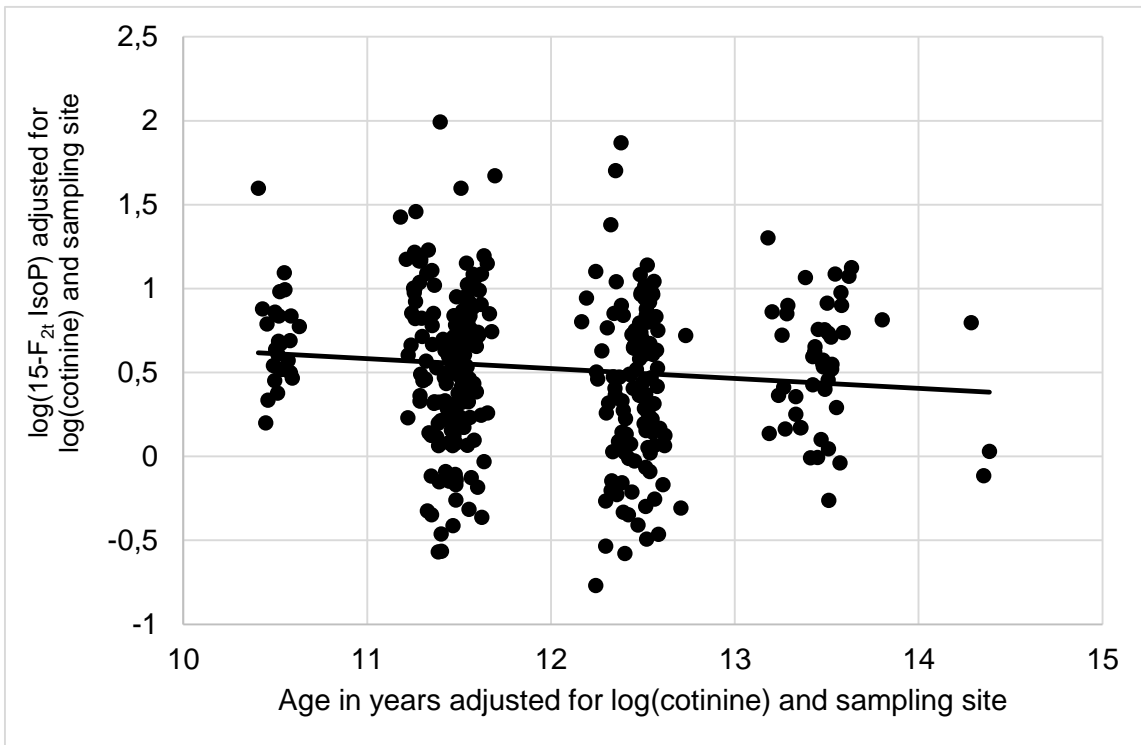


Figure 4. Marginal means and confidence intervals of 15-F_{2t} IsoP levels measured in the three sampling sites, adjusted for log (cotinine) and age by means of multiple regression model (with log link). Casalborgone is the rural site, Chivasso the medium size city, Torino the big city.

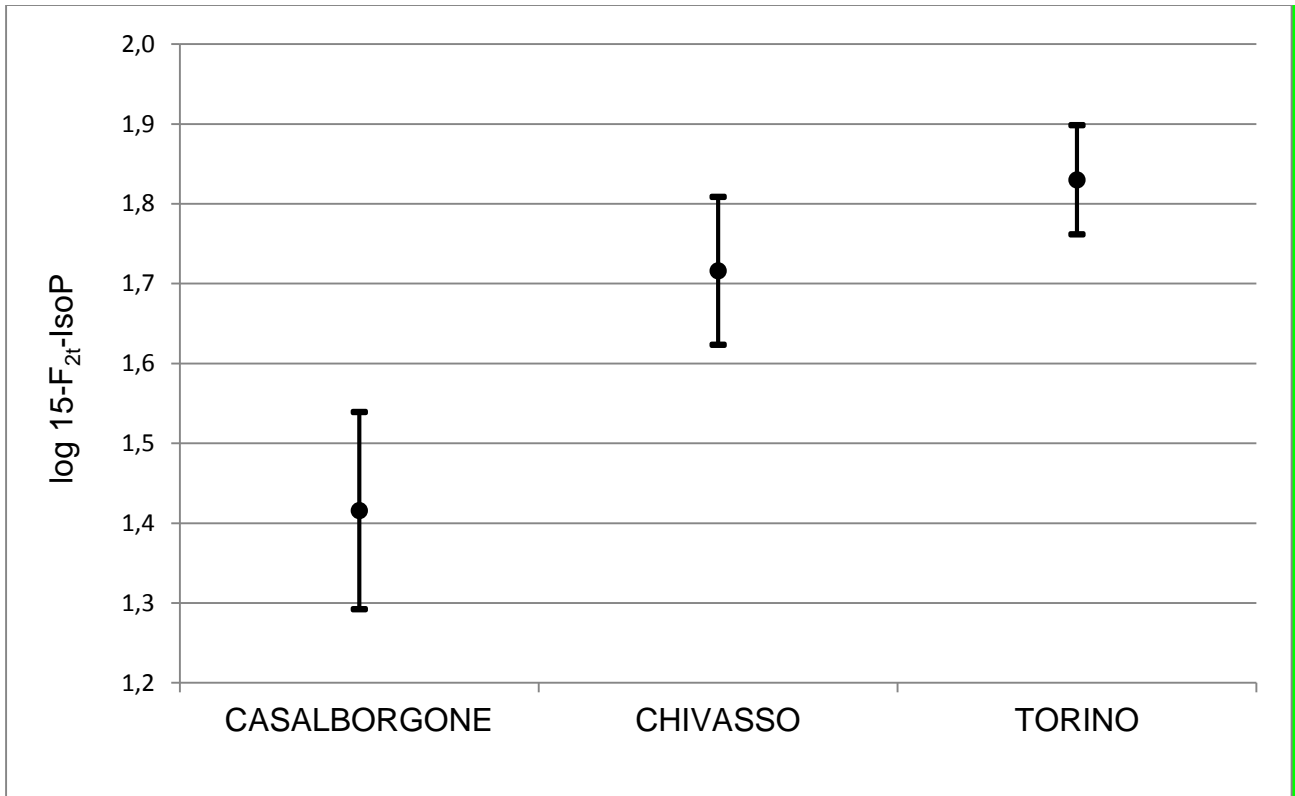


Figure 5. Marginal means and confidence intervals of lung function parameters by the three sampling sites, adjusted for age, height, gender, BMI and log (cotinine) by means of multiple regression model. A) FEF 50 - Forced Expiratory Flow at 50% of Forced Vital Capacity (FVC), B) FEF 25-75 mean forced expiratory flows at 25/75% of FVC, C) FEV1/FVC% = Forced expiratory volume in 1 second as % of FVC.

