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**Fetal undernutrition is associated with perinatal sex-dependent alterations in oxidative status**

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**Running title:** Fetal programming, sex and plasma oxidative status

## **Abstract**

Intrauterine growth retardation predisposes to hypertension development, known as fetal programming. Females are less susceptible, which has been mainly attributed to estrogen influence. We hypothesize that perinatal differences in oxidative status might also contribute. We studied 21-day old (prepuberal) and 6-month old male and female offspring from rats fed *ad libitum* during gestation (Control) or with 50% of Control daily intake from day 10 to delivery (Maternal Undernutrition, MUN). We assessed: *in vivo* blood pressure and the following plasma biomarkers of oxidative status: protein carbonyls, thiols, reduced glutathione (GSH), total antioxidant capacity (TAC), superoxide anion scavenging (SOSA) and catalase activities and calculated a global score (oxy-score) from them. Estradiol and melatonin concentration was measured in young rats. Prepuberal MUN males were normotensive but already exhibited increased carbonyls and lower thiols, GSH, SOSA and melatonin; oxy-score was significantly lower compared to Control males. Prepuberal MUN females only exhibited reduced SOSA compared to Control females. Adult rats from all experimental groups showed a significant increase in carbonyls and a decrease in antioxidants compared to prepuberal rats; oxy-score was negative in adult rats suggesting the development of a pro-oxidative status as rat age. Adult MUN males were hypertensive and exhibited the highest increase in carbonyls despite similar or even higher antioxidant levels compared to Controls. Adult MUN females remained normotensive and did not exhibit differences in any of the biomarkers compared to Controls. The better global antioxidant status developed by MUN females during perinatal life could contribute to their protection against hypertension programming.

**Keywords:** maternal undernutrition; sex-dependent fetal programming; hypertension; oxidative stress; antioxidants; melatonin; plasma; experimental models

## 1. Introduction

Non-communicable diseases (NCDs) are the main contributors to the global disease burden in developed societies and are also increasing in developing countries [1] [2]. Despite this fact, the causes of many NCDs are unknown and, therefore, ways to prevent them remain elusive. The conventional etiological model of NCDs based on a genetic predisposition and unhealthy lifestyle was challenged by Dr. Barker, who proposed that intrauterine life was an additional risk factor [3]. This hypothesis, known as fetal programming, is now well established through epidemiological and experimental studies evidencing the negative influence of intrauterine growth retardation (IUGR) in the development of NCDs, particularly cardiovascular diseases (CVD) and their risk factors obesity and hypertension [4,5].

It has been recently put forward that different NCDs might have common pathophysiological mechanisms, and oxidative stress might play a central role in their onset and development [6]. Therefore, it is plausible that exposure to stress conditions during intrauterine life could trigger an oxidative imbalance from early age, leading to long-term tissular damage and NCDs pathology. In fact, oxidative stress has been implicated in fetal programming [7] and several studies in animal models of IUGR have evidenced that oxidative damage is present in key organs for cardiovascular control such as the heart [8] the kidney [9] [10] and blood vessels [11]. However, the presence of oxidative damage in animals with already established pathology does not determine whether oxidative stress is the cause or consequence of the cellular alterations [12]. In addition, most studies have focused on individual biomarkers, and the complex and multifactorial nature of oxidative stress makes it difficult to assign a prevalent role to a particular biomarker and does not provide information on the global oxidative status. We and others have previously used the calculation of a global score, based on individual plasma biomarkers of antioxidant defense and oxidative damage, to assess the overall oxidative balance in several CVD [13-15].

Fetal stress seems to have a lower impact on females, particularly in the development of hypertension [16-18]. This difference has been mainly attributed to the modulatory role of sex hormones, but other innate sex differences have also been proposed [19]. Despite the fact that “sex” is a critical variable to be taken into consideration [20], the sex-dependent phenotype of fetal programming has not been thoroughly investigated, since most studies have been

performed on male animals. Based on the evidence that IUGR, induced by nutrient restriction to the fetus, is associated with oxidative stress, we hypothesize that females might counteract the deleterious effects of IUGR through the development of a better global oxidative balance during the critical developmental window of perinatal life. This early advantage, together with the cardiovascular protective effect of estrogens, could contribute to the milder consequences of IUGR on CVD programming on females.

To assess our hypothesis we have studied a rat model of IUGR induced by maternal undernutrition during the second half of gestation (MUN), which has been previously demonstrated to program for hypertension and obesity in adult age [5,21-24]. In male and female offspring from MUN and Control dams, we have assessed individual plasma biomarkers of oxidative damage and antioxidant capacity and calculated a global score (oxy-score) from them. We have chosen to study two age points: weaning, as a critical developmental period prior to sexual maturity, and adult age, where hypertension is already established in males.

## 2. Material and Methods

Experiments were performed in Sprague Dawley (SD) rats from the colony maintained at the Animal House facility of the Universidad Autónoma de Madrid. All experimental procedures were approved by the Ethics Review Board of Universidad Autónoma de Madrid and conformed to the Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised in 1996), the Spanish legislation (RD 1201/2005) and the Directive 2010/63/EU on the protection of animals used for scientific purposes.

The rats were housed in buckets 36.5/21.5/18.5 cm (length/width/height) on aspen wood bedding, under controlled conditions of 22 °C, 40% relative humidity and 12/12 light/dark photoperiod. The animal health monitoring indicated that they were free from pathogens that may interact with any of the parameters studied.

The rats were fed with breeding diet (SAFE A03) containing 51.7% carbohydrates, 21.4% protein, 5.1% lipids, 3.9% fibre, 5.7% minerals and 12.2% humidity (Safe Augy, France).

Drinking water was provided *ad libitum* in all cases.

### 2.1. Experimental model (MUN)

Maternal undernutrition (MUN) was induced as previously described [25]. Day 1 of gestation was determined by observation of sperm in the vaginal smear. Dams were then divided in two groups, one with *ad libitum* diet throughout pregnancy (Control group, C, n=6) and the other with *ad libitum* diet during the first 10 days of gestation and 50% of the daily intake from day 11 to the end of gestation (MUN, n=6). The maximum daily intake of rat chow was previously determined in a group of pregnant rats as 24 grams/day. Therefore, during the second half of gestation the MUN group received 12 grams of rat chow/day. After delivery, both C and MUN rats were offered food *ad libitum* during the suckling period.

24h after birth (postnatal day 1, P1) the pups were sexed and weighed individually, and the litter was standardized to 12 individuals, 6 males and 6 females, if possible (smaller litters were not used). At weaning (21 days, P21) 1-2 males and 1-2 females from every litter were used to test blood pressure, plasma parameters of oxidative status and estrogen levels.

One male and 1 female rat from each litter was kept for another 2 weeks under changed light/dark cycle to measure peak plasma levels of the antioxidant hormone melatonin. At the end of the 2 week period, sufficient for a change in melatonin pattern of secretion [26], the rats

were anesthetized by CO<sub>2</sub> and the blood was collected by cardiac puncture at 11.00h, peak time of melatonin secretion under the changed cycle. The rest of the litter was maintained until the age of 6 months to assess blood pressure, plasma biomarkers of oxidative status and for other studies.

## *2.2. Blood pressure measurements*

Systolic and diastolic blood pressures (SBP and DBP, respectively) and heart rate (HR) were measured in rats under anesthesia (ketamine 80 mg/kg-xylazine 10 mg/kg, i.p) as previously described [27]. Briefly, a cannula connected to a pressure transducer (Statham; Harvard Apparatus) was inserted in the right iliac artery and pressure wave was recorded in a PowerLab system (ADInstruments) during 60 minutes. SBP, DBP and HR were measured in the trace at the end of the 60 min, averaging the data from approximately 1 min recording period.

## *2.3. Biomarkers of oxidative status in plasma*

After blood pressure measurements, blood was collected from the cannula inserted in the iliac artery in eppendorff tubes containing 5% heparine. The blood was centrifuged for 10 min (900 g at 4°C) and the plasma was aliquoted and stored at -70°C until use.

*2.3.1. Total protein carbonyls.* Plasma protein carbonyls were assessed with a 2,4 dinitrophenylhydrazine-based assay [28], as previously described [27]. The protein carbonyl concentration was determined using extinction coefficient of 2,4-dinitrophenylhydrazine ( $\epsilon$  22000 M/cm) and expressed as nmol/mg protein. Protein content was assessed by Coomassie blue based microtiter plate assay, according to manufacturer's instructions (Bio-Rad). The absorbance was measured at 595 nm in a microplate reader (Synergy HT Multi-Mode, Biotek).

*2.3.2. Total antioxidant capacity (TAC).* TAC assay is based on enhanced horseradish peroxidase-catalyzed luminol chemiluminescence [29,29]. The relative luminescence (RL) was calculated by the following formula:  $RL = [1 - \text{luminescence}(t)] / [\text{luminescence}(t_0)]$ . RL was used to calculate the area under the curve (AUC) using GraphPad Prism software (GraphPad, San Diego, CA).

*2.3.3. Reduced glutathione (GSH) content.* Plasma GSH was assessed by a fluorimetric micromethod based on the reaction with ophthalaldehyde as previously described [27]. Fluorescence was measured in the microplate reader at 360±40 nm excitation and 460±40 nm emission wavelengths. GSH concentration of the samples was expressed as  $\mu\text{mol/mg}$  protein.

2.3.4. *Total thiols*. Plasma thiols were assessed by a 5,5'-dithiobis(2-nitrobenzoic acid) assay [28], adapted to a microplate reader, as previously described [27]. The absorbance was measured at 412 nm and thiol content was expressed as nM GSH/mg protein.

2.3.5. *Superoxide anion scavenging activity (SOSA)*. SOSA was determined using a luminescence assay with coelenterazine as detection probe [29,29], adapted to microplate reader [14]. SOSA values were quantified by comparing the luminescence inhibition of each sample with that observed from superoxide dismutase (SOD) activity standard curve (0-4 U/mL) and expressed as mU SOD/mg protein.

2.3.6. *Catalase activity*. Catalase activity was assessed by Amplex Red catalase assay (EnzChek Myeloperoxidase Assay Kit with Amplex Ultra Red reagent; Invitrogen). Catalase activity was expressed as Units/mg protein.

#### 2.4. *Calculation of a global score of oxidative status (oxy-score)*

The biomarkers of oxidative status mentioned above were used to calculate a global index (oxy-score) for each experimental group, using the statistical methodology previously described [13,14], using the following steps:

1. Analysis of the normality of the different biomarkers through the Kolmogorov-Smirnov test and Q-Q graphs.
2. Normalization of those parameters that did not show a normal distribution through a logarithmic transformation.
3. Parameter standardization.
4. Calculation of oxy-score based on the partial indexes (OXY and ANTIOX), according to the following equations:

$$\text{Oxy-score} = (\text{ANTIOX} - \text{OXY})$$

OXY = Standardized values of protein carbonyl, as biomarker of oxidative damage

ANTIOX = Sum (standardized antioxidant biomarkers)

Based on this calculation a positive oxy-score indicates prevalence of antioxidant capacity and a negative oxy-score indicates predominance of oxidative damage.

#### 2.5. *Determination of plasma hormones*

2.5.1. *Melatonin*. Plasma was evaporated to dryness with an evaporator centrifuge (Speed Vac SC 200, Savant, USA). The residues were dissolved in distilled water and melatonin levels were



determined by a competitive enzyme immunoassay kit (Melatonin ELISA, IBL International, Germany) according to manufacturer's instructions. The kit is characterized by an analytical sensitivity of 1.6 pg/mL and high analytical specificity (low cross-reactivity). Melatonin was expressed as pg/mL.

2.5.2. *Estradiol*. Plasma estradiol concentration was assessed by a competitive enzyme immunoassay kit (Estradiol ELISA Demeditec Diagnostics GmbH, Germany) according to manufacturer's instructions. Estradiol was expressed as pg/mL.

## 2.6. *Statistical analysis*

Data are expressed as mean  $\pm$  SEM and *Student's t* test was used to assess statistical differences between Control and MUN offspring (age and sex-matched). Statistical significant level was established at  $p < 0.05$ .

### 3. Results

#### 3.1. Growth pattern in MUN model

Body weight at P1 was significantly smaller in MUN offspring, both in males (Control=6.9±0.3 g, n=36; MUN=4.9±0.8 g, n=36; p<0.05) and females (Control=6.8±0.3 g, n=36; MUN=4.8±0.8 g, n=36; p<0.05). However, during the suckling period, MUN offspring had accelerated growth and by weaning (P21) they reached similar weight compared to control rats, both males (Control=51.5±0.9 g, n=11; MUN= 49.8±1.3 g, n=11) and females (Control=50.8±1.1 g, n=11; MUN= 49.4±1.4 g, n=11). At 6 months of age body weight was not different between MUN and Control offspring, either in males (Control=497.1±15.1g, n=10; MUN= 490.0±9.8g, n=10) or in females (Control=302.2±6.8g, n=10; MUN= 283.1±5.5, n=10). At this age females from both experimental groups had significantly smaller body weight compared to age-matched males.

#### 3.2. Sex differences in blood pressure

At the age of 21 days there were no significant differences in SBP or DBP between Control and MUN rats, either in males or in females (Figure 1A). Heart rate was also similar in male (Control=308±26, MUN= 319±18 bpm) and female offspring (Control=311±20, MUN= 327±18 bpm).

At the age of 6 months male MUN offspring exhibited a significantly higher SBP and DBP compared to controls. However, SBP and DBP were not statistically different in female MUN compared to sex-matched Control rats (Figure 1B). HR was not statistically different between control and MUN rats either in males (Control=258±8, MUN=254±10 bpm) or females (Control=222±10, MUN=221±11 bpm).

#### 3.3. Sex differences in individual parameters of oxidative status

21 day old MUN males exhibited significantly elevated carbonyl levels (42% higher), reduced GSH, thiols and SOSA and no significant differences in TAC and catalase activity compared to age and sex matched Control rats (Figure 2). 21 day old MUN females only exhibited significantly reduced SOSA compared to control age-matched females (Figure 3).

At the age of 6 months the carbonyl levels were further elevated in MUN male offspring (60% higher, compared to Control rats). Regarding the levels of individual antioxidants, TAC, GSH, thiols and catalase activity were not statistically different and SOSA was significantly elevated in

MUN males compared to Control rats (Figure 4). 6 month old female MUN exhibited no significant differences in any of the parameters studied compared to controls (Figure 5).

#### *3.4. Age differences in individual parameters of oxidative status*

At the age of 6 months protein carbonyl levels were significantly larger in all experimental groups compared to sex-matched 21 day old rats ( $p < 0.05$ ). On the other hand, SOSA, GSH and catalase were all significantly smaller in 6 month old rats compared to sex-matched 21 day old rats; this was observed in all experimental groups ( $p < 0.01$ ). Plasma thiol concentration was also smaller in 6 month old rats, with the exception of MUN males; in this group thiol levels were significantly elevated compared to 21 day old sex-matched counterparts ( $p < 0.01$ ).

#### *3.5. Oxy-Score*

At the age of 21 days, oxy-score was positive in all experimental groups (Figure 6A), while it was negative at the age of 6 months (Figure 6B).

Regarding sex differences, 21 day old MUN males, but not females, exhibited a significantly lower oxy-score compared to age and sex-matched Controls. Adult MUN male and female rats exhibited no significant differences in oxy-score, compare to sex-matched Controls.

#### *3.6. Plasma hormones.*

We assessed plasma concentrations of estradiol and melatonin in young rats. At P21 estradiol levels were similar in Control and MUN offspring, both in males and females. Melatonin levels (at their peak time of production) were significantly reduced in male MUN offspring compared to age-matched controls, while MUN and Control females exhibited similar values (Table 1).

#### 4. Discussion

Experimental and epidemiological research has evidenced that IUGR predisposes to fetal programming of CVD and hypertension, with a sex-dependent pattern. The less deleterious effects of IUGR on females have been mainly attributed to the cardiovascular protective actions of estrogens and the possible role of early alterations in oxidative stress has not been explored. Oxidative stress is present in several NCDs, such as CVD and it has been suggested it could be the common trigger initiating the pathogenesis of these diseases [6,30]. We hypothesize that males and females might exhibit different responses to fetal insult regarding oxidative-antioxidant balance which might contribute to sex differences in fetal programming of hypertension.

Maternal undernutrition has been widely used as experimental model of fetal programming, since it has been demonstrated that inadequate nutrient supply during intrauterine life is associated with low birth weight and subsequent development of obesity and hypertension in adult life [5] [22] [31]. Suboptimal fetal nutrition is usually followed by a period of accelerated weight gain during early postnatal life, known as catch-up growth. This is a compensatory mechanism to balance growth retardation *in utero*, but seems to be deleterious, contributing to the development of cardiovascular and metabolic alterations later on [32-34]. The rat model of IUGR used in the present study exhibits this accelerated growth process during suckling period; i.e. MUN offspring exhibited a lower birth weight, but reached a similar body weight as Control rats by weaning. We have previously demonstrated that this rapid weight gain is associated with an excess in white adipose tissue (WAT) at the end of lactation [25]. We have not assessed WAT in adult rats, but this early fat accumulation is likely to remain high throughout maturation. This has been previously demonstrated in adult offspring from similar IUGR models [23,24] and can be prevented by dietary interventions which avoid catch-up growth during suckling period [24]. These data indicate that perinatal life is a critical developmental window during which the alterations induced by IUGR might further develop. For this reason we chose to study oxidative balance at the age of weaning. Furthermore, our data indicates that at this age, the rats are still in the prepuberal stage, as evidenced by the low levels of estrogens in both males and females, similar to previous reports [35]. This enabled us to assess if sex differences in oxidative status take place before sexual maturity.

Under our experimental conditions male and female MUN rats were still normotensive at weaning and high blood pressure developed later and only in male MUN offspring. This is in agreement with previous findings in several models of IUGR, which demonstrate high blood pressure in males [16,17,36] while females are relatively protected from fetal programming [18]. Hypertension is associated with cardiovascular fibrosis, remodeling and endothelial dysfunction, alterations where oxidative stress plays a key role [37-39]. Similar cardiovascular alterations have been found in adult IUGR rats [40-42] and therefore oxidative stress has been proposed as a possible mechanism implicated in fetal programming of hypertension [7,43]. However, the presence of hypertension, fibrosis and endothelial dysfunction, together with oxidative damage, does not allow concluding whether oxidative stress is cause or consequence of the tissular injury [12]. Our study at weaning, when MUN offspring is still normotensive, enabled us to assess possible changes in oxidative status independent of damage induced by chronic high blood pressure. We found that prepuberal MUN males exhibited a generalized decrease in antioxidants, namely thiols, GSH, Superoxide Anion Scavenging Activity. These results are in agreement with previous reports which measure individual biomarkers in male offspring from various models of IUGR. For example, a reduction in GSH has been found in male fetus from low protein fed dams [42]. SOD activity is also decreased in perinatal life in goats [44] and rats [45] exposed to undernutrition in utero. We did not measure SOD activity directly, but indirectly through SOSA assay, a method that determines all plasma antioxidants capable to eliminate superoxide anion, including SODs and small molecular weight scavengers. Since we did not find a difference in TAC (a global parameter which measures non enzymatic, low molecular weight antioxidants), we suggest that a reduction in SOD activity accounts for the observed decrease in SOSA. We lacked to find differences in catalase activity in our model of global nutrient restriction, while it was reduced in the offspring from dams exposed to protein restriction [45]. This difference might be related to the fact that in the study of Fetoui and colleagues, protein was also restricted during lactation, which likely affected nutrient availability to offspring during the perinatal period. The decrease in plasma antioxidant status in male MUN offspring was associated to an early elevation of protein carbonyl levels, a biomarker of protein oxidation by reactive species, in agreement with other studies showing signs of oxidative damage in plasma and tissues from IUGR models during perinatal development [42,45].

The majority of studies regarding the role of oxidative stress in fetal programming have been performed in males and the possible contribution of a better oxidative balance in the protection of female offspring has not been carefully explored. We found that while MUN males exhibited a general reduction in plasma antioxidants at weaning, MUN females only showed a decrease in SOD levels and the rest of the antioxidants were normal, suggesting that the better antioxidant adaptation to the deleterious influence of fetal undernutrition. In addition, females did not show elevated carbonyl levels at weaning, possibly due to their better antioxidant system, protecting them from early oxidative damage.

The study at two age points enabled us to assess the evolution of oxidative balance from weaning (just after the catch-up growth process) to maturation (when the hypertensive phenotype is developed). From the age of 21 days to 6 months there was a decrease in practically all antioxidant systems, together with an increase in plasma carbonyls. Since this was found in Control male and female rats, we suggest that it is a physiological process which takes place along maturation and ageing. However, among all experimental groups, adult MUN males exhibited the highest increase in carbonyls (almost multiplied by 3) which suggests an excess in oxidative process. Taken these data together, it can be suggested that a robust antioxidant system at early stages of development could be an important advantage to neutralize tissular oxidative damage along ageing. Our data also indicate that a poor antioxidant system developed during fetal and perinatal life, cannot be counteracted later on. This is supported by the fact that MUN males exhibited the highest levels of protein oxidation and developed hypertension, despite normalization of antioxidants compared to sex-matched Controls. In the present study we could not measure enzymatic systems responsible for ROS production, since the most important are membrane bound-enzymes. Therefore, we cannot rule out the contribution of excess ROS to the development of a pro-oxidative status in MUN males.

The observed early global pro-oxidative status in plasma is likely to contribute to cardiovascular damage and hypertension. A good correlation has been previously found between plasma concentrations of catalase, TAC, GSH, SOD and protein carbonyls, and their levels in several tissues including the heart [46]. Therefore, it is reasonable to speculate that an increased heart oxidative damage from early age might lead to contractile dysfunction and increased risk of

heart failure later on. A generalized pro-oxidative status at early stages of development in MUN males might also contribute to the development of hypertension. Thus, an increased oxidative damage of blood vessels would lead to endothelial dysfunction and subsequent increase in peripheral resistance. In fact, there is evidence that adult offspring from IUGR models exhibit loss of arterial vasodilatation which is improved with SOD mimetics [40,43].

Adult female MUN offspring did not show evidence of protein oxidative damage and did not develop high blood pressure, at least at the age of 6 months. The relative protection of females against the unfavorable outcome of fetal programming, has been attributed to the effects of sex hormones [16,47]. In fact, there is evidence that testosterone is elevated in male rats exposed to IUGR and hypertension is abolished by castration [48]. On the other hand, there is also evidence that estradiol and estriol exhibit radical-scavenging activity and could play a role as protective hormones against oxidative stress, after puberty [49,49]. The present data suggest that there might be an additional influence of sex at earlier stages of development. It was beyond the aim of this study to explore the mechanisms through which nutrient deficit leads to a poorer development of antioxidant systems in males, but we can speculate that a possible contributor might be the placenta, which bears the same genetic information of sex as the fetus [50]. It is known that the sex of the embryo can affect placental size and its ability to respond to adverse stimuli [51]. Since IUGR is associated with a reduction of aminoacid transport across the placenta [52], the capacity for enzyme synthesis could be compromised. However, to the best of our knowledge there is no information regarding the influence of sex on placental expression of nutrient transport systems which could explain the observed differences between males and females. A second possible mechanism is that epigenetic modulation induced by fetal stress [53] might be sex-dependent, since sex-differences in gene expression appear very early during development [54]. Therefore, it is plausible that IUGR induces epigenetic modifications in genes related to oxidative stress, which might be modulated by the sex of the fetus. With the data available from the literature, the mechanisms discussed above remain speculative and future studies are required to gain insight in the sex-specific relationships between fetal insult and early alterations in oxidative status.

In the context of hormonal systems implicated in oxidative stress protection we found of interest to assess the levels of melatonin, a hormone able to act as direct free radical scavenger, through its ability to cross plasma membranes [55], as well as to stimulate several antioxidant enzymes, including SOD and catalase [55-58]. Furthermore, there is evidence that melatonin might be a key hormone in fetal life, improving utero-placental hemodynamics and reducing placental oxidative stress in pregnancies complicated with IUGR [59,60] and in animal models of maternal undernutrition [61,62]. The lower melatonin levels in male MUN offspring was not paralleled by a reduction in TAC values and therefore, its contribution in the pool of direct scavengers might not be relevant in this model. However, it is possible that it contributes to the lower SOD activity observed in 21 day old MUN males. In humans there is also evidence that IUGR and prematurity reduce antioxidant defense systems, including SOD, catalase and glutathione system [63,64] and melatonin has been proposed for use as an adjuvant in the treatment of several diseases related to oxidative stress in newborns [65].

In conclusion, the present study supports the hypothesis that a better antioxidant status during early life could contribute to the observed better adaptation of females to IUGR and might explain, at least in part, their protection against hypertension development. We also demonstrate the value of a score based on the combination of several plasma biomarkers to determine the global oxidative status of an individual. Further development of this tool could be of use in a clinical setting to assess if there is a relationship between maternal periconceptual oxidative status and IUGR or prematurity, as well as on longitudinal studies of nutritional or pharmacological interventions linked to these conditions.



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There are no conflicts of Interest

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## Figure Legends

**Figure 1.** Systolic and diastolic blood pressure (SBP, DBP) in anaesthetized 21 day old (A) and 6 month old (B) male and female offspring from rats exposed to maternal undernutrition during pregnancy (MUN) and rats fed *ad libitum* (Control). N=8-10 rats for each experimental group; \*p<0.05 compared to sex-matched control rats.

**Figure 2.** Plasma oxidative status biomarkers in 21 day old male offspring from rats exposed to maternal undernutrition during pregnancy (MUN) and rats fed *ad libitum* (Control). (A) carbonyls, (B) total antioxidant capacity (TAC), (C) reduced glutathione (GSH), (D) thiols, (E) superoxide anion scavenging activity (SOSA) and (F) catalase activity. N=8-10 rats for each experimental group; \*p<0.05 compared to sex-matched controls.

**Figure 3.** Plasma oxidative status biomarkers in 21 day old female offspring from rats exposed to maternal undernutrition during pregnancy (MUN) and rats fed *ad libitum* (Control). (A) carbonyls, (B) total antioxidant capacity (TAC), (C) reduced glutathione (GSH), (D) thiols, (E) superoxide anion scavenging activity (SOSA) and (F) catalase activity. N=9-11 rats for each experimental group; \*p<0.05 compared to sex-matched controls.

**Figure 4.** Plasma oxidative status biomarkers in 6 month old male offspring from rats exposed to maternal undernutrition during pregnancy (MUN) and rats fed *ad libitum* (Control). (A) carbonyls, (B) total antioxidant capacity (TAC), (C) reduced glutathione (GSH), (D) thiols, (E) superoxide anion scavenging activity (SOSA) and (F) catalase activity. N=8-10 rats for each experimental group; \*p<0.05 compared to sex-matched controls.

**Figure 5.** Plasma oxidative status biomarkers in 6 month old female offspring from rats exposed to maternal undernutrition during pregnancy (MUN) and rats fed *ad libitum* (Control). (A) carbonyls, (B) total antioxidant capacity (TAC), (C) reduced glutathione (GSH), (D) thiols, (E) superoxide anion scavenging activity (SOSA) and (F) catalase activity. N=9-10 rats for each experimental group; \*p<0.05 compared to sex-matched controls.

**Figure 6.** Global oxidative status score (Oxy-score) calculated from the plasma biomarkers of oxidative damage and antioxidant capacity in 21 day old and 6 month old male and female offspring from rats exposed to maternal undernutrition during pregnancy (MUN) and rats fed *ad libitum* (Control). \*p<0.05 compared to sex-matched controls.

**Table1. Plasma estradiol and melatonin (pg/mL) in young control and MUN offspring**

|                  | <b>Male</b>    |               | <b>Female</b>  |              |
|------------------|----------------|---------------|----------------|--------------|
|                  | <b>Control</b> | <b>MUN</b>    | <b>Control</b> | <b>MUN</b>   |
| <b>Melatonin</b> | 27.0±2.4 (6)   | 17.4±2.2 (6)* | 23.4±2.8 (6)   | 25.0±6.4 (6) |
| <b>Estradiol</b> | 40.8±3.0 (8)   | 36.0±1.4 (8)  | 38.9±1.1 (8)   | 36.6±0.9 (8) |

MUN, maternal undernutrition; in parenthesis the number of rats; \*p<0.05 compared to sex-matched control.

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