

MESTRADO INTEGRADO  
MEDICINA

# ***Impact of perinatal adverse factors on oxidative stress throughout childhood and adolescence***

Ana Cristina Almeida Oliveira Rodrigues

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childhood and adolescence***

**MESTRADO INTEGRADO EM MEDICINA**

**ARTIGO ORIGINAL**

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*Impact of perinatal adverse factors on oxidative stress throughout childhood  
and adolescence*

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Às duas melhores coisas da vida: a família e os amigos que se tornam parte dela.

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Nenhum dos autores possui conflito de interesses em relação a este estudo.

## RESUMO

**Introdução/Objetivos:** O stresse oxidativo resulta de um desequilíbrio entre espécies reativas de oxigénio/azoto e os mecanismos de defesa antioxidante, estando implicado em diversas doenças crónicas. As crianças e os adolescentes são particularmente vulneráveis ao stresse oxidativo devido às elevadas exigências metabólicas associadas ao crescimento. Fatores perinatais adversos, como obesidade materna, diabetes, hipertensão, tabagismo ou parto por cesariana, têm sido associados ao aumento do stresse oxidativo no período neonatal, mas os seus efeitos a longo prazo são ainda pouco conhecidos. Este estudo teve como objetivo avaliar o impacto de fatores maternos e perinatais nos biomarcadores de stresse oxidativo durante a infância e a adolescência, numa coorte populacional de nascimento, explorando possíveis diferenças associadas ao sexo.

**Metodologia:** Foram incluídas 313 crianças da coorte Geração XXI, avaliadas aos 8-9 anos (infância), das quais 167 foram reavaliadas aos 14-16 anos (adolescência). Recolheram-se dados antropométricos e avaliaram-se biomarcadores de stresse oxidativo em ambas as idades, nomeadamente isoprostanos e peróxido de hidrogénio urinários. A avaliação aos 8-9 anos incluiu ainda a quantificação de outros marcadores de stresse oxidativo/nitrosativo (isoprostanos plasmáticos, capacidade antioxidante total plasmática, mieloperoxidase sérica e nitratos/nitritos plasmáticos e urinários). Foram utilizados modelos de regressão linear multivariada estratificados por sexo, ajustados para idade gestacional, peso ao nascimento e índice de massa corporal (IMC) à data da avaliação, para estudar as associações das variáveis de stresse oxidativo com variáveis maternas e perinatais.

**Resultados:** A obesidade materna, hipertensão, tabagismo na gravidez e diabetes associaram-se a valores mais elevados de biomarcadores de stresse oxidativo nos descendentes, com padrões distintos consoante o sexo e a idade. Na infância, os rapazes cujas mães tinham maior IMC ou história de hipertensão apresentaram concentrações aumentadas de isoprostanos plasmáticos; as raparigas nascidas por cesariana mostraram menor capacidade antioxidante plasmática. A duração do aleitamento materno associou-se a maior excreção de nitratos/nitritos urinários nos rapazes. Na adolescência, observou-se uma maior excreção de isoprostanos urinários nas raparigas expostas ao tabagismo materno e nos rapazes expostos a diabetes materna ou a parto por cesariana. Observaram-se ainda diferenças marcadas entre

sexos, com valores geralmente mais elevados nos rapazes. Os isoprostanos urinários aumentaram com a idade, sobretudo no sexo masculino.

**Discussão e Conclusões:** Este é o primeiro estudo a explorar longitudinalmente a associação entre fatores perinatais e stresse oxidativo ao longo da infância e da adolescência. Os resultados apoiam a hipótese das Origens do Desenvolvimento da Saúde e da Doença, sugerindo que exposições intrauterinas e precoces podem modular o stresse oxidativo muito para além do período neonatal. Estes achados reforçam a importância de estratégias preventivas precoces e indicam que as bases de várias doenças crónicas podem estar presentes antes do surgimento de manifestações clínicas.

**Palavras-chave:** Adolescência; Complicações da Gravidez; Cesariana; Diabetes; Gravidez; Hipertensão; Infância; Obesidade; *Stress* Oxidativo.

## ABSTRACT

**Background:** Oxidative stress results from an imbalance between reactive oxygen/nitrogen species and antioxidant defenses, and it is implicated in various chronic diseases. Children and adolescents are especially vulnerable to oxidative stress due to high metabolic demands during growth. Perinatal adverse factors, such as maternal obesity, diabetes, hypertensive disorders, smoking, or cesarean delivery, have been associated with increased oxidative stress in the early postnatal period, but less is known about their long-term effects. This study aimed to evaluate the impact of maternal and perinatal factors on oxidative stress biomarkers during childhood and adolescence, within a population-based birth cohort, and to explore sex-specific differences in these associations.

**Methods:** The study included 313 children from the Generation XXI cohort evaluated at 8-9 years (childhood) and 167 of them re-evaluated at 14-16 years (adolescence). Anthropometric data and oxidative stress markers were collected at both time points, namely urinary isoprostanes and hydrogen peroxide. At the 8-9 years evaluation, we also measured other oxidative/nitrosative stress markers (plasma isoprostanes, plasma total antioxidant status, serum myeloperoxidase and nitrate/nitrite in the plasma and urine). Multivariate linear regression models stratified by sex were used to test associations between oxidative stress variables and maternal and perinatal variables, adjusting for gestational age, birthweight, and BMI at each assessment.

**Results:** Maternal obesity, hypertension, smoking during pregnancy, and diabetes were associated with higher oxidative stress biomarkers in offspring, with distinct patterns according to the sex and developmental stage considered. In childhood, boys whose mothers had higher BMI or hypertension showed increased plasma isoprostanes; girls born by cesarean had lower antioxidant status. Breastfeeding duration was associated with higher urinary nitrate/nitrite in boys. In adolescence, urinary isoprostanes were higher in girls exposed to maternal smoking and in boys exposed to maternal diabetes or cesarean delivery. Across both ages, sex differences were evident, with boys generally presenting higher oxidative stress levels. Notably, urinary isoprostanes increased with age, particularly among boys.

**Discussion and Conclusions:** This study is the first to longitudinally explore the association between perinatal factors and oxidative stress throughout childhood and adolescence. The

findings support the Developmental Origins of Health and Disease hypothesis, suggesting that intrauterine and early-life exposures can modulate redox homeostasis well beyond the neonatal period. These results highlight the importance of early preventive strategies and suggest that perinatal exposures may program oxidative stress pathways with long-term implications for health.

**Keywords:** *Adolescence; Cesarean Delivery; Childhood; Diabetes; Gestational Complications; Hypertension; Obesity; Oxidative Stress; Pregnancy.*

## **LISTA DE ABREVIATURAS**

**BMI** – Body mass index

**G21** – Generation XXI Cohort

**HPA** – Hypothalamic-pituitary-adrenal

**MDA** – Malondialdehyde

**MPO** – Myeloperoxidase

**NO** – Nitric oxide

**Nrf2** – Nuclear factor erythroid 2-related factor 2

**P-Isop** – Plasma isoprostanes

**P-NOx** – Plasma nitrates and nitrites

**RNS** – Reactive nitrogen species

**ROS** – Reactive oxygen species

**SD** – Standard deviation

**TAS** – Total antioxidant status

**U-H<sub>2</sub>O<sub>2</sub>** – Urinary hydrogen peroxide

**U-Isop** – Urinary isoprostanes

**U-NOx** – Urinary nitrates and nitrites

## ÍNDICE

|                        |    |
|------------------------|----|
| LISTA DE TABELAS ..... | ix |
| INTRODUCTION .....     | 1  |
| METHODS .....          | 2  |
| RESULTS .....          | 6  |
| DISCUSSION .....       | 10 |
| REFERENCES .....       | 17 |

## LISTA DE TABELAS

|                                                                                                                                       |    |
|---------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Table I.</b> Maternal and neonatal characteristics.....                                                                            | 23 |
| <b>Table II.</b> Oxidative stress parameters in childhood and adolescence.....                                                        | 24 |
| <b>Table III.</b> Spearman correlations between maternal and perinatal factors and oxidative stress parameters during childhood.....  | 25 |
| <b>Table IV.</b> Spearman correlations between maternal and perinatal factors and oxidative stress parameters during adolescence..... | 26 |
| <b>Table V.</b> Associations between maternal and perinatal factors and oxidative stress parameters during childhood .....            | 27 |
| <b>Table VI.</b> Associations between maternal and perinatal factors and oxidative stress parameters during adolescence.....          | 29 |
| <b>Table VII.</b> Multivariate linear regression models for each oxidative stress parameter during childhood.....                     | 30 |
| <b>Table VIII.</b> Multivariate linear regression models for each oxidative stress parameter during adolescence.....                  | 31 |

## INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are highly active and short-lived byproducts of cellular metabolism that can interact with several macromolecules (lipids, proteins, nucleic acids), causing severe damage<sup>1</sup>. However, living organisms have developed several enzymatic and non-enzymatic antioxidant defenses to adapt to the coexistence with these reactive species. Under physiological conditions, there is a delicate balance between oxidants and antioxidants, which not only protects the body from the detrimental effects of ROS and RNS, but also allows these species to function as important cell messengers<sup>2,3</sup>. When the production of oxidants exceeds the body's antioxidant capacity, oxidative stress ensues, potentially leading to macromolecule damage and disruption of redox signaling<sup>4</sup>. Oxidative stress is implicated in the pathogenesis of multiple chronic pathologies such as cardiovascular disease, chronic obstructive pulmonary disease, chronic kidney disease, neurodegenerative diseases, and cancer<sup>5</sup>. Due to the high rate of cell division necessary for growth, oxidative stress can be particularly harmful in children<sup>6</sup>. As far as pediatric pathology is concerned, there is also evidence of oxidative stress association with cardiovascular disease, kidney disease and obesity in children and adolescents<sup>6,7</sup>.

The significance of intrauterine life in postnatal health has been recognized for many years<sup>8</sup>. In recent decades, the “Developmental Origins of Health and Disease” hypothesis has been formulated, suggesting that events occurring in the periconceptual, fetal, and early childhood stages can influence the risk of chronic diseases<sup>9</sup>. Studies consistently link extremes of birth weight with a higher risk of metabolic syndrome, type 2 diabetes mellitus, and cardiovascular disease in adulthood<sup>8</sup>. However, the mechanisms underlying these predispositions are not yet fully understood. Oxidative stress has emerged as a potential mechanism to explain these associations, since many risk factors, established or potential, such as pre-eclampsia, diabetes, and smoking, are associated with increased oxidative stress<sup>8</sup>.

The present study aims to assess the association between various maternal and perinatal factors on oxidative stress parameters throughout childhood and adolescence, within a population-based birth cohort, and to explore potential sex-specific differences in these associations.

## METHODS

### Study design and sample

We used data collected from children at two different visits – at 8-9 years and again at 14-16 years – who have been followed since birth in a previously established cohort study (Generation XXI, Porto, Portugal – G21)<sup>10</sup>.

From the original cohort (n=8647), 4590 children attended a face-to-face follow-up visit at 7 years of age including an anthropometric evaluation and blood sample withdrawal, thus being eligible for the *ObiKid* project - a specific project aiming to clarify the impact of childhood obesity and associated comorbidities on the kidney<sup>11</sup>. It has been defined a minimum sample of 300 children for the *ObiKid* project's main objective; assuming that about 35% would be excluded due to refusal to participate, exclusion criteria or incomplete information, 463 children were preselected to be consecutively screened according to the date of their 7-year-old evaluation: 16 could not be contacted, 32 refused to participate, 23 although willing to participate were unable to schedule the study visits during the recruitment period and 68 met exclusion criteria (4 chronic diseases (genetic, renal or metabolic), 1 chronic usage of medication (affecting blood pressure or glucose or lipid metabolism), 51 with residence more than 30 km away from the study site and 6 pairs of twins). Three hundred and twenty-four participants were enrolled, between August 2013 and August 2014, but for the present analysis were additionally excluded 11 children due to incomplete evaluation, such as absence of blood or urine sample for oxidative stress and nitric oxide (NO) production/metabolism markers determination. A total of 313 children were included in the present analysis.

At age 14-16, participants from the *ObiKid* project were again contacted to be part of the *ObiKid* 2.0 reevaluation. Of those, 18 declined to participate due to personal restraints, 41 contacts could not be established, 77 were unable to plan visits during the recruitment period (more frequently due to the COVID-19 pandemic). Four *ObiKid* participants were excluded from the present reevaluation for chronic usage of medication (affecting either blood pressure, lipidic metabolism or glucose). Six additional participants were excluded for not having completed the evaluation (absence of blood or urine sample for oxidative stress and NO production/metabolism markers determination). A total of 167 were finally included in the present study. Tanner staging evaluation was performed for all participants, with all included being in a pubertal stage.

## **Data collection and variable definition**

Maternal data was obtained through interviews conducted within 72 hours after delivery. Data on maternal age, total years of education and pre-existing comorbidities were collected using questionnaires at the time of enrollment. Self-reported pre-pregnancy and pre-delivery weights were used to calculate gestational weight gain. Height was measured with wall-mounted stadiometers. Pre-pregnancy body mass index (BMI) was categorized as overweight/obesity if BMI  $\geq 25$  kg/m<sup>2</sup>. Self-reported active smoking and alcohol consumption during pregnancy were dichotomized. Medical records were reviewed to identify occurrences of gestational hypertension, eclampsia, and gestational diabetes. Chronic hypertension was defined as BP  $\geq 140/90$  mmHg diagnosed before pregnancy or until 20 weeks of gestation, whereas gestational hypertension was considered as new-onset hypertension occurring after 20 weeks of gestation. Perinatal data was also extracted from clinical records. Gestational age was considered as determined by ultrasonography. Birth weight and length were registered to the nearest 1g or 0.1cm, respectively. Birth weight for gestational age was classified into sex-specific categories based on the 2013 revised Fenton growth curves<sup>12</sup>.

The follow-up study visits took place at the Public Health and Forensic Sciences and Medical Education Department, Faculty of Medicine of University of Porto. Anthropometric and general physical examination were performed, according to standard procedures and as previously reported<sup>13</sup>. Body mass index (BMI) and BMI-for-age values were classified according to the World Health Organization reference data for BMI z-score into the following categories: normal weight [ $> -2$  and  $\leq 1$  standard deviation (SD)] and overweight/obesity ( $> 1SD$ )<sup>14</sup>.

## **Laboratory procedures**

A venous blood sample was collected at both visits, 8-9 years and 14-16 years, after an overnight fast of at least 8 hours. Regarding oxidative/nitrosative stress parameters, at the first visit (8-9 years), children were analyzed for serum myeloperoxidase (S-MPO), plasma isoprostanes (P-Isop), plasma nitrates and nitrites (P-NOx) and plasma total antioxidant status (P-TAS). Additionally, a 24-hour urine sample was collected and analyzed for creatinine, urinary isoprostanes (U-Isop), urinary hydrogen peroxide (U-H<sub>2</sub>O<sub>2</sub>) and urinary nitrates and nitrites (U-NOx). At the second visit (14-16 years), so far, we have only analyzed U-Isop and U-H<sub>2</sub>O<sub>2</sub> in 24-hour urine samples of the adolescents.

All the parents received information on the correct methods of 24-hour urine collection, and, upon sample delivery, compliance was rechecked by a brief questionnaire. The samples were

considered valid if urinary creatinine was within the range of 11.3–28.0 mg/kg per day (according to age and sex specific reference values<sup>15</sup>) and if the urinary volume was over 300 mL; on the basis of these criteria, 15 and 1 urine samples were excluded from the analysis, at the childhood and adolescence evaluation, respectively. All the standard laboratory analyses were performed in the Service of Clinical Pathology of Centro Hospitalar Universitário São João, Porto, Portugal. All the plasma, serum and urine samples used for the determination of oxidative stress and NO bioavailability biomarkers were stored at -80°C until assayed. MPO and oxidative stress and NO production/metabolism markers were assessed at the Department of Biomedicine – Unit of Pharmacology and Therapeutics of the Faculty of Medicine, University of Porto, using commercial kits and following the manufacturer's specific instructions. In brief, S-MPO was quantified by an immunoenzymatic assay (BioCheck, MPO Enzyme Immunoassay Test Kit; Oxis International Inc.). P-TAS was evaluated by a spectrophotometric assay (Antioxidant Assay Kit; Cayman Chemical Company) that measures the combined antioxidant activities of water and lipid soluble antioxidants, including vitamins, glutathione, uric acid, bilirubin, albumin and others. This assay depends on the ability of the antioxidants present in the sample to inhibit the absorbance of the radical cations of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). The antioxidant capacity of the sample is compared with that of Trolox, a water-soluble tocopherol analogue, and is expressed as mM Trolox equivalents. Free isoprostanes were quantified in plasma containing the preservatives butylated hydroxytoluene (0.005%, w/v) and indomethacin (10 µM), which were added before storage. Solid-phase extraction was performed before the measurement of P-Isop by a competitive enzyme immunoassay (15-Isoprostane F2t ELISA Kit; Oxford Biomedical Research, Inc.). U-Isop were quantified by a competitive enzyme immunoassay (Urinary Isoprostane ELISA Kit; Oxford Biomedical Research, Inc.) in nonextracted urine containing butylated hydroxytoluene (0.005 %, w/v) added before storage and incubated with β-glucuronidase before the assay, as a significant amount of isoprostanes is excreted in urine conjugated with glucuronide<sup>16</sup>. Total nitrates and nitrites were evaluated in P-NOx and U-NOx by a colorimetric assay (Nitrate/Nitrite Colorimetric Assay Kit; Cayman Chemical Company). Plasma samples were ultrafiltered before assay using 30-kDa filters. Urinary excretion of H<sub>2</sub>O<sub>2</sub> was evaluated by a microplate fluorimetric assay (Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit; Molecular Probes, AlfaGene).

## **Ethics**

This study complies with the Helsinki Declaration, the guidelines for the ethical conduct of medical research involving children<sup>17</sup> and the current national legislation. The Generation XXI and

*ObiKid* study were approved by the Ethics Committee of the Unidade Local de Saúde São João, Entidade Pública Empresarial. The Instituto de Saúde Pública da Universidade do Porto Ethics Committee approved the *ObiKid* 2.0 study. Data collection was approved by the National Data Protection Commission. Furthermore, the aim of the study and all procedures were explained to the participants and their parents (or their legal representatives). Permission to collect personal information and biological samples was secured through written informed consent from one of the parents and, at the last evaluation, also from the adolescents.

### **Statistical analysis**

Statistical analysis was performed using IBM SPSS Statistics, Version 29.0. The data is presented as mean and SD or as median and 25<sup>th</sup> and 75<sup>th</sup> percentiles (P25-P75). Differences between groups in continuous variables were evaluated using Mann–Whitney U tests. Spearman's correlations were used to test bivariate associations between oxidative stress parameters and continuous maternal and perinatal variables. The analyses were stratified by sex because there was a significant interaction with oxidative stress parameters and several maternal and perinatal factors. All maternal and perinatal variables whose association with oxidative stress parameters were significant or presented a p value < 0.20 at the univariate analysis were included in the multivariate models and a backwards stepwise approach was followed to obtain a final model of maternal and perinatal determinants for each oxidative stress parameter. Additionally, all final models were adjusted for gestational age at birth (weeks), birth weight (g) and BMI class (normal vs overweight/obese) at the respective age evaluation. All models were analyzed in both sexes, regardless of whether associations and correlations were statistically significant for one of them, and p value < 0.05 was considered statistically significant.

## RESULTS

The present study included 313 children aged 8-9 years (childhood), of whom 167 were subsequently reevaluated at 14-16 years of age (adolescence).

### Maternal and neonatal characteristics

Maternal and neonatal characteristics are presented in Table I, for the entire sample and stratified by sex. Twenty-three mothers (7.5%) were diagnosed with gestational diabetes, with this condition being more frequent in pregnancies with male offspring (10.4% vs 4.1%,  $p=0.036$ ). Forty-seven mothers (15.4%) smoked during pregnancy, with a significant difference between male and female pregnancies (20.4% vs. 9.7%, in boys and girls, respectively,  $p = 0.010$ ). Also, birthweight ( $3192.3 \pm 456.2\text{g}$  vs  $3305.2 \pm 452.2\text{g}$ ,  $p=0.024$ ) and birth length ( $48.6 \pm 2.4$  vs  $49.5 \pm 2.1$ ,  $p<0.001$ ) were significantly lower in girls.

### Anthropometric data and oxidative stress parameters in childhood and in adolescence

Table II presents anthropometric data and oxidative stress parameters values in childhood and in adolescence. Mean BMI z-score values were lower in adolescence than in childhood ( $0.77 \pm 1.20$  vs.  $0.95 \pm 1.24$ ,  $p<0.001$ , respectively), as was the percentage of individuals classified as overweight/obese (41.9% vs. 47.9%,  $p<0.001$ , respectively). The mean BMI was higher in adolescent girls compared to boys ( $23.7 \pm 4.8$  vs.  $22.1 \pm 4.5 \text{ kg/m}^2$ ,  $p=0.025$ ).

Among the oxidative stress parameters evaluated in childhood, only U-H<sub>2</sub>O<sub>2</sub> values differed between sexes, with boys showing higher levels than girls ( $1551.0$  [P25-P75: 872.7-2361.6] vs.  $937.9$  [P25-P75: 456.5-1880.8] nmol/d,  $p<0.001$ ). In adolescence, both evaluated parameters differed between sexes. Adolescent boys had higher U-Isop ( $5393.5$  [P25-P75: 3879.0-7437.3] vs.  $3880.5$  [P25-P75: 2991.6-5292.7] ng/d,  $p<0.001$ ) and higher U-H<sub>2</sub>O<sub>2</sub> ( $1656.3$  [P25-P75: 906.8-3131.4] vs.  $1110.2$  [P25-P75: 464.6-2056.8] nmol/d,  $p=0.002$ ). Among the parameters evaluated at both ages, only U-Isop was significantly higher in adolescents ( $1584.1$  [P25-P75: 1199.7-2271.2] vs.  $5182.3$  [P25-P75: 3436.3-6355.6] ng/d,  $p<0.001$ ).

### **Spearman correlations of maternal and perinatal factors with oxidative stress parameters in childhood and adolescence**

Spearman correlations of maternal and perinatal factors with oxidative parameters measured at childhood and adolescence, separately for boys and girls, are presented in Table III and Table IV, respectively.

In summary, during childhood, we found that U-Isop presented a positive correlation with mother's BMI prior to pregnancy ( $\rho=0.185$ ,  $p=0.041$ ) and a negative correlation with weight gain during pregnancy ( $\rho=-0.206$ ,  $p=0.027$ ), only in girls. P-Isop presented positive correlations with birth weight ( $\rho=0.178$ ,  $p=0.041$ ) and length ( $\rho=0.188$ ,  $p=0.030$ ), in girls, and with mother's weight ( $\rho=0.238$ ,  $p=0.005$ ) and BMI ( $\rho=0.289$ ,  $p=0.001$ ) prior to pregnancy, in boys. TAS presented a positive correlation with mother's age at child's birth ( $\rho=0.192$ ,  $p=0.020$ ), only in girls. P-NOx were positively correlated with weight gain during pregnancy ( $\rho=0.195$ ,  $p=0.031$ ) and negatively with birth length ( $\rho=-0.165$ ,  $p=0.046$ ), in girls, while in boys a negative correlation with mother's age at child's birth ( $\rho=-0.170$ ,  $p=0.028$ ) and a positive correlation with total time of breastfeeding ( $\rho=0.182$ ,  $p=0.037$ ), were found. U-NOx presented positive correlations with mothers' schooling years ( $\rho=0.205$ ,  $p=0.017$ ) and time of breastfeeding, both total ( $\rho=0.195$ ,  $p=0.045$ ) and exclusive ( $\rho=0.185$ ,  $p=0.042$ ), in girls. MPO was positively correlated with mother's schooling years ( $\rho=0.193$ ,  $p=0.021$ ) and BMI prior to pregnancy ( $\rho=0.226$ ,  $p=0.010$ ), in girls.

During adolescence, U-Isop presented a positive correlation with birth weight ( $\rho=0.243$ ,  $p=0.039$ ) and with length ( $\rho=0.295$ ,  $p=0.011$ ), in girls. U-H<sub>2</sub>O<sub>2</sub> showed a positive correlation with birth weight ( $\rho=0.234$ ,  $p=0.023$ ), only in boys.

### **Comparison of oxidative/nitrosative stress parameters, within offspring of each sex, for each maternal and perinatal variables, during childhood and adolescence**

The univariate analysis performed to test the association between oxidative stress parameters and categorical maternal and perinatal variables, both during childhood and adolescence, separately for boys and girls, are presented in Table V and Table VI, respectively.

In summary, during childhood, median U-Isop values were significantly lower among boys whose mothers had an excessive weight gain during pregnancy (1737.6 [P25-P75: 1309.4-2473.4] vs. 1538.6 [P25-P75: 1207.9-2051.6] ng/d,  $p=0.045$ ) and higher among those whose mothers had gestational diabetes (1609.3 [P25-P75: 1192.0-2205.7] vs. 2518.6 [P25-P75: 1652.7-3100.2] ng/d,  $p=0.003$ ). P-Isop were also significantly higher among boys whose mothers presented overweight

or obesity prior to pregnancy (0.28 [P25-P75: 0.14-0.51] vs. 0.51 [P25-P75: 0.25-0.73] ng/ml,  $p=0.001$ ). TAS was significantly lower in boys whose mothers had a diagnosis of diabetes, either pregestational (1.24 [P25-P75: 1.02-1.67] vs. 1.13 [P25-P75: 0.55-1.18] mM Trolox equivalents,  $p=0.034$ ) and gestational (1.24 [P25-P75: 1.02-1.71] vs. 1.20 [P25-P75: 0.65-1.25] mM Trolox equivalents,  $p=0.034$ ). U-H<sub>2</sub>O<sub>2</sub> median values were lower in girls born small for gestational age (1041.5 [P25-P75: 531.7-1994.6] vs. 291.1 [P25-P75: 132.1-1110.9] nmol/d,  $p=0.006$ ), while median U-NOx values were higher in girls delivered by cesarean section (712.0 [P25-P75: 539.1-1040.1] vs. 999.4 [P25-P75: 602.3-1343.2]  $\mu$ mol/d,  $p=0.010$ ). Median MPO levels were significantly higher in boys whose mothers had gestational diabetes (58.5 [P25-P75: 35.3-89.8] vs. 85.3 [P25-P75: 76.5-106.6] ng/ml,  $p=0.001$ ) and among those born by cesarean delivery (57.4 [P25-P75: 35.3-82.0] vs. 74.0 [P25-P75: 39.2-99.2] ng/ml,  $p=0.040$ ). No significant associations were observed between P-NOx and any categorical maternal or perinatal variables.

During adolescence, median U-Isop values were higher in girls whose mothers smoked during pregnancy (3687.7 [P25-P75: 2856.1-4523.9] vs. 5190.6 [P25-P75: 4248.1-5908.4] ng/d,  $p=0.011$ ), compared to girls whose mothers did not smoke during pregnancy.

### **Multivariate linear regression models for oxidative/nitrosative stress parameters during childhood and adolescence**

Several multivariate linear regression models for each oxidative stress parameter as dependent variable, at each age, were plotted (Table VII and Table VIII).

During childhood, P-Isop were found to be higher among boys whose mothers had higher BMI (by 0.02 ng/mL [95% CI 0.01 to 0.03],  $p<0.001$ ) and a history of hypertension prior to pregnancy (by 0.26 [95% CI 0.04 to 0.47],  $p=0.02$ ). Among girls, TAS increased with maternal age at birth (by 0.02mM Trolox equivalents [95% CI 0.01 to 0.04],  $p=0.009$ ) and decreased with cesarean delivery (by 0.20 mM Trolox equivalents [95% CI -0.37 to 0.04],  $p=0.015$ ). In boys, P-NOx increased with longer breastfeeding duration (by 0.51 nmol/ml [95% CI 0.33 to 0.70],  $p<0.001$ ). Also in boys, U-NOx also increased with longer breastfeeding duration (by 13.11  $\mu$ mol/day [95% CI 1.47 to 24.74],  $p=0.028$ ). Among girls, U-NOx increased in those born by cesarean (by 282.65  $\mu$ mol/day [95% CI 6.52 to 558.78],  $p=0.045$ ) and in those whose mothers had more than 9 years of scholarly (by 327.70  $\mu$ mol/day [95% CI 71.43 to 583.70],  $p=0.013$ ). MPO levels were higher in boys whose mothers had more than 12 years of education (by 19.40 ng/ml [95% CI 2.78 to 36.02],  $p=0.023$ ) and in those born by cesarean (by 22.46 ng/ml [95% CI 5.74 to 39.18],  $p=0.009$ ). We also found that

MPO increased among girls whose mothers had higher education (by 25.03 ng/ml [95% CI 4.69 to 45.37],  $p=0.016$ ).

During adolescence, we found that U-Isop increased among girls whose mothers smoked during pregnancy (by 1859.03 ng/day [95% CI 730.46 to 2987.61],  $p=0.002$ ). In boys, U-Isop increased among those whose mothers had diabetes, both pregestational and gestational, (by 2263.05 ng/day [95% CI 122.86 to 4403.24],  $p=0.039$ , for gestational diabetes) and who were born by cesarean (by 1422.65 [95% CI 110.28 to 2735.01],  $p=0.034$ ). The strength of the association was similar for those whose mothers had pregestational diabetes.

## DISCUSSION

To our knowledge, this is the first study to examine the longitudinal associations between perinatal factors and oxidative stress biomarkers in childhood and adolescence. Several maternal and perinatal factors were found to influence oxidative stress profiles. During childhood, increased P-Isop, in boys, were associated with higher maternal BMI and hypertension prior to pregnancy, while increased TAS was associated with higher maternal age at birth, in girls. Lower TAS and higher U-NOx, in girls, and higher MPO levels, in boys, were linked to cesarean delivery. Increased P-NOx and U-NOx, in boys, were associated with longer breastfeeding. Increased MPO levels, in boys, were associated with gestational diabetes and higher maternal education. During adolescence, higher U-Isop, in girls, were associated with maternal smoking. In boys, higher U-Isop were associated with maternal diabetes and cesarean delivery.

Our study revealed sex-related differences in several of the associations explored. As expected, we observed that female newborns had lower birth weight and length than males. This finding aligns with previous studies and may be attributed to sex-specific differences in placental function, including non-random X inactivation in females, gene expression of immune pathways, microRNAs, protein expression, and steroid profile, as proposed by Clifton<sup>18</sup>. We also found that some maternal and perinatal factors impact oxidative stress parameters differently in males and females during childhood and adolescence. Sex-related disparities in oxidative stress have previously been reported in animal models. For instance, an experimental study in rats demonstrated that oxidative damage to mitochondrial DNA was four times higher in males<sup>19</sup>. In humans, sex-related differences in oxidative stress have been reported in adult populations<sup>20</sup> and are also evident at birth. According to *Díaz-Castro et al.*, male newborns have higher values of oxidative stress markers, both in maternal blood during labor and in umbilical cord blood<sup>21</sup>.

Our study showed that U-Isop were significantly higher in adolescence compared to childhood. These findings are in line with those of *Pérez-Navero et al.*, who assessed oxidative stress markers in saliva samples from 70 healthy boys – 38 prepubertal and 32 pubertal – and reported lower levels of lipid peroxidation, measured by malondialdehyde (MDA) and 4-hydroxy-2-nonenal, in the prepubertal group. Although they used saliva samples, the study supports the correlation between salivary and plasma oxidative stress markers<sup>22</sup>. Similarly, *Bono et al.* observed an overall increase in U-Isop with age. However, when comparing prepubertal and pubertal children, they found lower levels in pubertal girls, with no significant difference when sex stratification was not applied<sup>23</sup>. In our cohort, adolescent boys exhibited higher U-Isop levels compared to girls of the

same age. This difference may reflect the influence of hormonal changes during puberty. Indeed, *Bono et al.* proposed that estrogens may modulate oxidative stress, particularly during this developmental stage<sup>23</sup>.

Maternal pre-gestational overweight has been associated with increased oxidative stress in newborns, as reported by *Gallardo et al.*<sup>24</sup>. In their study conducted in Mexico, higher levels of MDA and NO were detected in the umbilical cord blood of newborns of overweight and obese mothers. In our cohort, although no association was observed between maternal BMI and P-NOx, we did find that, in boys aged 8-9 years, increased P-Isop levels were associated with higher maternal pre-pregnancy BMI. It is possible that the mechanisms leading to increased lipid peroxidation and elevated P-Isop levels are influenced by alterations in the maternal reproductive system occurring prior to conception. Elevated triglyceride levels have been detected in the follicular fluid of obese women<sup>25</sup> and lipotoxicity – characterized by lipid accumulation and loss of mitochondrial membrane potential – has been observed in their oocytes. Loss of membrane potential disrupts oxidative phosphorylation, resulting in increased production of ROS<sup>26</sup>. These findings suggest that a higher maternal BMI may promote a pro-oxidative environment in the oocyte, potentially affecting the offspring's oxidative stress profile. However, we are unable to determine whether this association persists into adolescence and adulthood, as P-Isop levels were not available at the 14-16-year follow-up.

Our study identified an association between higher P-Isop levels in boys aged 8-9 years and chronic maternal hypertension. Although we did not find previous studies directly investigating this specific association, evidence from a large cohort study has shown a link between chronic maternal hypertension and the development of hypertension in adult offspring<sup>27</sup>. Potential mechanisms underlying this relationship include shared environmental and genetic factors, as well as direct intrauterine influences<sup>27</sup>. Oxidative stress has been implicated in the pathophysiology of hypertension through mechanisms such as endothelial dysfunction, altered renal physiology – including increased sodium reabsorption, reduced renal blood flow, activation of the renin-angiotensin-aldosterone system, and tissue damage – and enhanced sympathetic nervous system activity<sup>2</sup>. Previous findings of reduced vascular density in 3-month-old infants born to mothers with hypertensive disorders of pregnancy<sup>28</sup>, as well as evidence of vascular dysfunction in adolescents exposed in utero to preeclampsia, but not in their siblings from normotensive pregnancies<sup>29</sup>, underscore the relevance of intrauterine factors. While these findings do not exclude genetic and environmental contributions, they highlight the importance of prenatal exposures. Our results

suggest that these intrauterine mechanisms may persist throughout childhood, as evidenced by increased oxidative stress in this population.

Oxidative stress has been shown to increase with age in adults<sup>30</sup>. Aging is also associated with physiological changes, including in the reproductive system<sup>31</sup>, and advanced maternal age is linked to a higher risk of both maternal and fetal complications<sup>32</sup>. Based on this, we hypothesized that an increase in maternal age at the time of childbirth might be associated with elevated oxidative stress in the mother, including at the oocyte level, which could in turn influence oxidative stress parameters in the offspring. In our study, we found that higher maternal age was associated with increased TAS, but only in girls aged 8-9 years. This could suggest the activation of adaptive mechanisms in response to oxidative stress in this subgroup. Since we did not observe significant associations between maternal age and any of the other oxidative stress biomarkers assessed, it is also possible that maternal age exerts a protective effect on oxidative status in children. Becoming a mother later in life is often associated with greater emotional maturity, financial stability, and a more favorable psychosocial environment, which may benefit offspring development<sup>31</sup>. We also hypothesize that a transient increase in oxidative stress during the perinatal period might trigger long-lasting compensatory antioxidant responses. Supporting this idea, the Manchester Advanced Maternal Age Study found higher total antioxidant capacity in the maternal serum of older women with uncomplicated pregnancies<sup>33</sup>. Maternal age may function as a preconditioning factor that promotes adaptive antioxidant defenses. One possible mechanism that could explain this could be through activation of nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that regulates the expression of genes involved in cellular antioxidant responses<sup>34</sup>. Further studies are needed to explore whether maternal age is associated with activation of Nrf2 signaling pathways and whether this may contribute to adaptive antioxidant responses during pregnancy. In contrast, *Imanparast et al.* reported a decline in total antioxidant capacity with increasing maternal age, although their study focused on cord blood, reflecting the newborn's oxidative status rather than that of older children<sup>35</sup>. Additionally, a Spanish study showed that maternal age was negatively associated with melatonin levels in breast milk during the first month postpartum, suggesting potential age-related declines in specific maternal antioxidants<sup>36</sup>. Overall, these observations raise the possibility that maternal age may influence the oxidative status of the offspring, although the underlying mechanisms remain unclear and further research is needed to clarify the direction and persistence of this association.

On the other hand, we found that decreased TAS in girls aged 8-9 years was associated with cesarean delivery. This aligns, at least partially, with the findings of *Hacioglu et al.*, who reported

higher antioxidant capacity in cord blood – measured by glutathione and TAS – among newborns delivered vaginally<sup>37</sup>. Our data suggest that the lower antioxidant stimulation associated with cesarean delivery, in contrast to the heightened antioxidant response triggered by vaginal birth, may have long-term consequences, particularly among girls. Beyond this, cesarean delivery was also associated with elevated oxidative stress biomarkers across both sexes and age groups, including increased U-NOx in girls aged 8-9 years, higher MPO levels in boys of the same age, and elevated U-Isop in adolescent boys. The observation that several oxidative stress markers are elevated in association with cesarean delivery suggests that it may contribute to oxidative stress through multiple, possibly sex- and age-specific, pathways. One potential mechanism involves the early-life disruption of gut microbiota colonization. Cesarean-delivered infants are less exposed to maternal vaginal and intestinal microbiota and more often colonized by skin-associated microbes<sup>38,39</sup>, a shift that has been linked to dysbiosis<sup>40</sup> and heightened oxidative and inflammatory responses<sup>41</sup>. Although the effects of delivery mode on microbiota composition appear to diminish during the first three weeks of life, differences have been observed again at later stages, including at 2 and 7 years of age<sup>40</sup>. Previous studies comparing cord blood from vaginal and cesarean deliveries have consistently reported higher oxidative stress following cesarean birth<sup>42,43</sup>. Our findings indicate that such differences may extend beyond the perinatal period, although their expression appears to vary depending on developmental stage and sex. It is noteworthy, however, that *Hacioglu et al.* also reported higher levels of oxidative stress — including total oxidant status and MDA — in vaginally delivered newborns compared to those delivered by elective cesarean section, highlighting the complexity of these associations<sup>37</sup>. The authors suggest that cesarean delivery elicits a less pronounced endocrine stress response than vaginal delivery, as indicated by lower cortisol levels in both cesarean-born neonates and maternal peripheral blood. They also hypothesize that the observed increase in oxidative stress in vaginal deliveries may be due to fluctuations in placental oxygen levels caused by strong uterine contractions during labor, which can activate oxidative pathways<sup>37</sup>. Taken together, these findings underscore the need for further investigation into the potential long-term oxidative consequences of delivery mode, particularly considering sex-specific differences.

Although breast milk has been associated with increased lipid peroxidation — a marker of oxidative stress — it also appears to confer greater antioxidant capacity compared to formula milk<sup>44</sup>. Based on this, we hypothesized that longer breastfeeding duration would be protective against oxidative stress later in life. Contrary to this expectation, our results showed an association between longer breastfeeding duration and higher P-NOx and U-NOx levels in boys aged 8-9 years.

NO plays a multifaceted role in physiology, contributing to processes such as platelet aggregation, endothelial function, neuronal signaling, and immune modulation<sup>45</sup>. Given its short half-life – approximately 1.5 hours – and rapid excretion, elevated plasma nitrate and nitrite levels typically reflect recent or ongoing NO production<sup>3</sup>. In healthy children, urinary excretion of these metabolites tends to decrease with age, stabilizing around adolescence<sup>6</sup>. Notably, a prior study in the same cohort reported a positive association between urinary nitrate/nitrite levels and BMI z-score<sup>7</sup>. In our analysis, however, this potential confounding effect was accounted for by adjusting all models for BMI.

We initially hypothesized that higher maternal education would be associated with a more favorable oxidative profile in the offspring, given its established association with greater health consciousness and healthier lifestyle choices, such as lower rates of smoking and obesity<sup>46</sup>. However, our results showed that girls aged 8-9 years whose mothers had higher education levels (more than 9 years) had increased levels of U-NOx. Additionally, MPO, a heme peroxidase mainly expressed in neutrophils and monocytes and released during inflammatory processes, was also consistently elevated in association with maternal education levels above 12 years. MPO catalyzes the reaction between hydrogen peroxide and chloride ions, generating hypochlorous acid and contributing to increased ROS production<sup>47,48</sup>. Maternal education is widely used as a socioeconomic indicator in epidemiological studies like ours. A previous analysis of the same cohort (G21) found that atopic diseases, such as rhinitis and eczema, were more common in 4-year-old children whose mothers had higher education levels<sup>49</sup>. This could reflect differences in environmental exposures. Mothers with higher education often live in urban areas, where children are more likely to be exposed to air pollutants, known triggers of oxidative and inflammatory processes<sup>50</sup>. However, in our study, all participants lived within a 30-kilometer radius of the study site, corresponding to one of Portugal's largest urban centers. Although maternal education is generally associated with better health outcomes, our findings suggest a more complex relationship, likely shaped by interacting environmental and psychosocial factors. One possible hypothesis is that higher education may be associated with occupations involving greater responsibility, which in turn may contribute to increased psychosocial stress – an established contributor to oxidative stress<sup>51</sup>. It has been proposed that maternal psychosocial stress, reflected in alterations of the hypothalamic–pituitary–adrenal (HPA) axis, may influence the development of the fetal HPA axis in utero<sup>52</sup>. Higher levels of prenatal maternal cortisol have been associated with altered HPA axis activity and diminished sensitivity of immune responses to the inhibitory effects of cortisol in children at age 5<sup>53</sup>, as well as with elevated cortisol levels in adolescents at age 15<sup>52</sup>.

Further research is needed to better understand these associations and the underlying biological mechanisms.

Smoking is a well-established source of oxidative stress, with studies showing that it increases P-Isop, while smoking cessation leads to a reduction in these markers<sup>54</sup>. Moreover, maternal smoking during pregnancy is known to be associated with adverse perinatal outcomes<sup>33</sup>. At least one study has demonstrated that both active and passive exposure to tobacco smoke during pregnancy is positively correlated with increased oxidative stress index in cord blood<sup>55</sup>. In animal models, *Bruin* and colleagues found that rats exposed in utero to nicotine had elevated plasma isoprostanes at postnatal day 21 – the equivalent of approximately 6 months of age in humans<sup>56,57</sup>. In our study, we did not observe any significant association between maternal smoking during pregnancy and oxidative stress biomarkers in childhood. However, we found that adolescent girls whose mothers smoked during pregnancy had significantly higher U-Isop. This finding may suggest the presence of buffering mechanisms in early life that mitigate oxidative damage, with effects emerging later during adolescence. Interestingly, this association was not observed in adolescent boys. Further research is needed to identify and understand potential protective processes acting during childhood. It is also important to note that we did not collect information regarding whether maternal smoking persisted throughout pregnancy or continued during the postnatal period. Evidence from *Noakes et al.* suggests that maternal smoking habits are often maintained after childbirth, and they reported that infants of mothers who smoked during pregnancy had higher U-Isop at 3 months of age<sup>58</sup>. Furthermore, exposure to tobacco during pregnancy has been associated with a higher likelihood of daily smoking in the offspring during adolescence<sup>59,60</sup>, which may contribute to the oxidative profile observed in later life stages.

As highlighted by *Ruchat et al.* in their review, gestational diabetes is associated with an increased risk of chronic diseases in the offspring, including obesity, type 2 diabetes, and metabolic syndrome<sup>61</sup>. These conditions have all been linked to elevated oxidative stress<sup>62–64</sup>. In our study, we found an association between higher levels of U-Isop in the offspring of maternal diabetes – both pregestational and gestational – suggesting increased lipid peroxidation. Experimental evidence supports these findings. In a study where diabetes was induced in pregnant rats, adult offspring exhibited increased reactive oxygen species production and reduced superoxide dismutase activity, a key antioxidant enzyme. These alterations were accompanied by decreased NO bioavailability and elevated blood pressure, indicating long-term vascular consequences of intrauterine exposure to hyperglycemia<sup>65</sup>. Moreover, previous research has suggested that maternal metabolic factors, including diabetes and higher BMI, may exert a stronger effect on male offspring<sup>66</sup>.

A key strength of this study is the extensive profiling of oxidative stress biomarkers, together with detailed maternal and perinatal data, in a large, homogeneous cohort of healthy prepubertal and pubertal participants, within a birth cohort framework that enables future longitudinal analyses. At the time of analysis, not all parameters from the 14-16-year-old follow-up were processed and available for age-related comparisons. Additionally, lifestyle factors that may influence oxidative stress levels at the time of sample collection – such as physical activity and diet – were not accounted for. However, all models were adjusted for the current BMI. The findings of our study should, however, be interpreted with caution, as its design limits the ability to infer causal relationships.

Oxidative stress has been increasingly recognized as a key factor influencing both maternal and perinatal health, as well as a contributing element in various childhood diseases. In our study, conducted in healthy children and adolescents, we identified significant associations between elevated levels of oxidative stress biomarkers and several maternal and perinatal factors, including maternal BMI, pre-pregnancy chronic hypertension, diabetes (both pre-gestational and gestational), maternal age and education level, smoking during pregnancy, cesarean delivery, and breastfeeding duration. The elevation of different biomarkers may be explained by the activation of distinct biological pathways, potentially triggered by different origins. Hormonal – and other less explored mechanisms, like epigenetic and genetic – differences between sexes appear to be associated with different susceptibilities and the activation of specific pathways. Our findings reinforce the idea that early-life exposures, including maternal characteristics even before pregnancy, may be linked to disease development later in life. Furthermore, our results suggest that the mechanisms underlying these diseases may already be present long before clinical signs become evident. Interestingly, some of the associations observed seem to attenuate with increasing age, while others only become apparent later in life, particularly during adolescence, highlighting the dynamic nature of these processes over time. Although our study focused on maternal factors, future research should explore the role of paternal characteristics in influencing offspring oxidative stress and disease susceptibility. A prospective follow-up of this cohort would be invaluable in determining whether these associations persist over time.

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**Table I. Maternal and neonatal characteristics**

|                                                              | All<br>(n = 313) | Girls<br>(n = 147) | Boys<br>(n = 166) | P<br>value |
|--------------------------------------------------------------|------------------|--------------------|-------------------|------------|
| <b>Maternal characteristics</b>                              |                  |                    |                   |            |
| Age at child's birth (years)                                 | 29.7 ± 4.9       | 30.0 ± 4.8         | 29.3 ± 5.0        | 0.302      |
| Total time of education (years)                              | 10.9 ± 4.3       | 11.1 ± 4.2         | 10.8 ± 4.3        | 0.489      |
| > 9 years                                                    | 164 (52.4%)      | 79 (53.7%)         | 85 (51.2%)        | 0.514      |
| ≥ 12 years                                                   | 141 (45.0%)      | 70 (47.6%)         | 71 (42.8%)        | 0.299      |
| <b>Cardiovascular health status prior to pregnancy</b>       |                  |                    |                   |            |
| BMI (kg/m <sup>2</sup> )                                     | 24.2 ± 4.2       | 23.9 ± 3.9         | 24.4 ± 4.4        | 0.355      |
| Overweight / obesity                                         | 93 (33.5%)       | 43 (32.8%)         | 50 (34.0%)        | 0.834      |
| Hypertension                                                 | 16 (5.2%)        | 8 (5.5%)           | 8 (4.8%)          | 0.791      |
| Diabetes mellitus                                            | 24 (7.7%)        | 10 (6.8%)          | 14 (8.4%)         | 0.588      |
| <b>Nutritional status and complications during pregnancy</b> |                  |                    |                   |            |
| Pregnancy weight gain (kg)                                   | 12.6 ± 4.9       | 12.1 ± 4.7         | 12.9 ± 5.0        | 0.166      |
| Excessive weight gain in pregnancy                           | 99 (34.7%)       | 44 (32.8%)         | 55 (36.4%)        | 0.525      |
| Gestational hypertension                                     | 5 (1.6%)         | 4 (2.8%)           | 1 (0.6%)          | 0.137      |
| Eclampsia                                                    | 6 (1.9%)         | 3 (2.1%)           | 3 (1.8%)          | 0.885      |
| Gestational diabetes                                         | 23 (7.5%)        | 6 (4.1%)           | 17 (10.4%)        | 0.036      |
| Any active smoking during pregnancy                          | 47 (15.4%)       | 14 (9.7%)          | 33 (20.4%)        | 0.010      |
| Any alcohol consumption during pregnancy                     | 51 (16.3%)       | 24 (16.3%)         | 27 (16.3%)        | 0.988      |
| <b>Birth and perinatal characteristics</b>                   |                  |                    |                   |            |
| Gestational age at birth (weeks)                             | 38.8 ± 1.6       | 38.8 ± 1.7         | 38.9 ± 1.5        | 0.690      |
| Preterm (< 37 weeks)                                         | 15 (4.8%)        | 4 (2.7%)           | 11 (6.6%)         | 0.109      |
| C-section delivery                                           | 106 (35.9%)      | 41 (30.1%)         | 65 (40.9%)        | 0.055      |
| <b>Anthropometry at birth</b>                                |                  |                    |                   |            |
| Birth weight (g)                                             | 3252.2 ± 456.9   | 3192.3 ± 456.2     | 3305.2 ± 452.2    | 0.024      |
| Birth length (cm)                                            | 49.1 ± 2.3       | 48.6 ± 2.4         | 49.5 ± 2.1        | <0.001     |
| <b>Birthweight for gestational age</b>                       |                  |                    |                   |            |
| Small for gestational age                                    | 22 (7.0%)        | 7 (4.8%)           | 15 (9.0%)         | 0.140      |
| Large for gestational age                                    | 19 (6.1%)        | 6 (4.1%)           | 13 (7.8%)         | 0.166      |
| <b>Breastfeeding</b>                                         |                  |                    |                   |            |
| Total time of breast feeding (months)                        | 9.6 (3.0 – 13.0) | 10.2 (2.4 – 13.0)  | 9.1 (3.0 – 10.0)  | 0.866      |
| Time of exclusive breast feeding (months)                    | 4.2 (1.5 – 5.0)  | 4.1 (1.0 – 5.0)    | 4.3 (1.5 – 6.0)   | 0.187      |

BMI, body mass index; C-section, cesarean section.

Data are presented as mean ± standard deviations (SD), median (P25-P75) or n (%).

The p value presented refers to the comparison between girls and boys.

**Table II.** Oxidative stress parameters in childhood and adolescence

|                                          |  |  |  | Childhood       |                 |                 | Adolescence     |                 |                 | p value |
|------------------------------------------|--|--|--|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|---------|
|                                          |  |  |  | All             | Girls           | Boys            | All             | Girls           | Boys            |         |
|                                          |  |  |  | (n = 313)       | (n = 147)       | (n = 166)       | (n = 167)       | (n = 73)        | (n = 94)        |         |
| General characteristics                  |  |  |  |                 |                 |                 |                 |                 |                 |         |
| Age (years)                              |  |  |  | 8.8 ± 0.2       | 8.8 ± 0.2       | 8.8 ± 0.2       | 14.3 ± 0.7      | 14.2 ± 0.6      | 14.3 ± 0.8      | -       |
| BMI (kg/m²)                              |  |  |  | 18.4 ± 3.2      | 18.3 ± 3.2      | 18.5 ± 3.1      | 22.8 ± 4.7      | 23.7 ± 4.8      | 22.1 ± 4.5*     | <0.001  |
| z-score                                  |  |  |  | 0.95 ± 1.24     | 0.81 ± 1.21     | 1.07 ± 1.25     | 0.77 ± 1.20     | 0.93 ± 1.12     | 0.65 ± 1.25     | <0.001  |
| Overweight/Obesity                       |  |  |  | 150 (47.9%)     | 67 (45.6%)      | 83 (50.0%)      | 70 (41.9%)      | 31 (42.5%)      | 39 (41.5%)      | <0.001  |
| Oxidative stress parameters              |  |  |  |                 |                 |                 |                 |                 |                 |         |
| U-Isop (ng/d)                            |  |  |  | 1584.1          | 1484.3          | 1696.8          | 5182.3          | 3880.5          | 5393.5 **       | < 0.001 |
|                                          |  |  |  | (1199.7-2271.2) | (1170.9-2201.5) | (1240.5-2352.9) | (3436.3-6355.6) | (2991.6-5292.7) | (3879.0-7437.3) |         |
| P-Isop (ng/mL)                           |  |  |  | 0.36            | 0.35            | 0.36            | -               | -               | -               | -       |
|                                          |  |  |  | (0.19-0.59)     | (0.18-0.62)     | (0.20-0.58)     |                 |                 |                 |         |
| TAS (mM Trolox equivalents)              |  |  |  | 1.24            | 1.27            | 1.24            | -               | -               | -               | -       |
|                                          |  |  |  | (1.00-1.62)     | (1.01-1.58)     | (1.00-1.65)     |                 |                 |                 |         |
| U-H <sub>2</sub> O <sub>2</sub> (nmol/d) |  |  |  | 1278.1          | 937.9           | 1551.0 **       | 1399.5          | 1110.2          | 1656.3 *        | 0.569   |
|                                          |  |  |  | (694.5-2049.4)  | (456.5-1880.8)  | (872.7-2361.6)  | (694.3-2670.0)  | (464.6-2056-8)  | (906.8-3131.4)  |         |
| P-NOx (nmol/ml)                          |  |  |  | 12.2            | 12.3            | 12.2            | -               | -               | -               | -       |
|                                          |  |  |  | (9.7-16.3)      | (9.5-17.5)      | (10.1-15.6)     |                 |                 |                 |         |
| U-NOx (μmol/d)                           |  |  |  | 795.1           | 774.6           | 833.6           | -               | -               | -               | -       |
|                                          |  |  |  | (557.2-1159.9)  | (552.0-1090.5)  | (573.5-1220-6)  |                 |                 |                 |         |
| MPO (ng/mL)                              |  |  |  | 55.4            | 52.9            | 61.1            | -               | -               | -               | -       |
|                                          |  |  |  | (35.1-87.2)     | (33.1-85.1)     | (35.8-90.6)     |                 |                 |                 |         |

BMI, body mass index; U-Isop, urinary isoprostanes; P-Isop, plasma isoprostanes; TAS, total antioxidant status; U-H<sub>2</sub>O<sub>2</sub>, urinary hydrogen peroxide; P-NOx, plasma nitrates and nitrites; U-NOx, urinary nitrates and nitrites; MPO, myeloperoxidase.

Data are presented as mean ± SD, median (P25-P75) or as n (%).

The p value presented refers to the comparison between oxidative stress parameters levels in childhood and adolescence, for the whole sample. The statistically significant differences between sexes, at each age evaluation, are indicated with \*(\* p<0.05; \*\* p <0.001).

**Table III. Spearman correlations between maternal and perinatal factors and oxidative stress parameters during childhood**

|                                                                     | U-Isop<br>(ng/d) |        | P-Isop<br>(ng/ml) |         | TAS<br>(mM Trolox equivalents) |        | U-H <sub>2</sub> O <sub>2</sub><br>(nmol/d) |        | P-NOx<br>(nmol/ml) |         | U-NOx<br>(μmol/d) |        | MPO<br>(ng/ml) |        |
|---------------------------------------------------------------------|------------------|--------|-------------------|---------|--------------------------------|--------|---------------------------------------------|--------|--------------------|---------|-------------------|--------|----------------|--------|
|                                                                     | Girls            | Boys   | Girls             | Boys    | Girls                          | Boys   | Girls                                       | Boys   | Girls              | Boys    | Girls             | Boys   | Girls          | Boys   |
| <b>Maternal characteristics</b>                                     |                  |        |                   |         |                                |        |                                             |        |                    |         |                   |        |                |        |
| Age at child's birth (years)                                        | -0.032           | 0.118  | 0.86              | 0.053   | 0.192*                         | 0.044  | 0.129                                       | -0.094 | -0.132             | -0.170* | 0.038             | -0.024 | 0.152          | 0.128  |
| Schooling years                                                     | -0.049           | -0.039 | -0.090            | -0.068  | -0.035                         | 0.045  | 0.144                                       | 0.091  | -0.025             | 0.035   | 0.205*            | -0.008 | 0.193*         | 0.144  |
| <b><u>Cardiovascular health status prior to pregnancy</u></b>       |                  |        |                   |         |                                |        |                                             |        |                    |         |                   |        |                |        |
| Weight (kg)                                                         | 0.089            | 0.105  | 0.050             | 0.238** | -0.038                         | -0.051 | 0.042                                       | 0.053  | -0.072             | -0.012  | -0.041            | 0.081  | 0.154          | 0.116  |
| BMI (kg/m <sup>2</sup> )                                            | 0.185*           | 0.063  | 0.026             | 0.289** | -0.003                         | -0.078 | 0.063                                       | 0.048  | 0.002              | 0.027   | -0.080            | 0.091  | 0.226**        | 0.081  |
| <b><u>Nutritional status and complications during pregnancy</u></b> |                  |        |                   |         |                                |        |                                             |        |                    |         |                   |        |                |        |
| Pregnancy weight gain (kg)                                          | -0.206*          | -0.113 | 0.119             | -0.060  | 0.043                          | 0.005  | -0.058                                      | 0.065  | 0.195*             | 0.129   | 0.019             | 0.030  | -0.159         | -0.118 |
| <b>Birth and perinatal characteristics</b>                          |                  |        |                   |         |                                |        |                                             |        |                    |         |                   |        |                |        |
| Gestational age at birth (weeks)                                    | 0.044            | 0.009  | 0.090             | 0.027   | 0.035                          | 0.054  | 0.057                                       | 0.092  | -0.062             | 0.119   | 0.047             | -0.065 | -0.072         | -0.055 |
| <b><u>Anthropometry at birth</u></b>                                |                  |        |                   |         |                                |        |                                             |        |                    |         |                   |        |                |        |
| Birth weight (g)                                                    | 0.092            | 0.071  | 0.178*            | 0.002   | 0.041                          | 0.010  | 0.016                                       | 0.101  | 0.028              | 0.065   | 0.037             | 0.002  | -0.049         | 0.035  |
| Birth length (cm)                                                   | -0.035           | -0.022 | 0.188*            | 0.062   | 0.000                          | 0.086  | 0.010                                       | 0.073  | -0.165*            | -0.026  | 0.012             | -0.117 | 0.029          | -0.027 |
| <b><u>Breastfeeding</u></b>                                         |                  |        |                   |         |                                |        |                                             |        |                    |         |                   |        |                |        |
| Total time of breast feeding (months)                               | 0.072            | 0.076  | 0.031             | 0.094   | 0.066                          | -0.110 | -0.086                                      | 0.016  | 0.170              | 0.182*  | 0.195*            | 0.172  | 0.107          | -0.114 |
| Time of exclusive breast feeding (months)                           | -0.076           | 0.008  | 0.047             | 0.053   | 0.137                          | 0.031  | 0.013                                       | 0.008  | 0.081              | 0.124   | 0.185*            | 0.158  | -0.042         | -0.067 |

BMI, body mass index; U-Isop, urinary isoprostanes; P-Isop, plasma isoprostanes; TAS, total antioxidant status; U-H<sub>2</sub>O<sub>2</sub>, urinary hydrogen peroxide; P-NOx, plasma nitrates and nitrites; U-NOx, urinary nitrates and nitrites; MPO, myeloperoxidase.

\* p value < 0.05; \*\* p value < 0.01

**Table IV.** Spearman correlations between maternal and perinatal factors and oxidative stress parameters during adolescence

|                                                              | U-Isop<br>(ng/d) |        | U-H <sub>2</sub> O <sub>2</sub><br>(nmol/d) |        |
|--------------------------------------------------------------|------------------|--------|---------------------------------------------|--------|
|                                                              | Girls            | Boys   | Girls                                       | Boys   |
| <b>Maternal characteristics</b>                              |                  |        |                                             |        |
| Age at child's birth (years)                                 | 0.170            | 0.073  | 0.092                                       | 0.183  |
| Schooling years                                              | -0.149           | -0.023 | 0.112                                       | 0.042  |
| <b>Cardiovascular health status prior to pregnancy</b>       |                  |        |                                             |        |
| Weight (kg)                                                  | 0.064            | 0.101  | 0.065                                       | 0.086  |
| BMI (kg/m <sup>2</sup> )                                     | 0.041            | 0.134  | 0.083                                       | 0.042  |
| <b>Nutritional status and complications during pregnancy</b> |                  |        |                                             |        |
| Pregnancy weight gain (Kg)                                   | -0.192           | -0.093 | 0.047                                       | -0.046 |
| <b>Birth and perinatal characteristics</b>                   |                  |        |                                             |        |
| Gestational age at birth (weeks)                             | 0.105            | 0.041  | 0.031                                       | 0.086  |
| <b>Anthropometry at birth</b>                                |                  |        |                                             |        |
| Birth weight (g)                                             | 0.243*           | -0.109 | 0.063                                       | 0.234* |
| Birth length (cm)                                            | 0.295*           | -0.168 | 0.155                                       | 0.091  |
| <b>Breastfeeding</b>                                         |                  |        |                                             |        |
| Total time of breast feeding (months)                        | 0.133            | 0.102  | -0.023                                      | 0.094  |
| Time of exclusive breast feeding (months)                    | -0.048           | 0.058  | -0.079                                      | 0.142  |

BMI, body mass index; U-Isop, urinary isoprostanes; U-H<sub>2</sub>O<sub>2</sub>, urinary hydrogen peroxide.

\* p value < 0.05; \*\* p value < 0.01

**Table V. Associations between maternal and perinatal factors and oxidative stress parameters during childhood**

|                                                              | U-Isop<br>(ng/d)          |                           | P-Isop<br>(ng/ml) |                  | TAS<br>(mM Trolox equivalents) |                  | U-H <sub>2</sub> O <sub>2</sub><br>(nmol/d) |                           | P-NOx<br>(nmol/ml) |                  | U-NOx<br>(μmol/d)        |                         | MPO<br>(ng/ml)    |                   |
|--------------------------------------------------------------|---------------------------|---------------------------|-------------------|------------------|--------------------------------|------------------|---------------------------------------------|---------------------------|--------------------|------------------|--------------------------|-------------------------|-------------------|-------------------|
|                                                              | Girls                     | Boys                      | Girls             | Boys             | Girls                          | Boys             | Girls                                       | Boys                      | Girls              | Boys             | Girls                    | Boys                    | Girls             | Boys              |
| <b>Maternal characteristics</b>                              |                           |                           |                   |                  |                                |                  |                                             |                           |                    |                  |                          |                         |                   |                   |
| <b>Cardiovascular health status prior to pregnancy</b>       |                           |                           |                   |                  |                                |                  |                                             |                           |                    |                  |                          |                         |                   |                   |
| BMI class ( <i>p value</i> )                                 | 0.196                     | 0.781                     | 0.903             | 0.001            | 0.935                          | 0.522            | 0.358                                       | 0.855                     | 0.858              | 0.852            | 0.164                    | 0.924                   | 0.117             | 0.659             |
| Normal weight                                                | 1557.6<br>(1123.1-2196.9) | 1713.3<br>(1257.4-2347.2) | 0.38 (0.16-0.63)  | 0.28 (0.14-0.51) | 1.20 (0.87-1.57)               | 1.23 (1.94-1.67) | 972.2<br>(436.1-1876.3)                     | 1554.8<br>(936.2-2373.5)  | 13.0 (9.4-15.9)    | 11.5 (9.5-15.7)  | 791.1<br>(593.2-1158.1)  | 823.0<br>(545.0-1204.5) | 53.7 (28.7-88.4)  | 59.0 (37.6-92.8)  |
| Overweight / obesity                                         | 1425.9<br>(1227.9-2215.1) | 1702.8<br>(1283.6-2336.3) | 0.43 (0.19-0.65)  | 0.51 (0.25-0.73) | 1.05 (1.36-1.63)               | 1.21 (0.80-1.61) | 935.4<br>(562.8-1899.4)                     | 1587.5<br>(784.4-2539.6)  | 11.5 (9.4-15.9)    | 12.5 (9.9-15.6)  | 611.1<br>(593.2-1158.1)  | 903.9<br>(691.5-1292.1) | 66.9 (44.1-90.0)  | 66.9 (39.7-94.8)  |
| Hypertension ( <i>p value</i> )                              | 0.482                     | 0.063                     | 0.959             | 0.089            | 0.531                          | 0.205            | 0.592                                       | 0.422                     | 0.418              | 0.644            | 0.633                    | 0.446                   | 0.555             | 0.373             |
| No                                                           | 1557.3<br>(1172.1-2210.5) | 1677.5<br>(1248.6-2245.7) | 0.39 (0.18-0.65)  | 0.35 (0.18-0.55) | 1.22 (0.97-1.59)               | 1.24 (1.02-1.66) | 996.8<br>(490.2-1889.7)                     | 1595.2<br>(891.9-2458.6)  | 12.2 (9.1-15.9)    | 12.0 (9.5-15.7)  | 783.7<br>(543.1-1135.3)  | 865.2<br>(573.6-1251.5) | 56.6 (38.7-87.7)  | 62.1 (37.5-92.3)  |
| Yes                                                          | 1518.7<br>(955.4-3018.6)  | 2466.9<br>(2125.9-2883.9) | 0.34 (0.04-0.61)  | 0.73 (0.30-0.77) | 1.01 (0.77-1.52)               | 1.00 (0.91-1.24) | 807.3<br>(252.0-3210.8)                     | 1200.7<br>(661.1-1791.7)  | 16.3 (10.6-20.1)   | 11.4 (8.1-14.9)  | 880.7<br>(597.9-1495.7)  | 822.1<br>(469.8-942.6)  | 66.8 (38.6-119.9) | 89.5 (46.8-99.8)  |
| Diabetes mellitus ( <i>p value</i> )                         | 0.681                     | 0.120                     | 0.276             | 0.755            | 0.222                          | 0.034            | 0.582                                       | 0.456                     | 0.590              | 0.216            | 0.312                    | 0.711                   | 0.914             | 0.083             |
| No                                                           | 1557.0<br>(1167.5-2196.9) | 1677.5<br>(1228.0-2245.7) | 0.41 (0.19-0.66)  | 0.38 (0.19-0.56) | 1.27 (0.97-1.63)               | 1.24 (1.02-1.67) | 924.9<br>(488.8-1894.1)                     | 1558.6<br>(835.0-2454.0)  | 12.2 (9.4-16.9)    | 12.2 (9.4-15.7)  | 774.6<br>(540.1-1111.1)  | 885.9<br>(561.9-1251.5) | 56.2 (36.5-89.8)  | 61.5 (37.5-93.0)  |
| Yes                                                          | 1724.3<br>(1227.9-2585.1) | 1932.7<br>(1524.0-2738.0) | 0.22 (0.14-0.55)  | 0.26 (0.10-0.71) | 1.08 (0.79-1.10)               | 1.13 (0.55-1.18) | 1430.8<br>(291.6-2380.1)                    | 1618.5<br>(1190.8-2726.3) | 12.5 (8.7-17.9)    | 11.6 (10.1-13.0) | 1127.6<br>(560.4-1253.9) | 822.1<br>(725.2-938.9)  | 62.6 (46.2-73.2)  | 80.9 (62.1-99.8)  |
| <b>Nutritional status and complications during pregnancy</b> |                           |                           |                   |                  |                                |                  |                                             |                           |                    |                  |                          |                         |                   |                   |
| Weight gain ( <i>p value</i> )                               | 0.719                     | 0.045                     | 0.493             | 0.607            | 0.657                          | 0.557            | 0.919                                       | 0.953                     | 0.186              | 0.690            | 0.543                    | 0.500                   | 0.977             | 0.583             |
| Adequate                                                     | 1563.8<br>(1167.3-2251.3) | 1737.6<br>(1309.4-2473.4) | 0.37 (0.16-0.61)  | 0.35 (0.19-0.56) | 1.21 (0.96-1.54)               | 1.22 (1.02-1.62) | 953.8<br>(449.3-1873.1)                     | 1497.6<br>(863.1-2365.5)  | 12.3 (9.0-15.1)    | 11.8 (9.5-15.5)  | 790.7<br>(580.0-1154.4)  | 893.3<br>(575.9-1252.1) | 57.4 (40.5-88.7)  | 61.7 (38.7-92.8)  |
| Excessive                                                    | 1458.1<br>(1294.0-2091.8) | 1538.6<br>(1207.9-2051.6) | 0.47 (0.19-0.69)  | 0.39 (0.20-0.58) | 1.28 (1.04-1.71)               | 1.22 (0.75-1.86) | 1086.2<br>(486.4-2191.1)                    | 1660.1<br>(1023.1-2817.6) | 12.9 (9.9-19.7)    | 12.5 (9.8-15.8)  | 597.1<br>(485.6-1134.1)  | 819.2<br>(569.5-1059.5) | 55.0 (35.4-88.8)  | 69.3 (27.9-94.8)  |
| Gestational hypertension ( <i>p value</i> )                  | -                         | -                         | -                 | -                | -                              | -                | -                                           | -                         | -                  | -                | -                        | -                       | -                 | -                 |
| No                                                           | 1557.3<br>(1183.9-2227.2) | 1692.7<br>(1237.4-2336.5) | 0.36 (0.19-0.63)  | 0.35 (0.20-0.56) | 1.21 (0.99-1.60)               | 1.23 (1.00-1.68) | 1021.4<br>(482.2-1964.3)                    | 1567.8<br>(901.3-2357.8)  | 12.5 (9.7-17.4)    | 12.1 (9.7-15.6)  | 775.8<br>(552.9-1095.6)  | 839.2<br>(569.5-1209.7) | 54.7 (35.4-88.8)  | 62.3 (37.4-92.5)  |
| Yes                                                          | -                         | -                         | -                 | -                | -                              | -                | -                                           | -                         | -                  | -                | -                        | -                       | -                 | -                 |
| Gestational diabetes ( <i>p value</i> )                      | 0.824                     | 0.003                     | 0.109             | 0.272            | 0.861                          | 0.034            | 0.751                                       | 0.375                     | 0.785              | 0.690            | 0.328                    | 0.462                   | 0.156             | 0.001             |
| No                                                           | 1506.9<br>(1168.6-2210.5) | 1609.3<br>(1192.0-2205.7) | 0.38 (0.19-0.63)  | 0.35 (0.20-0.53) | 1.21 (0.97-1.59)               | 1.24 (1.02-1.71) | 1021.4<br>(490.2-1940.9)                    | 1567.8<br>(863.1-2338.9)  | 12.5 (9.6-17.3)    | 12.2 (9.52-15.6) | 775.8<br>(558.1-1080.9)  | 859.7<br>(564.4-1235.0) | 54.7 (38.7-89.3)  | 58.5 (35.3-89.8)  |
| Yes                                                          | 2064.5<br>(1155.5-2519.6) | 2518.6<br>(1652.7-3100.2) | 0.21 (0.13-0.31)  | 0.48 (0.20-0.73) | 1.10 (0.91-1.67)               | 1.20 (0.65-1.25) | 935.4<br>(226.0-2666.1)                     | 1646.5<br>(1011.8-2822.2) | 12.5 (10.1-15.6)   | 13.0 (11.4-15.6) | 753.8<br>(538.9-1355.7)  | 780.9<br>(695.0-993.6)  | 27.5 (14.4-89.4)  | 85.3 (76.5-106.6) |
| Active smoking ( <i>p value</i> )                            | 0.676                     | 0.577                     | 0.611             | 0.066            | 0.589                          | 0.681            | 0.682                                       | 0.496                     | 0.105              | 0.758            | 0.982                    | 0.727                   | 0.578             | 0.673             |
| No                                                           | 1557.3<br>(1161.1-2201.5) | 1739.7<br>(1263.0-2273.4) | 0.39 (0.17-0.65)  | 0.36 (0.19-0.55) | 1.21-0.97-1.56)                | 1.21 (0.99-1.64) | 996.8<br>(493.0-1913.2)                     | 1535.1<br>(833.1-2568.2)  | 12.7 (9.5-17.4)    | 11.9 (9.6-15.1)  | 783.7<br>(556.3-1150.8)  | 873.9<br>(572.4-1248.6) | 55.6 (39.8-88.8)  | 64.6 (38.9-96.6)  |
| Yes                                                          | 1634.1<br>(1233.3-2350.1) | 2565.6<br>(1265.1-2525.4) | 0.37 (0.18-0.58)  | 0.39 (0.18-0.65) | 1.21 (0.81-1.76)               | 1.23 (1.04-1.67) | 796.3<br>(414.0-1664.0)                     | 1640.3 (1169.5-1892.9)    | 9.9 (7.7-12.3)     | 12.6 (9.3-21.6)  | 897.0<br>(482.4-1141.2)  | 828.7<br>(572.4-1082.4) | 65.4 (32.4-85.9)  | 56.3 (19.5-77.3)  |

|                                            | U-Isop<br>(ng/d)          |                           | P-Isop<br>(ng/ml) |                  | TAS<br>(mM Trolox equivalents) |                  | U-H <sub>2</sub> O <sub>2</sub><br>(nmol/d) |                           | P-NOx<br>(nmol/ml) |                  | U-NOx<br>(μmol/d)       |                         | MPO<br>(ng/ml)    |                  |
|--------------------------------------------|---------------------------|---------------------------|-------------------|------------------|--------------------------------|------------------|---------------------------------------------|---------------------------|--------------------|------------------|-------------------------|-------------------------|-------------------|------------------|
|                                            | Girls                     | Boys                      | Girls             | Boys             | Girls                          | Boys             | Girls                                       | Boys                      | Girls              | Boys             | Girls                   | Boys                    | Girls             | Boys             |
| Alcohol consumption ( <i>p value</i> )     | 0.113                     | 0.081                     | 0.410             | 0.066            | 0.255                          | 0.946            | 0.866                                       | 0.530                     | 0.669              | 0.805            | 0.477                   | 0.844                   | 0.054             | 0.615            |
| No                                         | 1631.1<br>(1176.2-2284.1) | 1737.6<br>(1274.9-2433.5) | 0.42 (0.19-0.66)  | 0.35 (0.16-0.53) | 1.21 (0.96-1.54)               | 1.23 (1.03-1.65) | 1031.5<br>(490.2-1889.7)                    | 1586.1<br>(923.8-2455.2)  | 12.4 (9.6-17.0)    | 12.0 (9.5-15.6)  | 790.7<br>(552.3-1184.2) | 891.7<br>(570.6-1242.7) | 62.3 (41.6-94.0)  | 63.6 (37.8-93.3) |
| Yes                                        | 1298.6<br>(1161.1-1867.5) | 1517.1<br>(1238.7-1963.3) | 0.24 (0.11-0.56)  | 0.47 (0.23-0.72) | 1.28 (0.95-1.99)               | 1.19 (0.91-1.64) | 835.5<br>(371.2-2299.1)                     | 1415.2<br>(751.2-2403.9)  | 11.5 (8.8-15.3)    | 11.6 (9.1-15.5)  | 679.0<br>(552.1-1068.4) | 838.2<br>(574.7-1024.3) | 42.8 (22.6-58.9)  | 64.9 (41.2-92.3) |
| <b>Birth and perinatal characteristics</b> |                           |                           |                   |                  |                                |                  |                                             |                           |                    |                  |                         |                         |                   |                  |
| Gestational age class ( <i>p value</i> )   | -                         | 0.897                     | -                 | 0.404            | -                              | 0.815            | -                                           | 0.151                     | -                  | 0.617            | -                       | 1.000                   | -                 | 0.691            |
| Term (≥37 weeks)                           | 1157.0<br>(1167.5-2189.5) | 1688.6<br>(1283.7-2275.5) | 0.38 (0.17-0.64)  | 0.36 (0.19-0.55) | 1.23 (0.99-1.59)               | 1.24 (1.00-1.66) | 973.2<br>(488.8-1899.4)                     | 1577.0<br>(906.1-2374.9)  | 12.2 (9.4-16.9)    | 12.0 (9.5-15.6)  | 790.4<br>(553.2-1143.3) | 854.3<br>(572.0-1220.6) | 56.2 (38.1-85.5)  | 62.5 (37.5-93.1) |
| Preterm (<37 weeks)                        | -                         | 1845.2<br>(1148.5-2475.6) | -                 | 0.53 (0.14-0.81) | -                              | 1.14 (0.58-1.25) | -                                           | 1193.4<br>(385.1-2876.4)  | -                  | 11.6 (8.2-23.0)  | -                       | 993.6<br>(680.8-1602.2) | -                 | 76.3 (46.2-84.2) |
| Delivery method ( <i>p value</i> )         | 0.106                     | 0.979                     | 0.506             | 0.391            | 0.061                          | 0.384            | 0.913                                       | 0.328                     | 0.170              | 0.700            | 0.010                   | 0.607                   | 0.918             | 0.040            |
| Vaginal delivery                           | 1700.1<br>(1261.7-2284.1) | 1651.6<br>(1343.7-2269.0) | 0.44 (0.20-0.65)  | 0.31 (0.15-0.54) | 1.28 (1.02-1.73)               | 1.26 (1.06-1.63) | 1021.4<br>(469.1-1783.3)                    | 1370.1<br>(826.9-2251.5)  | 12.0 (9.5-16.7)    | 12.0 (9.5-16.1)  | 712.0<br>(539.1-1040.1) | 799.2<br>(556.3-1209.7) | 66.1 (41.7-94.0)  | 57.4 (35.3-82.0) |
| C-section delivery                         | 1240.0<br>(827.3-2098.8)  | 1783.1<br>(1097.6-2473.4) | 0.31 (0.14-0.63)  | 0.39 (0.21-0.61) | 1.14 (0.78-1.38)               | 1.14 (0.89-1.67) | 881.1<br>(592.4-2131.4)                     | 1766.6<br>(990.8-2700.2)  | 12.8 (8.9-18.3)    | 11.9 (9.2-14.8)  | 999.4<br>(602.3-1343.2) | 934.8<br>(664.9-1252.1) | 49.7 (23.4-67.9)  | 74.0 (39.2-99.2) |
| Birthweight for gestational age            |                           |                           |                   |                  |                                |                  |                                             |                           |                    |                  |                         |                         |                   |                  |
| AGA                                        | 1557.0<br>(1167.4-2206.0) | 1611.4<br>(1212.9-2116.7) | 0.35 (0.19-0.62)  | 0.34 (0.19-0.55) | 1.22 (1.00-1.61)               | 1.23 (1.01-1.67) | 1041.5<br>(531.7-1994.6)                    | 1444.2<br>(872.7-2372.1)  | 12.3 (9.5-17.2)    | 11.8 (9.5-15.6)  | 790.4<br>(567.7-1119.3) | 844.8<br>(578.2-1213.3) | 53.0 (34.6-89.1)  | 62.1 (35.8-93.6) |
| ( <i>p value</i> )                         | 0.597                     | 0.189                     | 0.098             | 0.249            | 0.307                          | 0.904            | 0.006                                       | 0.519                     | 0.375              | 0.123            | 0.658                   | 0.869                   | 0.549             | 0.356            |
| SGA                                        | 1740.3<br>(1192.6-4788.6) | 2125.9<br>(1347.0-3640.2) | 0.16 (0.12-0.33)  | 0.50 (0.23-0.58) | 0.99 (0.74-1.36)               | 1.20 (1.00-1.87) | 291.1<br>(132.1-1110.9)                     | 1777.2<br>(1595.2-2354.0) | 13.0 (8.9-15.7)    | 14.5 (9.3-25.0)  | 602.5<br>(463.2-1042.3) | 788.4<br>(552.5-1800.5) | 66.9 (32.6-119.5) | 64.6 (34.2-73.0) |
| ( <i>p value</i> )                         | 0.653                     | 0.029                     | 0.159             | 0.979            | 0.836                          | 0.952            | 0.762                                       | 0.903                     | 0.506              | 0.209            | 0.494                   | 0.537                   | 0.252             | 0.819            |
| LGA                                        | 1398.2<br>(1000.7-2240.3) | 2602.4<br>(1968.4-3574.8) | 0.53 (0.27-0.80)  | 0.45 (0.16-0.64) | 1.10 (0.82-1.59)               | 1.23 (0.86-1.64) | 655.8<br>(452.8-1897.3)                     | 1516.4<br>(682.7-2574.8)  | 13.9 (9.7-22.6)    | 13.7 (11.1-14.9) | 553.2<br>(489.2-959.9)  | 861.4<br>(453.5-1045.3) | 73.2 (51.5-101.3) | 63.7 (44.6-88.9) |

AGA, adequate for gestational age; BMI, body mass index; C-section, cesarean section; LGA – large for gestational age; SGA – small for gestational age; U-Isop, urinary isoprostanes; P-Isop, plasma isoprostanes; TAS, total antioxidant status; U-H<sub>2</sub>O<sub>2</sub>, urinary hydrogen peroxide; P-NOx, plasma nitrates and nitrites; U-NOx, urinary nitrates and nitrites; MPO, myeloperoxidase.

Data are presented as median (P25-P75). Summary statistics and non-parametric tests *p* values are not shown for variables with less than 5 individuals per class.

**Table VI. Associations between maternal and perinatal factors and oxidative stress parameters during adolescence**

|                                                                     | U-Isop<br>(ng/d)        |                        | U-H <sub>2</sub> O <sub>2</sub><br>(nmol/d) |                        |
|---------------------------------------------------------------------|-------------------------|------------------------|---------------------------------------------|------------------------|
|                                                                     | Girls                   | Boys                   | Girls                                       | Boys                   |
| <b>Maternal characteristics</b>                                     |                         |                        |                                             |                        |
| <b><u>Cardiovascular health status prior to pregnancy</u></b>       |                         |                        |                                             |                        |
| BMI class ( <i>p value</i> )                                        | 0.570                   | 0.330                  | 0.440                                       | 0.789                  |
| Normal weight                                                       | 3728.5 (2819.3-4944.4)  | 5492.8 (3710.4-7214.5) | 1221.6 (455.2-2392.2)                       | 1671.8 (886.5-2781.6)  |
| Overweight/ obesity                                                 | 3849.8 (3111.5-5018.2)  | 6080.9 (3495.6-7980.7) | 1161.4 (537.7-2529.5)                       | 1613.6 (883.8-3337.0)  |
| Hypertension ( <i>p value</i> )                                     | 0.495                   | -                      | 0.249                                       | -                      |
| No                                                                  | 3795.6 (2953.3-4944.4)  | 5711.8 (3733.3-7709.3) | 1203.6 (430.3-2132.5)                       | 1646.2 (902.7-3031.6)  |
| Yes                                                                 | 3338.1 (26000.1-5384.1) | -                      | 1648.5 (778.1-3120.2)                       | -                      |
| Diabetes mellitus ( <i>p value</i> )                                | -                       | 0.122                  | -                                           | 0.560                  |
| No                                                                  | 3728.5 (2921.7-4909.0)  | 5509.7 (3683.1-7416.9) | 1203.6 (469.0-2283.6)                       | 1646.2 (911.0-3093.8)  |
| Yes                                                                 | -                       | 6459.4 (4098.1-8500.1) | -                                           | 1962.4 (651.0-2193.6)  |
| <b><u>Nutritional status and complications during pregnancy</u></b> |                         |                        |                                             |                        |
| Weight gain ( <i>p value</i> )                                      | 0.789                   | 0.633                  | 0.863                                       | 0.517                  |
| Adequate                                                            | 3772.2 (2851.9-4963.9)  | 5393.5 (3567.4-7479.6) | 1202.5 (422.1-2207.0)                       | 1729.2 (1090.0-3252.7) |
| Excessive                                                           | 3739.7 (2984.4-4424.2)  | 6447.7 (4332.6-7927.0) | 1751.1 (653.0-3071.6)                       | 1486.6 (608.3-2145.8)  |
| Gestational hypertension ( <i>p value</i> )                         | -                       | -                      | -                                           | -                      |
| No                                                                  | 3739.7(2880.6-4950.6)   | 5610.8 (3692.2-7757.8) | 1202.5 (477.3-2375.5)                       | 1656.3 (898.6-2906.8)  |
| Yes                                                                 | -                       | -                      | -                                           | -                      |
| Gestational diabetes ( <i>p value</i> )                             | -                       | 0.166                  | -                                           | 0.851                  |
| No                                                                  | 3717.2 (2880.6-4880.2)  | 5438.4 (3692.2-7323.2) | 1202.5 (477.3-2207.8)                       | 1603.5 (928.9-2711.4)  |
| Yes                                                                 | -                       | 7106.3 (3829.0-8286.8) | -                                           | 2020.7 (590.6-4290.9)  |
| Active smoking ( <i>p value</i> )                                   | 0.011                   | 0.203                  | 0.716                                       | 0.938                  |
| No                                                                  | 3687.7 (2856.1-4523.9)  | 5386.0 (3683.1-7416.9) | 1221.6 (536.5-2417.8)                       | 1560.8 (862.7-2719.0)  |
| Yes                                                                 | 5190.6 (4248.1-5908.4)  | 6436.1 (4568.7-9219.1) | 664.8 (76.4-2212.7)                         | 1760.9 (1346.5-3374.8) |
| Alcohol consumption ( <i>p value</i> )                              | 0.885                   | 0.794                  | 0.137                                       | 0.763                  |
| No                                                                  | 3795.6 (2963.5-4851.5)  | 5711.8 (3719.4-7870.2) | 1203.6 (430.3-2166.3)                       | 1560.8 (894.5-2719.0)  |
| Yes                                                                 | 3458.0 (2567.6-5627.7)  | 4826.4 (3621.5-7071.1) | 1335.3 (613.2-2464.2)                       | 2021.7 (835.4-3244.4)  |
| <b>Birth and perinatal characteristics</b>                          |                         |                        |                                             |                        |
| Gestational age class ( <i>p value</i> )                            | -                       | 0.210                  | -                                           | 0.051                  |
| Term (≥37 weeks)                                                    | 3756.0 (2921.7-4957.1)  | 5492.8 (3692.2-7406.6) | 1203.6 (469.0-2384.0)                       | 1681.9 (1008.8-3062.7) |
| Preterm (<37 weeks)                                                 | -                       | -                      | -                                           | -                      |
| Delivery method ( <i>p value</i> )                                  | 0.907                   | 0.108                  | 0.619                                       | 0.743                  |
| Vaginal delivery                                                    | 3739.7 (3119.9-4822.7)  | 4826.4 (3552.7-6871.0) | 1202.5 (438.5-2493.6)                       | 1490.0 (999.0-2829.0)  |
| C-section delivery                                                  | 4210.8 (2619.7-5531.2)  | 6391.0 (4382.7-8053.3) | 1204.8 (623.5-1970.6)                       | 1760.9 (802.2-3519.2)  |
| Birthweight for gestational age                                     |                         |                        |                                             |                        |
| AGA                                                                 | 3894.1 (3001.1-5305.9)  | 5509.7 (4315.9-7416.9) | 1110.2 (480.6-2012.8)                       | 1611.7 (862.7-3093.8)  |
| ( <i>p value</i> )                                                  | 0.498                   | 0.526                  | 0.542                                       | 0.506                  |
| SGA                                                                 | 3658.2 (2293.0-5439.0)  | 4411.2 (3326.9-8118.1) | 345.4 (332.6-2745.6)                        | 1522.1 (1034.1-1771.7) |
| ( <i>p value</i> )                                                  | -                       | 0.215                  | -                                           | 0.192                  |
| LGA                                                                 | -                       | 3929.6 (3352.9-7016.6) | -                                           | 2123.5 (1423.4-5113.6) |

AGA, adequate for gestational age; BMI, body mass index; C-section, cesarean section; LGA – large for gestational age; SGA – small for gestational age; U-Isop, urinary isoprostanes; U-H<sub>2</sub>O<sub>2</sub>, urinary hydrogen peroxide.

Data are presented as median (P25-P75). Summary statistics and non-parametric tests p values are not shown for variables with less than 5 individuals per class.

**Table VII.** Multivariate linear regression models for each oxidative stress parameter during childhood

|                                        | Girls            |                 |       | Boys             |               |         |
|----------------------------------------|------------------|-----------------|-------|------------------|---------------|---------|
|                                        | Adjusted $\beta$ | 95% CI          | p     | Adjusted $\beta$ | 95% CI        | p       |
| <b><u>P-Isop</u></b>                   |                  |                 |       |                  |               |         |
| Overweight/obesity prior to pregnancy  | -                | -               | -     | 0.02             | (0.01, 0.03)  | <0.001  |
| Hypertension prior to pregnancy        | -                | -               | -     | 0.26             | (0.04, 0.47)  | 0.02    |
| <b><u>TAS</u></b>                      |                  |                 |       |                  |               |         |
| Maternal age at child's birth (years)  | 0.02             | (0.01, 0.04)    | 0.009 | -                | -             | -       |
| C-section delivery                     | -0.20            | (-0.37, 0.04)   | 0.015 | -                | -             | -       |
| <b><u>P-Nox</u></b>                    |                  |                 |       |                  |               |         |
| Total time of breastfeeding (months)   |                  |                 |       | 0.51             | (0.33, 0.70)  | < 0.001 |
| <b><u>U-Nox</u></b>                    |                  |                 |       |                  |               |         |
| C-section delivery                     | 282.65           | (6.52, 558.78)  | 0.045 | -                | -             | -       |
| More than 9 years of school (mother)   | 327.70           | (71.43, 583.97) | 0.013 | -                | -             | -       |
| Total time of breastfeeding (months)*  | -                | -               | -     | 13.11            | (1.47, 24.74) | 0.028   |
| <b><u>MPO</u></b>                      |                  |                 |       |                  |               |         |
| More than 12 years of school (mother)* | 25.03            | (4.69, 45.37)   | 0.016 | 19.40            | (2.78, 36.02) | 0.023   |
| C-section delivery                     |                  |                 |       | 22.46            | (5.74, 39.18) | 0.009   |

CI, confidence interval; P-Isop, plasma isoprostanes; TAS, total antioxidant status; C-section, cesarean section; P-NOx, plasma nitrates and nitrites; U-NOx, urinary nitrates and nitrites; MPO, myeloperoxidase.

Adjusted linear regression coefficients ( $\beta$ ) and 95% confidence intervals are presented, estimated by linear regression models with each oxidative stress parameter as the dependent variable, adjusted for all variables in the table and additionally for gestational age at birth and birth weight, and BMI class (normal vs overweight/obese) at the assessment (8-9 years). Only models with significant associations identified are shown.

**Table VIII.** Multivariate linear regression models for each oxidative stress parameter during adolescence

|                                      | Girls            |                   |       | Boys             |                   |       |
|--------------------------------------|------------------|-------------------|-------|------------------|-------------------|-------|
|                                      | Adjusted $\beta$ | 95% CI            | p     | Adjusted $\beta$ | 95% CI            | p     |
| <b>U-Isop</b>                        |                  |                   |       |                  |                   |       |
| <b>U-Isop model 1</b>                |                  |                   |       |                  |                   |       |
| Any active smoking during pregnancy  | 1859.03          | (730.46, 2987.61) | 0.002 | -                | -                 | -     |
| <b>U-Isop Model 2</b>                |                  |                   |       |                  |                   |       |
| Diabetes mellitus prior to pregnancy |                  |                   |       | 2202.38          | (196.37, 4208.38) | 0.032 |
| C-section delivery                   |                  |                   |       | 1432.08          | (129.87, 2734.28) | 0.032 |
| <b>U-Isop Model 3</b>                |                  |                   |       |                  |                   |       |
| Gestational diabetes                 |                  |                   |       | 2263.05          | (122.86, 4403.24) | 0.039 |
| C-section delivery                   |                  |                   |       | 1422.65          | (110.28, 2735.01) | 0.034 |

CI, confidence interval; U-Isop, urinary isoprostanes; C-section, cesarean section.

Adjusted linear regression coefficients ( $\beta$ ) and 95% confidence intervals are presented, estimated by linear regression models with each oxidative stress parameter as the dependent variable, adjusted for all variables in the table and additionally for gestational age at birth and birth weight, and BMI class (normal vs overweight/obese) at the assessment (14-16 years). Only models with significant associations identified are shown.

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