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Research Paper

Oxidative stress and DNA repair and detoxification gene expression in adolescents exposed to heavy metals living in the Milazzo-Valle del Mela area (Sicily, Italy)

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ABSTRACT

Background: The area of Milazzo-Valle del Mela (Sicily, Italy) is considered at high risk of environmental crisis by regional authorities.

Objective: To measure oxidative-stress, DNA repair and detoxification genes in school children living near the industrial area and in age-matched controls.

Methods: The parent study was a biomonitoring investigation evaluating heavy metal urine levels in 226 children aged 12–14 years, living in the high risk area, and in 29 age-matched controls living 45 km far from the industrial site. In the present study 67 exposed adolescents and 29 controls were included. Samples were analyzed for urinary 8-hydroxydeoxyguanosine (8OHdG) levels, and gene expression of OGG1 (DNA repair gene), NQO1, ST13, and MT1A (detoxifying genes).

Results: Urinary cadmium was higher ($p = 0.0004$) in exposed [geometric mean, 0.46 μg/L; 25th–75th percentile: 0.3–0.56] than in control adolescents [geometric mean, 0.26 μg/L; 25th–75th percentile: 0.2–0.3]. Chromium was also significantly elevated in exposed [geometric mean, 1.52 μg/L; 25th–75th percentile: 1.19–1.93] compared with controls [geometric mean, 1.25 μg/L; 25th–75th percentile: 1.05–1.48; $p = 0.02$]. Urinary 8-OHdG concentration was greater in exposed than in controls (71.49 vs 61.87 μg/L, $p = 0.02$), and it was correlated with cadmium levels ($r = 0.46$, $p < 0.0001$), and with the combined exposure index ($r = 0.43$, $p < 0.0001$). Moreover, cadmium levels showed a robust correlation with OGG1 and MT1A gene expression levels ($r = 0.44$, $p < 0.0001$; $r = 0.39$, $p < 0.0001$, respectively). Finally, OGG1 and MT1A were over-expressed in adolescents from Milazzo-Valle del Mela area compared with controls ($p = 0.0004$; $p < 0.0001$, respectively).

Conclusions: Continuous exposure at relatively low concentrations of heavy metals is associated with increased oxidative DNA damage and impaired expression of DNA repair and detoxification genes in adolescents.

Introduction

The area surrounding the industrial plants of Milazzo-Valle del Mela (Sicily, Italy) is considered to be at high risk of environmental crisis by regional authorities, because of the presence of several industrial plants (i.e. refinery, batteries recycling implant, and power plant) nearby the residential area.

All these plants contribute to the emission of pollutants and heavy metals that represent one of the major threat to human health. These metals accumulate in ecosystem components, such as air, soil, water and also food chain; thus the risk of human exposure increases not only for industrial workers but also for people living near polluted areas [1–3].

Heavy metals as mercury, cadmium, chromium, arsenic, nickel, and lead are considered capable of disrupting the activity of several proteins involved in the reproductive and endocrine system [4–6].

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These metals might also alter the expression pattern of numerous genes involved in detoxifying processes. Moreover, heavy metals exposure is able to cause an increase in the oxidative DNA damage, as reported by Sughis and co-workers [7] who showed that urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentrations were significantly correlated with both chromium exposure and the combined heavy metals exposure index in working children compared with school children. These and other biomarkers have been previously investigated by other authors [8–13] and are considered useful to monitor the health status of exposed populations.

In light of these observations and taking also in consideration the lack of data about the healthy status of adolescents living in the Milazzo-Valle del Mela area, we investigated urinary levels of heavy metals, oxidative stress, and the gene expression of DNA repair (8-Oxoguanine glycosylase; OGG1) and detoxifying (NAD(P)H dehydrogenase (quinone 1); NQO1, Metallothionein-1A; MT1A, and suppression of tumorigenicity 13; ST13) genes in this exposed population. We also compared the results with the same data obtained from a control population living 45 km from the Milazzo-Valle del Mela area.

Materials and methods

Population

In the Milazzo-Valle del Mela area there are emissions by oil refinery and thermal-power plants. The parent study was a human biomonitoring investigation evaluating heavy metals urinary concentration in 226 children aged 12–14 years, living close to the industrial plants, and in age-matched controls ($n=29$) living 45 km far from the industrial site. Starting from December 2012 and up to January 2013, we met the parents in the different schools and after an exhaustive explanation of the project, we asked for their consent to enrol their children in our study. For this investigation only 67 parents (both sex, 12–14 years old) from two schools in Milazzo, and all the 29 control parents gave their informed consent and therefore, the adolescents were included in this study.

Sample collection, storage and analysis

Samples collection of both populations was performed during the same season and the same month (Spring; May 2013); all the children were provided with urine collection containers for 24-h specimens, and their parents were instructed for appropriate procedure and storage. Urine collection was performed 1 or 2 days before the medical visit and stored at 2–6 °C, to avoid any loss of sample or contamination. The 24-h collection was done on Sunday, when the children were not at school. All the participants underwent medical visits that were performed on Monday and Tuesday afternoon in the outpatient of Milazzo Hospital. All the enrolled children were in a good healthy status. As part of the visit, urine containers were collected and blood was withdrawn from a peripheral vein of the forearm. Furthermore all children underwent a complete clinical evaluation. Height and weight were routinely recorded and BMI was calculated using the following formula: BMI = mass (kg)/height $(m)^2$; the BMI *z*-score was calculated as difference between the BMI of each children and the 50th percentile BMI reference of age and sex matched controls as reported by Cacciari et al. [14].

In addition, both parents and children were administered a multiple-choice questionnaire to assess their lifestyle, smoke habits, quality of life, and risk perception.

Following collection, the biological samples were delivered and stored at the University of Messina, Pharmacology and Toxicology Laboratory. For heavy metals evaluation urine total volume was recorded, and aliquots (100 mL) were treated with 2 mL of 90% pure HNO_3, and stored at −20 °C until analysis. Additional urine aliquots (10 mL) were stored for 8-OHdG evaluation at −20 °C. Blood samples were immediately treated for mRNA extraction, as detailed below.

8-OHdG urinary concentration

8-OHdG urinary concentration was the main outcome of the study and was assayed using the DNA Damage EIA kit (Enzo Life Sciences, Switzerland), according with the manufacturer's protocol. All samples were loaded in duplicate and the mean absorbances of each sample were interpolated with those obtained from a standard curve. Data were expressed as ng/mL.

Heavy metals analysis

Urine samples were analyzed for the following heavy metals: arsenic, cadmium, chromium, mercury, nickel, vanadium, and determined by blinded technicians on coded samples. The Perkin-Elmer® (Norwalk, CT) AAnalyst™ 300 atomic absorption spectrometer (AAS) with an automated turret, deuterium arc background correction (AA-BG) and HGA®-800 graphite furnace were used for all analyses. The furnace was equipped with an AS-72 autosampler. Hollow cathode lamps were used as light sources.

When the results exceeded the reference value, a repeated sampling was performed as suggested by guidelines [15]. According to the IUPAC guideline [16] the reference value is defined within the 95% confidence interval of the 95th population percentile of the distribution of concentrations of a specific compound or element in a body fluid of a reference population [17,18]. The results in the form of descriptive statistics were expressed in μg/L. In fact the presentation of creatinine-based analytical data and reference values is not anymore recommended [15].

Gene expression analysis

Gene expression was evaluated by quantitative Real-Time PCR. The extraction of total mRNA was performed from blood specimens using TRIZOL reagent (Life Technologies, Foster City, CA) and organic extraction under sterile conditions, according to the manufacturer's protocol. For each sample 5 μg of mRNA were reverse transcripted into cDNA using SuperScript® III Reverse Transcriptase kit (Life Technologies) and cDNA was amplified in duplicate using the TaqMan Universal PCR Master Mix containing primers and specific TaqMan probes (Life Technologies), using the instrument QuantStudio™ 6 Flex Real-Time PCR System (Life Technologies). The results were expressed as number of copies of the target gene and compared with the housekeeping gene (β-actin); the $2^{-\Delta\Delta Ct}$ mean values of both not-exposed and exposed adolescents were compared with those of an arbitrary calibrator.

Statistical analysis

Statistical analysis was performed with XLStat 2013. To compare parametric data between groups Student's *t*-test was used, while for non-parametric data we used the Mann–Whitney *U*-test. A *p* value of <0.05 was considered statistically significant. A post-hoc power calculation revealed that the power of the study was 84.2% assuming 8OHdG as the main outcome. The test was performed according with Rosner [19], using the following formula: Power $= \{-Z_{1-\alpha/2} + \Delta/\sqrt{{\sigma_1}^2/n_1 + {\sigma_2}^2/n_2}\}$, where n_1 was the sample size for the group of adolescents living in Milazzo-Valle del Mela, n_2 was the sample size for the group of adolescents living in Montalbano Elicona, Δ was the absolute difference between the means relative to the primary outcome of our study (8-OHdG concentration), σ_1 and σ_2 were the variances of such means in our two study groups (Milazzo-Valle del Mela and Montalbano Elicona, respectively), α was the probability of

type I error (set to 0.05), β was the probability of type II error (set to 0.2), Z was the critical value for α and Φ was the function converting a critical Z value to power.

In order to express the Composite Exposure Index, a Principal Component Analysis was performed and the resulted F1 factor was assumed as representative of global exposure to arsenic, cadmium, chromium and nickel. Correlation and linear regression were calculated using Spearman's method for non-parametric data, or Pearson's test for the parametric.

3. Results

Demographic characteristics and heavy metals concentrations

A total of 96 adolescents (56 males and 40 females) were included in this analysis; of these 67 (40 males and 27 females) were born and resident in Milazzo municipality and 29 (16 males and 13 females) were born and resident in the control area of Montalbano Elicona. The general characteristics of the whole population are shown in Table 1. No significant difference in age, BMI and BMI z-score was observed between the two groups.

Table 2 depicts the limit of quantification of the analytical method used for determining heavy metal levels in biological matrix samples, and the percentage of samples below the limit. Mercury and vanadium showed the highest percentage of values below the limit of detection in the analyzed samples; therefore both metals were excluded from further statistical analysis.

Table 3 shows heavy metals concentration measured in biological matrices of the whole population resident in the high risk area and in the control population. Data were expressed as geometric means and 95% confidence intervals. The concentration of each analyzed metal was compared between exposed and not-exposed adolescents (Table 3). Arsenic, cadmium, and chromium levels were significantly higher in exposed than in control population ($p < 0.0001$, $p = 0.0004$, and $p = 0.02$ vs not-exposed, respectively). Heavy metals levels in both exposed and not-exposed children of the present study were compared with the reference values for not-exposed adolescents as identified in previously published German surveys (Table 3). From this comparison it appears that cadmium and chromium levels in our exposed population were higher than suggested reference values.

In exposed children a statistical significant increase in arsenic levels was observed when gender differences were considered (male vs female $p = 0.03$; Table 4). In not-exposed adolescents cadmium levels were higher in males than in females ($p = 0.0009$; Table 4).

Finally we performed a correlation between heavy metals levels and either BMI or BMI z-score. No significant correlation was found ($p = 0.7$ and $p = 0.58$, respectively; data not shown), thus ruling out the hypothesis that the increased heavy metals might be influenced by the nutritional status in our population.

Oxidative DNA damage

8-OHdG urinary concentration, a marker of oxidative DNA damage, was significantly higher ($p = 0.026$) in exposed [geometric mean 71.49, (64.16–79.65)] than in not-exposed ones [geometric mean 61.87, (56.13–68.20)] (Fig. 1A).

8-OHdG concentration was significantly correlated with the cadmium levels of the whole population of exposed and not-exposed adolescents (Fig. 1B; $r = 0.46$, $p < 0.0001$). Similarly, a strong correlation was also found for 8-OHdG and arsenic (Fig. 1C; $r = 0.27$, $p = 0.006$). Furthermore, we evaluated the combined effect of all heavy metals by calculating the composite exposure index. Composite exposure index was significantly correlated with 8-OHdG urinary levels (Fig. 1D; $r = 0.43$, $p < 0.0001$). For all the above mentioned correlations, we found a linear regression pattern with the slopes that in all cases were significantly non-zero and an r^2 of 0.35 for cadmium, 0.06 for arsenic, and 0.26 for the composite exposure index, respectively.

No gender differences were detected in 8-OHdG levels in either exposed and not-exposed adolescents.

Gene expression

We assessed the expression levels of OGG1, NQO1, ST13 and MT1A and we compared the results obtained from exposed with those of not-exposed ones (Fig. 2). A significant up-regulation of OGG1 mRNA expression was observed in exposed compared with not-exposed adolescents (Fig. 2A, $p = 0.0004$). Gene expression analysis for MT1A, a protein involved in metal excretion, showed a significant up-regulation in the exposed population (Fig. 2B, $p < 0.0001$). A slight, but not-significant up-regulation of NQO1 and ST13 was measured in the adolescents living in the Milazzo area (Fig. 2C and D).

The expression levels of the analyzed genes were correlated with the urinary metal concentrations. Linear regression analysis showed that both OGG1 and MT1A expression levels correlated with urinary cadmium ($r = 0.45$, $p < 0.0001$; $r = 0.39$, $p < 0.0001$, respectively) with a significant non-zero slope in a liner regression model ($r^2 = 0.15$; $r^2 = 0.05$, respectively; Fig. 3A and B). In addition, a significant correlation between urinary arsenic values and both OGG1 and MT1A gene expression levels ($r = 0.22$ and $r = 0.31$, respectively) was observed. Assuming arsenic urinary levels as the independent variable, a linear regression for OGG1 ($r^2 = 0.05$), but not for MT1A, was detected (Fig. 3C). Considering gender differences, we did not observe any statistically significant change in gene expression levels.

General habits and risk perception questionnaire

We administered a questionnaire to the adolescents and their parents, for the identification of modifying factors, including socio-economic and lifestyle factors which could provide important information on susceptibility factors. Table 5 shows the most relevant answers provided by either exposed and not-exposed groups regarding quality of life, risk perception, and food habits. From the obtained answers it is possible to observe that none of the enrolled kids stated to be a smoker, and those living in Milazzo use to drink bottled water.

Discussion

Exposure to heavy metals can be due to inhalation of such pollutants transported by dust through the air, or by consumption of contaminated drinking water and food. Exposure to heavy metals is known to exert toxic effects even at relatively low concentrations. Such toxicity could result in gene expression alteration potentially able to cause an increase in the susceptibility to various diseases [20–23]. Thus, the main goal of our study was to assess whether living near to heavy metal-polluted area of Milazzo-Valle del Mela (Sicily, Italy) could produce any change in gene expression profile of some detoxifying and DNA repair genes. Children living in Milazzo had higher levels of cadmium, and chromium compared to reference values and to the children living in Montalbano Elicona. It can be hypothesized that the elevated levels of heavy metals found in the urine samples of adolescents living in Milazzo area (that has been recognized at high risk of environmental crisis), are due to a high burden of pollutants in both the eco-system and the food chain; indeed these cannot be ascribed to the smoking habit, as suggested by the results of our questionnaires. However, cadmium and chromium levels in the adolescents living in Montalbano Elicona were slightly higher than reference values suggested by Schultz et al. [24], and Heitland and Koster [25] (Table 3). This could be due to the peculiar environmental background of Sicily, as compared to other geographic areas; in fact the presence of the

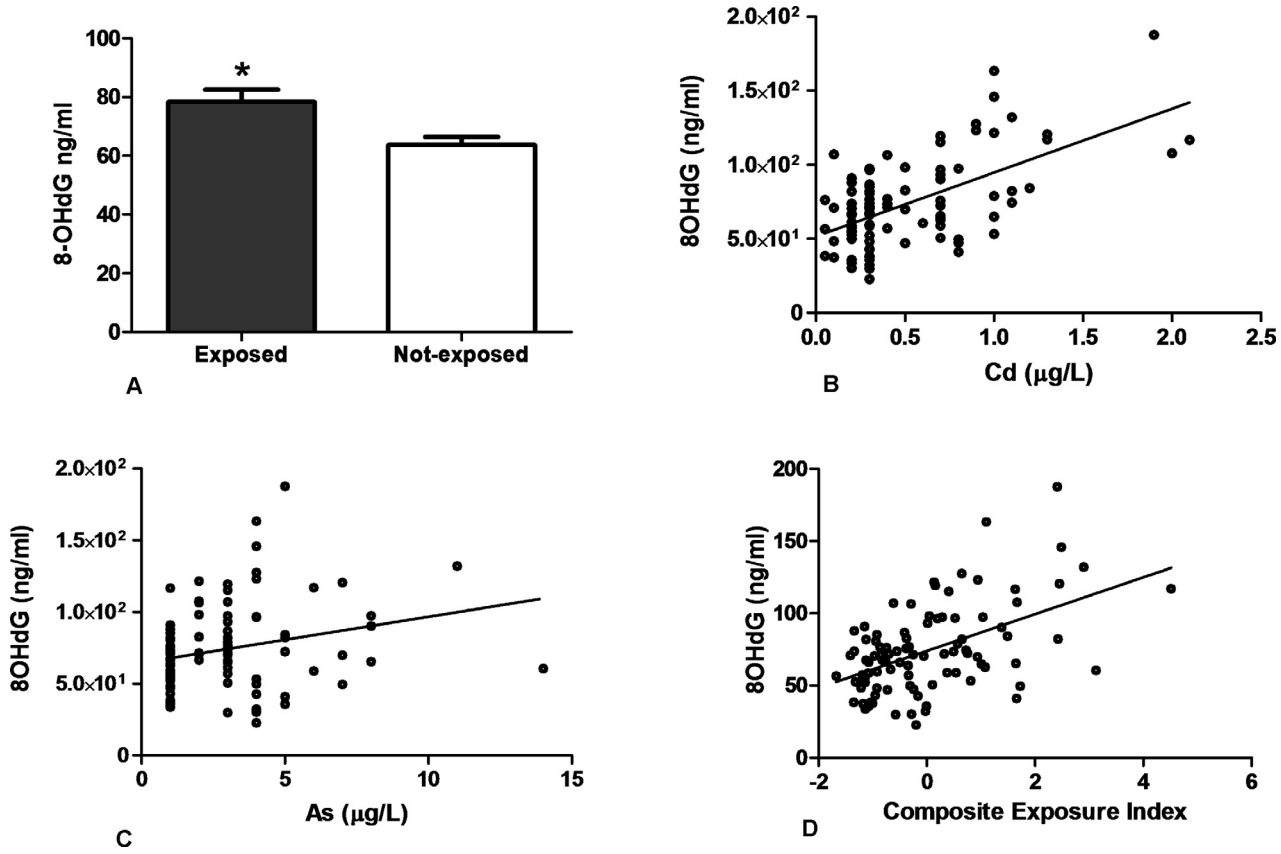

Fig. 1. 8-OHdG urinary concentration (A) in exposed and not-exposed adolescents ($^{*}p = 0.026$) and association of 8-OHdG in urine with urinary concentration of Cd (B), As (C) and composite exposure index (D). Spearman $r = 0.46$, $p < 0.0001$; $r = 0.27$, $p = 0.006$; $r = 0.43$, $p < 0.0001$ respectively.

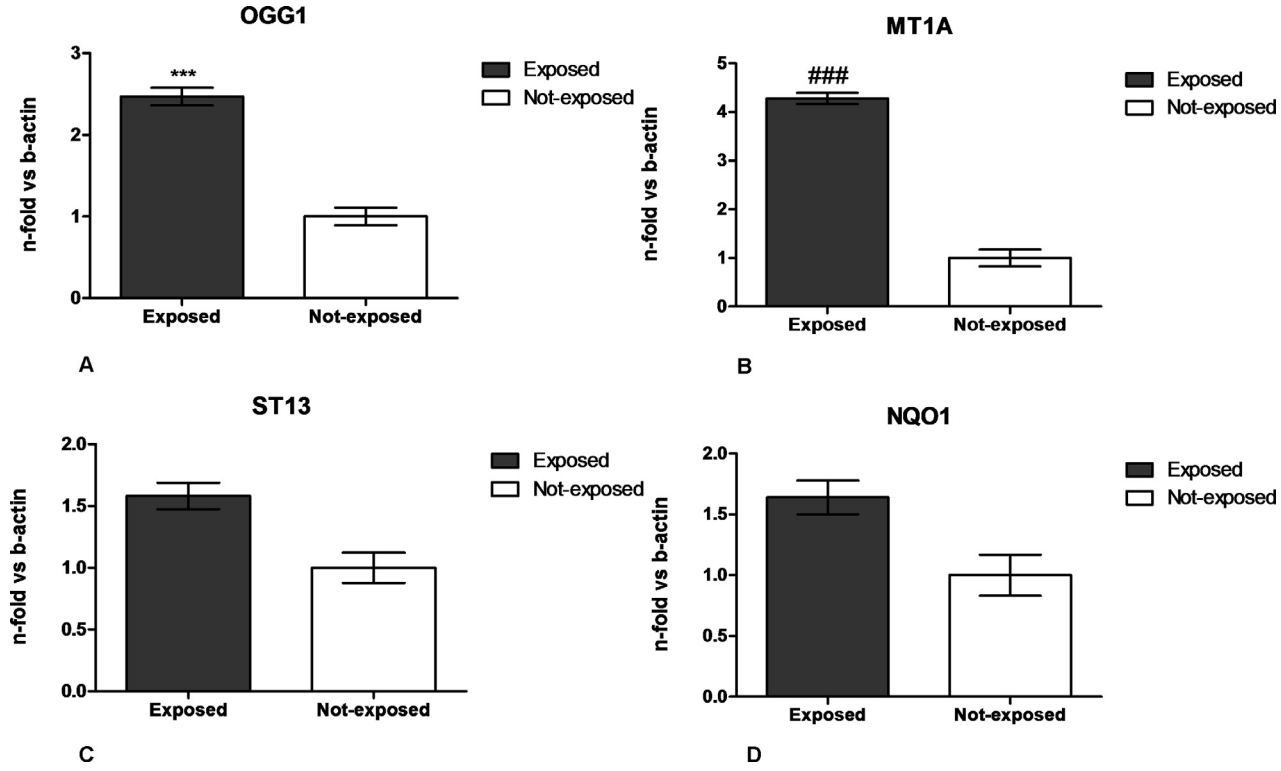

Fig. 2. OGG1 (A), MT1A (B), ST13 (C) an NQO1 (D) gene expression values in exposed and non-exposed children, expressed as mean ± SEM. $^{***}p = 0.0004$, $^{###}p < 0.0001$.

Table 1
General characteristics of the studied population.

Characteristics	Exposed	Not-exposed
Male:female	40:27	16:13
Age (mean ± SD)	13.05 ± 0.62	13.46 ± 0.69
BMI (mean ± SD)	21.33 ± 4.79	21.44 ± 4.87
BMI *z*-score (mean ± SD)	1.66 ± 4.9	0.89 ± 4.84

Table 2
Limits of detection of heavy metals.

Heavy metals μg/L	Cd	Cr	Hg	Ni	As	V
Matrix	Urine	Urine	Urine	Urine	Urine	Urine
LOD	0.1	0.2	1	0.1	2	0.1
% LOD	3.12	4.16	83.3	36.46	40.6	89.58

Table 3
Urinary metals concentrations [μg/L; geometric means (Lower-CI, Upper-CI)] in exposed and non-exposed adolescents.

Metals	Exposed adolescents ($n = 67$)	Not-exposed adolescents ($n = 29$)	*p*-Value	GerES IV report (2010) (μg/L)	RV (μg/L)
Cd	0.46 (0.37–0.56)	0.26 (0.22–0.30)	0.0004	0.08	0.2[a]
Cr	1.52 (1.19–1.93)	1.24 (1.05–1.48)	0.0266	–	0.59[b]
Hg	0.58 (0.53–0.63)	0.52 (0.47–0.57)	0.0695	0.11	0.4[a]
Ni	0.65 (0.40–1.08)	0.27 (0.12–0.61)	0.0535	1.23	4.5[a]
As	2.69 (2.25–3.20)	1.38 (1.11–1.71)	<0.0001	4.11	15[a]
V	0.053 (0.050–0.057)	0.057 (0.049–0.066)	0.4469	–	0.1[b]

Data are geometric means (GM). CI: 95% confidence interval. R.V. Reference Value.
[a]Schultz et al. [24].
[b]Heitland and Koster. [25]

Table 4
Gender differences in heavy metals concentration.

Heavy metals	Exposed		Not-Exposed	
	n	**μg/L**	*n*	**μg/L**
Arsenic	67	2.68	29	1.38
Male	40	3.09[#]	16	1.32
Female	27	2.19	13	1.45
P value male vs female	#'0.03		*n*. s.	
Cadmium	67	0.46	29	0.26
Male	40	0.48	16	0.32[#]
Female	27	0.43	13	0.20
P value male vs female	*n*. s.		#'0.0009	
Chromium	67	1.18	29	1.25
Male	40	1.31	16	1.36
Female	27	1.90	13	1.12
P value male vs female	*n*. s.		*n*. s.	
Nickel	67	0.65	29	0.52
Male	40	0.65	16	0.54
Female	27	0.66	13	0.50
P value male vs female	*n*. s.		*n*. s.	

volcanic rocks and gas from Mount Etna account for elevated traces of heavy metals in soil and water springs [26].Considering gender differences in the exposed population, we observed that arsenic seems to be more accumulated by male than female adolescents (3.09 vs 2.19 μg/L; $p = 0.03$); by contrast, cadmium concentrations were not significantly different between males and females. Cadmium levels were higher in males than in females (0.32 vs 0.2 μg/L; $p = 0.0009$) living in the control area of Montalbano Elicona. Indeed, gender differences in the accumulation of cadmium have been already reported in other populations [27]; nevertheless our observation let us to speculate that cadmium exposure in a non-smoker adolescent population at relatively high dose and for long time could lead to similar cadmium accumulation in males and females (0.48 vs 0.43 μg/L). 8-OHdG is one of the predominant forms of free radical-induced oxidative lesions in DNA structure, and has been widely used as a biomarker of oxidative stress. Urinary 8-OHdG has been reported as a good biomarker for (i) risk assessment of both cancers and degenerative diseases; (ii) for measuring the effect of both endogenous and exogenous oxidative damage to DNA; and (iii) as a factor of initiation and promotion of carcinogenesis [28]. This biomarker has been also used to estimate DNA damage in humans after exposure to cancer-causing agents, such as tobacco smoke, asbestos fibres, heavy metals, and polycyclic aromatic hydrocarbons [28]. Considering the ability of heavy metals to generate reactive oxygen species (ROS) [29], we quantified 8-OHdG concentration in urine samples with the aim of assessing oxidative stress-related DNA damage caused by such pollutants. We found a strong

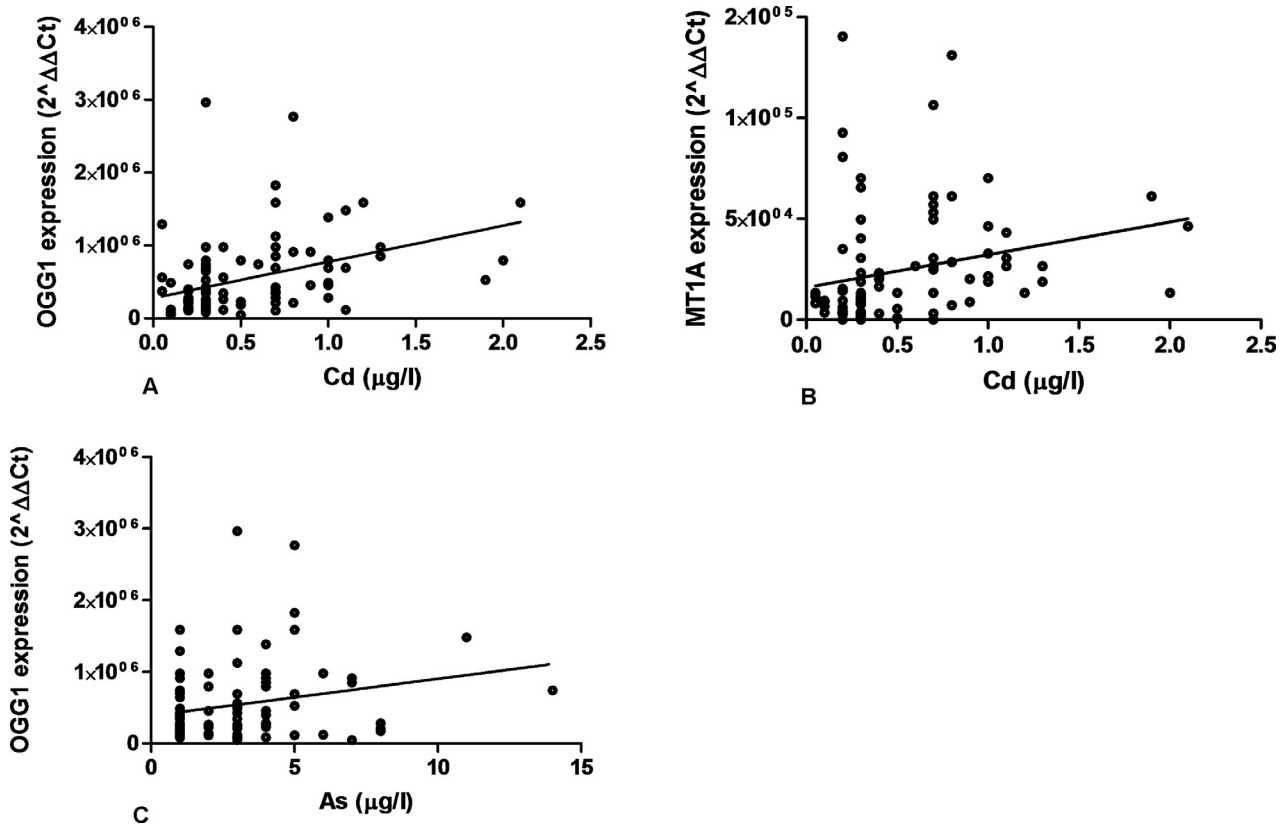

Fig. 3. Association between urinary Cd concentrations and OGG1 (A), MT1A (B) gene expression levels. Spearman $r = 0.45$; $p < 0.0001$ and $r = 0.39$; $p < 0.0001$ respectively (A and B). Association between urinary As concentration and OGG1 gene expression level (C). Spearman $r = 0.22$; $p = 0.02$.

Table 5
Smoking and food habits questionnaire administered to either parents and adolescents involved in the study. Results are expressed as percentage.

	Milazzo (%)	Montalbano Elicona (%)
Parental questionnaire		
Do you smoke?		
No, I do not	**82**	**59**
Less than 10/day	**11**	**7**
Up to 20/day	**6**	**27**
More than 20/day	**1**	**7**
Do you buy fruit and vegetables from the local market?		
Yes, I prefer fresh products	**30**	**7**
Yes, however sometimes I go to the grocery stores	**29**	**48**
No, I use to buy in the grocery stores	**31**	**14**
I grow my own vegetable garden	**10**	**31**
Do you use tap water for food purposes?		
Yes, I drink it everyday	**14**	**72**
Yes, but only for cooking	**38**	**25**
No, I do not	**48**	**3**
Children questionnaire		
Do you smoke?		
No, I do not	**100**	**100**
Less than 10/day	**0**	**0**
Up to 20/day	**0**	**0**
More than 20/day	**0**	**0**
How many times a week do you eat fish?		
Once a week	**42**	**14**
Twice a week	**34**	**48**
1–2 times a month	**20**	**38**
I do not eat fish	**4**	**0**
Do your parents let you drink tap water?		
Yes, I drink it everyday	**14**	**70**
No, never	**86**	**30**

correlation between cadmium exposure and 8-OHdG urinary concentration ($r = 0.46$; $p < 0.0001$), as previously reported by Al Bakheet and collaborators [8]; moreover, we also showed similar relationship for arsenic exposure, even if at lower significant level ($r = 0.27$; $p = 0.006$). These observations are also in agreement with previous data [29] about the putative toxicity of both cadmium and arsenic that appears to be the consequence of an imbalance between pro-oxidant and antioxidant homeostasis, the so called oxidative stress. Since the enrolled subjects were exposed to several metals, we investigated the impact of such multiple exposure by the calculation of the composite metal exposure index. We found a significant correlation between 8-OHdG and composite metal exposure index ($r = 0.43$; $p < 0.0001$), as previously reported in exposed children and workers [7,30]. Previous studies have reported the impairing effects of heavy metals on gene expression profile [31]. Therefore, to assess the impact of such pollutants we investigated gene expression levels of OGG1, MT1A, NQO1, and ST13 in adolescents living in the Milazzo area and in the control group living 45 km far from the polluted area. Our results showed a significant up-regulation (more than 2.5 fold increase; $p = 0.0004$) of OGG1 expression in the exposed population compared with controls; similar results were found for MT1A expression levels that was 4-fold higher in exposed than in the control ones ($p < 0.0001$). Moreover, OGG1 and MT1A expression correlated with urinary cadmium concentration ($r = 0.45$; $p < 0.0001$ and $r = 0.39$; $p < 0.0001$, respectively), while arsenic urinary concentration showed a correlation only with OGG1 expression ($r = 0.22$; $p = 0.02$).As far as NQO1 and ST13 expression were concerned, we did not observe any significant difference, even though both genes were over-expressed in exposed group compared with controls (>1.5 fold in either cases). Taken together, our data suggest a relevant burden of heavy metals exposure on oxidative stress-related DNA damage process, as shown by the significant increase in 8-OHdG concentration in the exposed population. Furthermore, heavy metals exposure up-regulated not only genes directly involved in heavy metals metabolism and DNA repair, but also those involved in the detoxifying process, even if not significantly. Since a linear regression pattern was observed between 8-OHdG showed and composite metal exposure index, we propose the measurement of these adduct as inexpensive biomarker to evaluate the health risk in adolescents living nearby polluted area. In conclusion, our findings point out to the need of a continuous monitoring not only for workers potentially exposed to high dose of heavy metals or other pollutants, but also for those populations living near industrial plants. In fact, our data strongly suggest that heavy metals increase oxidative stress-related DNA damage during continuous exposure at relatively low concentrations.

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