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Evaluation of a urinary multi-parameter biomarker set for oxidative stress in children, adolescents and young adults

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Abstract
The involvement of reactive oxygen species (ROS) and oxidative stress in pediatric diseases is an important concern, but oxidative stress status in healthy young subjects and appropriate methods for its measurement remain unclear. This study evaluated a comprehensive set of urinary biomarkers for oxidative stress in healthy children, adolescents and young adults. Results show that urinary excretion of acrolein–lysine, 8-hydroxy-2′-deoxyguanosine (8-OHdG), nitrite/nitrate and pentosidine were highest in the youngest subjects and decreased to constant levels by early adolescence. Urinary acrolein–lysine, 8-OHdG, nitrite/nitrate and pentosidine showed significant inverse correlations with age, but pyrraline did not change significantly with age. No significant differences in biomarkers were apparent between males and females. Younger subjects grow rapidly and sustain immune activation, and are probably exposed to high concentrations of ROS and nitric oxide. Consequently, they are more vulnerable to oxidation of lipids, proteins, DNA and carbohydrates. Normal reported values in this study are a basis for future studies of disease mechanisms involving oxidative stress and for future trials using antioxidant therapies for oxidative stress-related diseases in the pediatric field.

Keywords: *Biomarkers, oxidative stress, urine, young subjects*

Abbreviations: *AD, atopic dermatitis; AGE, advanced glycation endproduct; NO, nitric oxide; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; ROS, reactive oxygen species*

Introduction

Reactive oxygen species (ROS) are generated as by-products of cellular metabolism, primarily in mitochondria. Small (physiological) amounts of ROS are a cellular requirement because they are involved in signaling pathways and in the defense against invading pathogens. However, because ROS can induce considerable damage, cells possess many antioxidant systems for scavenging or otherwise eliminating them. Under physiological conditions, a well-managed balance exists between formation and neutralization of ROS by these systems. Oxidative stress can occur when ROS production is accelerated or when the mechanisms involved in maintaining the normal reductive cellular *milieu* are impaired. Oxidative stress is likely to be associated with various pathological phenomena including aging, atherosclerosis, hypertension, renal failure, immune alterations, neurodegeneration, reperfusion injury, radiation damage,

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carcinogenesis and many other inflammatory and degenerative conditions [1,2].

All types of biomolecules can be damaged by ROS. Oxidative damage to lipids, proteins, nucleic acids and carbohydrates can be deleterious and concomitant [1]. The primary cellular target of oxidative stress depends on the cell type, the nature of the stress imposed, the site of generation, the proximity of ROS to a specific target, and the stress severity. Direct measurement of ROS *in vivo* is difficult because the half-lives of ROS are usually short. So-called "oxidative stress biomarkers" are often measured using stable adducts that are produced as a result of the oxidative processes *in vivo* [3–7]. These include malondialdehyde–lysine, 4-hydroxy-2-nonenal-lysine, acrolein–lysine (markers of lipid peroxidation and oxidative protein damage), 8-hydroxy-2′-deoxyguanosine (8-OHdG) (a marker of oxidative DNA damage), carboxymethyl-lysine, pentosidine (markers of glyco-oxidation), nitrite/nitrate (a marker of nitric oxide (NO) production), nitrotyrosine (a marker of nitro-oxidation) and so forth. Measurement of these specific biomarkers in body fluids or breath condensate enables repeated monitoring of the oxidative stress status *in vivo*, which is otherwise not possible with invasive tests.

Circumstantial evidence that ROS and oxidative damage are involved in various diseases in young people has recently stimulated much interest and concern. These diseases include sepsis, meningitis, encephalitis, brain injury, HIV infection, Kawasaki disease, bronchial asthma, atopic dermatitis (AD), chronic tonsillitis, chronic arthritis, hypertension, renal failure, hyperthyroidism, diabetes mellitus, obesity, malnutrition, non-alcoholic fatty liver disease, Wilson disease, leukemia, sickle cell anemia, muscular dystrophy, mitochondrial disorder, epilepsy, psychiatric disorder, Down syndrome, and premature-birth-related diseases [8–34]. Oxidative stress might also contribute to tissue damage induced by certain drugs [35–37]. Despite this fact, the oxidative stress levels of healthy children and adolescents have never been characterized comprehensively. Normal values for oxidative stress biomarkers remain limited [38–42].

Such limitations in the literature led us to evaluate oxidative stress levels in healthy young subjects using a set of biomarkers reflecting oxidative damage to lipids, proteins, DNA and carbohydrates. In the present study, we determined age-related changes of urinary excretion of acrolein–lysine, 8-OHdG, nitrite/nitrate, pentosidine and pyrraline in healthy young subjects. Herein, we measured two types of advanced glycation endproducts (AGEs), i.e. pentosidine (a marker of oxidative glycation) and pyrraline (a marker of non-oxidative glycation), to estimate glycation and oxidation separately [25,43].

Materials and methods

Subjects and procedures

Subjects of this study were 100 healthy Japanese subjects of a broad age range (age, 9.3 ± 4.9 (mean ± SD) years; range, 1.5–21.0 years) including children, adolescents and young adults. They included 50 males and 50 females. No significant difference in age was found between males (age, 8.7 ± 4.3 years; range, 1.5–20.7 years) and females (age, 9.9 ± 5.4 years; range, 1.5–21.0 years) by unpaired *t*-test ($p = 0.21$). All subjects were non-smokers. None of them suffered from any acute illness or chronic condition at the time of study or were taking any medication. Their dietary intake of nutrients was sufficient and considered to be representative of healthy young populations in Japan. The study protocol conformed to the guidelines of the Helsinki Conference for research on human subjects. The nature and purpose of the study were explained to the subjects and their parents. Informed consent was obtained from individuals or parents prior to enrollment.

Early-morning void urine samples were obtained from each subject. The samples were centrifuged, and the supernatants were stored at −20°C until analyses, which were all performed in duplicate. The examiners were blinded to the clinical and laboratory results.

Laboratory tests

The concentrations of acrolein–lysine and 8-OHdG were determined in all subjects using competitive enzyme-linked immunosorbent assay kits (ACR-Lysine Adduct ELISA; NOF Corp., Tokyo, Japan [7,23,25]; 8-OHdG Check, Institute for the Control of Aging, Shizuoka, Japan [7,11,23,25], respectively). The former ELISA kit uses a monoclonal antibody, designated as mAb5F6, which is specific for the acrolein–lysine adduct, N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine [3,44]. The 8-OHdG ELISA uses a monoclonal antibody, designated as N45.1 [1,45]. N45.1 has the following characteristics: it recognizes both the hydroxy (keto) function of 8-hydroxyguanine and the 2′ portion of deoxyribose; and it is scarcely reactive with other DNA adducts and thus highly specific for 8-OHdG. The urine samples were diluted with Tris–HCl buffer (pH 7.4) prior to ELISA detection. These two assays showed similar tolerance to the changes of pH (from 5 to 10) in human urine. The plates were read at 450 nm with a microplate reader. The determination range was 2–50 nmol/ml for acrolein–lysine and 0.5–200 ng/ml for 8-OHdG. Nitrite/nitrate was measured in all subjects using colorimetric, non-enzymatic assay (Bioxytech Nitric Oxide Non-Enzymatic Assay; Oxis International Inc., Portland, OR, USA) [11,23]. Pentosidine and pyrraline were determined in 96 subjects (50 males and 46

females) using high-performance liquid chromatography with fluorometric detection, as detailed previously [25,43]. All urinary markers were expressed relative to urinary Cr concentration, which was measured enzymatically using the Creatinine HR-II Test kit (Wako Pure Chemical Industries Ltd, Osaka, Japan). Assay variances of all methods described above were $<10\%$.

Statistical analyses

Data are presented as mean ± SD and range. Comparisons between groups were validated using unpaired *t*-test or ANOVA using Scheffe's method as appropriate. Correlations between variables were assessed by linear regression. Statistical significance was inferred for $p < 0.05$.

Results

The levels of urinary acrolein–lysine, 8-OHdG, nitrite/nitrate, pentosidine and pyrraline in healthy young subjects are provided in Table I. No significant differences were found between males and females in any oxidative stress parameters.

The levels of urinary acrolein–lysine, 8-OHdG, nitrite/nitrate and pentosidine demonstrated significant inverse correlations with age ($r = -0.54, -0.66, -0.43, -0.56$, respectively; $p < 0.001$ in all) (Figures 1 and 2). More specifically, these urinary biomarkers were highest in the youngest subjects and decreased through aging to reach constant levels by early adolescence. The levels of these four parameters correlated significantly with each other (acrolein–lysine vs. 8-OHdG: $r = 0.53$, $p < 0.001$; acrolein–lysine vs. nitrite/nitrate: $r = 0.29$, $p < 0.005$; acrolein–lysine vs. pentosidine: $r = 0.42$, $p < 0.001$; 8-OHdG vs. nitrite/nitrate: $r = 0.22$, $p < 0.05$; 8-OHdG vs. pentosidine: $r = 0.33$, $p < 0.005$; nitrite/nitrate vs. pentosidine: $r = 0.44$, $p < 0.001$). In contrast, the levels of pyrraline did not correlate significantly with age in the subjects ($r = -0.19$).

We classified our subjects into the following four groups to verify the influence of age on the oxidative stress parameters: 1–6 years ($n = 33$; 19 males and 14 females), 6–11 years ($n = 34$; 11 males and 23 females), 11–16 years ($n = 20$; 10 males and 10 females), and 16–21 years ($n = 13$; 10 males and 3 females) (Table II). The levels of urinary acrolein–lysine, 8-OHdG, nitrite/nitrate and pentosidine in the youngest age group (1–6 years) were significantly higher than those for the respective parameters in the older age groups.

Discussion

Overloads of ROS that exceed the capacity of antioxidant systems induce oxidative stress in the body [1,2]. Increased production of ROS is thought to occur more frequently than diminished antioxidant defense and has been postulated to play a pivotal role in the pathogenesis of various diseases and aging. In clinical practice, therefore, estimation of the degree of oxidative damage by appropriate techniques appears to be a useful pursuit.

Oxidative tissue injury from pathological conditions might have more serious consequences in young people (especially children) than in older people because of the need for subsequent tissue growth to match somatic growth and because survival is longer in young people than in older people. Primary and secondary prevention against oxidative damage might therefore be important, especially in young people. Furthermore, the use of antioxidants has presented new therapeutic perspectives for diseases that are related to enhancement of oxidative stress. We are feeling that it is time to pursue intensive research on oxidative stress in pediatric patients with a wide range of diseases.

Investigation of the role of oxidative stress in pediatric diseases requires information about the oxidative stress status of young populations. However, only a few reports on oxidative stress status exist in healthy children and adolescents. The studied subjects in those studies are small in number or fit a narrow age range [38–42]. In most studies, blood was collected from the subjects for analyses of reduced/oxidized glutathione, glutathione peroxidase and glutathione

Table I. Urinary levels of acrolein–lysine, 8-OHdG, nitrite/nitrate, pentosidine and pyrraline in 100 healthy young people.

	Total ($n = 100$)	Male ($n = 50$)	Female ($n = 50$)
Acrolein–lysine (nmol/mg Cr)	168 ± 69 (62–428)	160 ± 65 (62–428)	175 ± 72 (65–319)
8-OHdG (ng/mg Cr)	13.3 ± 5.2 (4.6–27.2)	13.1 ± 4.8 (4.6–22.8)	13.5 ± 5.7 (5.0–27.2)
Nitrite/nitrate (μmol/mg Cr)	2.55 ± 1.55 (0.56–7.57) ($n = 96$)	2.79 ± 1.67 (0.75–7.57) ($n = 50$)	2.30 ± 1.39 (0.56–7.02) ($n = 46$)
Pentosidine (pmol/mg Cr)	26.4 ± 9.8 (8.0–64.1)	27.4 ± 10.8 (15.4–64.1)	25.3 ± 8.5 (8.0–53.1)
Pyrraline (nmol/mg Cr)	34.9 ± 27.1 (7.7–152)	33.7 ± 22.7 (7.7–108)	36.3 ± 31.4 (7.8–152)

Data are presented as mean ± SD and range. No significant differences are present between males and females in any urinary oxidative stress parameters (unpaired *t*-test).

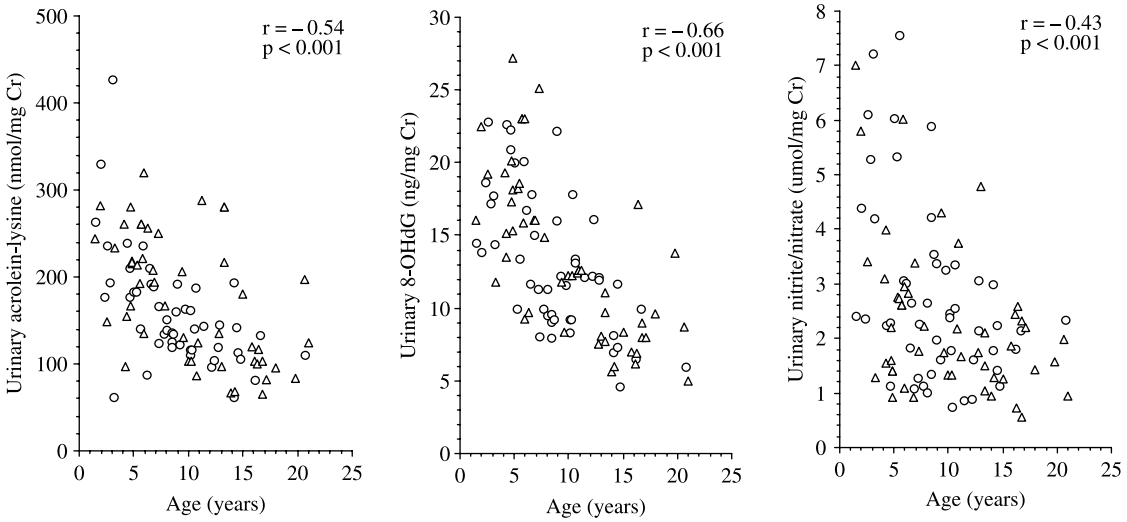

Figure 1. Age-related changes of urinary levels of acrolein–lysine, 8-OHdG and nitrite/nitrate. Circular and triangular symbols respectively indicate males and females. When both data are combined, urinary levels of acrolein–lysine, 8-OHdG and nitrite/nitrate show significant inverse correlations with age ($r = -0.54$, -0.66, -0.43, respectively; $p < 0.001$ in all).

reductase activities, selenium [38], antioxidant vitamins [39], reduced/oxidized coenzyme Q10 [40], thiobarbituric acid reactive substances and superoxide dismutase and catalase activities [42]. Other studies used small groups of healthy children and adolescents as age- and sex-matched controls, rather than the focus of those studies [8–13,15–18,20–25,28–31,34]. Kauffman et al. [39] determined urinary levels of F2-isoprostanes in 342 healthy children under 7 years old. To our knowledge, this is the only study that evaluates oxidative stress in a large population of healthy children using urine samples. Therefore, normal values for urinary biomarkers of oxidative stress are still lacking in young people.

Urine collection is simple, quick, comfortable, non-invasive, and therefore particularly easy to perform in children. We chose urine as the sample of choice in the present study. Collection of spot urine samples is much more feasible than 24-hour collection and standardization by Cr excretion corrects for variation in water intake. Early-morning urines were analyzed. Therefore, we assume that the urinary levels determined by us reflected the stable condition of the subjects.

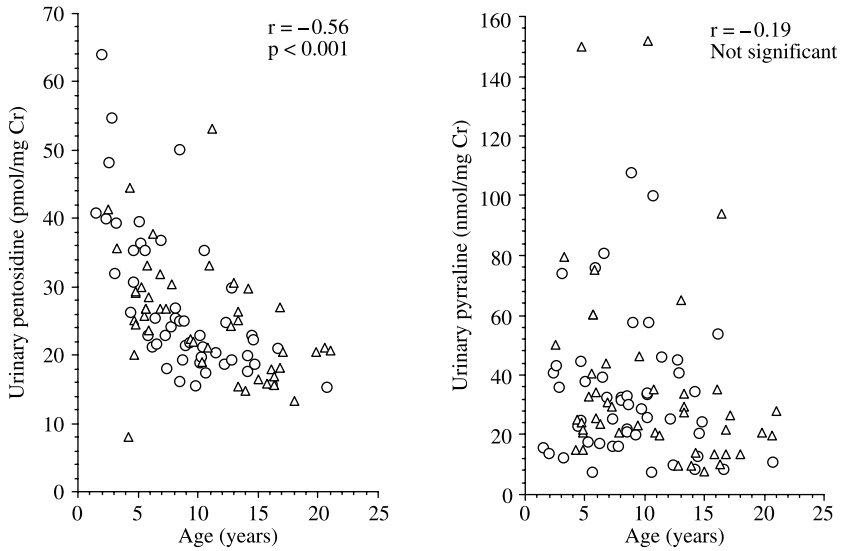

Figure 2. Age-related changes of urinary levels of pentosidine and pyrraline. Circular and triangular symbols respectively indicate males and females. Urinary levels of pentosidine show significant inverse correlation with age when both data are combined ($r = -0.56$; $p < 0.001$). Urinary levels of pyrraline show no significant correlation with age ($r = -0.19$).

Table II. Age-related changes of urinary levels of acrolein–lysine, 8-OHdG, nitrite/nitrate, pentosidine and pyrraline.

	1–6 years ($n = 33$)	6–11 years ($n = 34$)	11–16 years ($n = 20$)	16–21 years ($n = 13$)
Acrolein–lysine	218 ± 70⋆	153 ± 43	148 ± 71	107 ± 33
(nmol/mg Cr)	(62–428)	(86–256)	(62–288)	(65–197)
8-OHdG	18.0 ± 4.1⋆	12.7 ± 4.0†	9.3 ± 3.0	8.8 ± 3.4
(ng/mg Cr)	(9.2–27.2)	(8.0–25.1)	(4.6–16.1)	(5.0–17.1)
Nitrite/nitrate	3.46 ± 1.98‡	2.39 ± 1.15	1.82 ± 0.93	1.78 ± 0.68
(μmol/mg Cr)	(0.93–7.57)	(0.75–5.89)	(0.88–4.78)	(0.56–2.60)
	($n = 30$)	($n = 33$)	($n = 20$)	($n = 13$)
Pentosidine	33.3 ± 10.9¶	25.0 ± 7.3	23.4 ± 8.6	18.8 ± 3.6
(pmol/mg Cr)	(8.0–64.1)	(15.6–50.1)	(14.7–53.1)	(13.3–27.0)
Pyrraline	40.0 ± 29.4	39.4 ± 30.3	25.0 ± 15.6	27.4 ± 23.5
(nmol/mg Cr)	(7.7–150)	(7.7–152)	(7.8–65.3)	(8.6–93.9)

Data are presented as mean ± SD and range. Statistically significant inter-group differences (ANOVA using Scheffe's method): ⋆ $p < 0.001$ vs. any of the older age groups; † $p < 0.05$ vs. any of the older age groups; ‡ $p < 0.05$ vs. any of the older age groups; ¶ $p < 0.005$ vs. any of the older age groups.

Acrolein (CH_2═CH–CHO) is a major lipid peroxidation product with cytotoxic and mutagenic activities. Acrolein–lysine is used as a sensitive marker of lipid peroxidation and oxidative protein damage [3,7,23,25]. Among the base modifications induced by ROS, 8-OHdG is an abundant oxidative DNA product. Many studies have measured 8-OHdG as a sensitive marker of oxidative DNA damage [1,7,11–13,18,23,25,33]. NO may act as a pro-oxidant as well as an antioxidant, depending on the degree, site and timing of its generation [6,37]. Specifically, superoxide (O_2^-) toxicity is attributable to formation of peroxynitrite ($ONOO^-$) via its reaction with NO. Nitrite/nitrate is used as a marker for endogenous NO formation [6,11,16,21,23]. Oxidative stress is usually involved in AGE formation, and AGEs induce oxidative stress. Pentosidine is a major marker of oxidative glycation, whereas pyrraline is a marker of pure glycation [25,43]. Recently, we examined whether oxidative stress and AGE production were augmented in young patients with type 1 diabetes [25]. Urinary levels of 8-OHdG, acrolein–lysine and pentosidine, but not pyrraline, were significantly higher in diabetic patients than in age-matched control subjects. For the patient group, urinary 8-OHdG, acrolein–lysine and pentosidine, but not pyrraline, correlated significantly with urinary albumin excretion. These results show that enhanced oxidative damage to lipids, proteins, DNA and carbohydrates are linked to early renal complication in young patients with type 1 diabetes.

Our present study demonstrated that the urinary biomarkers for oxidative stress, i.e. acrolein–lysine, 8-OHdG, nitrite/nitrate and pentosidine, were highest in the youngest subjects and decreased through aging to reach constant levels by early adolescence, although individual alterations were present. The levels of urinary 8-OHdG, acrolein–lysine, nitrite/nitrate and pentosidine showed significant inverse correlations with age. These four parameters correlated significantly with each other. In contrast, the levels of pyrraline did not change significantly through aging in the subjects. The mechanisms for the high levels of urinary oxidative stress biomarkers in younger subjects remain to be clarified. Younger subjects, who grow rapidly and sustain immune activation, are likely to be exposed to high concentrations of ROS and NO and are therefore more vulnerable to oxidation of lipids, proteins, DNA, and carbohydrates. This interpretation of the above data can be defended by the lack of age-related changes of a non-oxidative glycation marker, pyrraline [25,43]. Kauffman et al. [39] also studied healthy children and found that urinary F2-isoprostane levels were highest in infancy and decreased until 7 years old. Of interest, the reduced/oxidized ratios of glutathione and coenzyme Q10 were reported to be higher in children when compared to adults [38,40]. Glutathione and coenzyme Q10 might function as protective antioxidants in young people. These results probably represent physiological changes associated with normal aging, although we still need a better understanding of oxidative processes in children and adolescents. No significant differences in the biomarkers were apparent between males and females in this study. Therefore, we should devote attention to the age of the subjects when interpreting data of urinary oxidative stress biomarkers, i.e. acrolein–lysine, 8-OHdG, nitrite/nitrate and pentosidine, in young people. We should also evaluate whether potential changes in oxidative stress status are attributable to disease progression or merely an effect of aging *per se*. The wide range of individual alterations might have resulted from genetic or environmental differences. Research is necessary to understand the critical relationship between oxidative stress on the body and genetic or environmental factors.

Oxidative damage might take place in a selective manner. For instance, lipid peroxidation is not always accompanied by oxidative DNA damage, and the accumulation of the damage depends on the combined effectiveness of the antioxidant and repair systems. It must be emphasized that the detection of more than one marker for oxidative stress is a key because a single marker might give misleading results. It might also be crucial to determine which particular markers, alone or in combination with others, can serve as a true indicator of the contribution of oxidative stress to the disease, thereby allowing the success (or the failure) of the treatment to be monitored. A good example is shown in the following. We examined the involvement of oxidative stress and antioxidant defense in children with acute exacerbation of AD [23]. Urinary levels of 8-OHdG and acrolein–lysine, but not nitrite/nitrate, were significantly higher in AD children on admission than those in control subjects. Response to treatment was associated with significant decreases in levels of 8-OHdG and acrolein–lysine from the day of admission to the 7th–9th hospital day. However, urinary levels of acrolein–lysine, but not 8-OHdG, were still significantly higher in AD children on the 7th–9th hospital day relative to the control. Urinary bilirubin oxidative metabolites (a marker of heme oxygenase activity under oxidative stress) remained almost constant and significantly high in AD children during hospitalization. These findings might indicate that the antioxidant and repair systems were able to eliminate the increased levels of 8-OHdG more efficiently than those of acrolein–lysine in these patients.

A number of clinical trials involve the administration of antioxidants in the pediatric field. Therapeutic antioxidant strategies reported previously are the following: melatonin for neonatal asphyxia [46] and for epilepsy [47]; vitamins E and C for endothelial dysfunction in hyperlipidemia [48]; L-arginine for endothelial dysfunction in cardiac transplantation [49]; amifostine for total body irradiation [50] and for anticancer drug use [51]; vitamin E and coenzyme Q10 for Friedreich ataxia [52]; angiotensin II type-1 receptor antagonist for endothelial dysfunction in diabetes mellitus [53]. The authors found some favorable effects of the above antioxidant strategies. However, these results should be interpreted cautiously and confirmed with studies that have been conducted with more numerous patients and with other techniques to measure oxidative stress status because these studies analyzed samples using only a few parameters from only a few subjects. Large-scale, prospective, controlled clinical trials using a multiparameter set of oxidative stress biomarkers are necessary to establish both the efficacy and safety of antioxidant strategies in clinical practice.

In summary, we identified age-related changes of urinary oxidative stress biomarkers in young people. The normal values reported in this study might be useful in subsequent comparisons evaluating oxidative stress progression in pediatric diseases. Means of reducing oxidative stress (e.g. antioxidant supplementation) must be investigated when oxidative stress is deemed to be important to the clinical outcome of certain diseases. These non-invasive parameters might also be useful in supporting future antioxidant therapies that will prevent disease progression and improve clinical outcomes in pediatric patients with oxidative stress-related diseases.

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