Biochemical and Biophysical Research Communications 426 (2012) 71–75

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Erythrocyte glutathione transferase: A novel biomarker to check environmental pollution hazardous for humans

Raffaele Fabrini [a,1], Alessio Bocedi [a,1], Erica Del Grosso [a], Laura Morici [a], Giorgio Federici [b], Antonio Palleschi [a], Giorgio Ricci [a,*]

[a] Department of Chemical Sciences and Technologies, University of Rome "Tor Vergata", 00133 Rome, Italy
[b] Childrens Hospital IRCCS "Bambin Gesù", 00165 Rome, Italy

ARTICLE INFO

Article history:
Received 25 July 2012
Available online 14 August 2012

Keywords:
Biomarkers
Pollution
Contamination
Erythrocyte glutathione transferase
Sacco river valley

ABSTRACT

Glutathione transferase (GST) is an enzyme capable of protecting the body from a lot of toxic compounds. Previous studies demonstrated that the erythrocyte GST (e-GST) expression increases as the level of circulating toxins increases. Aim of the present study is to verify if e-GST may represent a biomarker able to signalize an environmental pollution hazardous for humans. The study involved about 500 healthy volunteers living in eight distinct areas at or near the Sacco river valley, a region of the Frosinone district (Lazio-Italy) well known for its environmental pollution. Subjects of six areas displayed increased levels of e-GST ranging from 18% to 44% compared to 400 volunteers living in the Rome hinterland. Higher levels of GSTs are present in the areas where the risk of pollution is higher (areas 7 and 8). Interestingly, women living in the Sacco valley display much higher expression of e-GST than men, possibly due to a greater time exposition to the environmental contamination. Possible oxidative alteration of GST activity has not been observed. In conclusion, e-GST may represent an early and sensitive bio-signal of dangerous pollution for humans.

1. Introduction

Environmental pollution is a world scale problem. Virtually, most water or air pollutants are hazardous to humans as well as lesser species; sodium is implicated in cardiovascular disease, nitrates in blood disorders. Mercury, lead, cadmium and other heavy metals can cause nervous disorders. Many pesticides are toxic for humans and can alter chromosomes as well as toxic volatile compounds like dioxins and polycyclic aromatic compounds. Unfortunately, thousands of compounds are really dangerous "contaminants" but we know and recognize only a few of them and sometimes their impact on human health is not completely known. Today, the identification of polluted areas is normally made by means of chemical analysis of air, water and soil. Alternatively, epidemiological analysis on the levels of specific toxins in human body or blood or the incidence of specific diseases or the increase of mortality may reveal an hazardous pollution. In this context, it will be of great interest the discovery of sensitive biomarkers that could reveal precociously an hazardous contamination before its deleterious impact on human health. The present study proposes the erythrocyte glutathione transferase (e-GST) for this role.

Glutathione transferases are a well known enzyme superfamily devoted to protect organisms from many toxic endogenous and exogenous compounds [1–3]. Their protective activity is achieved by promoting the conjugation of GSH to the electrophilic centre of a lot of toxic organic compounds or, alternatively, acting like ligandins, i.e. binding many toxic compounds and promoting their excretion from the cell. These enzymes are present in all organisms from bacteria and plants to humans and are classified in about sixteen gene-independent isoenzyme classes. In humans GSTs are mainly represented by the Alpha, Pi and Mu classes that are abundantly expressed in many different organs [4]. A specific GST is also present in blood and in particular in erythrocytes. The prevalent erythrocyte isoenzyme is GSTP1-1, a homodimeric enzyme of about 46,000 Da [4]. In the last few years it has been observed that e-GST is over-expressed in uremic patients under dialytic therapy as a defense response to an increased level of circulating toxins [5,6]. More recently, we have discovered that also patients with renal disease under conservative therapy display similar or even higher hyper-expression of this enzyme [7]. In other words, GST appears like a natural and endogenous marker of blood toxicity being over-expressed as a defense response of the organism

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DTT, dithiothreitol; GSH, glutathione; e-GST, erythrocyte glutathione transferase; Hb, hemoglobin.

* Corresponding author. Fax: +39 0672594328.
E-mail address: riccig@uniroma2.it (G. Ricci).

[1] These authors equally contributed to this work.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.08.037

against the threat of increased levels of circulating toxins. An important property of e-GST is that this enzyme represents a long life biomarker that does not reveal an acute toxicity, but more usefully, an average exposition to toxins in a temporal range of about two months. In fact, the erythrocyte is an enucleated cell, and then its enzymatic content remains unchanged after the cell maturation during the entire cell life. Data shown here represent a preliminary study performed in a region which covers part of the Sacco river valley, a region well known for its pollution [8] and the neighbouring territory at the confluence of Sacco and Liri rivers.

2. Materials and methods

2.1. Blood samples

Venous blood samples were collected from healthy subjects and stored in vacutainer tubes containing EDTA as anticoagulant. The tubes were immediately stored at 4 °C and analyzed within one week from recovery.

2.2. Volunteers

Five hundred and ten healthy volunteers were assayed for e-GST. Women were 46% with an average age of 45 ± 15 years. Men displayed an average age of 46 ± 13 years. Four hundred healthy volunteers living in Rome (city and hinterland) were assayed for e-GST as a "reference" value. Women were 35% with an average age of 40 ± 17 years. Men were 65% with an average age of 43 ± 16 years. Subjects with acute or chronic liver and kidney diseases were excluded from the study.

2.3. e-GST activity

Activity of e-GST in total blood was determined spectrophotometrically essentially as described previously [7]. Briefly, 0.04 ml of blood were diluted with 1 ml of bi-distilled water. After 2 min, 0.1 aliquots of hemolyzed samples were incubated with 1 mM GSH and 1 mM of 1-chloro-2,4-dinitrobenzene in 1 ml of 0.1 M potassium-phosphate buffer, pH 6.5. The enzymatic activity was followed (37 °C) at 340 nm where the enzymatic product, the *S*-glutathionyl-2,4-dinitrobenzene, absorbs ($\varepsilon_{340nm} = 9600\ M^{-1} \cdot cm^{-1}$). Each spectrophotometric determination was subtracted by the spontaneous reaction of GSH with CDNB. Activity determinations were expressed as enzymatic unit (*U*) at 37 °C and normalized to the amount of hemoglobin (Hb) determined in the same blood sample as reported in literature [7]. The procedure can be also performed using an automated apparatus as described previously [7]. Importantly, the activity of e-GST is linearly related to its expression [6,7]. Thus the increase of e-GSTs activity or the hyper-expression of e-GST can be considered synonyms.

Recombinant human GSTP1-1, equivalent to e-GST, has been expressed in *Escherichia coli* and purified through affinity chromatography as described previously [9].

2.4. Areas of the study

Eight distinct areas within the Sacco river valley and after the confluence of Sacco and Liri rivers in the Frosinone district have been selected for this study. Geographic features and possible source of pollution have been briefly described in Table 1. A number of healthy volunteers ranging from 50 to 80 have been analyzed for each area. About 400 health volunteers living in the city of Rome and hinterland have given the health reference value for e-GST.

Table 1
Geographic features of selected areas.

Selected areas in Frosinone disctrict	Territorial extension (km^2)	Geographic features
Area 1	25	Nearby confluence of Sacco and Liri rivers
Area 2	10	Close to Liri river
Area 3	90	After confluence of Sacco and Liri rivers
Area 4	40	Near the Sacco river – presence of industrial site
Area 5	40	Liri river flows through the area – presence of regolarized landfill and compost sites
Area 6	30	Close to important industrial site
Area 7	60	Sacco river flows through the area
Area 8	40	Close to Sacco and Liri rivers – presence of incineration plant

Data referred to the mortality of the Frosinone and Rome areas have been recovered by the data given by the Italian Institute of Statistics (ISTAT) in 2009.

2.5. Reduction by dithiothreitol

Hemolyzed blood samples were incubated with 10 mM dithiothreitol (DTT) for 30 min at 37 °C. After incubation, a sample of 0.1 ml was analyzed for e-GST activity.

2.6. Statistical analyses

Data were processed using GraphPad Prism software and MatLab (Natick, USA). Data are reported as mean ± standard deviation.

3. Results

3.1. e-GST in healthy subjects in the Sacco river areas

The Sacco river valley is a wide territory between the two provinces of Rome and Frosinone in the center of Italy. The exploitation of the valley by companies without scruples and complaisant institutions has been going on for several decades, thus producing an environmental and social disaster without precedent. In March 2005 the emergency status was granted for this geographic area.

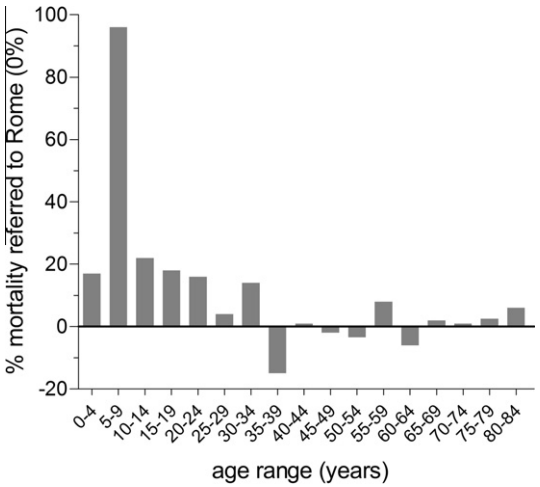

Fig. 1. Increase of mortality in the Frosinone area compared to that of the Rome area. Data are given by ISTAT 2009. The Sacco river valley is only a portion of the entire Frosinone area.

For example, environmental contamination by hexachlorocyclohexanes has been well documented [9]. Moreover, in the last few years a number of illegal dumpings have been also discovered. As reported in Fig. 1, the chemical contamination has dramatic effects on the % of mortality in infancy and in youth. Note that ISTAT data reported in Fig. 1 refer to the entire Frosinone area of which the Sacco river valley is only a portion. Other specific diseases have a relevant increase in this area, causing a drop in the quality of life [10–12]. Thus this region represents a dramatic but useful model to check the effectiveness of a novel biomarker possibly capable of detecting and assessing an environmental pollution dangerous for humans.

Eight different areas of the Sacco river valley and also the neighbouring area after the confluence of the Sacco and Liri rivers have been selected and numbered from 1 to 8. Each area displays distinct and specific geographic features and possibly different contamination risks (see Table 1). As described under Section 2, 50–80 health volunteers for each area were analyzed for their e-GST activity using the standard assay procedure [7]. The reference value of e-GST has been obtained checking 400 health volunteers living in the Rome hinterland and it results to be 5.6 ± 0.4 U/gHb. Most likely, this value cannot be considered the value for "uncontaminated" areas as even these health subjects may over-express e-GST to some extent due to the environmental pollution of the city. As reported in Fig. 2 six of the eight areas showed remarkable increased levels of e-GST when compared to the "reference" value of Rome. They reach the impressive levels of 7.6 ± 0.6 and 8.5 ± 0.6 U/gHb in the area 7 and 8, respectively (about 35–45% increase) (Fig. 2). Only just area 1 shows e-GST level similar to that of the roman area. Interestingly, areas 7 and 8 display the highest risk of pollution as the former is entirely crossed by the Sacco river and the latter, after the confluence of Sacco and Liri rivers, displays an additional source of contamination, i.e. an incineration plant (see Table 1).

3.2. Different expression of e-GST in men and women

It has been shown that healthy men display about 10% lower e-GST activity than healthy women [5]. Our data for the volunteers of Rome confirmed this difference, indicating an increase of 12% for women (5.9 ± 0.2 and 5.3 ± 0.2 U/gHb for women and men, respectively). We also confirmed previous data indicating that smoking does not perturb the expression of this enzyme [13] (data not shown). In any case smoking subjects were randomly present in all the groups of the selected area, and do not exceed 20%. Data analyzed for men and women gave surprising and interesting indications. In fact in six areas the increase of e-GST level for women (compared to men) is higher than 12%. Except for area 3, the mean of e-GST for women is 28% higher than that for men (see Fig. 2). The origin of this phenomenon could be merely due to a different intrinsic responsiveness of men and women to the environmental pollution. Alternatively, it could be the result of a different exposition time to pollutants. In fact most of the analyzed women spent much more time in the residence area than most men that work 10–12 h far from the contaminated areas. Surprisingly, subjects of area 3 display an opposite behavior. In fact, while the women

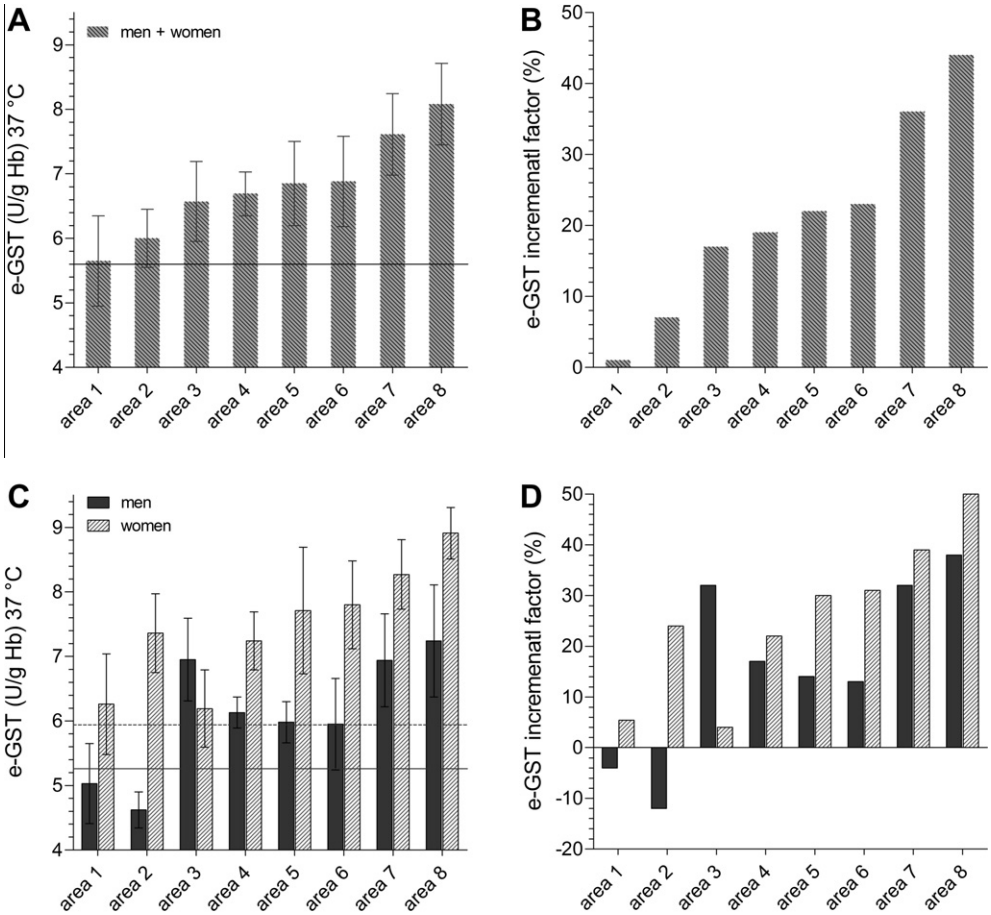

Fig. 2. e-GST activity in the Sacco river valley and its relative increase compared to the Rome area. (A) Cumulative (men + women) e-GST activity. Data are reported as units (U_{GST}) per gram of Hb. Continuous line is the reference value for the Rome area. (B) e-GST increase in the Sacco river valley referred to the Rome area taken as 0%. (C) As in (A) but with the distinct data for men and women. Continuous line is the e-GST activity found in men of the Rome area. Dotted line is the e-GST activity of women in the Rome area. (D) As in (B) with distinct data for men and women.

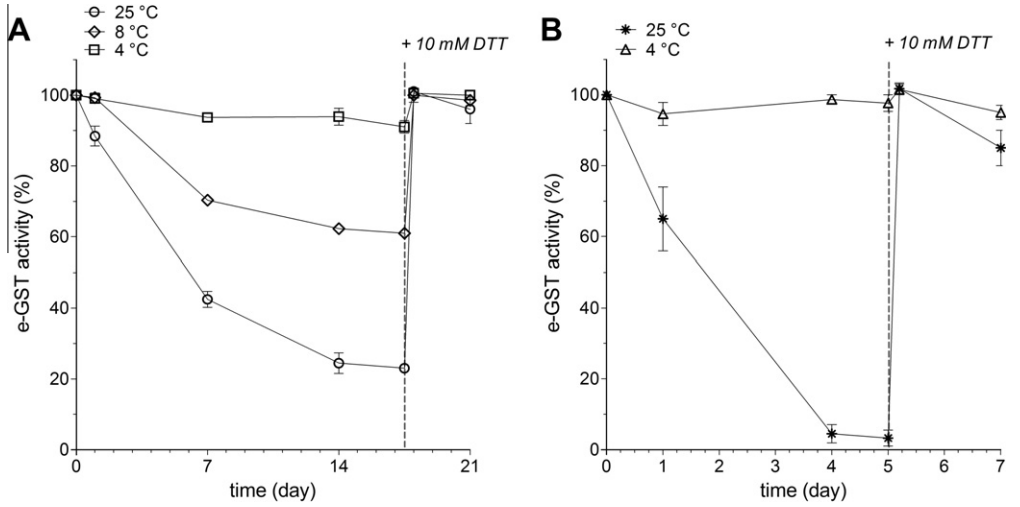

Fig. 3. Oxidative inactivation of e-GST under different storage conditions. (A) Stability of e-GST in not hemolyzed blood samples stored at different temperatures. After 17 days each sample was reactivated with 10 mM DTT (20–30 min, 37 °C). (B) As in (A) but with blood samples hemolyzed in bi-distilled water (dilution 1:25). Values reported in the figure are the mean of three independent experiments on at least 10 blood samples ± standard deviation.

show a normal e-GST profile, all the analyzed men show very high GST levels (Fig. 2). A more careful investigation revealed that most of the screened men work in the same industrial district far from the Sacco area.

3.3. e-GST activity is not affected by possible redox inactivation

It has been found that workers exposed to 1,3-butadiene, a well known oxidizing compound also present as contaminant in the air of a few industrial areas, display lower e-GST activity probably due to oxidative stress [14]. Human e-GST is characterized by the presence of two crucial cysteine residues places near the active site. Cys-47 and Cys-101 can be easily oxidized and yield an intrasubunit disulphide bridge that inactivate completely the enzymatic activity [15]. This oxidative event can be observed when intact erythrocytes are stored for days at 25 °C or, by following a faster kinetics, when erythrocytes were lysed. This inactivation is completely reversed using 10 mM DTT for 30 min at 37 °C (Fig. 3). However, at 4 °C e-GST is particularly stable both in the entire blood and in the hemolyzed blood (Fig. 3). We explored the possibility that the e-GST of the subjects living in these areas could be partially oxidized as a consequence of oxidative stress due to toxins, and thus our analysis underestimated to some extent. For this purpose, we randomly treated both normal and hyper expressing e-GST samples with 10 mM DTT for 30 min as described in Section 2 and no difference has been observed. In conclusion we have no evidence that our analysis may have been affected by intracellular oxidative events. In addition, recovery experiments made with human blood implemented with recombinant GSTP1-1 before the standard analysis confirmed the absence of activity modifications due to unknown inhibitors (not shown).

4. Discussion

This study indicates that a relevant increase of e-GST expression occurs in healthy subjects living in the Sacco river valley and also in the neighbouring territories compared to those living in Rome, suggesting that the expression of this enzyme could be related to the degree and impact of pollution on the population. Indeed, the existence of an hazardous pollution in this area is indicated by a mortality in young subjects higher than in Rome (Fig. 1).

Obviously e-GST cannot define the chemical nature of contaminants, but only reveals a defense responsiveness of the organism to a toxic chemical contamination.

In this context, e-GST reminds us of white blood cells, whose increased levels reveal the presence of a bacterial disease. The specific bacteria could be then identified and localized in the body with further microbiological analysis. Similarly, an increased level of e-GST in a specific geographic area could be followed by a more accurate analysis to assess the presence and the nature of specific contaminants. Interestingly, the assay for e-GST is very simple, not expensive, and can be performed with only a few microliters of blood [7], thus it may be employed for large epidemiological studies. One important novelty inherent e-GST is that this enzyme possibly reveals the occurrence of environmental toxins that really threaten the human health. Conversely all chemical analysis discovers dangerous chemicals in air, soil or water, without revealing the actual impact of these substances on human living systems. Indeed, the presence of dangerous compounds in a specific area does not yield necessarily a damage for humans if they are not absorbed or ingested. On the contrary, not detectable traces of a toxic compound which is actively absorbed could induce a relevant damage and likely a relevant hyper-expression of e-GST. In this context, what has been observed in area 1 and area 3 is interesting. In fact area 1 is located near the confluence of the Sacco and Liri rivers, but the "normal" value of GST seems to indicate a scarce deleterious impact on the population. Area 3 shows an anomalous pattern, i.e. a normal value of e-GST for women and a relevant hyper-expression for men. This finding seems to be caused by the fact that most of the screened men worked in the same industrial district with possible exposition to many organic solvents. Thus Area 3 must be considered similar for healthiness to the roman area even if the mean of e-GST (men + women) is high.

Although this study fulfills only a preliminary indication for the possible use of e-GST like biomarker of environmental pollution, data are encouraging and worthy to be extended to design an "healthiness map" in wider areas.

Acknowledgments

This study has been supported by a grant of "Fondazione Roma" Terzo Settore that supported the project "Erythrocyte glutathione transferase, biomarker for multiple chemical sensitivity and environmental health". Additional funds were also given by the AVIS

section of Ceprano. Its President Mr Tommaso D'Aversa provided samples from blood donors living in the Sacco river valley.

References

[1] R.N. Armstrong, Structure, catalytic mechanism, and evolution of the glutathione transferases, Chem. Res. Toxicol. 10 (1997) 2–18.
[2] W.B. Jakoby, J.H. Keen, A triple-threat in detoxification: the glutathione-S-transferase, Trends Biochem. Sci. 2 (1997) 229–231.
[3] W.B. Jakoby, The gluthatione-S-transferase: a group of multifunctional detoxification proteins, Adv. Enzymol. 46 (1978) 383–414.
[4] Y.C. Awasthi, R. Sharma, S.S. Singhal, Human glutathione S-transferase, Int. J. Biochem. 26 (1994) 295–308.
[5] F. Carmagnol, P.M. Sinet, J. Rapin, H. Jerome, Glutathione-S-transferase of human red blood cells; assay, values in normal subjects and in two pathological circumstances: hyperbilirubinemia and impaired renal function, Clin. Chim. Acta 117 (1981) 209–217.
[6] F. Galli, S. Rovidati, S. Benedetti, U. Buoncristiani, C. Covarelli, A. Floridi, F. Canestrari, Overexpression of erythrocyte glutathione-S-transferase in uremia and dialysis, Clin. Chem. 45 (1999) 1781–1788.
[7] M. Dessì, A. Noce, K.F. Dawood, F. Galli, M. Taccone-Gallucci, R. Fabrini, A. Bocedi, R. Massoud, G. Fucci, A. Pastore, S. Manca di Villahermosa, V. Zingaretti, G. Federici, G. Ricci, Erythrocyte glutathione transferase: a potential new biomarker in chronic kidney diseases which correlates with plasma homocysteine, Amino Acids 43 (2012) 347–354.
[8] V. Pompi, L. Donnarumma, S. Rosati, E. Conte, Levels of hexachlorocyclohexanes in agricultural environment of Sacco river valley, Commun. Agric. Appl. Biol. Sci. 75 (2010) 183–190.
[9] M. Lo Bello, A. Battistoni, A.P. Mazzetti, P.G. Board, M. Muramatsu, G. Federici, G. Ricci, Site-directed Mutagenesis of Human Glutathione Transferase P1-1. Spectral, kinetic and structural properties of Cys-47 and Lys-54 mutants, J. Biol. Chem. 270 (1995) 1247–1253.
[10] S. Palange, V. Ascoli, C. Carnovale-Scalzo, F. Forastiere, D. D'Ippoliti, E. Lo Presti, R. Di Domenicantonio, R. Pasetto, C.A. Perucci, Stime di incidenza del mesotelioma pleurico nel Lazio, 1997–2000, Med. Lav. 95 (2004) 45–54.
[11] E. Rapiti, F. Fantini, V. Dell'Orco, V. Fano, F. Blasetti, C. Bracci, F. Forastiere, P. Comba, Cancer mortality among chemical workers in an Italian plant, Eur. J. Epidemiol. 13 (1997) 281–285.
[12] Mortality and hospitalization of the Sacco Valley population (1997–2004) – Epidemiological relation of the Department of Epydemiology ASL Rome E.
[13] F. Kuralay, T. Yildiz, Urinary thioether excretion and erytrocyte glutathione S-transferase activities in smokers and patients with squamous-cell laryngeal cancer, J. Toxicol. Environ. Health 64 (2001) 447–452.
[14] A. Primavera, S. Fustinoni, A. Biroccio, S. Ballerini, A. Urbani, S. Bernardini, G. Federici, E. Capucci, M. Manno, M. Lo Bello, Glutathione transferases and glutathionylated hemoglobin in workers exposed to low doses of 1,3-butadiene, Cancer Epidemiol. Biomarkers Prev. 17 (2008) 3004–3012.
[15] G. Ricci, G. Del Boccio, A. Pennelli, M. Lo Bello, R. Petruzzelli, A.M. Caccuri, D. Barra, G. Federici, Redox forms of human placenta glutathione transferase, J. Biol. Chem. 266 (1991) 21409–21415.