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TITLE PAGE

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ABSTRACT

Urinary biomarkers of oxidative stress have been little studied in adults with Down syndrome (DS), usually no more than two biomarkers have been measured in the population studied and controversial results are reported in literature. Thus, we aimed to assess a set of oxidative and nitrosative stress biomarkers in urine samples of adolescents and adults with DS, with and without hypothyroidism, which comprise: 8-hydroxy-2'-deoxyguanosine (8-OHdG), isoprostane 15-F_{2t}-IsoP, thiobarbituric acid-reacting substances (TBARS), advanced glycation end products (AGEs), dityrosine (diTyr), hydrogen peroxide (H₂O₂) and nitrite/nitrate (NOx). Fluorimetric and spectrophotometric assays were performed in DS (*n*=78), some of them taking levothyroxine for hypothyroidism (*n*=24), and in their healthy age-matched controls (*n*=65).

We found that levels of AGEs, diTyr, H₂O₂ and NOx are increased in DS patients in any or in all age groups, whereas Cr levels were lower in DS than in controls in all age groups. Besides, correlations with age in DS were positive for diTyr and negative for Cr, TBARS, 15-F_{2t}-IsoP and NOx. We also found lower levels of Cr from 15 to 19 years, higher levels of TBARS and AGEs from 20 to 40 years and higher levels of diTyr from 15 to 40 years in DS patients receiving levothyroxine than in DS without hypothyroidism diagnosed.

We conclude that AGEs, diTyr, H₂O₂ and NOx could be used as oxidative stress biomarkers in DS in contrast to 8-OHdG, 15-F_{2t}-IsoP and TBARS, at least with the methods used. However, renal impairment could occur in DS and Cr adjustment may bias the results, particularly in hypothyroid patients.

Research highlights

- Urinary AGEs, diTyr, H₂O₂ and NOx could be used as oxidative stress biomarkers in DS.
- Urinary levels of 8-OHdG, 15-F_{2t}-IsoP and TBARS are not altered in DS.

- Correlations with age were positive for diTyr and negative for Cr, TBARS, 15-F_{2t}-IsoP and NOx in DS.
- Renal impairment could occur in DS and Cr adjustment may bias the results.
- Renal impairment due to hypothyroidism may bias the results in DS.

Keywords: Down syndrome; oxidative stress; nitrosative stress; biomarker; hypothyroidism; urine.

1. Introduction

Down syndrome (DS)¹, caused by the triplication of chromosome 21 (HSA21) or part of it, is found to be one of the more complex genetic conditions compatible with a large survival. However, individuals with DS suffer from accelerate aging, being DS considered as a progeroid syndrome, which may be the result of an increased oxidative stress observed in these patients. The trisomy, affecting more than 300 genes, is associated with a variety of manifestations, including pathologies which are possibly related to aging such as Alzheimer disease,

¹

Abbreviations

8-OHdG, 8-hydroxy-2'-deoxyguanosine; 15-F_{2t}-IsoP, isoprostane; AFU, arbitrary fluorescence units; AGEs, advanced glycation end-products; BHT, butylated hydroxytoluene; Cr, creatinine; DiTyr, dityrosine; DS, Down syndrome; ELISA, enzyme-linked immunosorbent assay; FOX-2, xylenol orange version-2; H₂O₂, hydrogen peroxide; HCl, hydrochloric acid; HPLC, high-performance liquid chromatography; HSA21, chromosome 21; JaiCA, Japan Institute for the Control of Aging; MDA, 1,1,3,3-tetraethoxypropane; NO, nitric oxide; NOx, total nitrite and nitrate; NS, not significant; SOD1, Cu/Zn superoxide dismutase; TBA, 2-thiobarbituric acid; TBARS, thiobarbituric acid-reacting substances; VCl₃, vanadium(III) chloride.

cataracts, leukemia, diabetes mellitus, hypogonadism, vascular disease, amyloidosis and premature graying or loss of hair.

The amount of studies reporting a number of dysfunctions associating oxidative stress with DS phenotype is increasing. An excess of the enzyme Cu/Zn superoxide dismutase (SOD1) activity has been considered as the main responsible of the increased oxidative stress found in patients with DS [1]. Besides, several abnormalities in mitochondrial function have been found in DS and also in mouse models of this pathology. Indeed, in addition to SOD1, there are at least 15 genes or predicted genes on HSA21 with a role in mitochondrial energy generation and reactive oxygen species metabolism [2].

There are considerable studies providing evidence about the free radical involvement in the pathogenesis of DS based on biomarkers of oxidative stress measurements [3-5], which are stable adducts that are produced as a result of the oxidative processes *in vivo*. However, urinary biomarkers of oxidative stress have been little studied in this condition, the set of markers measured in each work is very limited and studies are normally performed in a reduced age range. In fact, most works are performed only in children, being the biomarkers of oxidative stress poorly studied in DS adults or in the elderly stage.

The use of urine samples provides many advantages over plasma or serum in population studies as their collection does not require any invasive technique. Since it is particularly difficult to obtain reliable 24 hour urine samples and sampling is often incomplete (which may bias the results), spot urine samples could be employed and compliance is high. Moreover, urine is the preferred body fluid for the measurement of isoprostanes [6]. For all these reasons, we chose urine as the specimen of choice in this study.

In order to assess the reliability of a set of urinary biomarkers of oxidative and nitrosative stress in DS, we studied the urinary levels of: 8-hydroxy-2'-deoxyguanosine (8-OHdG), isoprostane 15-F_{2t}-IsoP (also 8-*epi*-PGF_{2α}, 8-*iso*-PGF_{2α} or iPF_{2α}-III), thiobarbituric acid-reacting substances (TBARS), advanced glycation end products (AGEs), dityrosine (diTyr), hydrogen peroxide (H₂O₂) and nitrite/nitrate (NOx) in a sample of adolescents, adults and senescent subjects with DS and in those of healthy age-matched controls. Moreover, we compare these parameters in DS treated with levothyroxine with DS without thyroid dysfunction diagnosed to find out whether hypothyroidism is related to oxidative stress in DS.

2. Materials and methods

2.1. Subjects

The study was performed in 78 patients with DS and 65 healthy controls. Clinical characteristics of participants are shown in Table 1. Three age groups were established: from 15 to 19 years (age group 1), from 20 to 40 years (age group 2) and >40 years (age group 3). These groups represent respectively: early adulthood, adulthood and senescence (based on DS individuals according to the classification of Bittles et al. [7]). Participants filled in a comprehensive questionnaire to obtain the following information: a) sociodemographic parameters: age and gender; b) use of drugs or dietary supplements; c) exercise habits; and d) smoking habits. All of them were non-smokers and received no dietary or medicinal vitamin supplementation. Subjects were not affected by diabetes mellitus, myeloproliferative disorders, any type of dementia such as Alzheimer disease, malnutrition or treated with drug possibly interfering

with oxidative metabolism, being 24 subjects with DS taking levothyroxine (synthetic T₄ hormone) for hypothyroidism treatment. Informed consent was obtained from the participants or was given by parents. The study was approved by The Ethics Committee of the Spanish National Research Council.

2.2. Urine sample collection and preparation

First morning urine samples on an empty stomach were collected in a sterile flask covered with aluminium foil to keep out stray light and processed within 2 hours of the collection. Samples were 1 mL-aliquoted and frozen at -80 °C until analysis. Creatinine (Cr) determination was performed the day of collection, without previous freezing of the sample.

2.3. Biochemical determinations

2.3.1. Creatinine

Cr was determined according to the spectrophotometric Jaffé method [8] which is based on the reaction of Cr with picric acid in alkaline pH, described by Varley and Gowenlock [9].

2.3.2. 8-hydroxy-2'-deoxyguanosine (8-OHdG)

Urinary 8-OHdG was determined by a competitive enzyme-linked immunosorbent assay (ELISA) kit from the Japan Institute for the Control of Aging (JalCA, Shizuoka, Japan) according to the manufacturer's instruction. Opaque samples were centrifuged at 4000 g for 10 min. Incubations at 37 °C and absorbance measurements were carried out using a microplate reader (ELx808, Bio-Tek Instruments, Winooski, VT, USA).

2.3.3. Urinary isoprostane (15-F_{2t}-IsoP)

Free isoprostane 15-F_{2t}-IsoP was analyzed using a commercially ELISA kit (Oxford Biomedical Research, Oxford, MI, USA). The procedure was performed following the manufacturer's instruction. Urine samples were diluted 1:4 in the buffer supplied and a microplate reader (ELx808, Bio-Tek Instruments, Winooski, VT, USA) with a 450 nm filter was used for spectrophotometric measurements.

2.3.4. Thiobarbituric acid-reacting substances (TBARS)

TBARS were measured by the method of Uchiyama and Mihara [10] with modifications. Briefly, 33 μ L of 0.01% BHT (in absolute ethanol), 1 mL of 1% phosphoric acid and 300 μ L of 42 mmol/L TBA (dissolved in water and heating) were added to 140 μ L of urine, mixing in vortex. The mixtures were incubated in boiling water for 45 min and, after cooling tubes on ice, 1.4 mL of 1-butanol was added in each tube. Following a 15 min centrifugation (2000 g), the absorbance of supernatant was read at 535 nm using a UVmini-1240 Shimadzu spectrophotometer (Shimadzu, Tokyo, Japan). The standard absorption curve of MDA was prepared by dissolving it in phosphate buffer (20 mmol/L, pH 7.0).

2.3.5. Advanced glycation end-products (AGEs)

AGEs was estimated essentially as described by Yanagisawa et al. [11]. Fluorescence intensity of urine diluted 20-fold in phosphate buffered saline was measured at 440 nm after excitation at 370 nm, using a Varioskan Flash

microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) operating at room temperature. Results were reported as arbitrary fluorescence units (AFU).

2.3.6. *Dityrosine (diTyr)*

For diTyr measurement the assay was performed essentially as described by Witko-Sarsat et al. [12]. In brief, urine was diluted 20-fold in 50 mmol/L phosphate buffer, pH 7.4, containing 6 mol/L urea. Fluorescence intensity was measured at excitation and emission wavelengths of 315 and 410 nm respectively, using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) operating at room temperature, after 30 min at room temperature. Results were reported as AFU.

2.3.7. *Hydrogen peroxide (H_2O_2)*

H_2O_2 was measured using the ferrous ion oxidation xylénol orange version-2 (FOX-2) method of Banerjee et al. [13]. In brief, 90 μ L of urine was mixed with 10 μ L of methanol and 900 μ L of FOX-2 reagent (100 μ mol/L xylénol orange, 250 μ mol/L ammonium ferrous sulfate, 90% methanol, 4 mmol/L BHT and 25 mmol/L sulfuric acid). Tubes were vortexed and kept at room temperature for 30 min. Following a 10 min centrifugation (15000 g) absorbance of supernatant was read at 560 nm against a methanol blank using a UVmini-1240 Shimadzu spectrophotometer (Shimadzu, Tokyo, Japan). Then, the same procedure was followed except for 10 μ L of methanol that were replaced by 10 μ L of catalase solution (2200 U/mL in 25 mmol/L phosphate buffer, pH 7.0). Urinary H_2O_2 concentrations were calculated from the absorbance difference (with and without catalase) at 560 nm using a standard curve prepared with H_2O_2 .

2.3.8. Total nitrate and nitrite (NO_x)

Total nitrate and nitrite concentration (NO_x) was measured by the acidic Griess reaction according to the method of Miranda et al. [14]. In brief, 100 µL of sample were applied to a microplate well. Following the addition of 100 µL VCl₃ (8 mg/mL in 1 mol/L HCl) to each well 100 µL of the Griess reagent (premixed 50 µL sulfanilamide (2% in 5% HCl) and 50 µL N-(1-Naphthyl) ethylenediamine dihydrochloride (0.1%)) were added immediately. Microplate was then incubated at 37 °C for 30 min and absorbance was read at 540 nm using a microplate reader (ELx808, Bio-Tek Instruments, Winooski, VT, USA). Nitrate was determined from a linear standard curve established with sodium nitrate. Nitrite was measured in a similar manner except for the fact that samples and nitrite standards were only exposed to Griess reagent.

2.4. Statistical analysis

Data are presented as mean ± standard deviation (SD) and range. Normal distribution and homogeneity of variance of the data were tested by the Shapiro-Wilk's and Levene's tests, respectively. For parametric variables Student's unpaired *t* test was used whereas Mann-Whitney U test was used for nonparametric data. Comparisons between age groups in both DS and control groups were performed by one-way analysis of variance (ANOVA) and Scheffé post-hoc test. Correlation analyses were performed using Pearson's or Spearman's correlation tests when appropriate and then linear regression analyses were carried out. Statistical significance was set at *P*<0.05. Data were

processed using IBM SPSS Statistics 18.0 software (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

In the present study, a set of urinary biomarkers of oxidative and nitrosative stress in a sample of adolescents, adults and senescent subjects with DS, which comprise: 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H₂O₂ and NO_x, has been analysed. Clinical characteristics of DS and control subjects are summarized in Table 1. No significant differences in age were observed between patients with DS and controls for any age group ($P=0.369$ for age group 1, $P=0.623$ for age group 2, $P=0.678$ for age group 3 and $P=0.117$ for all ages). When the influence of gender was analyzed in DS and control groups, no significant differences were found for any parameter in any age group ($P>0.05$ for all). Besides, when subjects with Rb translocation or mosaicism were excluded similar results were obtained.

In Table 2 are listed the urinary levels of Cr, 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H₂O₂ and NO_x in patients with DS and controls for all age groups. Table 3 and Figure 1 show correlations between these variables and age.

3.1. 8-OHdG

8-OHdG is the most commonly used biomarker of oxidative DNA damage. We did not find differences in urinary levels of 8-OHdG between DS and controls for any age group (Table 2). These results agree with those obtained by Pallardó et al. [15] and Seidl et al. [16], studying 8-OHdG levels in leukocytes and in the cerebral cortex respectively. They found no significant differences between

adults with DS and controls in accordance with our results. Moreover, we found no correlation with age neither in DS nor in controls (Table 3 and Figure 1) as it has been reported in previous works using the ELISA assay of JAlCA [17]. However, Jovanovic et al. [1] analyzed 8-OHdG by HPLC obtaining higher levels in urine samples of children with DS than controls. The results obtained in the present work, including the absence of correlation with age in DS and in controls, could be due to cross-reaction of the commercial ELISA assay of JAlCA which we used, since urea and other substances contribute to the overestimation of urinary 8-OHdG [18]. Therefore, these results should be taken with caution and more studies are necessary in order to prove the suitability of the ELISA assays used for the DNA damage assessment. Since HPLC and other accurate techniques are currently limited in population studies, novel spectrophotometric assays must be developed for the accurate determination of 8-OHdG.

3.2. Lipid peroxidation biomarkers (15-F_{2t}-IsoP and TBARS)

Levels of lipid peroxidation biomarkers, 15-F_{2t}-IsoP and TBARS, were significantly lower in DS than in controls for age group 3 (Table 2) and both biomarkers correlated negatively with age in DS (Table 3 and Figure 1). Possible explanations to these results include that altered levels of lipids and lipoproteins have been reported in DS [19, 20]. Moreover, carbonyl reductase 1, which gene (*CBR1*) is located on HSA21, protects cells against lipid peroxidation [21] and is increased in DS individuals [22]. However, our findings will require further investigation. On the other hand, serum lipid resistance to oxidation has been found significantly higher in subjects with DS compared to

controls [23] and a concomitant increase in serum uric acid was also observed by the same authors. In fact, Nyssönen et al. [24] previously reported that ascorbate and urate are the strongest determinants of plasma lipid peroxidation resistance capacity. Although in a previous study of our research group with a representative sample of the population used in this work, no significant increase in urinary uric acid levels was found in adults with DS [4], Pallardó et al. [15] found increased levels of plasmatic uric acid in a group of adolescents and adults (from 15 to 57 years) with DS. Thus, we proposed as another possible explanation for our results that negative correlations with age in DS, resulting in lower levels of both biomarkers in DS than in controls for age group 3, could be the result of an increased lipid resistance to oxidation in DS due to higher levels of uric acid in blood. It has been recently reported, however, that plasma lipid resistance to oxidation is not significantly different in DS than in non-Down subjects [25], although the correlation between uric acid and plasma lipid resistance to oxidation has not been studied in this work and further studies are clearly needed in this area.

One major problem with TBARS assay is the fact that it is performed in numerous variations making comparisons of results between laboratories difficult. Moreover, much controversy has appeared in studies concerning the specificity of TBARS towards composites other than MDA. In fact, results in DS have indicated conflicting results. In a similar manner, commercially available ELISA assays against 15-F_{2t}-IsoP have not been tested for cross reactivity with most isoeicosanoids and most of their metabolites so they are of uncertain quantitative reliability in biological fluids. Hence, although chromatographic

techniques are currently limited in population studies, they might provide better results.

3.3. AGEs

High levels of AGEs occur in diabetes, cataracts and neurodegenerative diseases, such as Alzheimer disease, and all of them occur more frequently in DS. It has been reported that oxidative stress is involved in the accelerated formation of AGEs, which contribute to lower retardation, reduced motor activity and decreased immune response, meaning an accelerated aging process [26]. AGEs also depress superoxide production by stimulated polymorphonuclear leukocytes [27] and reduce SOD1 function. Therefore, AGEs could play an important role in the oxidative status of DS patients.

In this study, we found significant differences in urinary levels of AGEs only between adolescents, from 15 to 19 years, with DS and controls (Table 2). Since differences in Cr between DS and controls are the highest in this age group, Cr adjustment could bias the results. Moreover, positive correlation between AGEs and age was found only in the control group (Table 3 and Figure 1). By all of them, urinary AGEs measured with the wide used fluorescence assay of Yanagisawa et al. [11] do not seem to be a suitable biomarker of oxidative stress in DS. Pallardó et al. [15] measured glyoxal and methylglyoxal levels in plasma samples of DS patients and found higher levels of glyoxal in DS children than in controls and no significant differences in adolescents and adults group. Opposite to glyoxal, plasma levels of methylglyoxal were significantly lower in adolescents and adults with DS than controls and no significant differences were reported in children. Thus, different types of AGEs

instead of the overall AGEs of a sample must be analyzed with more specific techniques in DS patients, in a wider age range and comparing plasma and urine samples in order to clarify the usefulness of a specific AGE as a biomarker of oxidative stress in DS.

3.4. DiTyr

DiTyr, also *o,o'*-dityrosine, is a fluorescent molecule formed as a result of normal posttranslational processes affecting specific structural proteins. Tyrosine is oxidized to diTyr in response to oxidative stress by peroxidase-catalyzed mechanisms, being found in a number of structural (lysozyme, calmodulin, myoglobin, and haemoglobin) and non-structural proteins (insulin, RNase, and chymotrypsin) [28]. Moreover, tyrosine dimerization as well as nitration can be affected by ONOO^- (a powerful oxidant and nitrating agent), which can be formed by the reaction between active NO metabolites and superoxide [29]. Therefore, diTyr could be considered as a biomarker of both oxidative and nitrosative stress. In fact, it has been used as an important biomarker for oxidatively modified proteins during UV and γ -irradiation, aging, and exposure to oxygen free radicals, nitrogen dioxide, peroxynitrite, and lipid hydroperoxides [30], and has been implicated in a variety of physiologic and pathologic processes. However, urinary levels of diTyr have been previously measured neither in adolescents nor in adults with DS.

Our results showed significantly higher levels of this biomarker in DS patients than in controls for all age groups. Moreover, positive correlation with age was found in DS and in controls from 20 years old (Table 3 and Figure 1). Therefore, urinary diTyr could be considered as biomarker of oxidative stress in

adolescents and adults with DS and the methodology for its determination is fast, simple and inexpensive to perform.

3.5. H_2O_2

Urinary H_2O_2 was postulated to be a biomarker of oxidative stress [31]. An excess of H_2O_2 is expected in DS patients due to overexpression of SOD1, however urinary levels of this biomolecule have been previously measured neither in adolescents nor in adults with DS. Our results demonstrate that DS patients excreted more H_2O_2 in urine than healthy controls aged from 15 to 40 years. However, we found a large interindividual variations in urine H_2O_2 concentrations, similar to what occurred in previous works [32], and it may bias the results. Thus, a lack of correlation between H_2O_2 and age was found in both DS and control groups (Table 3). Moreover, no significant differences in H_2O_2 levels have been found between both groups in age group 3 (Table 2) probably due to the large biological variation, which limits the usefulness of urinary H_2O_2 as a biomarker of oxidative stress. In addition, it has been reported that drinking coffee, exercise and salt loading affect H_2O_2 excretion [31]. Therefore, urinary levels of H_2O_2 could be more determined by metabolic and nutritional influences than by changes in oxidative stress. In summary, our results suggest that urinary H_2O_2 could be a suitable biomarker of oxidative stress in DS, as it has also been proposed for malignancy [33], although more studies are needed to explore the causes of its biological variation.

3.6. Nitrite and nitrate (NO_x)

Nitric oxide (NO) is a free radical gas which has been involved in a wide range of physiological functions, including the modulation of renal hemodynamics and excretory function [34]. Since NO rapidly degrades to nitrite and nitrate (NO_x), which are relatively slowly excreted in urine, urinary levels of NO_x were estimated as an index of NO production. Although neurodegeneration in DS has been associated with aberrant expression of nitric oxide synthase 3 gene [35] and nitrosative stress is linked to oxidative stress, biomarkers of nitrosative stress have been little studied in DS.

In the present work we found higher levels of NO_x in adolescents and adults (from 15 to 40 years) with DS than in the age-matched controls (Table 2). Besides, correlation of NO_x with age was found negative in DS from 14 years and positive in controls from 20 years (Table 3). It has been reported that the tendency of urinary nitrate levels to decrease with age in healthy children may be a reflection of an age-related increase in glomerular filtration rate [36]. Therefore, glomerular filtration rate could be impaired in DS as it is also shown by the differences in Cr levels (Table 2), being urinary levels of NO_x in DS more influenced by urinary excretion than by oxidative status contrary to what was observed in controls.

It must be taken into account that active NO metabolites can react with superoxide to form peroxynitrite (ONOO⁻) a powerful oxidant and nitrating agent. Tyrosine dimerization as well as nitration can be affected by ONOO⁻, so the same oxidant could be responsible for the accumulation of both 3-NO₂-Tyr (3-nitrotyrosine) and diTyr [29]. Therefore, although NO_x is a widely used

biomarker of nitrosative stress, 3-nitrotyrosine and diTyr would have been better indicators of nitrosative damage.

3.7. Analysis of hypothyroidism in DS

Hypothyroidism is common in adults with DS [37], being the frequency of patients with DS treated with levothyroxine in the present work of 31%. A decrease in free radical production is expected in hypothyroidism due to the metabolic suppression brought about by the decreased levels of thyroid hormone, however data are controversial and several works reported increased oxidative stress in hypothyroidism [38]. In the present study, patients with DS receiving levothyroxine for hypothyroidism treatment showed increased levels of TBARS and AGEs for age group 2 and increased levels of diTyr for age groups 1 and 2 than those without hypothyroidism diagnosed (Table 4). However, impaired renal function has been reported in patients with hypothyroidism [39]. It enhances serum Cr levels because it reduces glomerular filtration rate and increases production of Cr. Our results showed lower levels of urinary Cr in DS patients receiving levothyroxine than DS without hypothyroidism diagnosed for age group 1, being levels in the rest of age groups slightly decreased in DS patients receiving levothyroxine although without significant differences (Table 4). Therefore, renal impairment due to hypothyroidism may bias the results in these patients.

Signs and symptoms of hypothyroidism can be difficult to discriminate from those found in the natural course of DS itself. Moreover, untreated subclinical hypothyroidism is present in DS at birth and persists throughout life [40]. Some abnormalities reported in DS are related to thyroid function: 1) decreased levels

of selenium [41] which acts as an antioxidant protecting the thyrocyte from peroxides 2) an impairment in the activity of phenylalanine hydroxylase [42], which converts the phenylalanine in tyrosine, and 3) overexpression of DYRK1A kinase [43], which could reduce the availability of tyrosine. Hence, underlying factors may influence thyroid function in DS. For all these reasons, it could be possible that a group of DS patients without thyroid dysfunction diagnosed but having untreated subclinical hypothyroidism are biasing the results, in the present study and maybe in others researching clinical aspects of DS.

3.8. Analysis of Cr adjustment

Renal impairment has been described in DS based on decreased Cr clearance [44]. Interestingly, lower levels of urinary Cr have been found in DS than in controls in all age groups (Table 2) and similar results were obtained in DS vs controls comparisons for all the variables studied when patients with hypothyroidism were excluded. Besides, negative correlation between Cr and age was found in DS patients (Table 3 and Figure 1), so a decline in urinary excretion with aging could occur in DS patients. In fact, with increasing survival, an increasing number of DS patients with renal disease are being reported [45]. In summary, Cr used for standardized urinary levels of biomarkers studied may bias the results, as it has been discussed above for all the biomarkers studied, and it could be due to hypothyroidism in DS.

3.9. Post-hoc comparisons between age groups

Post-hoc comparisons between age groups for both DS and control groups are shown in Table 2. Significant differences were found in urinary AGEs between

age groups 2 and 3 ($P=0.032$) in controls. In DS, significant differences were found in urinary 15-F_{2t}-IsoP between age groups 1 and 3 ($P=0.004$) and between age groups 2 and 3 ($P=0.042$), and also in urinary NOx between age groups 1 and 2 ($P=0.005$) and between age groups 1 and 3 ($P=0.035$). These results are in accordance with correlation results. However, we found some correlations between urinary parameters with age, but no significant differences were found in post-hoc comparisons between age groups in both DS and control groups. Further studies must be performed before age-related changes in urinary biomarkers of oxidative/nitrosative stress can be established.

3.10. Future approaches

Previous studies addressing biomarkers of oxidative stress in DS have shown conflicting results. Methodological aspects are not usually taken into account in the approach to discussion. Moreover, since oxidative conditions have been suggested to be of importance in developmental and maturational processes, biomarkers of oxidative stress may need to be differently interpreted in childhood and adulthood. Nonetheless, we can find several works studying them in disease states, including DS, in which age groups are formed by both children and adolescents or even children and adults, so discrepancies in results are frequently found.

On the other hand, adults with DS over 40 years are under higher risk for dementia. However, the diagnosis of Alzheimer disease in people with DS is far from an easy task and oxidative damage may increase as a consequence of this disease process in addition to aging, biasing the results. Improvements in this area are required before the causes of increased oxidative stress in senescent DS subjects can be well established.

DS subjects have a high prevalence to have or develop hypothyroidism. In our study, we found clinically important changes in hypothyroid DS patients taking levothyroxine compared to those without hypothyroidism diagnosed, suggesting that medical treatment does not normalize alterations caused by thyroid dysfunction in these subjects. Hence, thyroid hormones levels must be assessed together with oxidative stress biomarkers in future works, even more when DS subjects can suffer from untreated subclinical hypothyroidism.

In our study, we obtained *P*-values higher than the conventional cut-off of 0.05 but close to this value in several of the between-group comparisons. Such data are *suggestive* of an effect. Thus, there could be some results of clinical relevance, although no statistical differences have been obtained (type II error). Moreover, high variability in urinary levels of all biomarkers was obtained. Sample size and variability are important primarily because of its effect on statistical power. Hence, statistical power may be limited not only in subgroup analyses, but also by the statistical variability. Therefore, to assess urinary biomarkers of oxidative/nitrosative stress in a large number of adolescent and/or adult individuals with DS must be a major goal in future works.

Individuals with DS could suffer from renal impairment, leading to a diminished excretion of metabolites such as oxidative/nitrosative stress biomarkers. Therefore, a comparative study assessing a comprehensive set of these biomarkers in both urine and plasma or serum samples must be performed in DS to clarify this hypothesis.

Finally, potential biasing factors, such as dietary or exercise habits, exposure to pollutants, etc., must be considered in future works studying oxidative/nitrosative stress biomarkers in DS. In fact, in our study most

individuals with DS practised moderate physical activity weekly. Therefore, improvements in life patterns of DS individuals could contribute to reduce oxidative stress, biasing the results.

In summary, similar studies in a larger number of DS subjects, in which thyroid dysfunctions have been described and performed in various biological matrices, such as serum, plasma and urine, and where potential biasing factors, such as dietary or exercise habits, exposure to pollutants, etc., are considered, would help to clarify the role of oxidative stress in DS phenotype and subsequently devising intervention strategies which improve the quality of life of these patients.

4. CONCLUSION

This work shows that AGEs, diTyr, H_2O_2 and NOx could be used as biomarkers of oxidative/nitrosative stress in urine samples of adolescents and adults with DS in contrast to 8-OHdG, 15-F_{2t}-IsoP and TBARS, at least with the methods described in the present study. However, intraindividual variability was high for most of the variables studied, mainly in DS patients. Besides, the present work shows evidence of a probable renal impairment in DS and Cr adjustment may bias the results, particularly in hypothyroid patients.

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Figure captions

Figure 1. Scatter plots of Cr, 8-OHdG/Cr, 15-F_{2t}-IsoP/Cr, TBARS/Cr, AGEs/Cr, diTyr/Cr, H₂O₂/Cr and NOx/Cr vs. age in Down syndrome (DS) and controls (C) in the age groups 2 and 3 (from 20 to 59 years). Correlation lines are shown for DS (●, solid lines) and C (□, dashed lines). Individuals with DS receiving levothyroxine for hypothyroidism treatment are identified in each graph as HT.

Table 1. Clinical characteristics of patients.

	Controls	Down syndrome
Age*		
All ages (15-59 years)	32.1±13.1, <i>n</i> = 65	28.8±12.4, <i>n</i> = 78
Age group 1 (15-19 years)	17.3±1.5, <i>n</i> = 15	17.0±1.4, <i>n</i> = 29
Age group 2 (20-40 years)	28.7±5.4, <i>n</i> = 31	29.6±5.9, <i>n</i> = 33
Age group 3 (41-59 years)	49.5±6.5, <i>n</i> = 19	48.5±4.6, <i>n</i> = 16
Sex (male/female)	26/39	35/43
Hypothyroidism	0	24
Karyotype		
Primary trisomy		72
Robertsonian translocation trisomy		5
der(14 ;21)(q10 ;q10)		
Mosaicism (normal cellular line 94%)		1

*Data are mean±SD and number of subjects (*n*).

Table 2. Urinary levels of Cr and biomarkers of oxidative and nitrosative stress (8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H₂O₂ and NOx) in patients with Down syndrome (DS) and their age-matched controls. The data are expressed as mean \pm standard deviation (SD) and ranges.

Urinary biomarker	Controls			DS			P
	n	Mean \pm SD	Range	n	Mean \pm SD	Range	
Cr (mg/dL)							
All ages (15-59 years)	65	193 \pm 79	37-470	78	127 \pm 63***	25-298	<0.001 ^b
Age group 1 (15-19 years)	15	198 \pm 63	92-334	29	119 \pm 55***	25-276	<0.001 ^a
Age group 2 (20-40 years)	31	206 \pm 86	91-470	33	140 \pm 65**	34-298	0.001 ^a
Age group 3 (>40 years)	19	169 \pm 78	37-308	16	115 \pm 72*	27-290	0.045 ^a
8-OHdG (ng/mg Cr)							
All ages (15-59 years)	45	8.3 \pm 3.5	2.1-17.7	77	8.9 \pm 3.5	2.1-24.5	0.181 ^b
Age group 1 (15-19 years)	10	8.4 \pm 2.2	4.6-12.8	29	9.2 \pm 3.2	4.9-19.0	0.623 ^b
Age group 2 (20-40 years)	23	8.8 \pm 4.1	2.1-17.7	33	8.8 \pm 3.9	2.8-24.5	0.809 ^b
Age group 3 (>40 years)	12	7.2 \pm 3.1	4.3-15.4	15	8.3 \pm 2.9	2.1-12.7	0.152 ^b
15-F_{2t}-IsoP (ng/mg Cr)							
All ages (15-59 years)	44	0.93 \pm 0.44	0.21-2.18	78	0.94 \pm 0.57	0.19-2.98	0.924 ^a
Age group 1 (15-19 years)	10	0.78 \pm 0.48	0.28-1.46	29	1.14 \pm 0.65	0.26-2.98	0.127 ^b
Age group 2 (20-40 years)	21	1.00 \pm 0.40	0.21-1.88	33	0.93 \pm 0.49	0.19-2.35	0.608 ^a
Age group 3 (>40 years)	13	0.98 \pm 0.46	0.41-2.18	16	0.57 \pm 0.39*,†,‡	0.22-1.61	0.016 ^a
TBARS (μg/mg Cr)							
All ages (15-59 years)	65	0.77 \pm 0.32	0.00-1.71	78	0.73 \pm 0.35	0.00-2.36	0.457 ^b
Age group 1 (15-19 years)	15	0.85 \pm 0.37	0.46-1.71	29	0.81 \pm 0.41	0.14-2.36	0.990 ^b
Age group 2 (20-40 years)	31	0.70 \pm 0.26	0.30-1.37	33	0.72 \pm 0.26	0.00-1.41	0.861 ^a
Age group 3 (>40 years)	19	0.80 \pm 0.35	0.00-1.59	16	0.60 \pm 0.36*	0.08-1.58	0.029 ^b
AGEs (AFU/mg Cr)							
All ages (15-59 years)	61	134 \pm 47	76-359	75	150 \pm 53	64-324	0.063 ^b
Age group 1 (15-19 years)	14	118 \pm 33	76-188	29	143 \pm 38*	64-234	0.034 ^b
Age group 2 (20-40 years)	30	123 \pm 25	81-175	31	143 \pm 51	86-276	0.215 ^b
Age group 3 (>40 years)	17	166 \pm 68 [‡]	105-359	15	178 \pm 73	99-324	0.653 ^b
DiTyr (AFU/mg Cr)							
All ages (15-59 years)	61	494 \pm 149	249-1200	75	794 \pm 526***	165-3877	<0.001 ^b
Age group 1 (15-19 years)	14	512 \pm 133	258-731	29	816 \pm 479**	165-2473	0.004 ^b
Age group 2 (20-40 years)	30	450 \pm 106	302-659	31	745 \pm 630***	259-3877	<0.001 ^b
Age group 3 (>40 years)	17	559 \pm 205	249-1200	15	850 \pm 377**	349-1858	0.012 ^b
H₂O₂ (μg/mg Cr)							
All ages (15-59 years)	58	0.39 \pm 0.40	0.00-1.71	72	0.74 \pm 0.69***	0.00-3.14	<0.001 ^b
Age group 1 (15-19 years)	14	0.59 \pm 0.55	0.09-1.71	29	0.91 \pm 0.71	0.22-3.14	0.042 ^b
Age group 2 (20-40 years)	29	0.33 \pm 0.31	0.05-1.56	31	0.51 \pm 0.41	0.00-1.39	0.046 ^b
Age group 3 (>40 years)	15	0.33 \pm 0.35	0.00-1.20	12	0.89 \pm 1.03	0.01-2.78	0.152 ^b
NOx (μg/mg Cr)							
All ages (15-59 years)	64	46 \pm 34	14-186	74	81 \pm 79***	18-503	<0.001 ^b
Age group 1 (15-19 years)	15	50 \pm 36	21-158	29	121 \pm 112**	28-503	0.001 ^b
Age group 2 (20-40 years)	31	38 \pm 24	14-121	31	55 \pm 27**,*†	18-116	0.005 ^b
Age group 3 (>40 years)	18	58 \pm 45	16-186	14	58 \pm 26 [‡]	26-119	0.377 ^b

^aby Student's unpaired *t* test.

^bby Mann-Whitney U test.

P*<0.05, *P*<0.01 and ****P*<0.001 (significantly different) from the control group.

†,‡Indicates *P*<0.05 (significantly different) from the age group 1 and the age group 2, respectively.

Table 3. Correlations between age and biochemical parameters in Down syndrome (DS) and their age-matched controls.

Correlated variables with age	Controls		DS	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Cr				
All ages (14-59 years)	-0.207	0.098 ^a	-0.093	0.418 ^a
Age groups 2 and 3 (20-59 years)	-0.265	0.063 ^a	-0.300	0.036 ^a
8-OHdG				
All ages (14-59 years)	-0.204	0.179 ^b	-0.050	0.667 ^b
Age groups 2 and 3 (20-59 years)	-0.211	0.223 ^b	-0.021	0.886 ^b
15-F_{2t}-IsoP				
All ages (14-59 years)	0.147	0.342 ^a	-0.328	0.003 ^a
Age groups 2 and 3 (20-59 years)	-0.053	0.766 ^a	-0.295	0.040 ^a
TBARS				
All ages (14-59 years)	0.113	0.368 ^b	-0.287	0.011 ^b
Age groups 2 and 3 (20-59 years)	0.286	0.044 ^b	-0.242	0.044 ^b
AGEs				
All ages (14-59 years)	0.297	0.021 ^b	0.114	0.339 ^b
Age groups 2 and 3 (20-59 years)	0.326	0.025 ^b	0.252	0.094 ^b
DiTyr				
All ages (14-59 years)	0.106	0.420 ^b	0.063	0.594 ^b
Age groups 2 and 3 (20-59 years)	0.364	0.013 ^b	0.375	0.010 ^b
H₂O₂				
All ages (14-59 years)	-0.175	0.190 ^b	-0.165	0.167 ^b
Age groups 2 and 3 (20-59 years)	0.016	0.918 ^b	0.239	0.123 ^b
NOx				
All ages (14-59 years)	0.127	0.318 ^b	-0.288	0.013 ^b
Age groups 2 and 3 (20-59 years)	0.350	0.014 ^b	0.171	0.261 ^b

^aCompared by Pearson's correlation coefficient analysis.

^bCompared by Spearman's correlation coefficient analysis.

Table 4. Urinary levels of Cr and biomarkers of oxidative and nitrosative stress (8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H₂O₂ and NOx) in patients with Down syndrome (DS) who were receiving levothyroxine for hypothyroidism and those without hypothyroidism diagnosed. The data are expressed as mean \pm standard deviation (SD) and ranges.

Urinary biomarker	DS without hypothyroidism			DS with treated hypothyroidism			P [#]
	n	Mean±SD	Range	n	Mean±SD	Range	
Cr (mg/dL)							
All ages (15-59 years)	54	138±65	27-298	24	102±51*	25-209	0.026
Age group 1 (15-19 years)	20	133±57	45-276	9	88±36*	25-164	0.038
Age group 2 (20-40 years)	22	155±66	50-298	11	112±56	34-209	0.086
Age group 3 (>40 years)	12	118±75	27-290	4	108±72	35-207	0.903
8-OHdG (ng/mg Cr)							
All ages (15-59 years)	54	8.8±3.2	2.1-19.0	23	9.0±4.1	3.0-24.5	0.781
Age group 1 (15-19 years)	20	9.8±3.6	4.9-19.0	9	8.0±1.8	5.7-11.5	0.220
Age group 2 (20-40 years)	22	8.4±2.7	2.8-14.2	11	9.7±5.7	3.0-24.5	0.819
Age group 3 (>40 years)	12	8.0±3.1	2.1-12.7	3	9.5±1.4	8.1-10.9	0.386
15-F_{2t}-IsoP (ng/mg Cr)							
All ages (15-59 years)	54	0.95±0.56	0.22-2.98	24	0.90±0.60	0.19-2.78	0.458
Age group 1 (15-19 years)	20	1.13±0.65	0.26-2.98	9	1.16±0.68	0.46-2.78	0.962
Age group 2 (20-40 years)	22	0.98±0.45	0.25-1.87	11	0.83±0.56	0.19-2.35	0.169
Age group 3 (>40 years)	12	0.60±0.44	0.22-1.61	4	0.50±0.19	0.30-0.73	0.903
TBARS (µg/mg Cr)							
All ages (15-59 years)	54	0.51±0.30	0.00-1.58	24	0.76±0.44	0.14-2.36	0.622
Age group 1 (15-19 years)	20	0.79±0.28	0.34-1.50	9	0.86±0.64	0.14-2.36	0.944
Age group 2 (20-40 years)	22	0.67±0.26	0.00-1.41	11	0.80±0.26*	0.18-1.12	0.047
Age group 3 (>40 years)	12	0.65±0.40	0.08-1.58	4	0.43±0.06	0.36-0.48	0.090
AGEs (AFU/mg Cr)							
All ages (15-59 years)	53	138±45	64-276	22	179±60**	93-324	0.003
Age group 1 (15-19 years)	20	140±39	64-205	9	152±37	116-234	0.696
Age group 2 (20-40 years)	21	129±43	86-276	10	175±53*	93-261	0.014
Age group 3 (>40 years)	12	154±55	99-270	3	267±65*	196-324	0.022
DiTyr (AFU/mg Cr)							
All ages (15-59 years)	53	721±508	165-3877	22	969±538**	309-2473	0.008
Age group 1 (15-19 years)	20	678±267	165-1208	9	1122±694*	557-2473	0.034
Age group 2 (20-40 years)	21	740±756	259-3877	10	757±231*	309-1103	0.043
Age group 3 (>40 years)	12	759±232	349-1032	3	1214±670	520-1858	0.248
H₂O₂ (µg/mg Cr)							
All ages (15-59 years)	48	0.66±0.63	0.00-3.14	24	0.89±0.78	0.07-2.78	0.239
Age group 1 (15-19 years)	20	0.82±0.71	0.22-3.14	9	1.12±0.73	0.44-2.27	0.131
Age group 2 (20-40 years)	20	0.51±0.40	0.00-1.39	11	0.52±0.45	0.07-1.36	0.967
Age group 3 (>40 years)	8	0.63±0.87	0.01-2.68	4	1.42±1.25	0.15-2.78	0.174
NOx (µg/mg Cr)							
All ages (15-59 years)	52	77±81	19-503	22	91±76	18-384	0.119
Age group 1 (15-19 years)	20	117±118	28-503	9	129±104	47-384	0.396
Age group 2 (20-40 years)	21	51±24	19-101	10	62±34	18-116	0.473
Age group 3 (>40 years)	11	54±27	26-119	3	72±23	48-93	0.186

[#]Indicates *P*-value obtained in the comparisons between DS and controls by Mann-Whitney U test.

P*<0.05 and *P*<0.01 (significantly different) from the control group.

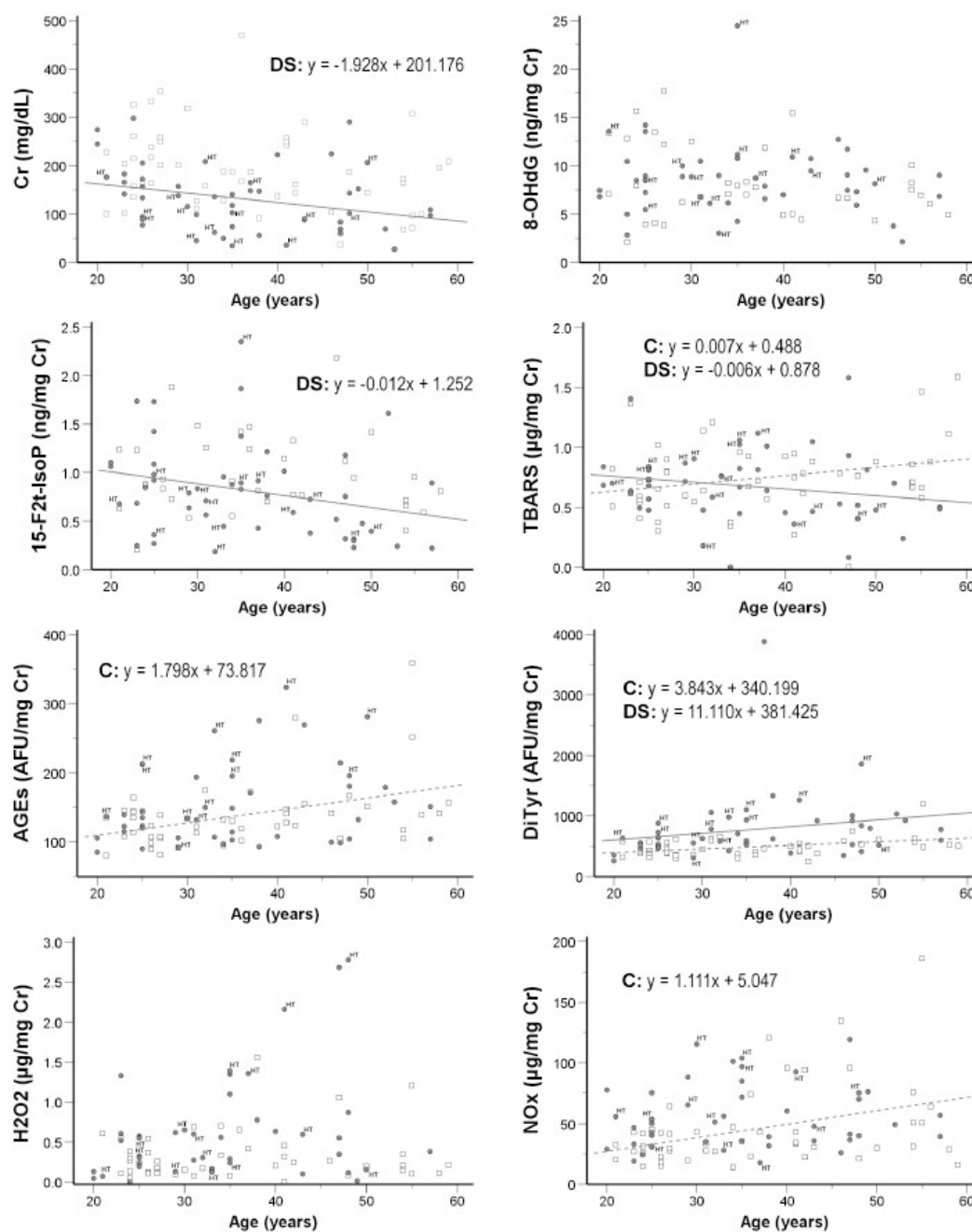


Fig. 1