



# Implications of maternal nutrient restriction in transgenerational programming of hypertension and endothelial dysfunction across F1–F3 offspring

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## ABSTRACT

**Aims:** An extensive variety of prenatal insults are associated with an increased incidence of metabolic and cardiovascular disorders in adult life. We previously demonstrated that maternal global nutrient restriction during pregnancy leads to increased blood pressure and endothelial dysfunction in the adult offspring. This study aimed to assess whether prenatal exposure to nutritional insult has transgenerational effects in F<sub>2</sub> and F<sub>3</sub> offspring.

**Main methods:** For this, female Wistar rats were randomly divided into two groups on day 1 of pregnancy: a control group fed standard chow *ad libitum* and a restricted group fed 50% of the *ad libitum* intake throughout gestation. At delivery, all animals were fed a standard laboratory chow diet. At 11 weeks of age, one female and one male from each restricted litter were randomly selected and mated with rats from another restricted litters in order to generate the F<sub>2</sub> offspring. The same procedure produced F<sub>3</sub> generation. Similarly, the rats in the control group were bred for each generation.

**Key Findings:** Our findings show that the deleterious effects of maternal nutrient restriction to which the F<sub>0</sub> mothers were exposed may not be limited to the male first generation. In fact, we found that elevated blood pressure, an impaired vasodilatory response to acetylcholine and alterations in NO production were all transferred to the subsequent males from F<sub>2</sub> and F<sub>3</sub> generations.

**Significance:** Our data show that global nutrient restriction during pregnancy results in a specific phenotype that can be passed transgenerationally to a second and third generation.

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## Introduction

It has been postulated that deleterious effects during fetal life that occur during critical windows in development may have an impact on cellular structure and function and, consequently, on the development of diseases in later life (Gluckman and Hanson, 2004; Gluckman et al., 2005). The most striking aspect is the evidence that the consequences of an unfavorable intrauterine environment can be passed transgenerationally from the mother to subsequent generations (Harrison and Langley-Evans, 2009; Zambrano et al., 2005). These non-genomic effects have been described in several animal models (Bertram et al., 2008; Pinheiro et al., 2008; Thamocharan et al., 2007; Torrens et al., 2008).

In the first generation of adult offspring exposed to global nutrient restriction *in utero*, the vascular adaptations with regard to endothelial function consist of a reduction in both NO production and bioavailability

(Franco et al., 2003, 2004). A recent study demonstrated that a low protein diet through gestation leads to changes in vascular reactivity in the first and second generations of adult male offspring, despite normal F<sub>1</sub> postnatal nutrition (Torrens et al., 2008). These deleterious effects on the vascular system, however, were demonstrated only in a single generation after the maternal insult. Here we extend those findings with a study examining the transgenerational effects of global nutrient restriction on the developmental programming of vascular reactivity and blood pressure levels in the F<sub>1</sub> through F<sub>3</sub> generations. To distinguish the mechanisms underlying the transgenerational persistence of vascular perturbations, we evaluated the inheritance of NO and superoxide production.

## Methods

All of the procedures described below were approved by the São Paulo University Animal Use and Care Committee. The study design can be seen in Fig. 1. The F<sub>0</sub> dams were 30 female Wistar rats aged 11 weeks and were maintained in a room at 22 ± 1 °C on a 12-h light cycle with 60% humidity. The female rats were mated overnight with male breeders, and the day on which spermatozoa were found in the vaginal smear was designated as the day of conception (day 0).

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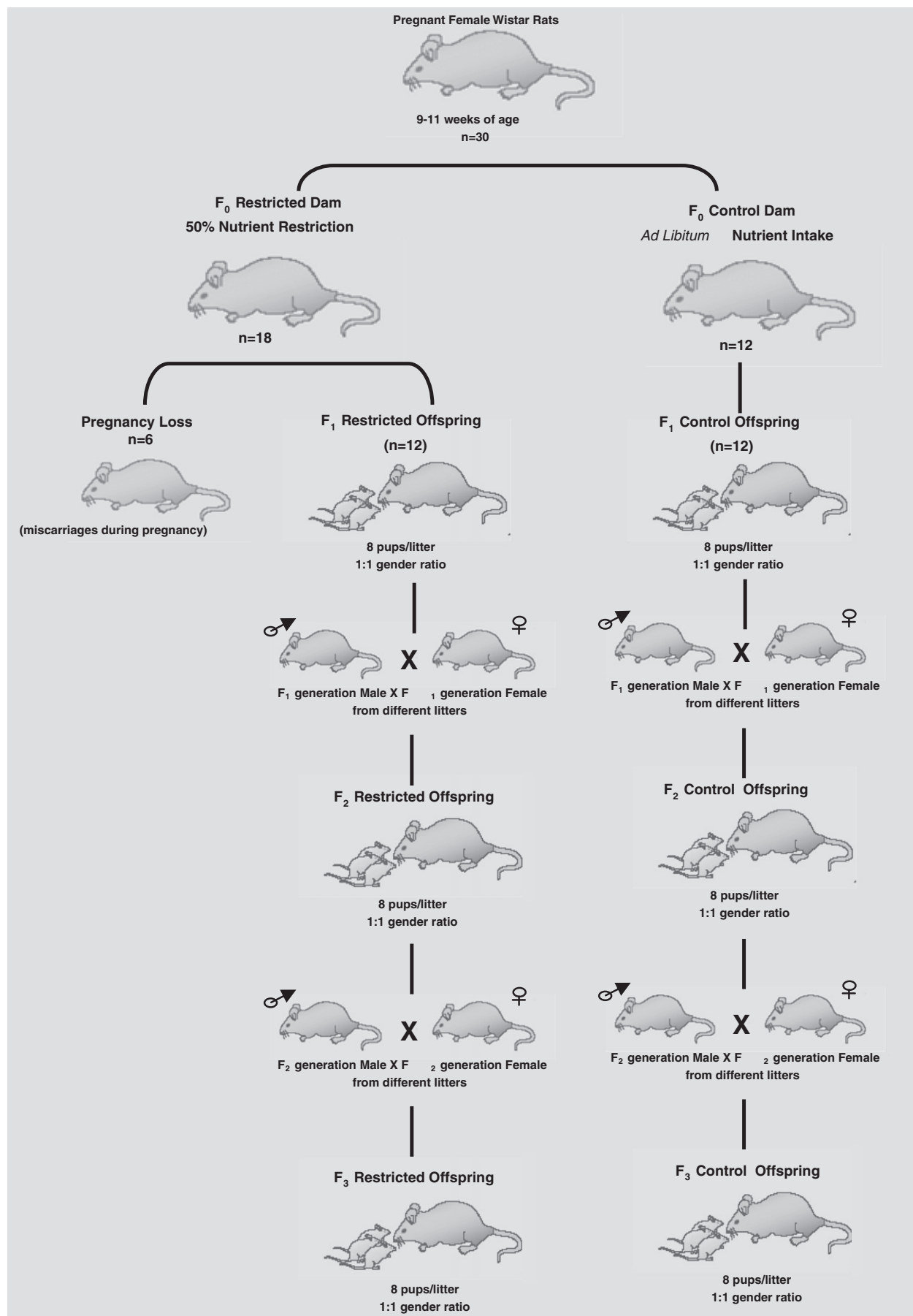


Fig. 1. Schematic showing study design.

Pregnant rats were transferred to individual standard cages and randomly allocated into one of two groups: the control group (C,  $n = 12$ ) was fed with a standard chow laboratory animal diet (Nuvilab® CR1 – based on the recommendation of the National Research Council and National Institutes of Health, USA) *ad libitum*, and the restricted group (RT,  $n = 18$ ) was fed with 50% of the typical daily food intake determined by the amount of food consumed by the C group through the gestation period. The amount of food provided daily was weighed, and food intake was monitored. At least six females from the  $F_0$  restricted group were unable to complete the gestation period due to miscarriages during pregnancy and/or occurrence of fetal resorption, and were excluded from the present study. All of the  $F_0$  female rats delivered the  $F_1$  generation by spontaneous vaginal delivery. Following parturition, all offspring were returned to a standard chow diet *ad libitum* and continued to receive this diet for the remainder of the experimental protocol. The litter size and birth weight of the  $F_1$  offspring were recorded. The rats were accompanied until weaning when the offspring were divided by gender and placed into separate cages. At 11 weeks of age, one female and one male from each restricted litter were randomly selected and mated with rats from another restricted litters in order to generate the  $F_2$  offspring. After confirmation of mating the female rats were singly housed and fed standard chow until delivery of the litter. Male rats were placed back into single sexed housing with their littermates for future studies. Following the procedure described above, the  $F_2$  offspring were bred and crossed to generate the  $F_3$  generation. No inbreeding or sibling crosses were generated. The rats in the control group were similarly bred for each generation.

To prevent any variation in neonatal growth due to the availability of milk intake during suckling, in all generation ( $F_1$  to  $F_3$ ) the litter size was standardized to eight pups, and the gender ratio was kept as close to 1:1 as possible. In addition, neither the litter size nor the gender composition was affected in the restricted and control groups. At 16 weeks of age male offspring of each generation from both the control and restricted groups were randomly selected for each litter and used in the experimental protocols to assess blood pressure, vascular function and NO/superoxide production.

### Determination of arterial blood pressure

Blood pressure levels were determined in conscious rats by an indirect tail-cuff method using the Powerlab/4S System (AD Instruments Ltd., Sydney, Australia). Briefly, the rats were housed in a room heated to 30 °C to stimulate tail blood flow, and placed in a restraint tube with an appropriate-sized cuff placed over the tail and inflated. Blood pressures were determined in triplicate for each animal and the mean recorded. To minimize the stressful response to this procedure, rats were handled throughout life and were made familiar with the recording equipment before the measurements were made.

### Aorta Excision

At 16 weeks of age, the rats were anesthetized with pentobarbital (50 mg/kg, i.p.). The thorax was opened, and the descending aorta was excised and divided into three segments used to measure vascular reactivity, the presence of superoxide anion and NO production.

### Isolated Aorta Preparation

Isolated fragments of the descending thoracic aorta (4 mm in length) were connected to a force transducer to record the isometric force and placed in organ baths filled with 15 mL of Krebs solution (37 °C - 94%  $O_2$ /6%  $CO_2$  - pH 7.4). Vessels were submitted to a tension of 1.5 g, which was adjusted during 60 minutes before the addition of a given drug. Concentration-dependent response curves to acetylcholine (ACh) ( $10^{-9}$ – $10^{-5}$  M) were obtained during submaximal contractions in response to phenylephrine ( $10^{-7}$  M) (concentration that

induces 60–80% of the maximum effect). Isometric tension was recorded by using an isometric force displacement transducer connected to a data acquisition system (PowerLab 8/S, AD Instruments Pty Ltd, Castle Hill, Australia).

### Measurement of NO Production in Transverse Aortic Sections

The production of NO was assessed using 4,5-diaminofluorescein diacetate (DAF-2), an NO-specific fluorescent dye, as previously described (Franco et al., 2009). The fluorescence ratio was evaluated in at least three locations in each image and in at least six aortic segments obtained from different animals in each group. Aortic segments from all groups were processed in parallel. Data for each experiment were normalized to a reference image of the basal state and expressed as a percentage increased in NO production following ACh stimulation.

### Detection of superoxide anion in transverse aortic sections

Hydroethidine (HE), an oxidative fluorescent dye, was used to evaluate the level of superoxide anion as previously described (Franco et al., 2009; Miller et al., 1998). Cells are permeable to HE, and in the presence of superoxide anion, HE is oxidized to fluorescent ethidium bromide, which is then trapped by intercalation with DNA. In fact, the HE oxidation to ethidium bromide is caused more rapidly by superoxide anion than by hydroxyl radical or hydrogen peroxide (Benov et al., 1998). Transverse cross-sections (7  $\mu$ m) of unfixed frozen ring segments were obtained and incubated with PBS containing HE ( $2 \times 10^{-6}$  mol/L). After that, sections were incubated into a light-protected humidified chamber at 37 °C for 30 minutes. Fluorescence was detected with a 585–590 nm long-pass filter. Relative increases in ethidium bromide fluorescence were detected with a Zeiss Axiovert S100 inverted microscope and, the images were analyzed with image software by measuring the mean optical density of the fluorescence. The fluorescence ratio was evaluated in at least three locations in each image and at least six aortic segments obtained from different animals in each group. Aortic segments from all experimental groups were processed in parallel.

### Statistical analysis

The results are shown as the mean  $\pm$  S.E.M. Relaxant responses are expressed as the percentage inhibition of phenylephrine-induced contraction. Cumulative concentration–effect curves in response to agonists were analyzed by fitting to a four-parameter logistic equation using nonlinear regression to obtain the  $pEC_{50}$  and maximum response. All continuous variables were examined for normality with the Kolmogorov-Smirnov test. Analyses of variance (One-way ANOVA) followed by Tukey's test were performed to evaluate the role of the maternal diet across the three generations groups. Statistical tests were two-tailed, and the significance level was set at  $P < 0.05$ .

### Results

Maternal nutrient restriction during pregnancy resulted in fetal growth restriction, as evidenced by a marked reduction in the birth weight of  $F_1$  offspring. In fact, birth weight was 68% lower in the  $F_1$  restricted ( $4.8 \pm 0.1$  g) when compared to the control ( $7.0 \pm 0.2$  g) offspring. In the second and third generations of the restricted offspring, the birth weight did not differ from that observed in the control offspring (Restricted:  $F_2$ :  $7.1 \pm 0.1$  g and  $F_3$ :  $7.0 \pm 0.2$  g; Control:  $F_2$ :  $7.3 \pm 0.3$  g and  $F_3$ :  $7.5 \pm 0.2$  g).

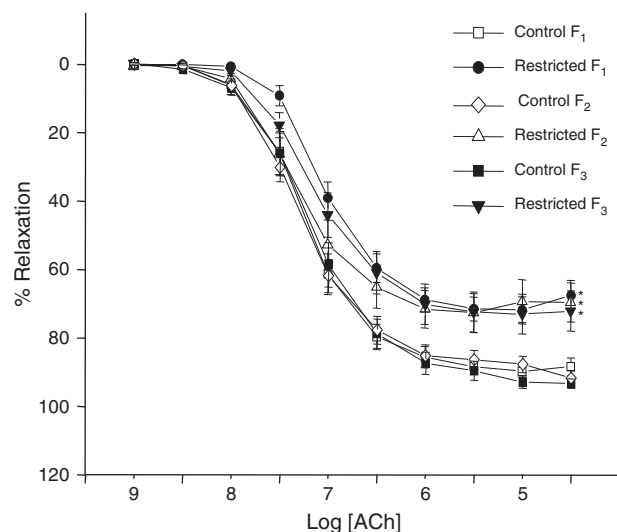
One important finding was the persistently high blood pressure level observed in the restricted offspring. In fact, restricted offspring of  $F_1$  through  $F_3$  generation had significantly higher blood pressure than controls, suggesting that the increased blood pressure was

transmitted and preserved through at least three generations (Table 1). At 16 weeks of age, vascular parameters were analyzed. The pre-constrictor tension development in response to phenylephrine was not significantly different between the controls and restricted generations (Restricted: F<sub>1</sub>:  $3.3 \pm 0.12$  g/tension, F<sub>2</sub>:  $3.1 \pm 0.15$  g/tension and F<sub>3</sub>:  $3.0 \pm 0.09$  g/tension; Control: F<sub>1</sub>:  $3.2 \pm 0.12$  g/tension, F<sub>2</sub>:  $3.1 \pm 0.11$  g/tension and F<sub>3</sub>:  $3.4 \pm 0.23$  g/tension). In all restricted generations, vasodilatation in response to ACh was significantly impaired when compared to the control (Table 1) (Fig. 2). The maximal response to ACh was ~20% less in the three generations of restricted offspring, suggesting that the effects of fetal undernutrition on vascular reactivity could be passed from the F<sub>1</sub> to the F<sub>2</sub> and F<sub>3</sub> generations. To investigate the mechanism involved in this impairment of vascular function, we evaluated NO and superoxide concentration. As shown in Fig. 3, the ACh-stimulated NO production was significantly lower in the aortic segments of F<sub>1</sub> restricted offspring than in controls. NO production was also significantly lower in both subsequent generations of the restricted offspring (Fig. 3A and B). Using an epifluorescent inverted microscopy, aortic sections isolated from F<sub>1</sub> restricted rats showed significant increase in ethidium bromide fluorescence. A similar increase was found in the other restricted generations, suggesting that superoxide anion might be the primary reactive oxygen species in the restricted offspring, since this method is especially sensitive to superoxide anion. This effect was also noted when analyzing the restricted generations. It was apparent that superoxide concentration was higher in the F<sub>1</sub> offspring than those F<sub>2</sub> and F<sub>3</sub> generation (Fig. 4A and B).

## Discussion

Our findings show that the deleterious effects of nutrient restriction during pregnancy to which the F<sub>0</sub> mothers were exposed may not be limited to the first generation. We found that elevated blood pressure, impaired vasodilatory response to ACh and perturbations in both NO and superoxide pathways were all transferred to the F<sub>2</sub> and F<sub>3</sub> generations.

It is clear from several studies that F<sub>1</sub> offspring exposed to early nutrient restriction are characterized by the presence of high blood pressure levels and endothelial dysfunction in adulthood (Brawley et al., 2003; Franco et al., 2004; Torrens et al., 2003, 2008). The present data showed that the blood pressure was increased 23 mmHg in the F<sub>1</sub> restricted offspring, and this phenotype was passed to the second and third generations, suggesting that the early nutrient restriction causes transgenerational effects on blood pressure levels. Moreover, we assessed the impact of global nutrient restriction in utero on vascular function across several generations. We found that the impairment of vascular function observed in F<sub>1</sub> was also detected in the F<sub>2</sub> and F<sub>3</sub> male restricted offspring. The fact that disturbances in the vascular function was also observed in both second and third generations of the restricted offspring suggests that adverse uterine environment has a critical role in the transgenerational transmission of endothelial dysfunction. The second part of our statement on the vascular function carries a special interest. Previous studies demonstrated that fetal programming has a greater effect on NO/superoxide



**Fig. 2.** Vascular relaxation in response to acetylcholine (ACh) in aortic rings isolated from F<sub>1</sub> (n = 12), F<sub>2</sub> (n = 12) and F<sub>3</sub> (n = 9) control and restricted male offspring. Data are expressed as a percentage of initial phenylephrine precontraction. Values are the mean  $\pm$  S.E.M. \* Mean values for each generation were significantly different from the matched control offspring generation ( $P < 0.05$ , One-way ANOVA followed by Tukey's test).

pathways, which contributes to endothelial dysfunction observed in the F<sub>1</sub> restricted offspring (Franco et al., 2003, 2004). Our current data demonstrated that levels of NO were reduced in aorta rings isolated from both F<sub>2</sub> and F<sub>3</sub> restricted offspring, and this decrease occurs in association with elevated superoxide concentration. Our findings are in agreement with idea that F<sub>2</sub> and F<sub>3</sub> restricted offspring developed the same characteristics of their parents and grandparents and, the global nutrient restriction plays a critical role in the transgenerational transmission.

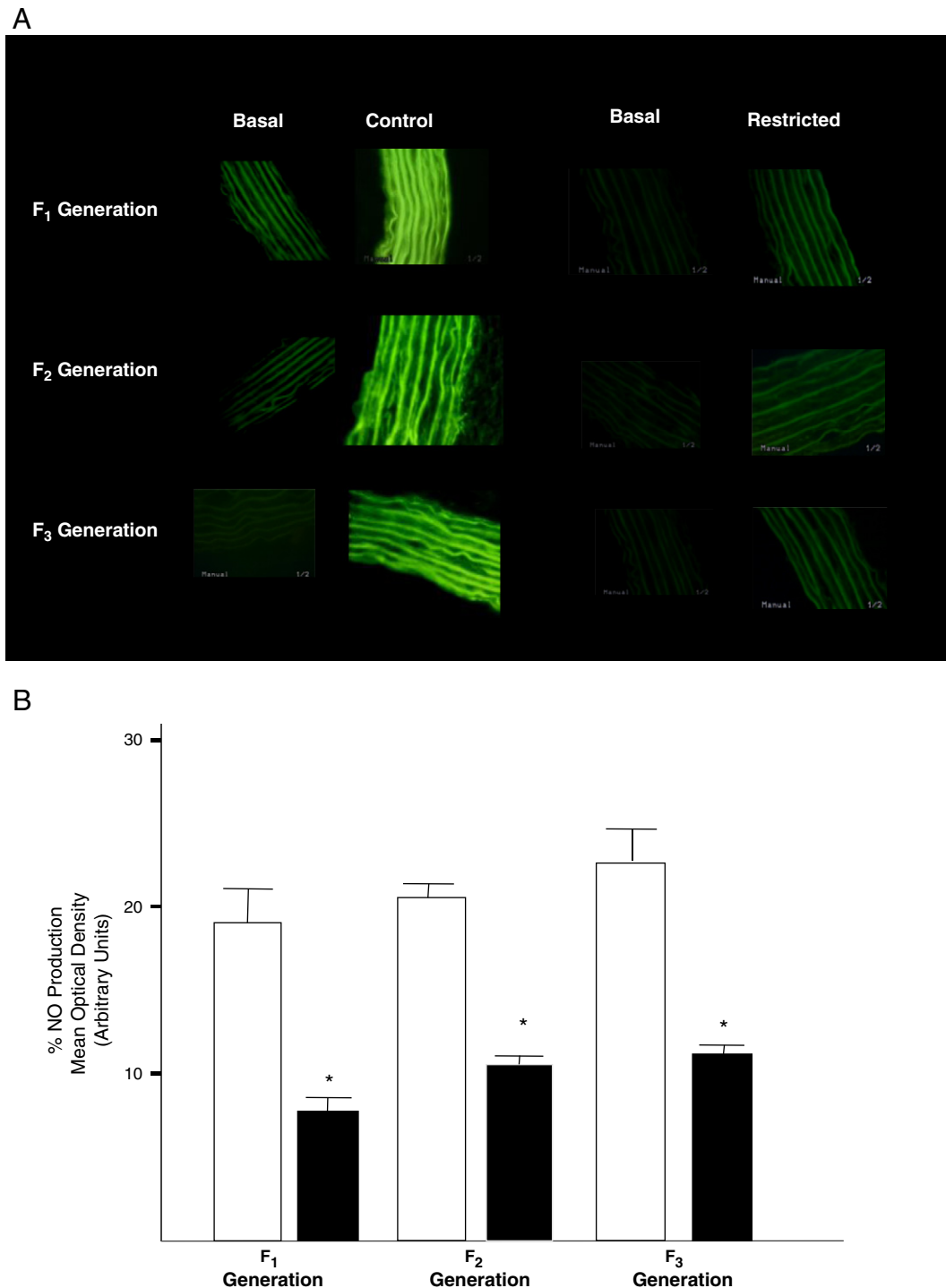
It is recognized that environmental stimulus (e.g., diet manipulation, hormonal treatment or surgical procedure) during fetal life has ability to promote the development of chronic diseases in the next generations. In fact, some literature evidences have demonstrated that maternal protein restriction results in higher blood pressure levels, endothelial dysfunction and abnormal glucose tolerance in both first- and second-generation offspring, however, no effect was observed in the F<sub>3</sub> generation (Oh et al., 1991; Aerts and Van Assche, 2006; Brawley et al., 2003; Harrison and Langley-Evans, 2009; Torrens et al., 2008; Zambrano et al., 2005). Our findings confirm and extend other experimental data, which have shown transgenerational effects of maternal nutrient restriction during pregnancy on altered blood pressure and vascular function in F<sub>2</sub> offspring. (Bertram et al., 2008; Torrens et al., 2008) However, we observed that this phenotype persist into the third generation. This apparent discrepancy can be explained by specific symmetric mating approach, where both sexes are the same experimental group. Moreover, previous studies used a moderate protein-restriction diet, whereas we have used 50% global nutrient restriction diet throughout gestation. It is possible that the impact on the transgenerational programming on phenotype is transmitted through generations only in specific experimental conditions.

While the exact mechanism to explain how prenatal environment can promote transgenerational consequences cannot be determined from this study, it is thought that alterations in early nutrition can affect the establishment of DNA methylation or acetylation, leading to persistent changes in gene expression that can be transmitted to the next generation and affect postnatal development and health later in life, by transgenerational transmission of epigenetic modification (Jaenisch and Bird, 2003; Waterland and Jirtle, 2004; Youngson and Whitelaw, 2008). In fact, there is evidence from some studies that

**Table 1**  
General characteristics.

	Body weight (g)	Blood PRESSURE (mmHg)	Maximal Response to ACh (%)
Control F <sub>1</sub>	390.2 $\pm$ 4.0 (12)	110.6 $\pm$ 2.3 (12)	91.3 $\pm$ 1.6 (12)
Restricted F <sub>1</sub>	393.7 $\pm$ 2.3 (12)	133.7 $\pm$ 4.8 * (12)	72.8 $\pm$ 3.5 * (12)
Control F <sub>2</sub>	394.3 $\pm$ 2.2 (12)	113.1 $\pm$ 2.2 (12)	89.9 $\pm$ 2.1 (12)
Restricted F <sub>2</sub>	392.4 $\pm$ 3.6 (12)	128.5 $\pm$ 3.3 * (12)	73.9 $\pm$ 5.1 * (12)
Control F <sub>3</sub>	393.2 $\pm$ 2.8 (9)	109.8 $\pm$ 1.8 (9)	91.4 $\pm$ 1.3 (9)
Restricted F <sub>3</sub>	392.8 $\pm$ 4.6 (9)	124.9 $\pm$ 3.2 * (9)	78.5 $\pm$ 5.8 * (9)

Values expressed as the mean  $\pm$  S.E.M. (n) Number of observations. \* Mean value was significantly different from that of the control offspring generation.



**Fig. 3.** (A) and (B) Digital images and histogram showing the level of NO production in DAF-treated sections of aortic rings from male rats under basal and acetylcholine-stimulated conditions from F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> control (open bars) and restricted (close bars) male offspring. Data are expressed as the percentage increase in NO production following acetylcholine (ACh) stimulation (means  $\pm$  S.E.M) in six different experiments. \* Mean values for each generation were significantly different the matched control offspring generation ( $P < 0.05$ , One-way ANOVA followed by Tukey's test).

maternal protein restriction and placental insufficiency have the ability to promote epigenetic programming of the germ line, induction of imprinted-like genes, and subsequent transmission to progeny (Pham et al., 2003; Lillycrop et al., 2005, 2007, 2008; Thompson et al., 2010).

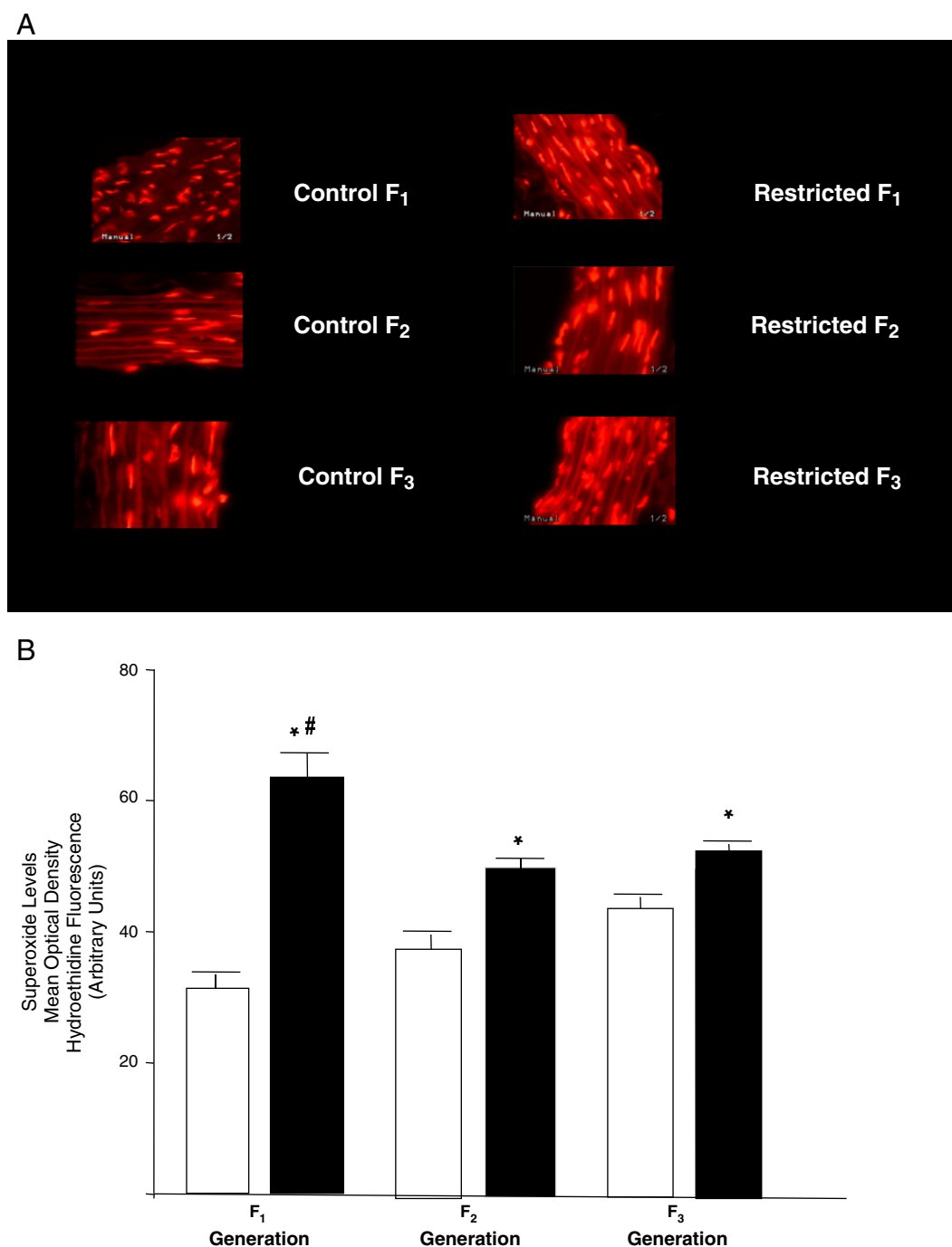
In conclusion, our data show that global nutrient restriction during pregnancy results in a specific phenotype that can be passed transgenerationally to a second and third generation. This transgenerational phenotype is characterized elevated blood pressure, endothelial dysfunction

and an NO/superoxide imbalance. Future studies will be designed to identify the specific mechanisms behind the transgenerational nature of this vascular damage and to establish possible gene targets involved in endothelial cells.

#### Conflict of Interest

None.





**Fig. 4.** (A) and (B) Digital images and histogram showing the presence of superoxide in hydroethidine (HE)-treated sections of aortic rings from F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> control (open bars) and restricted (close bars) male offspring. Values are the mean  $\pm$  S.E.M of six different experiments. \* Mean values for each generation were significantly different the matched control offspring generation and # Mean values for F<sub>1</sub> restricted generation were significantly different than F<sub>2</sub>, F<sub>3</sub> restricted offspring ( $P < 0.05$ , One-way ANOVA followed by Tukey's test).

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