menopausal women ($\chi^2 = 10.988$; $P = 0.0009$), whereas hormone replacement therapy in postmenopausal women did not significantly alter ferritin concentrations. Whether this is because of different dosage schedules of the estrogens used or because of less blood loss in postmenopausal women has to be further evaluated.

When subjects donated blood on a regular basis, a significantly lower ferritin concentration could be observed for men ($\chi^2 = 23.242$; $P = 0.0001$) as well as for premenopausal women (not shown). This correlates well with the results of Punnonen and Rajamäki *(23)*, who showed that 17% of Finnish women who frequently donated blood had completely lost their iron stores. Unlike the findings of Vernet and Doyen *(18)*, who found increased sTfR concentrations in males who regularly donated blood, we found that blood donation did not affect sTfR concentrations. This may be caused by compensation of the chronic blood loss by mobilizing iron from storage pools. These findings suggest that serum sTfR concentrations will only be increased when erythropoiesis becomes deprived of iron, whereas a decrease in serum ferritin will reflect changes over a broad range of body iron stores. In premenopausal women, we found significantly higher sTfR concentrations compared with postmenopausal women ($\chi^2 = 6.5076$; $P = 0.0107$), which is in contrast with the earlier findings of Allen et al. *(11)*. In our opinion, this difference may be a result of the more rigid exclusion criteria or the low number of postmenopausal women involved in their study.

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Determination of Blood Total, Reduced, and Oxidized Glutathione in Pediatric Subjects, *Anna Pastore,[1*] Fiorella Piemonte,[2] Mattia Locatelli,[3] Anna Lo Russo,[1] Laura Maria Gaeta,[2] Giulia Tozzi,[2] and Giorgio Federici[1]* ([1] Laboratory of Biochemistry, [2] Molecular Medicine Unit, and [3] Scientific Directorate, Children's Hospital and Research Institute "Bambino Gesù", Piazza S. Onofrio, 4, 00165 Rome, Italy; * author for correspondence: fax 39-0620902270, e-mail apastore@opbg.net)

Glutathione (L-γ-glutamyl-L-cysteinylglycine), which is present in virtually all mammalian tissues, provides reducing capacity for several reactions and plays an important role in detoxification of hydrogen peroxide, other peroxides, and free radicals *(1)*.

The synthesis and degradation of glutathione are controlled by reactions of the γ-glutamyl cycle; a decrease in blood reduced glutathione (GSH) has been reported in patients affected by deficiencies of the enzymes involved in the synthesis of glutathione *(1)*.

In cells, total glutathione can be free or bound to proteins; measurement of free glutathione in blood samples is essential for evaluation of the redox and detoxification status of cells in relation to its protective role against oxidative and free radical-mediated cell injury; moreover, GSH measurement is important for the diagnosis of γ-glutamyl cycle disorders.

Recently, several methods to measure glutathione in blood have been described, but little is known about the concentrations of various forms of blood glutathione in pediatric subjects *(2–6)*. We report a rapid and fully automated HPLC method for determining total (tGSH), reduced (GSH), and oxidized glutathione (GSSG) in

whole blood. Moreover, we report values for the different forms of blood glutathione in pediatric subjects.

The reference sample group included 227 healthy, randomly selected children who attended the outpatient clinic of our hospital. The children were selected to provide approximately equal numbers of each sex across the age range of 0–15 years. The study was performed according to the recommendations of the Ethics Committee of Children's Hospital and Research Institute "Bambino Gesù".

Blood was collected into a Vacutainer Tube (Becton Dickinson) containing EDTA. The hemoglobin (Hb) concentration was determined with a hemocytometer, and 100 μL of the blood was immediately mixed with 12 μL of 10 mmol/L phosphate buffer, pH 7.2 (for tGSH and GSH) or with 12 μL of 0.1 mol/L *N*-ethylmaleimide (NEM; for GSSG). A 100-μL aliquot of each mixture was hemolyzed by adding 900 μL of ice-cold distilled water; 100 μL of each hemolyzed sample was then deproteinized by adding 200 μL of sulfosalicylic acid (120 mL/L), and the glutathione content in the acid-soluble fraction was determined.

The derivatization and chromatography procedures were performed, with minor modifications, as reported previously *(7)*. Briefly, the autosampler collected 3 μL of 4 mol/L $NaBH_4$, 2 μL of 2 mmol/L EDTA-dithiothreitol (DTT), 1 μL of 1-octanol, and 2 μL of 1.8 mol/L HCl and placed the mixture in the derivatization vial containing 10 μL of sample and 10 μL of internal standard (50 μmol/L cysteamine). After the mixture was incubated for 3 min, 10 μL of 1.5 mol/L *N*-ethylmorpholine buffer (pH 8.0), 40 μL of distilled water, and 2 μL of 25 mmol/L bromobimane were added. After an additional 3-min incubation, 4 μL of acetic acid was added, and 40 μL of this mixture was injected into the column. For GSH determinations, $NaBH_4$ was substituted with 2 mmol/L EDTA-DTT.

Known concentrations of GSH or GSSG at three different concentrations (100, 500, and 2500 μmol/L) were added to blood samples. The concentrations in samples with added glutathione were determined in five replicates, and analytical recoveries were calculated. The intraassay precision was obtained by analyzing 10 replicates of the biological samples in the same day. The interassay precision was determined by analyzing the same biological samples on 10 different days over 1 month.

The CVs for repeatability of sample measurements were 0.5% for tGSH, 7.7% for GSSG, and 1.1% for GSH. The CVs for reproducibility, determined by assaying on 10 different days, were 2.8% for tGSH, 7.9% for GSSG, and 1.8% GSH. The mean recoveries were 89–102.2% for GSH and 96.1–114% for GSSG.

Calibration curves for glutathione (5–100 μmol/L glutathione) were prepared in duplicate by diluting the stock solutions with 0.1 mol/L HCl containing 100 μmol/L DTT. The linearity of the assays was also assessed at glutathione concentrations of 0–100 μmol/L. The limit of detection for the calibrators, defined as the concentration that produces a signal-to-noise ratio >5, was ~50 nmol/L.

A linear relationship was obtained between peak area and glutathione concentration in the ranges studied in an aqueous matrix. The correlation coefficient was >0.99. The equation for the regression line (n = 5) was: $y = 0.5x + 0.26$. The lowest concentrations of the linearity studies were all above the limit of detection of 50 nmol/L. No significant matrix effect was observed when linearity studies were performed in samples diluted up to 1:32 with water containing 100 μmol/L DTT (data not shown).

In a typical HPLC-fluorescence detection chromatogram of a sample from a healthy subject (Fig. 1A), the retention time for glutathione-*S*-bimane was 4.037 min. The glutathione peak in Fig. 1A was produced by a sample containing 8.6 nmol GSH/mg of Hb. A chromatogram from a subject with suspect glutathione synthetase deficiency is shown in Fig. 1B. The glutathione peak in Fig. 1B was produced by a sample containing 0.2 nmol GSH/mg of Hb.

All statistical analyses were performed by the statistical package SPSS for Windows, Ver. 8.0.0 (SPSS). Reference intervals were calculated as recommended by IFCC *(8)*. Kolmogorov–Smirnov and Lilliefors tests for normality were performed for all data. The two-tailed nonparametric Mann–Whitney test was used for comparison between groups, and correlations were calculated as Spearman

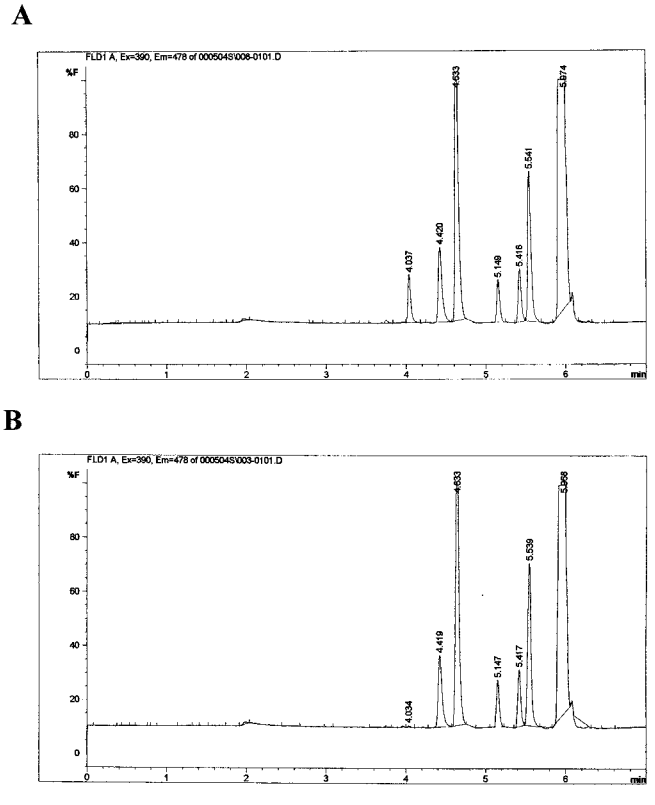

Fig. 1. Chromatograms obtained for a healthy subject (*A*) and for a patient with suspect glutathione synthetase deficiency (*B*).

Glutathione eluted at 4.0 min, and cysteamine (internal standard, 50 μmol/L) eluted at 4.4 min. Peaks eluting at 4.6, 5.5, and 5.9 min are nonspecific compounds related to bromobimane fluorescence.

Table 1. Blood concentrations[a] of tGSH, GSSG, and GSH in infants and children.

	tGSH	GSSG	GSH	GSSG/GSH	n
Male	8.34 (3.8–16.87)	0.85 (0.23–2.4)	7.07 (3.0–15.27)	0.11 (0.003–0.5)	112
Female	7.79 (4.3–22.2)	0.81 (0.24–2.1)	7.1 (2.97–21.8)	0.1 (0.0016–0.5)	115
Total	8.0 (4.2–20.3)	0.82 (0.23–2.1)	7.1 (3–19.6)	0.11 (0.0018–0.5)	227

[a] Median values are expressed as nmol/mg of hemoglobin; 95% confidence intervals of the distributions are indicated in parentheses.

correlation coefficients. $P < 0.05$ was considered statistically significant.

As shown in Table 1, tGSH, GSSG, and GSH were determined in 227 healthy donors; when subjects were distributed for age classes and sex, no significant differences in blood glutathione concentrations were found.

Several laboratory studies have suggested that GSH is a critical factor in protecting organisms against toxicity and disease. A study has recently reviewed the role of glutathione in the brain, reporting a glutathione deficiency in some neurologic disorders *(9)*. Thus, blood GSH concentrations may serve as an indicator of GSH status and disease risk in human subjects. However, no information is available on various blood GSH values within pediatric subjects. In the present work, we analyzed the different forms of glutathione in 227 healthy pediatric subjects (0–15 years).

In a large-scale screening study, Richie et al. *(3)* assayed 715 adults for tGSH and reported a value of 7.17 μmol/g of Hb, which is similar to our data for adults (8.73 ± 2.9 nmol/mg of Hb; data not shown). The authors stated that the variations in glutathione concentrations observed between age and sex groups were not significant in subjects <65 years of age. Smokers tended to have higher GSH concentrations than nonsmokers, suggesting that GSH concentrations are an adaptive response to chronic oxidative challenge. Therefore, glutathione may be a sensitive marker of the redox status in physiologic and pathologic conditions. Michelet et al. *(2)* reported blood tGSH and GSH values in 64 healthy pediatric (10–17 years) and 201 adult subjects (18–73 years) and obtained results similar to ours. When these investigators studied GSH concentrations in smokers, alcohol consumers, oral contraceptive users, and subjects who performed regular physical exercise, they concluded that blood GSH concentrations seem to be affected by some life habits, but not by sex or age.

We also analyzed GSSG in pediatric subjects and obtained a median GSSG concentration of 0.82 nmol/mg of Hb; when we analyzed NEM-treated samples (final concentration, 1.2 mmol/L), the GSSG values were similar to those obtained from the calculated differences between tGSH and GSH values. Paroni et al. *(4)* found lower blood GSSG concentrations, probably because of higher final NEM concentrations (10 mmol/L). Indeed, as reported by Mills et al. *(6)*, NEM treatment can produce artifactually low GSSG values.

In conclusion, this study is the first large-scale study reporting pediatric values of tGSH, GSH, and GSSG; our data may be useful in monitoring glutathione status in physiologic and pathologic conditions and can be applied for future therapeutic trials.

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Rapid Analysis of Mitochondrial DNA Heteroplasmy in Diabetes by Gel-Microchip Electrophoresis, *András Guttman,[1*] Hong-Guang Gao,[2] and Richard Haas[2]* ([1] Torrey Mesa Research Institute, 3115 Merryfield Row, La Jolla, CA 92121; [2] Department of Neurosciences, University of California San Diego and the UCSD Mitochondrial Disease Laboratory, CTF C103, 214 Dickinson Street, San Diego, CA 92103-8467; * author for correspondence: fax 858-812-1097, e-mail andras.guttman@syngenta.com)

Gel-microchip electrophoresis is a novel combination of the well-established methods of slab-gel electrophoresis and capillary-gel electrophoresis *(1)*. The gel-microchip format provides a multilane separation platform (a plurality of virtual channels up to 96 lanes) with excellent heat dissipation characteristics, allowing application of high voltages necessary to obtain rapid and high-performance analysis of DNA fragments. The system readily accommodates fluorescent labeling during the electrophoresis separation process (in migratio), such as intercalation with ethidium bromide or complexation with other novel, high-sensitivity DNA staining dyes, in addition to the use of conventional covalently labeled primers (i.e., before the separation process). Sample injection onto the gel microchip is accomplished by membrane-mediated loading technology *(2)*, which also enables robotic spotting of multiple samples. The method is readily applicable