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Integrated Master's Degree in Medicine

Gender-related differences in cardiometabolic risk factors and oxidative stress among prepubertal children with obesity

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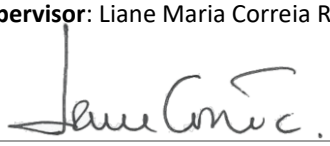

Co-supervisor: Teresa Maria de Jesus Teixeira de Sousa

Walking this path was only possible with the unconditional support and energy of several people, to whom I dedicate this dissertation. Especially to my parents, brother, friends and Sara.

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Resumo

Introdução/Objetivos: Existe evidência que sugere que o stress oxidativo, a biodisponibilidade do óxido nítrico (NO) e os fatores de risco cardiometabólico, bem como a sua associação na presença de obesidade, podem já ser influenciados pelo género em crianças pré-púberes. No presente estudo, pretendeu-se avaliar se a associação entre diversos marcadores de stress oxidativo/biodisponibilidade do NO e variáveis cardiometabólicas é diferente entre géneros, de acordo com o seu estado nutricional, em crianças entre 8 e 9 anos de idade.

Metodologia: Foi realizada análise transversal de 313 crianças de 8-9 anos, inseridas na coorte de nascimento Geração XXI (Portugal). Foram avaliados dados antropométricos, pressão arterial ambulatória de 24h, velocidade da onda de pulso (PWV), colesterol total, colesterol não HDL, colesterol HDL, triglicerídeos, resistência à insulina, isoprostanos plasmáticos e urinários (P-Isop, U-Isop), capacidade antioxidante total no plasma (TAS), mieloperoxidase sérica (MPO), ácido úrico sérico, nitratos e nitritos no plasma e na urina (P-NO_x, U-NO_x) e peróxido de hidrogénio na urina (U-H_2O_2).

Resultados: Os valores de colesterol total, colesterol não-HDL, triglicerídeos e HOMA-IR foram significativamente mais elevados nas raparigas. Relativamente aos biomarcadores de estado oxidativo e de biodisponibilidade do NO, encontrámos apenas diferenças significativas nos valores de U-H_2O_2, que foram consideravelmente menores nas raparigas. Quando estes marcadores foram analisados por classes de índice de massa corporal (IMC) e género, verificámos que os valores de MPO e de U-Isop aumentavam significativamente ao longo das classes de IMC, tanto em raparigas como em rapazes ($p<0,001$ para MPO e $p<0,01$ para U-Isop), a concentração de ácido úrico era superior em raparigas com excesso de peso e obesas do que em raparigas com peso normal ($p<0,001$), sem diferenças detetadas em rapazes, e os valores de U-NO_x diferiram apenas em rapazes, sendo significativamente superiores nos com excesso de peso ($p=0,005$). Na análise multivariada, em modelos ajustados para idade e z-score do IMC, verificaram-se associações inversas significativas entre os valores de U-H_2O_2 e PWV e entre U-NO_x e colesterol total ou não-HDL, apenas em rapazes, enquanto que em raparigas constatou-se uma associação positiva significativa entre os valores de U-Isop e HOMA-IR.

Discussão e Conclusões: O género afeta de forma diferente o stress oxidativo, a biodisponibilidade do NO e os fatores de risco cardiometabólico, bem como a associação entre estes parâmetros. As raparigas pré-púberes parecem ser mais vulneráveis ao desenvolvimento de disfunção metabólica induzida pelo stress oxidativo, enquanto que nos rapazes a elevação dos valores dos marcadores

de stress oxidativo e de biodisponibilidade de NO parece ter um impacto protetor e não deletério na rigidez arterial e na homeostase lipídica.

Palavras-chave: Gender differences; Oxidative stress; Cardiometabolic risk factors; Childhood obesity

Gender-related differences in cardiometabolic risk factors and oxidative stress among prepubertal children with obesity

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Short title (50 caract.): Sex differences in cardiometabolic risk and redox status

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Keywords: Gender differences; Oxidative stress; Cardiometabolic risk factors; Childhood obesity

ABSTRACT

BACKGROUND: Recent evidence suggests that oxidative stress, nitric oxide (NO) bioavailability and cardiometabolic risk factors, as well as their association in the setting of obesity, might already be influenced by gender in prepubertal children. In this study we aimed to evaluate if the association between several redox/NO bioavailability markers and cardiometabolic variables is different between genders, and according to nutritional status, in 8 to 9-year-old children.

METHODS: We performed a cross sectional evaluation of 313 children aged 8 to 9 years old, followed in the birth cohort Generation XXI (Portugal). We evaluated anthropometrics, 24-h ambulatory blood pressure, pulse wave velocity (PWV), total cholesterol, non-HDL-cholesterol, HDL-cholesterol, triglycerides, insulin resistance (homeostasis model assessment index, HOMA-IR), plasma and urinary isoprostanes (P-Isop, U-Isop), plasma total antioxidant status (TAS), serum myeloperoxidase (MPO), serum uric acid, plasma and urinary nitrates and nitrites (P-NO_x, U-NO_x) and urinary hydrogen peroxide (U-H_2O_2).

RESULTS: Total cholesterol, non-HDL-cholesterol, triglycerides and HOMA-IR values were significantly higher in girls. Regarding the biomarkers of redox status and NO bioavailability, we only found significant differences in U-H_2O_2 values, which were significantly lower in girls compared to boys. When these markers were analysed by classes of body mass index (BMI) and gender, we found that MPO concentration and U-Isop values were significantly higher across BMI classes, both in girls and boys ($p<0.001$ for MPO and $p<0.01$ for U-Isop), uric acid concentration was considerably higher in overweight and obese girls than in normal weight girls ($p<0.001$), with no differences detected for boys across BMI classes, and U-NO_x values only differed in boys, being markedly higher in those who were overweight and obese than in

normal weight subjects (p=0.005). Multivariate analysis, adjusted for age and BMI z-score, detected significant inverse associations between U-H_2O_2 and PWV values and between U-NO_x and total or non-HDL-cholesterol, only in boys, while in girls there was a significant positive association between U-Isop and HOMA-IR values.

CONCLUSIONS: Gender differentially affects oxidative stress, NO bioavailability and cardiometabolic risk factors, as well as the association between these parameters. Prepubertal girls seem to be more vulnerable to the development of oxidative stress-induced metabolic dysfunction, while in boys the rise in the values of redox and NO bioavailability markers seems to have a protective rather than a deleterious impact on arterial stiffness and lipid homeostasis.

INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of mortality in developed countries[1]. During the last decades, the prevalence of childhood overweight and obesity has been increasing at an alarming pace. Childhood obesity is known to track into adulthood and to be associated with several cardiovascular risk factors, which later in life might increase the risk of cardiovascular events and premature mortality[2]. In today´s society we face the reality that the majority of people reside in countries where one is more expected to die from the outcomes of being overweight than underweight[3]. Thus, it is essential to address adverse cardiometabolic risk profiles early in life in order to lessen the burden of adult disease.

Obesity has been described as a metaflammation process, which is a chronic low-grade inflammatory response triggered by excess nutrients in metabolic cells, that differs from the classic paradigm of inflammation. Besides being addressed as a proinflammatory condition, it is also described as a prooxidant state and both contribute to dysfunctional cell signalling and macromolecular damage, promoting the functional and structural impairment of adipose tissue, pancreas, liver, muscle, gut and vascular endothelium, as well as central dysregulation of food intake, body mass and systemic metabolism[3–6]. Oxidative stress is a major contributor to the development of co-morbidities related to obesity. This obesity-induced oxidative stress involves multiple biochemical mechanisms, such as reactive oxygen species (ROS) generation from NADPH oxidases (NO_x), protein kinase C (PKC) activation, hyperleptinemia, chronic inflammation, postprandial ROS generation, etc.[5] Among the obese population, the increased CVD risk related to oxidative stress is one of the biggest concerns. For example, there is evidence that oxidative stress decreases endothelium-derived nitric oxide (NO) bioavailability, further promoting the development of arterial hypertension, and

that oxidized LDL (Ox-LDL)-induced changes in adipocyte proliferation and adipokine secretion potentially contribute to CVD.[5] Oxidative stress, along with a low-grade inflammatory state, is already present in young obese children with no evidence of other medical conditions at the onset of obesity, possibly playing a critical role in the early development of vascular damage and other cardiometabolic complications[7–9].

The consideration of sex/gender differences in clinical practice is increasingly being viewed as an important issue to improve health care and patient outcomes[1,10,11]. Gender differences are known to exist in cardiometabolic diseases, as well as in oxidative stress processes. There are many studies pointing to a higher vulnerability of males with these pathophysiological conditions. In western countries, ischaemic heart disease (IHD) develops approximately 7 to 10 years later in women compared to men[1]. Similar differences are also suggested to exist in hypertension, with significantly lower prevalence in women below the age of 30[1], with sex steroids (e.g. estrogen) and chromosomes playing a role in BP control[12,13]. Women with metabolic syndrome have also been reported to have better antioxidant status (TAS) and lipid profiles, in comparison to men[14]. However, there are also descriptions of worse cardiometabolic profiles in overweight/obese or diabetic females. For example, a study in young healthy adults, reported that oxidative stress markers such as plasma Ox-LDL and TAS values were significantly more influenced by central adiposity indicators in women than in men[15]. The adverse cardiometabolic risk profile related to obesity is observed from early adolescence, with cardiometabolic risk factors being more prevalent with the increasing degree of obesity and with expected differences between genders starting to arise[16]. Nevertheless, in early childhood, and particularly in the prepubertal period, few studies have focused on gender differences in obesity-related comorbidities[17,18], perhaps because the small differences on the levels of sex hormones at this age might be expected to have a small

impact on the metabolic and cardiovascular function[18,19]. However, increasing evidence indicates that prepubertal boys and girls, at a similar age, may already present significantly different concentrations of sex hormones[19–21] and that these differences may already be clinically relevant, specifically in what concerns to cardiovascular risk. Previous studies reported significant associations between obesity parameters and blood pressure or fasting blood glucose levels in prepubertal boys, but not in girls[18]. Noteworthy, a study in healthy term neonates reported gender specific differences in oxidative stress and inflammatory signalling thus suggesting that gender differences need to be considered even in early childhood[22].

In light of this body of evidence, we hypothesize that oxidative stress, cardiometabolic risk factors and their association in the setting of obesity might already be influenced by gender in prepubertal children. Therefore, we aim to evaluate if the association between several redox parameters and cardiometabolic variables is different between genders, and according to nutritional status, in 8 to 9-year-old children included in the Generation XXI cohort.

METHODS

Study design and sample

We studied children aged 8–9 years that have been followed since birth in a previously established cohort study (Generation XXI, Porto, Portugal)[23]. 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[24]. We 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 30km away from the study site and 6 pairs of twins). We enrolled 324 participants, between August 2013 and August 2014, but for the present analysis we additionally excluded 11 children due to incomplete evaluation, such as absence of blood or urine sample for oxidative stress and NO production/metabolism markers determination. A total of 313 children were included in the present analysis.

Data collection and variable definition

The 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[25]. 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 ≤ +1 standard deviation (SD)] and overweight/obesity (> 1SD)[26].

Ambulatory blood pressure monitoring (ABPM) for 24 hours was performed in all children with a portable non-invasive oscillometric blood pressure recorder (Spacelabs Healthcare®, model 90207, Snoqualmie, WA, USA). The non-dominant arm was used in all children with an appropriate cuff size. BP measurements were taken automatically at 20-min intervals during the daytime and at 30-min intervals during the night-time. A minimum monitoring duration of 24h with gaps of less than 2h was required for acceptance; five exams were excluded due to insufficient readings. Hypertension was defined as an average systolic (SBP) and/or diastolic blood pressure (DBP) measurements ≥ 95th percentile, during the day or the night, according to the reference values[27]. The absence of dipping pattern was considered as a fall in the mean arterial pressure (MAP) during night-time of less than 10% of the corresponding daytime BP. Carotid-femoral pulse wave velocity (PWV) analysis was performed by a single trained cardiopneumology technician with a portable device (Micro Medical®, model PulseTrace PWV PT4000, Kent, UK); digital volume pulse waveform had to fill 2/3 of the display with little or no noise and artefact to be considered and three measurements of PWV were performed and averaged for analysis.

Laboratory procedures

A venous blood sample was collected after an overnight fast of at least 8 hours and analysed for uric acid, glucose, insulin, lipids, myeloperoxidase (MPO), isoprostanes (P-Isop),

nitrates and nitrites (P-NO_x) and total antioxidant status (TAS). Insulin resistance was determined using the homoeostasis model assessment index (HOMA-IR). All participants collected a 24-h urine sample, which was analysed for creatinine, isoprostanes (U-Isop), urinary hydrogen peroxide (U-H_2O_2) and nitrates and nitrites (U-NO_x). All the parents received information on the correct methods of 24-h 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 11.3–28.0mg/kg per d (according to age- and sex-specific reference values[28]) and if the urinary volume was over 300mL; on the basis of these criteria, 15 urine samples were excluded from the analysis. All the standard laboratory analyses were performed in the Clinical Pathology Department of Centro Hospitalar 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 Pharmacology and Therapeutics of the Faculty of Medicine, University of Porto, using commercial kits and following the manufacturer's specific instructions. In brief, serum MPO was quantified by an immunoenzymatic assay (BioCheck, MPO Enzyme Immunoassay Test Kit; Oxis International Inc.). Plasma 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, etc. 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 (P-Isop) containing the

preservatives butylated hydroxy toluene (BHT, 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 BHT (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[29]. Total nitrates and nitrites (NO_x) were evaluated in P-NO_x and U-NO_x 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_2O_2 (U-H_2O_2) was evaluated by a microplate fluorimetric assay (Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit; Molecular Probes, AlfaGene). Data concerning oxidative stress and NO bioavailability markers have been recently used by our group as part of studies on the same cohort to evaluate the association of these biomarkers with cardiometabolic risk, renal function and angiotensinogen[8,9,30].

Ethics

The ObiKid study was approved by the Ethics Committee of Centro Hospitalar São João, Entidade Pública Empresarial (E.P.E.) and Faculty of Medicine, University of Porto, and complies with the Helsinki Declaration, the guidelines for the ethical conduct of medical research involving children[31] and the current national legislation. Written informed consent from parents (or their legal substitute) and verbal assent from children were obtained regarding information and biological samples gathering.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics, Version 25.0 (Armonk, NY). The data is presented as mean and standard deviation (SD) or, if skewed, as median with percentiles (P25-P75). The distribution of oxidative stress and NO production/metabolism markers by classes of body mass index (normal weight, overweight and obese) in both genders were shown in box plot graphs and compared using Kruskal-Wallis tests. Spearman's correlations were used to test bivariate associations between oxidative stress and NO production/metabolism markers and cardiometabolic variables.

Linear multivariate regression models were fitted with each cardiometabolic variable as the dependent variables to test their association with oxidative stress and NO production/metabolism markers. All linear regression models were stratified by gender because there was a significant interaction between gender and some oxidative stress and NO production/metabolism markers. All models were additionally adjusted for age (months) and BMI z-score. The oxidative stress and NO production/metabolism markers had an asymmetric distribution, so those included in the regression models were logarithmized (base 10), allowing to obtain a normal distribution. P values were considered statistically significant if <0.05.

RESULTS

A total of 313 children with a mean (SD) age of 8.8 (0.2) years, 166 boys, were included in the present study. General characteristics of the study sample are shown, for girls (n=147) and boys (n=166) in Table 1. The mean BMI z-score was higher in boys, but the difference was not statistically significant (1.07 vs. 0.81, in boys and girls respectively, p=0.067). Daytime and night-time MAP values, both absolute values and z-scores, and PWV values were similar in girls and boys. Total cholesterol, non-HDL-cholesterol, triglycerides, and HOMA-IR levels were considerably higher in girls, whereas the HDL-cholesterol levels were not significantly different between boys and girls. The values of oxidative stress markers, including uric acid, and NO bioavailability parameters, were identical between groups, except for U-H_2O_2, with girls presenting significantly lower amounts (1021 vs. 1577 nmol/day, in girls and boys respectively, p<0.001) (Table 1).

The distribution of oxidative stress and NO synthesis/metabolism markers by classes of BMI in boys and girls, separately, is depicted in Figure 1. P-Isop, P-NO_x, P-TAS and U-H_2O_2 were not significantly different across BMI classes for both genders. MPO concentration and U-Isop values were significantly higher across BMI classes, both in girls and boys (p<0.001 for MPO and p<0.010 for U-Isop). Uric acid concentration was considerably higher in overweight and obese girls than in normal weight girls (p<0.001), whilst in boys no difference was found between the BMI classes. No substantial difference was found in girls for U-NO_x values across BMI groups, whereas in overweight and obese boys the U-NO_x values were significantly higher than in normal weight boys (p=0.005).

Correlations between oxidative stress and NO bioavailability markers and cardiometabolic variables are shown in Table 2. In girls, we found positive correlations

between MPO and HOMA-IR, uric acid and HOMA-IR, U-Isop and triglycerides or U-Isop and HOMA-IR. In boys, MPO and uric acid were positively correlated with night-time MAP *z*-score and U-Isop values presented a positive correlation with daytime MAP *z*-score. Boys also exhibited positive correlations between P-TAS and PWV and between U-NO_x and triglycerides, but negative correlations between U-H_2O_2 and PWV or between U-NO_x and total cholesterol or non-HDL-cholesterol.

Considering the associations found between oxidative stress and NO bioavailability markers and cardiometabolic variables in the univariate analysis, multiple linear regression models were plotted for each sex, adjusted for age and BMI *z*-score, with each cardiometabolic variable as the dependent variable and with each oxidative stress and NO synthesis markers as covariates. The following models were plotted (model 1 – daytime MAP z-score and U-Isop; model 2 and 3 – night-time MAP *z*-score and MPO or uric acid, respectively; model 4 and 5 – PWV and TAS or U-H_2O_2, respectively; model 6 – total cholesterol and U-NO_X; model 7 – non-HDL-cholesterol and U-NO_X; model 8 and 9 – triglycerides and U-Isop or U-NO_X, respectively; model 10, 11 and 12 – HOMA-IR and MPO or uric acid or U-Isop, respectively). No statistical significance was found in models 1, 2, 3, 4, 8, 9, 10 and 11. In model 5, U-H_2O_2 was significantly associated with lower PWV values only in boys, decreasing by 0.059 m/s (95% confidence interval: -0.109 to -0.008, p=0.023) per unit of pmol/day of U-H_2O_2. In model 6 and 7, in boys, but not in girls, U-NO_x was associated with lower values of total cholesterol and non-HDL cholesterol which decreased by 0.008 mg/dL (95% confidence interval: -0.014 to -0.001, p=0.020) and 0.006 mg/dL (95% confidence interval: -0.012 to -0.001, p=0.021) per unit of µmol/day of U-NO_x, respectively. In model 12, U-Isop was extensively associated with higher levels of HOMA-IR, in girls, increasing the

HOMA-IR SCORE by 0.155 (95% confidence interval: 0.049 to 0.261, p=0.005) per unit of (ng/day) /1000 of U-Isop, whereas in boys no association was found.

DISCUSSION

The results of our study show that gender plays a significant role in the effect of several oxidative stress/NO bioavailability markers, such as U-H_2O_2, U-NO_x and U-Isop, on cardiometabolic risks factors, namely PWV, total and non-HDL cholesterol and insulin resistance assessed by HOMA-IR, respectively. Significant inverse associations between U-H_2O_2 and PWV, as well as between U-NO_x and total/non-HDL cholesterol were observed amongst boys, whereas in girls there was a significant positive association between U-Isop and HOMA-IR. This suggests that girls are more prone to oxidative stress-induced metabolic disorders than boys. Furthermore, in boys, higher values of redox/NO bioavailability markers appear to be protective, rather than deleterious, regarding their impact on arterial stiffness and cholesterol homeostasis.

Noteworthy, we demonstrated that boys had significantly higher excretion of U-H_2O_2 compared to girls and that there was a negative association between these values and PWV, an index of arterial stiffness. There is currently evidence describing H_2O_2 as a potential regulator of aortic stiffness. Zou et al. reported that aged mice with a deficiency in superoxide dismutase 2 and consequent low concentrations of H_2O_2 showed marked changes in vascular wall, namely increases in PWV, collagen I expression and medial smooth muscle cell apoptosis and disruption of elastic lamellae integrity[32]. A further study in a different mutant mice model associated with decreased H_2O_2 production also demonstrated an enhanced extracellular matrix, vascular stiffness and impaired vascular smooth muscle contraction[33]. Moreover, the influence of gender on oxidative stress markers or ROS bioavailability has also been described, with male gender presenting higher values of ROS or oxidative stress biomarkers, both in animal models and in humans[12,34,35]. For example, it has been reported that H_2O_2 production

is significantly higher in vascular cells from males than in cells from females and that male spontaneously hypertensive rats (SHR) have increased urinary excretion of H_2O_2 when compared with female SHR[12,34,35]. These results are in line with those found in our study and may help to explain the higher values of U-H_2O_2 found in boys, as well as their negative association with PWV.

Besides the putative protective impact of H_2O_2 in the vascular structure and function, boys also seem to benefit from increased U-NO_x values, as inferred from the inverse correlation of U-NO_x with total/non-HDL cholesterol. Our group had previously observed this association between U-NO_x and total or non-HDL cholesterol values among obese children[8], which is in accordance with prior studies in experimental animals showing that hypercholesterolemia contributed to low renal NO production and possibly explains the renal dysfunction in obesity[36,37]. Moreover, the incubation of murine peritoneal macrophages with both cholesterol and oxidized cholesterol was shown to induce a dose-dependent decrease in NO values[38]. Noteworthy, the inverse association between NO and cholesterol values may result not only from the negative impact of cholesterol on NO production, but also from an inhibitory effect of NO on plasma cholesterol concentration. Indeed, a study in mice overexpressing human endothelial NO synthase showed that these animals exhibited lower plasma cholesterol concentrations than control mice[39]. Obesity is a condition involving an overaccumulation of body fat and is strictly associated with hyperlipidemia. Endothelial and inducible NO synthases have been shown to be present in adipose tissue of the rat, suggesting that adipose tissue may be a possible source of NO synthesis[40]. This was reinforced by our previous study where oxidative status and NO were shown to be increased in overweight and obese prepubertal children[8], and is also consistent with our present findings of significantly higher values of U-NO_x in overweight and obese boys compared to those with normal weight.

Furthermore, in the present evaluation, boys also exhibited lower concentrations of total and non-HDL cholesterol than girls. Nevertheless, a study performed in adolescents reported that serum NO_x concentrations were markedly higher in those presenting augmented values of total cholesterol and triglycerides, thus suggesting that NO production is positively correlated with serum lipid levels. Additionally, the authors also showed that the association between NO_x and serum lipids were stronger in male than in female adolescents[41]. In our study, the only similarity with these results was the positive association, detected by univariate linear regression, between U-NO_x and triglycerides. However, this effect was observed only among boys, whereas in the study involving adolescents this association was evident for both genders. Furthermore, the study in adolescents detected a positive and stronger correlation between U-NO_x and total or non-HDL cholesterol in males, while we found a negative association between these parameters in boys. Since high lipid concentrations promote the development of atherosclerosis and coronary heart disease which are known to be frequently associated with low systemic NO_x values, it is possible that in initial stages of hyperlipidemia there is a compensatory rise in NO_x concentration, which is followed by a decline in NO production in later stages of atherosclerosis development[41].

While in prepubertal boys the increase of redox status and NO bioavailability markers appears to be protective, regarding cardiovascular and metabolic parameters, in girls there seems to be a higher vulnerability to oxidative stress-induced metabolic derangements, as evidenced by the positive association between U-Isop and insulin resistance which was detected only for female gender. Evidence shows that isoprostanes are directly related to insulin resistance, even when adjusted for percent body fat[42,43]. Accordingly, our group previously observed a significant positive association between these parameters[8]. Murphy et al. also reported that prepubertal girls are intrinsically more prone to insulin resistance than

boys and that sex-linked genes could be the reason for this difference[44]. In 2015, a meta-analysis showed likewise, that girls seemed to have higher prevalence rates of insulin resistance than boys, and this would be explained by their earlier pubertal development[45]. This is in line with the findings of our present work, where we were able to demonstrate higher values of HOMA-IR amongst girls, when compared to boys. Moreover, we also evidenced a positive association between U-Isop values and insulin resistance in girls, which persisted after adjustment in multivariate models.

Strengths and Limitations

Major strengths of this study reside in the broad evaluation of redox status/NO bioavailability biomarkers, as well as in the detailed characterization of cardiometabolic risk factors, in a large and homogeneous sample of healthy prepubertal children in the setting of a birth cohort sample, which allows the possibility of further evaluations. Regarding limitations, it is important to note that the protocol of our study did not consist of any dietary restraints during the 48 hours prior to collecting the sample, as well, nor meals nor physical activity of children were considered in our analysis. Furthermore, the connections found in the cross-sectional evaluation, although they are relevant, they must be interpreted with vigilance, especially regarding causality inferences, which is portrayed as a disadvantage for the interpretation of our results.

Conclusions

Our results show that gender differentially impacts oxidative stress, NO bioavailability and cardiometabolic risk factors, as well as the association between these parameters. Noteworthy, prepubertal girls appear to be more prone to the development of oxidative stress-induced metabolic dysfunction, while in boys the rise in the values of redox and NO

bioavailability markers seems to be beneficial, rather than deleterious, for vascular structure/function and lipid homeostasis. These findings suggest that primary prevention of cardiovascular and metabolic diseases should start early in childhood, and that gender differences in the susceptibility for these disorders should be considered to improve health care. A future prospective evaluation of this cohort will be important to determine the prognostic significance of the differences found in biomarkers of redox status and NO bioavailability, in terms of cardiometabolic disease development.

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Table 1 – Characteristics of the study sample by gender.

	GIRLS n= 147	**BOYS** n= 166	***p***
Demography and anthropometry			
Age (years)	8.8 ± 0.2	8.8 ± 0.2	0.875
Weight (kg)	32.5 ± 7.6	33.5 ± 7.7	0.275
Height (cm)	133 ± 6	134 ± 6	0.075
BMI ($kg.m^{-2}$)	18.3 ± 3.2	18.5 ± 3.1	0.596
BMI *z*-score	0.81 ± 1.21	1.07 ± 1.25	0.067
24-h ambulatory blood pressure and PWV			
Daytime MAP (mmHg)	85.2 ± 5.9	85.4 ± 4.9	0.690
z-score	0.14 ± 0.90	0.13 ± 0.93	0.910
Night-time MAP (mmHg)	74.0 ± 6.1	74.2 ± 4.5	0.688
z-score	0.70 ± 0.89	0.60 ± 0.92	0.347
PWV (m/s)	5.06 ± 0.51	5.01 ± 0.50	0.414
Biochemical parameters			
Total cholesterol (mg/dL)	162 ± 26	156 ± 25	0.038
Non-HDL-cholesterol (mg/dL)	109 ± 25	102 ± 2	0.004
HDL-cholesterol (mg/dL)	53 ± 10	55 ± 11	0.211
Triglycerides (mg/dL)	63 ± 30	55 ± 22	0.006
HOMA-IR	1.44 (1.03-2.08)	1.18 (0.93-1.50)	0.001
Oxidative stress and NO bioavailability markers			
P-Isop (ng/mL)	0.352 (0.173-0.616)	0.356 (0.200-0.568)	0.980
P-NO_x (nmol/mL)	12.4 (9.5-17.1)	12.2 (9.7-25.5)	0.781
TAS (mM Trolox equivalents)	1.20 (0.99-1.60)	1.22 (1.00-1.65)	0.799
MPO (ng/mL)	54 (35-89)	63 (36-93)	0.503
Uric acid (mg/dL)	3.65 ± 0.76	3.65 ± 0.70	0.988
U-Isop (ng/day)	1535 (1171-2227)	1689 (1234-2303)	0.083
U-NO_X (µmol/day)	764 (556-1115)	834 (573-1213)	0.232
U-H_2O_2 (nmol/day)	1021 (493-1964)	1577 (898-2372)	<0.001

The values presented are mean ± standard deviation or median (25^{th} percentile - 75^{th} percentile).

BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; MAP, mean arterial pressure; MPO, myeloperoxidase; P-Isop, plasma isoprostanes; P-NO_x, plasma nitrates and nitrites; PWV, pulse wave velocity; TAS, total antioxidant status; U-Isop, urinary isoprostanes; U-H_2O_2, urinary hydrogen peroxide; U-NO_x, urinary nitrates and nitrites.

Table 2 – Spearman correlations between oxidative stress and NO production/metabolism markers and cardiometabolic parameters in girls and boys.

	P-Isop (ng/mL)		**P-NO_X** (nmol/mL)		**TAS** (mM Trolox equiv.)		**MPO** (ng/mL)		**Uric acid** (mg/dL)		**U-Isop** (ng/day)		**U-NO_X** (µmol/day)		**U-H_2O_2** (nmol/day)	
	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys
Daytime MAP *z*-score	0.069	0.092	-0.010	0.038	-0.022	-0.071	0.053	0.101	-0.007	0.150	0.019	0.206*	-0.024	0.041	-0.004	0.032
Night-time MAP *z*-score	-0.031	0.152	0.025	0.014	0.018	-0.121	0.111	0.206**	0.049	0.160*	0.157	0.157	0.078	-0.033	0.060	-0.072
PWV (m/s)	-0.004	-0.078	-0.137	0.064	0.104	0.173*	0.101	0.021	0.028	-0.049	-0.106	-0.007	0.035	0.057	0.107	-0.169*
Total cholesterol (mg/dL)	0.074	-0.028	0.112	-0.150	-0.016	0.064	-0.087	0.080	-0.003	0.056	-0.025	-0.007	-0.083	-0.240**	-0.053	-0.044
Non-HDL-cholesterol (mg/dL)	0.063	-0.036	0.116	-0.101	0.017	0.076	-0.071	0.083	0.063	0.142	-0.029	-0.019	-0.079	-0.200*	-0.049	-0.020
HDL-cholesterol (mg/dL)	0.056	-0.040	0.022	-0.037	-0.073	0.018	-0.078	-0.030	-0.110	-0.114	-0.028	0.019	0.021	-0.083	0.034	-0.019
Triglycerides (mg/dL)	-0.020	-0.024	0.084	0.034	-0.052	-0.046	0.044	0.068	0.125	0.122	0.178*	0.091	-0.013	0.167*	0.071	0.130
HOMA-IR	0.075	-0.033	-0.073	-0.095	-0.083	-0.061	0.214**	0.029	0.266**	0.119	0.294**	0.137	-0.037	0.041	0.016	0.010

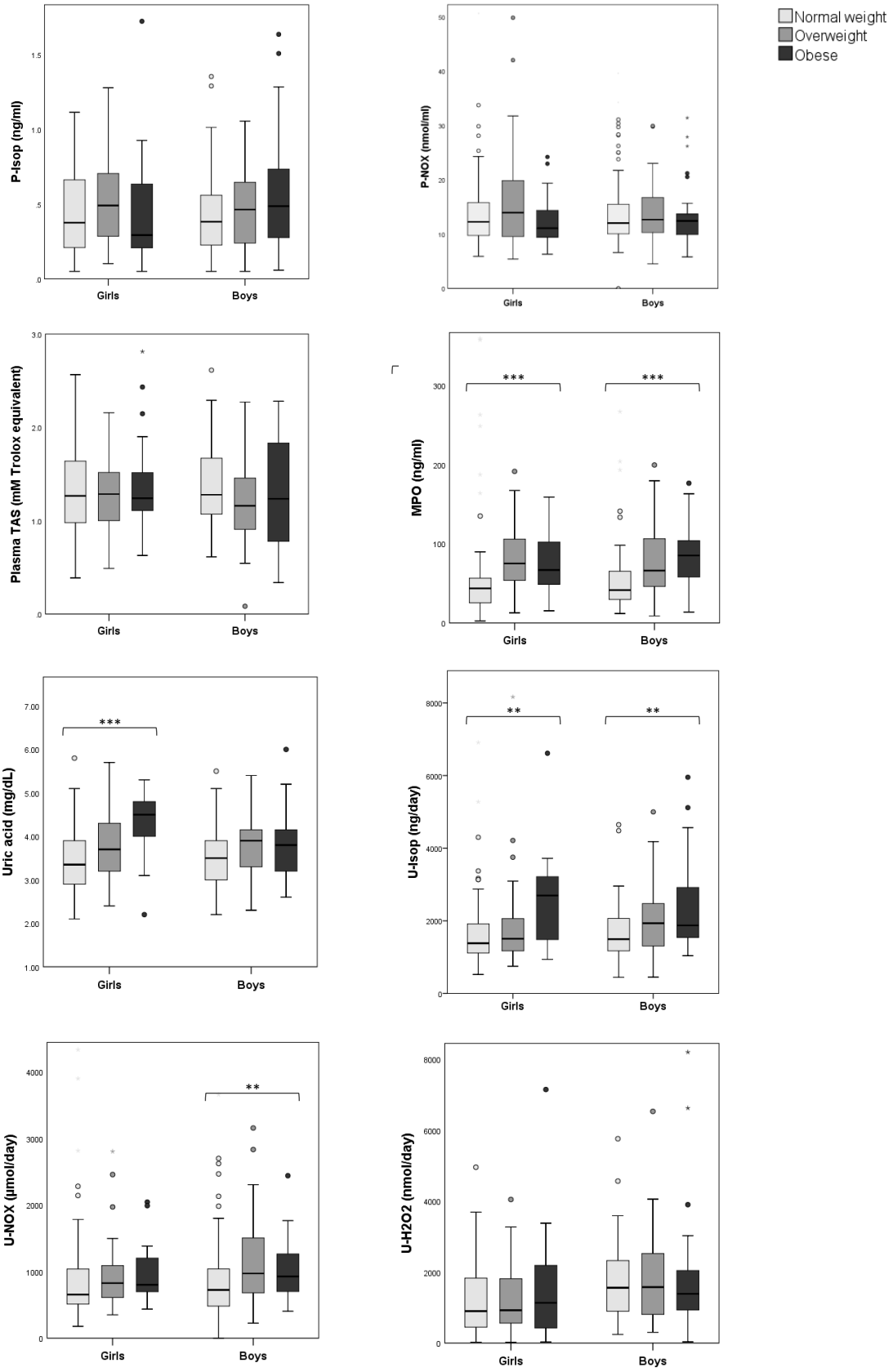
HOMA-IR, homoeostasis model assessment of insulin resistance; MAP, mean arterial pressure; P-Isop, plasma isoprostanes (in ng/mL); P-NO_x, plasma nitrates and nitrites (in nmol/mL); PWV, pulse wave velocity (in m/s); TAS, total antioxidant status (in mM Trolox equivalents); U-H_2O_2, urinary hydrogen peroxide (in nmol/day);); U-Isop, urinary isoprostanes (in ng/day); U-NO_x, urinary nitrates and nitrites (in µmol/day).
* p value <0.05, ** p value <0.01.

Table 3 – Multivariate linear regression models for each cardiometabolic parameter, as the dependent variables.

		GIRLS	BOYS
		Adjusted β (95% CI)	**Adjusted β (95% CI)**
	Daytime MAP *z*-score		
Model 1	U-Isop (per ng/day) /1000	0.011 (-0.139 to 0.161)	0.001 (-0.115 to 0.116)
	p	0.885	0.991
	Night-time MAP *z*-score		
Model 2	MPO (ng/mL)	0.001 (-0.002 to 0.003)	0.001 (-0.003 to 0.004)
	p	0.570	0.662
Model 3	Uric acid (mg/dL)	0.010 (-0.201 to 0.222)	0.206 (-0.001 to 0.414)
	p	0.922	0.051
	PWV (m/s)		
Model 4	TAS (per Mm Trolox equivalents)	-0.039 (-0.222 to 0.144)	0.059 (-0.127 to 0.246)
	p	0.676	0.532
Model 5	U-H_2O_2 (per nmol/day) /1000	0.034 (-0.025 to 0.093)	-0.059 (-0.109 to -0.008)
	P	0.251	0.023
	Total cholesterol (mg/dL)		
Model 6	U-NO_X (per µmol/day)	-0.001 (-0.008 to 0.005)	-0.008 (-0.014 to -0.001)
	P	0.697	0.020
	Non-HDL-cholesterol (mg/dL)		
Model 7	U-NO_X (per µmol/day)	-0.001 (-0.007 to 0.005)	-0.006 (-0.012 to -0.001)
	P	0.826	0.021
	Triglycerides (mg/dL)		
Model 8	U-Isop (per ng/day)	0.004 (0.000 to 0.008)	0.001 (-0.002 to 0.003)
	P	0.074	0.620
Model 9	U-NO_X (per µmol/day)	0.004 (-0.003 to 0.011)	0.003 (-0.002 to 0.009)
	P	0.266	0.251
	HOMA-IR		
Model 10	MPO (ng/mL)	0.000 (-0.002 to 0.003)	-0.001 (-0.003 to 0.001)
	p	0.774	0.444
Model 11	Uric acid (mg/dL)	0.054 (-0.141 to 0.249)	0.051 (-0.069 to 0.171)
	p	0.584	0.403
Model 12	U-Isop (per ng/day) /1000	0.155 (0.049 to 0.261)	0.053 (-0.013 to 0.119)
	p	0.005	0.114

The values presented are adjusted linear regression coefficients (β) and 95% confidence intervals for each oxidative stress and NO production/metabolism marker, estimated by linear regression models with each cardiometabolic variable as the dependent variables. All models are additionally adjusted for age (months) and BMI *z*-score. The results are presented separately by gender.

Figure 1 – Distribution of oxidative stress and NO production/metabolism markers among BMI *z*-scores classes (normal weight, overweight and obese), by gender.



* p value <0.05, ** p value <0.01, *** p value <0.001.