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# Uteroplacental insufficiency causes a nephron deficit, modest renal insufficiency but no hypertension with ageing in female rats

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**In rats, uteroplacental insufficiency induced by uterine vessel ligation restricts fetal growth and impairs mammary development compromising postnatal growth. In male offspring, this results in a nephron deficit and hypertension which can be reversed by improving lactation and postnatal growth. Here, growth, blood pressure and nephron endowment in female offspring from mothers which underwent bilateral uterine vessel ligation (Restricted) on day 18 of pregnancy were examined. Sham surgery (Control) and a reduced litter group (Reduced at birth to 5, equivalent to Restricted group) were used as controls. Offspring (Control, Reduced, Restricted) were cross-fostered on postnatal day 1 onto a Control (normal lactation) or Restricted (impaired lactation) mother. Restricted-on-Restricted offspring were born small but were of similar weight to Control-on-Control by postnatal day 35. Blood pressure was not different between groups at 8, 12 or 20 weeks of age. Glomerular number was reduced in Restricted-on-Restricted offspring at 6 months without glomerular hypertrophy. Cross-fostering a Restricted pup onto a Control dam resulted in a glomerular number intermediate between Control-on-Control and Restricted-on-Restricted. Blood pressure, along with renal function, morphology and mRNA expression, was examined in Control-on-Control and Restricted-on-Restricted females at 18 months. Restricted-on-Restricted offspring did not become hypertensive but developed glomerular hypertrophy by 18 months. They had elevated plasma creatinine and alterations in renal mRNA expression of transforming growth factor-$\beta_1$, collagen IV ($\alpha$1) and matrix matelloproteinase-9. This suggests that perinatally growth restricted female offspring may be susceptible to onset of renal injury and renal insufficiency with ageing in the absence of concomitant hypertension.**

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**Abbreviations** $AT_nR$, angiotensin type *n* receptor; BAX, BCL-2 associated X protein; BCL-2, B-cell leukaemia/lymphoma 2; ECM, extracellular matrix; FN1, fibronectin 1; IGF-1, insulin-like growth factor 1; MMP, matrix metalloproteinase; P53, protein 53; RAS, renin–angiotensin system; TIMP, tissue inhibitors of metalloproteinase; TGF-$\beta_1$, transforming growth factor-$\beta_1$; VEGF-A, vascular endothelial growth factor A.

Intrauterine growth restriction occurs in approximately 10% of pregnancies in the Western world and is a major cause of perinatal morbidity and mortality (Barker *et al.* 1989). Being born small also increases the predisposition to many adult diseases, including hypertension (Law & Shiell, 1996; Eriksson *et al.* 2000; Adair & Cole, 2003; Barker & Bagby, 2005). Growth during the early postnatal period has also been documented to independently predict the risk of adult diseases, with early catch-up growth conferring some protection, but accelerated growth later in childhood increasing the risk of disease (Lucas *et al.* 1997; Eriksson *et al.* 2000). Although the


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mechanisms through which the conditions that alter early growth can result in adult disease are unclear, evidence suggests that development of particular organs, including the kidney, may be affected. Many studies have now shown that early life perturbations induce later hypertension in this manner, with a reduced nephron endowment implicated as a contributory factor (Brenner, 1985; Wlodek *et al.* 2007, 2008).

A common cause of intrauterine growth restriction in the human is a poorly formed or functioning placenta. To delineate the mechanisms by which poor placental function programmes later phenotype, we have used bilateral uterine vessel ligation in the rat. This results in uteroplacental insufficiency and reduces oxygen and nutrient supply to the fetus, restricting its growth as well as litter size (Rajakumar *et al.* 1998; Lane *et al.* 1998; Jansson & Lambert, 1999). Uteroplacental insufficiency in the rat increases blood pressure and reduces the number of glomeruli (nephron number) in offspring (Wlodek *et al.* 2005, 2007, 2008; Schreuder *et al.* 2005, 2006), as also occurs following maternal undernutrition or glucocorticoid treatment (Langley-Evans *et al.* 1999; Ortiz *et al.* 2001; Singh *et al.* 2007), although not all of these studies have examined offspring of both sexes. Uteroplacental insufficiency in the rat also impairs mammary development during pregnancy in preparation for lactation, causing reduced milk production and altered milk composition (Wlodek *et al.* 2007; O'Dowd *et al.* 2008*a*,*b*). This causes postnatal growth restriction and may independently contribute to the later adverse cardiovascular outcomes (Wlodek *et al.* 2007, 2008).

We have taken the novel approach of using the bilateral uterine vessel (artery and vein) ligation rat model to induce growth restriction (Restricted group) and then performed cross-foster studies in order to clearly define the separate contributions of the prenatal and postnatal environments in the programming of adult phenotype. Exposure of male pups to a nutritionally restricted environment prenatally and postnatally (that is, pups born to a Restricted dam and cross-fostered onto a Restricted dam) impaired postnatal growth and caused a nephron deficit and hypertension in adulthood (Wlodek *et al.* 2007). However, cross-fostering a male pup from a Restricted dam onto a Control dam after birth improved postnatal growth and prevented the nephron deficit and hypertension (Wlodek *et al.* 2007).

Recently, studies have suggested the same prenatal insult may result in markedly different disease outcomes for male and female offspring (Denton & Baylis, 2007; Grigore *et al.* 2008). For many prenatal insults, males tend to be affected to a greater degree than females. Alternatively, it may be that disease emerges at an earlier age in males compared to females. Therefore, our initial aim in the current study was to assess growth, nephron endowment and blood pressure at 6 months of age in female offspring born to uteroplacentally restricted mothers and compare them to the outcomes in male offspring that we have reported previously (Wlodek *et al.* 2007). We hypothesised that similar to males, female offspring born to a restricted dam and cross-fostered onto a restricted dam after birth would have a significant nephron deficit. Furthermore, we hypothesised that altering the postnatal (lactational) environment by cross-fostering a female growth restricted pup onto a Control mother at birth would overcome the nephron deficit by restoring early postnatal nutrition and growth. We predicted females may not be as severely affected as male offspring at 6 months (Wlodek *et al.* 2007), and that disease may take longer to develop in female offspring. Thus, a subset of normally grown female pups cross-fostered onto a normal mother (Control-on-Control), as well as pups born small cross-fostered onto a mother with lactational restraint (Restricted-on-Restricted), were examined at 18 months of age for evidence of hypertension and renal damage. In particular, we examined the kidneys histologically for signs of glomerulosclerosis and interstitial fibrosis as well as for alterations in the gene expression of key regulators of renal extracellular matrix (ECM) remodelling.

## Methods

### Animals and cross-fostering groups

All experiments were approved by The University of Melbourne Pharmacology, Physiology, Biochemistry & Molecular Biology and Bio21 Institute Animal Ethics Committee prior to commencement. Wistar–Kyoto rats (9–13 weeks of age) were mated and surgery performed on day 18 as described previously (Wlodek *et al.* 2005, 2007; O'Dowd *et al.* 2008*a*). In brief, under general anaesthesia (ketamine (Parnell Laboratories, Alexandria, NSW, Australia; 50 mg (kg body wt)$^{-1}$) and ilium xylazil-20 (Troy Laboratories, Smithfield, NSW, Australia; 10 mg (kg body wt)$^{-1}$)), a midline abdominal incision was made and the cervical end of the uterus exposed. The uterine artery and vein vessels were ligated on both left and right sides using 4–0 silk suture. Sham surgery for the control group was performed in the same manner, except uterine vessels were not ligated. At birth, half of the litters from the Control (sham surgery) group had their litter size randomly reduced to five to match the Restricted group (reduced litter size of Control from 10–14 to 5 pups) (Wadley *et al.* 2008; Wlodek *et al.* 2008; O'Dowd *et al.* 2008*a*).

Pups from each of the three groups, Control, Reduced and Restricted (uteroplacental insufficiency) were cross-fostered 1 day after birth onto a Control (Sham surgery) or Restricted (uteroplacental insufficiency surgery) mother (Wlodek *et al.* 2007; Siebel *et al.* 2008).

All pups in the Control and Restricted groups were cross fostered regardless of litter size. This resulted in six experimental groups: Control-on-Control, Control-on-Restricted, Reduced-on-Restricted, Reduced- on-Control, Restricted-on-Control and Restricted-on-Restricted ($n = 7$–10 mothers per group). Pups were allowed to wean naturally and were removed from the dam on day 35 after birth. One or two female pups from each litter were used resulting in 10 females being studied per group up to 6 months. A subset of Control-on-Control (9 offspring from 5 mothers) and Restricted-on-Restricted (9 offspring from 4 mothers) females were studied at 18 months. Eighteen months was selected to allow a considerable time for any potential disease including hypertension or renal insufficiency to develop.

### Body weight, blood pressure measurements and renal excretion studies

Body weight was measured on postnatal days 1, 14 and 35 and at post-mortem (6 or 18 months). Systolic blood pressure was measured at 8, 12 and 20 weeks by a tail-cuff method (Wlodek *et al.* 2000, 2003, 2007). At 18 months, mean arterial blood pressure was measured using an indwelling tail-artery catheter. Under brief general anaesthesia (isoflurane, Abbott, Australia), a catheter was inserted into the caudal artery and animals allowed to recover for at least 2 h. The catheter was connected to a pressure transducer, and the Powerlab data acquisition system and Chart 5 (ADInstruments, Australia) were used to record blood pressure (systolic, diastolic and mean arterial) in the conscious, unrestrained rat over a 1 h period (Bergstrom *et al.* 1998).

### Measurement of urinary and plasma electrolytes

Plasma samples were collected at post-mortem from all animals at 6 and 18 months of age. In addition, 18-month-old female rats were weighed and placed individually in metabolic cages for 24 h to obtain measurements of food and water intake, along with urine production. Rats were acclimatized to the metabolic cages by placing them in for a short daytime period on two separate occasions. Urine and faeces were collected, weighed and urine frozen at −20°C. Measurements of sodium, potassium, chloride, urea, creatinine, glucose, uric acid (Beckman Synchron CX-5 clinical system, Beckman Instruments Inc.) and osmolality (Advanced Model 2020 Osmometer, Advanced Instruments, Norwood, MA, USA) were performed.

### Tissue collection

At post-mortem (6 or 18 months), rats were anaesthetized with an intraperitoneal injection of a mixed solution containing ketamine (Parnell Laboratories, Pty. Ltd, Alexandria, NSW, Australia, 50 mg (kg of body weight)$^{-1}$ and ilium xylazil – 20 (Troy Laboratories, Pty Ltd, Smithfield, NSW, Australia, 10 mg (kg of body weight)$^{-1}$. The right kidney was weighed and fixed in 10% neutral-buffered formalin for subsequent analysis of nephron number. The left kidney was frozen in liquid nitrogen and stored at −80°C for subsequent extraction of RNA.

### Renal stereology and morphology

Glomerular number ($N_{\mathrm{glom,kid}}$) and volume ($V_{\mathrm{kid}}$) were determined using the physical disector/fractionator principle (Wlodek *et al.* 2007). Five to seven kidneys were counted per group. Total kidney volume ($V_{\mathrm{kid}}$) was estimated using the Cavalieri principle (Wlodek *et al.* 2007). In the 18-month-old females, glomerulosclerosis was estimated by the method of Weibel & Gomez (1962). Samples of the kidney not taken for glomerular counting were embedded in paraffin and sectioned at 5 $\mu$m thickness. Sections were stained with haematoxylin and eosin, periodic acid–Schiff (PAS) or Masson's trichrome, and examined by light microscopy.

### Gene expression analysis

Total RNA was extracted and reverse transcription and the real-time polymerase chain reaction (PCR) was performed as previously described using the Rotor-Gene v6 (Corbett Research, Mortlake, Australia) (Wlodek *et al.* 2005, 2007). Gene expression of the $AT_{1A}$, $AT_{1B}$ and $AT_2$ receptors was examined at both 6 and 18 months of age. Genes involved in ECM remodelling, growth and apoptosis were examined in kidneys of the 18-month-old groups only. Fluorescence-based real-time PCR primers and TaqMan probes were designed using the real-time software by Biosearch Technologies (Biosearch Technologies, Novato, CA, USA). The GenBank database was used for the cDNA sequences for each gene. The primer-probe design strategy was to situate primers/probes within the protein-coding region, exon spanning where possible to avoid genomic DNA contamination. Taqman® Probes were modified at the 5′ end with the reporter dye (FAM; 6-carboxyfluorescein), and at the 3′ end with the quencher dye (BHQ1; black hole quencher 1; Biosearch Technologies). A full list of these genes and their respective primer and probe sequences are shown in Table 1 or have been reported previously (Wlodek *et al.* 2007, 2008). Optimal concentrations for primers and probes were 300 and 100 nM, respectively. Relative quantification of gene expression was performed by the comparative $C_{\mathrm{T}}$ ($\Delta\Delta C_{\mathrm{T}}$)

**Table 1. Real-time PCR Taqman primers and probes**

| Gene | Sequence (5′ to 3′) | Gene/Bank accession no. |
|---|---|---|
| 18S | | |
| Forward | GCATGGCCGTTCTTAGTTGG | V01270.1 |
| Reverse | TGCCAGAGTCTCGTTCGTTA | |
| Probe | TGGAGCGATTTGTCTGGTTAATTCCGA | |
| Collagen IV ($\alpha$1) | | |
| Forward | GACAGCCAGGACCTAAAGGT | XM_001067473 |
| Reverse | ACCTGGCAAGCCCATTCCTC | |
| Probe | CCCAGGCCTTAGTGGAATACCAGGA | |
| FN1 | | |
| Forward | AGCCCGGATGTCAGAAGCTATAC | NM_019143 |
| Reverse | AGCGTGTACAGGTGGATCTTG | |
| Probe | ACAGGTTTACAGCCAGGCACTGA | |
| IGF-1 | | |
| Forward | CCAGCGCCACACTGACATG | X06043 |
| Reverse | GGGAGGCTCCTCCTACATTC | |
| Probe | CCCAAGACTCAGAAGGAAGTACACTTGA | |
| VEGF-A | | |
| Forward | GGAGCAGAAAGCCCATGAAGT | X06043 |
| Reverse | GATGTCCACCAGGGTCTCAA | |
| Probe | TCATGGACGTCTACCAGCGCA | |
| BAX | | |
| Forward | CGTGTGGCAGCTGACATG | NM_031836 |
| Reverse | AGGGCCTTGAGCACCAGTTTG | |
| Probe | TTGCAGACGGCAACTTCAACTGG | |
| BCL-2 | | |
| Forward | AGCGTCAACAGGGAGATGTCA | NM_016993 |
| Reverse | GATGCCGGTTCAGGTACTCA | |
| Probe | CCCTGGTGGACAACATCGCTCTG | |
| P53 | | |
| Forward | TGAGCGTTGCTCTGATGGTG | NM_030989 |
| Reverse | AGTCTGCCTGTCGTCCAGATAC | |
| Probe | CCTGGCTCCTCCCCAACATCTTATCC | |

18S, ribosomal RNA; collagen IV$\alpha$1 ($\alpha$1); FN1, fibronectin 1; IGF-1, insulin-like growth factor 1; VEGF-A, vascular endothelial growth factor A; BAX, BCL-2 associated X protein; BCL-2, b-cell leukaemia/lymphoma 2; P53, protein 53. All primers/probes concentrations are 300nM/100nM respectively.

method with ribosomal 18S RNA as the endogenous control.

### Data analysis

For group comparisons, data were analysed by Student's unpaired *t*-test or one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test for *post hoc* comparisons (SPSS-X, SPSS Inc., Chicago, IL, USA). Data are presented as means ± S.E.M. and $P < 0.05$ was taken as statistically significant.

## Results

### Litter size and body and organ weight

Table 2 shows the effects of prenatal and postnatal restraint with cross-fostering on litter sizes and female offspring body weights. Uteroplacental insufficiency reduced litter size ($P < 0.05$) by approximately 50%. Control-on-Restricted offspring were of similar weight to Control-on-Control at all ages examined. Restricted-on-Restricted females were lighter during early lactation (days 1–14; by 15–20%, $P < 0.05$; Table 2) compared to Control-on-Control offspring. Restricted-on-Control females were lighter than Control-on-Control at day 1 ($P < 0.05$), but were intermediate between Control-on-Control and Restricted-on-Restricted by day 14 ($P < 0.05$). By 35 days of age, all groups were of a similar weight. There were no effects of prenatal or postnatal restraint and cross-fostering at 6 or 18 months for body weight (Table 2), total and relative kidney weight, kidney volume, or relative heart weight (Table 3).

**Table 2. Litter size and body weight**

| | | Body weight (g) | | | | |
|---|---|---|---|---|---|---|
| | Litter size | Day 1 | Day 14 | Day 35 | 6 months | 18 months |
| Control-on-Control | $10.3 \pm 0.5^{b}$ | $4.2 \pm 0.1^{b}$ | $20.8 \pm 0.3^{b}$ | $78.9 \pm 1.5$ | $226 \pm 4$ | $288 \pm 7$ |
| Control-on-Restricted | $8.5 \pm 0.3^{b}$ | $4.0 \pm 0.1^{b}$ | $20.9 \pm 0.6^{b}$ | $81.2 \pm 1.9$ | $241 \pm 4$ | — |
| Reduced-on-Restricted | $4.9 \pm 0.1^{a}$ | $4.0 \pm 0.1^{b}$ | $19.2 \pm 1.3^{ab}$ | $77.1 \pm 3.2$ | $235 \pm 4$ | — |
| Reduced-on-Control | $5.0 \pm 0.0^{a}$ | $4.0 \pm 0.1^{b}$ | $19.7 \pm 0.9^{ab}$ | $72.6 \pm 2.4$ | $236 \pm 8$ | — |
| Restricted-on-Control | $5.7 \pm 0.8^{a}$ | $3.4 \pm 0.1^{a}$ | $18.5 \pm 1.4^{ab}$ | $71.9 \pm 3.2$ | $227 \pm 5$ | — |
| Restricted-on-Restricted | $5.2 \pm 0.7^{a}$ | $3.4 \pm 0.1^{a}$ | $16.7 \pm 1.2^{a}$ | $71.2 \pm 2.2$ | $223 \pm 5$ | $280 \pm 2$ |

Litter size at birth and female offspring body weight during lactation and at post-mortem (6 and 18 months) in the cross-foster groups. Data are expressed as means ± S.E.M. ($n = 10$ per group at 6 months and $n = 9$ per group at 18 months). Significant differences ($P < 0.05$) across the groups at a given age are indicated by different letters; for example *a* is different from *b*, but not different from *ab*.

**Table 3. Kidney and heart weight**

| Pup-on-Mother | Total kidney weight (g) | Kidney weight (% body weight) | Total kidney volume ($mm^3$) | Heart weight (% body weight) |
|---|---|---|---|---|
| 6 months | | | | |
| Control-on-Control | $1.43 \pm 0.03$ | $0.63 \pm 0.02$ | $561.8 \pm 36.2$ | $0.39 \pm 0.01$ |
| Control-on-Restricted | $1.45 \pm 0.03$ | $0.60 \pm 0.01$ | $589.5 \pm 16.2$ | $0.36 \pm 0.01$ |
| Reduced-on-Restricted | $1.43 \pm 0.03$ | $0.61 \pm 0.01$ | $580.3 \pm 31.9$ | $0.38 \pm 0.01$ |
| Reduced-on-Control | $1.47 \pm 0.02$ | $0.62 \pm 0.02$ | $578.0 \pm 22.8$ | $0.40 \pm 0.02$ |
| Restricted-on-Control | $1.39 \pm 0.04$ | $0.61 \pm 0.02$ | $545.1 \pm 20.5$ | $0.38 \pm 0.01$ |
| Restricted-on-Restricted | $1.36 \pm 0.02$ | $0.61 \pm 0.01$ | $502.6 \pm 21.6$ | $0.40 \pm 0.01$ |
| 18 months | | | | |
| Control-on-Control | $1.77 \pm 0.08$ | $0.62 \pm 0.03$ | $994.3 \pm 56.3$ | $0.33 \pm 0.01$ |
| Restricted-on-Restricted | $1.77 \pm 0.06$ | $0.63 \pm 0.02$ | $990.6 \pm 51.9$ | $0.33 \pm 0.01$ |

Total and relative kidney weight (% body weight), total kidney volume and heart weight (% body weight) at 6 ($n = 10$ per group) and 18 months ($n = 9$ per group). Data are expressed as means ± S.E.M.

**Table 4. Blood pressure**

| | Blood Pressure (mmHg) | | | |
|---|---|---|---|---|
| Pup-on-Mother | 8 weeks | 12 weeks | 20 weeks | 18 months |
| Control-on-Control | $117 \pm 4$ | $123 \pm 4$ | $122 \pm 1$ | $120 \pm 2$ |
| Control-on-Restricted | $127 \pm 3$ | $117 \pm 2$ | $120 \pm 4$ | |
| Reduced-on-Restricted | $115 \pm 3$ | $129 \pm 2$ | $120 \pm 5$ | |
| Reduced-on-Control | $123 \pm 2$ | $129 \pm 2$ | $119 \pm 2$ | |
| Restricted-on-Control | $124 \pm 3$ | $118 \pm 4$ | $116 \pm 3$ | |
| Restricted-on-Restricted | $120 \pm 1$ | $123 \pm 4$ | $120 \pm 5$ | $126 \pm 3$ |

Systolic blood pressure measured at 8, 12 and 20 weeks and mean arterial pressure at 18 months. There were no differences in female blood pressure across the cross-foster group studies to 6 ($n = 10$ per group) or 18 ($n = 9$ per group) months. Data are expressed as means ± S.E.M.

## Blood pressure

Prenatal and postnatal restraint with cross-fostering did not alter systolic blood pressure at 8, 12 and 20 weeks of age (Table 4). At 18 months, mean arterial blood pressure (measured by arterial catheter) in the female Control-on-Control and Restricted-on-Restricted offspring was not different (Table 4). Similarly, at 18 months, there were no differences between Control-on-Control and Restricted-on-Restricted offspring in systolic ($138 \pm 3$ *versus* $144 \pm 5$ mmHg) or diastolic ($105 \pm 2$ *versus* $110 \pm 3$ mmHg) blood pressures, respectively.

### Glomerular number and size

At 6 months, glomerular number was similar in the Control and Reduced litter cross-fostered offspring (Fig. 1*A*). Restricted-on-Restricted females had decreased total glomerular number (by 22%) compared with Control-on-Control offspring (Fig. 1*A*, $P < 0.05$), but there was no effect on individual glomerular volume (Fig. 1*B*). Total glomerular volume tended to be lower

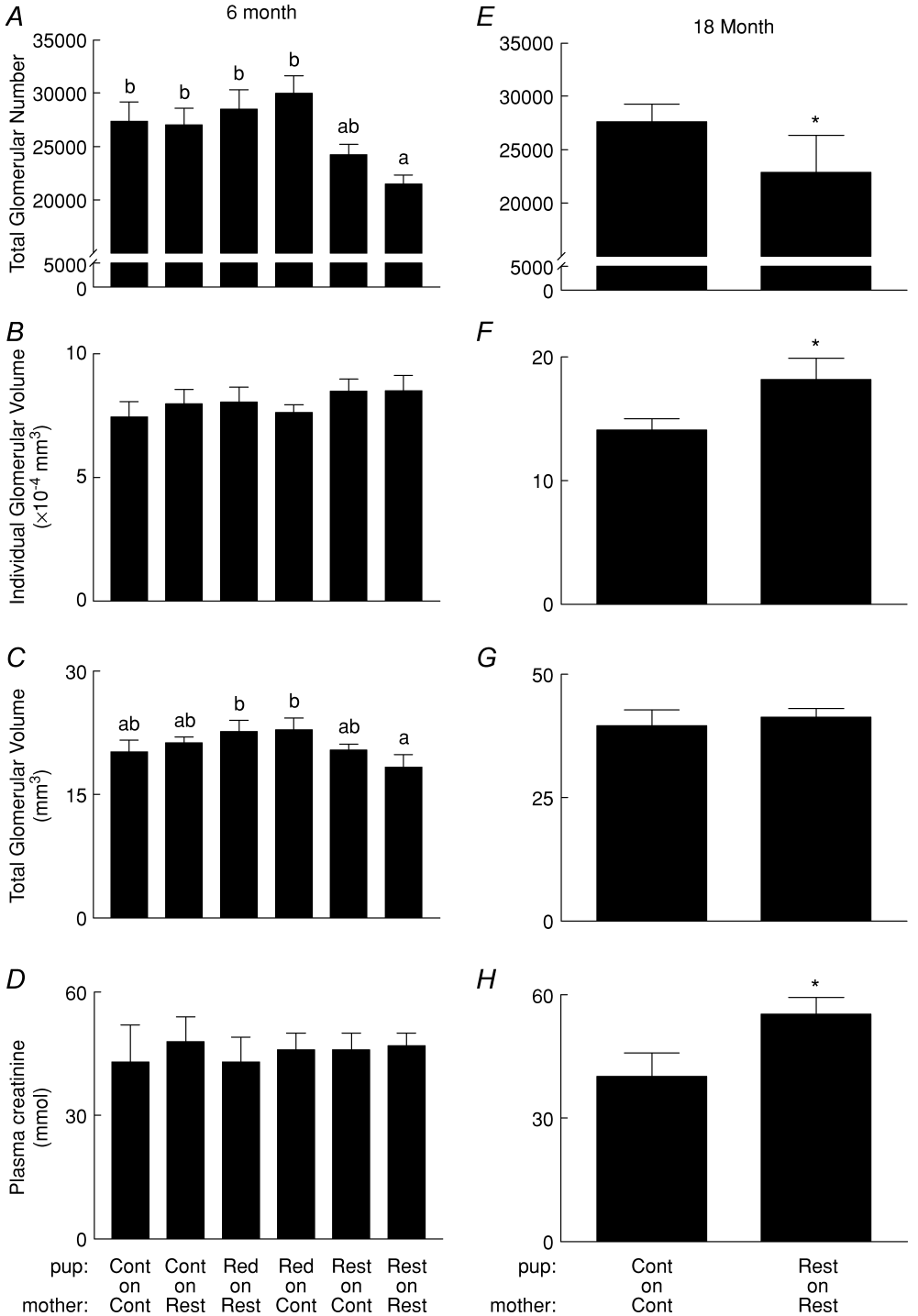


**Figure 1. Total glomerular number, individual, total glomerular volume and plasma creatinine**
Total glomerular number, indicative of nephron number at 6 months (*A*, left) and 18 months (*E*, right). Individual glomerular volume (*B*) at 6 months and at 18 months (*F*) along with total glomerular volume at 6 months (*C*) and 18 months of age (*G*). Data are expressed as means ± S.E.M. ($n = 4$–6). Significant differences ($P < 0.05$) across the groups are indicated by different letters for example *a* is different from *b*. *$P < 0.05$ between groups.

in the Restricted-on-Restricted group, but this only reached statistical significance when compared to the Reduced-on-Control and Reduced-on-Restricted groups ($P < 0.05$). Restricted-on-Control animals had a total glomerular number intermediate between Control-on-Control and Restricted-on-Restricted offspring (Fig. 1*A*). There were no effects of prenatal or postnatal restraint on corpuscle volumes (data not shown). At 18 months of age, nephron number was reduced (by 18%) in the Restricted-on-Restricted group compared to Control-on-Control (Fig. 1*E*, $P < 0.05$). Individual glomerular volume increased significantly (by 29%) (Fig. 1*F*) in the Restricted-on-Restricted group resulting in similar total glomerular volumes in the two groups (Fig. 1*G*).

### Kidney histology

Kidneys from a Restricted-on-Restricted and Control-on-Control female at 18 months of age were examined but no gross histological differences were observed between the groups (data not shown). There was no evidence of renal interstitial fibrosis or overt kidney pathology in any offspring in either group. Levels of glomerulosclerosis at 18 months of age were very low and not different between groups (data not shown).

### Plasma electrolytes and renal function

Water and food intake, urine and faeces output, urine osmolality and urinary excretions were not different between Control-on-Control and Restricted-on-Restricted females at 18 months (Table 5). Prenatal and postnatal restraint and cross-fostering did not alter plasma sodium, potassium and urea, as well as osmolality, at 6 or 18 months (data not shown). Plasma creatinine was not different across these groups at 6 months (Fig. 1*D*), but was increased in the Restricted-on-Restricted females at 18 months compared with the Control-on-Control females (Fig. 1*H*, $P < 0.05$).

### Renal gene expression

Renal angiotensin type 1 receptor ($AT_{1A}R$) and $AT_{1B}R$ mRNA expression were not different between any of the groups at 6 or 18 months of age (Table 6). Renal $AT_2R$ mRNA expression was not detectable after 40 cycles of PCR in any of the kidney tissues examined at either age. At 18 months, Restricted-on-Restricted females had increased renal gene expression for TGF-$\beta_1$ (+31%), MMP-9 (+78%) and collagen IV ($\alpha$1) (+43%) compared to the Control-on-Control offspring (Fig. 2, $P < 0.05$). Renal mRNA expression of collagen I ($\alpha$1) (+48%), MMP-2 (+33%, Fig. 2), TIMP-2 (+25%, Table 6) and fibronectin (+38%, Table 6) also tended to be increased in Restricted-on-Restricted compared to Control-on-Control females although these did not reach statistical significance. However, there were no differences in mRNA expression levels of TIMP-1 (Fig. 2) or collagen III ($\alpha$1) between groups at 18 months (Table 6). There were no differences in mRNA levels for apoptotic markers (BAX, BCL-2, P53; Table 6) or specific growth factors (IGF-1, VEGF-A; Table 6) between groups at 18 months.

## Discussion

This study demonstrates that uteroplacental insufficiency and the associated impaired lactation leads to growth restriction and a low nephron number in female offspring. These deficits were of a similar magnitude to those seen in male offspring subjected to the same intrauterine perturbation (Wlodek *et al.* 2007). In contrast to male offspring which developed raised blood pressure by 5–6 months of age (Wlodek *et al.* 2007), the Restricted-on-Restricted female offspring did not develop hypertension even by 18 months of age when there are subtle indications of functional renal insufficiency. However, elevations in plasma creatinine along with alterations in key markers of extracellular matrix composition and the emergence of glomerular hypertrophy with ageing suggest growth restricted female offspring may be predisposed to renal injury and renal failure.

Many studies are now showing there is sexual dimorphism in the programming of disease, particularly hypertension, with males generally exhibiting more severe outcomes than females (Denton & Baylis, 2007; Grigore *et al.* 2008). Growth restriction at birth due to exposure to a maternal low protein diet (9% protein rather than 18%) during pregnancy caused hypertension in male (Woods *et al.* 2001), but not female, offspring (Woods *et al.* 2005). Prenatal exposure to glucocorticoids increases blood pressure to a greater extent in male than female offspring despite a similar nephron deficit (Ortiz *et al.* 2001). Alexander (2003) showed in another model of uteroplacental insufficiency in the rat, only male offspring are hypertensive in adulthood (Alexander, 2003). In that study, the elevated blood pressure was due, in part, to testosterone as gonadectomy reduced blood pressure in growth restricted but not control offspring (Ojeda *et al.* 2007). Conversely, in females, oestrogen may play a protective role in prevention of hypertension (Huang & Kaley, 2004). In humans it has been shown that prior to menopause, women have lower rates of hypertension than age matched males but after menopause rates of hypertension are generally greater in women (Wiinberg *et al.* 1995). Furthermore, in growth restricted female rats which do not become hypertensive in adulthood, ovariectomy resulted in increases in blood

**Table 5. Urinary measures**

| Renal Parameters | Control-on-Control | Restricted-on-Restricted |
|---|---|---|
| Urine volume (l (24 h)$^{-1}$ kg$^{-1}$) | 0.075 ± 0.007 | 0.066 ± 0.009 |
| $U_K V$ (mmol l$^{-1}$ (24 h)$^{-1}$ kg$^{-1}$) | 7.80 ± 0.50 | 6.37 ± 0.81 |
| $U_{Na} V$ (mmol l$^{-1}$ (24 h)$^{-1}$ kg$^{-1}$) | 3.75 ± 0.33 | 3.55 ± 0.68 |
| $U_{Cl} V$ (mmol l$^{-1}$ (24 h)$^{-1}$ kg$^{-1}$) | 5.27 ± 0.33 | 4.58 ± 0.83 |
| $U_{urea} V$ (mmol l$^{-1}$ (24 h)$^{-1}$ kg$^{-1}$) | 35.9 ± 2.1 | 32.3 ± 3.6 |
| $U_{glucose} V$ (mmol l$^{-1}$ (24 h)$^{-1}$ kg$^{-1}$) | 0.07 ± 0.01 | 0.07 ± 0.01 |
| $U_{uricacid}$ $V$ (mmol l$^{-1}$ (24 h)$^{-1}$ kg$^{-1}$) | 0.21 ± 0.02 | 0.25 ± 0.04 |
| Osmolality (mosm (kg $H_2O$)$^{-1}$) | 794 ± 44 | 764 ± 51 |
| $U_{Creatinine} V$ ($\mu$mol l$^{-1}$ (24 h)$^{-1}$ kg$^{-1}$) | 0.28 ± 0.01 | 0.25 ± 0.02 |
| Total food intake (g (24 h)$^{-1}$) | 15.32 ± 0.52 | 14.52 ± 1.62 |
| Total water intake (ml (24 h)$^{-1}$) | 35.5 ± 3.4 | 35.3 ± 2.8 |
| Total faeces weight (g (24 h)$^{-1}$) | 5.69 ± 0.66 | 4.38 ± 0.61 |

Urinary measures along with food and water intake in rats at 18 months. There were no differences between the groups. Data are expressed as means ± S.E.M. ($n = 9$ per group).

**Table 6. Renal mRNA expression**

| Genes | Control-on-Control | Restricted-on-Restricted |
|---|---|---|
| Renin–angiotensin system | | |
| $AT_{1A}R$ | 1.055 ± 0.124 | 1.264 ± 0.113 |
| $AT_{1B}R$ | 1.080 ± 0.145 | 1.211 ± 0.084 |
| $AT_2R$ | not detected | not detected |
| ECM proteins | | |
| FN1 | 1.041 ± 0.101 | 1.434± 0.200 |
| TIMP-2 | 1.047 ± 0.096 | 1.309 ± 0.122 |
| Collagen III ($\alpha$1) | 1.031 ± 0.088 | 0.976 ± 0.079 |
| Apoptotic genes | | |
| BAX | 1.033 ± 0.029 | 1.188 ± 0.106 |
| BCL-2 | 1.078 ± 0.124 | 1.037 ± 0.111 |
| P53 | 1.057 ± 0.118 | 1.315 ± 0.116 |
| Growth factors | | |
| IGF-1 | 1.045 ± 0.113 | 0.988 ± 0.151 |
| VEGF-A | 1.102 ± 0.166 | 0.954 ± 0.079 |

Relative renal gene expression of $AT_{1A}R$, $AT_{1B}R$, $AT_2R$, FN1, TIMP-2, Collagen III ($\alpha$1), IGF-1, VEGF-A, BAX, BCL-2 and P53 in the kidney at 18 months of age in Control-on-Control and Restricted-on-Restricted females. Data are expressed as mean ± S.E.M. ($n = 9$).

pressure while there was no change in normally grown offspring (Grigore *et al.* 2008). Together these data suggest an important role for sex hormones in determining the likelihood of a hypertensive phenotype following a prenatal insult. Another factor to be taken into consideration in all the animal studies is the methodology used to measure blood pressure. Non-invasive methods such as tail cuff measurements are useful when large numbers of animals are to be tested repeatedly over a period of time, such as performed in this study up until 6 months of age. However, it is acknowledged that this method may induce stress and some measurements obtained may not reflect true basal blood pressures (O'Regan *et al.* 2008). In all our studies, great care is taken to minimise stress including adequate habituation of the animal to the measurement apparatus. Indeed, in rats habituated to the tail cuff, blood pressure is not different from that recorded via arterial cannulation (Kett *et al.* 2004). Nevertheless, we cannot totally discount that the hypertension observed in Restricted-on-Restricted males (Wlodek *et al.* 2007) may be due to hyper-responsiveness to stress. However, if this is the case, we still observe sex differences as this stress effect on blood pressure is not present in Restricted-on-Restricted females. Future studies incorporating radiotelemetry to measure blood pressure would be of value in our model.

The renal renin–angiotensin system (RAS) may also play an important mediating role in the differential disease outcomes in males and females. In our previous study in male offspring following uteroplacental insufficiency, the reduced nephron endowment and elevated blood pressure in Restricted-on-Restricted offspring was associated with elevations in renal expression of the $AT_1$ receptor gene. No such changes in renal $AT_1$ expression were observed in female offspring in the current study. Changes in $AT_1$ receptor mRNA expression in response to maternal low protein (McMullen & Langley-Evans, 2005) or glucocorticoid exposure (Singh *et al.* 2007) in the rat are also sex specific. This suggests that the RAS responds to prenatal perturbation in a sex specific manner and may play a key role in the development of hypertension in male offspring.

In our model, the development of hypertension only in male offspring following uteroplacental insufficiency may also be due to their different responses to the postnatal environment as shown by the different growth profiles of the two sexes. Restricted-on-Restricted males and females showed a similar degree of growth restriction at day 1 but females underwent accelerated

growth during lactation, such that by 5 weeks they were of similar size to Control-on-Control females. The Restricted-on-Restricted females did not become obese and were of similar size to Control-on-Control animals throughout their adult lives. In contrast, males had not caught up by 4–5 weeks of age and in fact were still slightly smaller at 6 months of age (Wlodek *et al.* 2007). Further to this, Restricted males cross-fostered onto Control mothers were the same weight as Control-on-Control males by weaning, and these Restricted males were also normotensive (Wlodek *et al.* 2007). In humans, weight gain during the first 2 years in babies born small, decreases the risk of developing hypertension whilst a low birth weight associated with poor infant growth but weight gain after 2 years increases susceptibility to hypertension (Eriksson *et al.* 2007). This suggests that early 'catch-up' growth, as seen in the Restricted-on-Restricted females and Restricted-on-Control males in our model, may protect against the onset of hypertension.

Interestingly, although we did not observe changes in arterial blood pressure, we did detect increases in plasma creatinine in Restricted-on-Restricted female offspring at 18 months of age suggesting a modest decline in renal function. Eighteen months was selected as the age for study as many diseases, particularly chronic renal disease, take considerable time to develop and may only become apparent with ageing. Increased plasma creatinine is one of the first clinical markers of chronic renal disease.

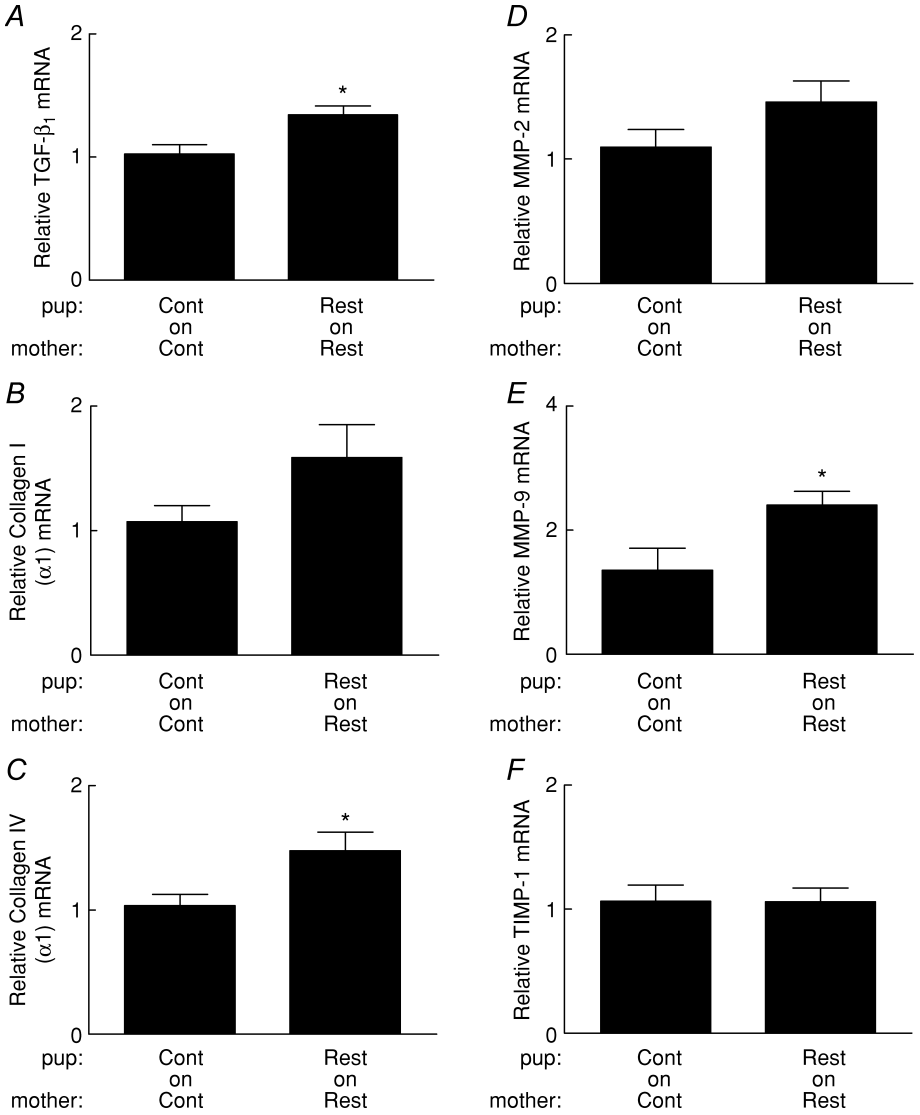


**Figure 2. Markers of renal damage at 18 months**
Relative renal gene expression of TGF-$\beta_1$ (*A*), collagen I ($\alpha$1) (*B*), collagen IV ($\alpha$1) (*C*), MMP-2 (*D*), MMP-9 (*E*) and TIMP-1 (*F*) in the kidney at 18 months of age in Control-on-Control and Restricted-on-Restricted females. Data are expressed as means ± S.E.M. ($n = 9$). Significant differences ($P < 0.05$) between the groups are indicated by *.

Although the data obtained from 24 h metabolic cage studies suggest no overt decline in renal function, the increased plasma creatinine and the glomerular hypertrophy, which developed between 6 and 18 months suggest the low nephron endowment has resulted in moderate renal insufficiency which may eventually contribute to renal disease, particularly upon an additional challenge.

We examined the effects of uteroplacental insufficiency combined with lactational restraint on apoptosis and alterations in ECM remodelling in the kidneys of aged female offspring. While perinatal restraint did not affect renal expression of apoptotic genes, there was increased mRNA expression of TGF-$\beta_1$. TGF-$\beta_1$ is a member of a superfamily of multifunctional cytokines that participate in a variety of biological activities such as development and wound repair, as well as pathological processes. TGF-$\beta_1$ is a potent inducer of ECM protein remodelling and has been implicated as a key mediator of renal fibrogenesis. The increased renal expression of TGF-$\beta_1$ mRNA may have in turn caused the concomitant changes in MMP-9 and collagen IV ($\alpha$1) mRNA. In other animal models of renal injury, mRNA levels for TGF-$\beta_1$ and ECM components including MMPs, tissue inhibitors of MMPs (TIMPs) and collagens are often altered soon after the induction of renal damage, and these changes precede the development of glomerulosclerosis and interstitial fibrosis (Norman & Lewis, 1996; Border & Noble, 1997; Visse & Nagase, 2003). Thus, although we saw no marked fibrosis or glomerulosclerosis in the kidneys of Restricted-on-Restricted animals, it does suggest the kidneys may be more susceptible to glomerular damage and subsequent sclerosis in the future. The increased TGF-$\beta_1$ expression is likely to have resulted in the observed increased collagen IV ($\alpha$1) mRNA expression. This may have important consequences for renal function, as an upregulation of collagen IV expression has previously been shown to predict the progression from normoalbuminuria to microalbuminuria in diabetic nephropathy (Adler *et al.* 2001).

Altered renal abundance of MMPs has been found in many renal pathophysiologies (Lenz *et al.* 2000; Catania *et al.* 2007). In animal models of established chronic kidney disease, the observed tubulointerstitial fibrosis and glomerulosclerosis is often associated with increased MMP-2 expression and decreased MMP-9 activity (Sharma *et al.* 1995; Maric *et al.* 2004). In humans with chronic kidney disease, increased serum creatinine concentrations were inversely correlated with MMP-9 expression (Chang *et al.* 2006). However, in other renal disease models, particularly in inflammatory glomerular disease and some models of diabetic nephropathy, increased MMP-2 and MMP-9 mRNA levels are found (Lenz *et al.* 2000; Chang *et al.* 2006). There is also evidence that MMPs are elevated in human patients with some forms of glomerulonephritis (Koide *et al.* 1996). Thus, the increased MMP-9 mRNA in the Restricted-on-Restricted females at 18 months may be suggestive of early glomerular damage. Alterations in MMPs can be regionally specific with decreased cortical MMP-2 and MMP-9, but increased medullary MMP-9 observed in the spontaneously hypertensive rat (Camp *et al.* 2003). In our study we examined whole kidney so it is not known where the alterations in MMP-9 induced in aged females by perinatal restraint occurred.

Finally, of great interest, cross-fostering a growth restricted female pup onto a control dam at birth resulted in partial restoration of nephron endowment at 6 months. This is likely to be due, in part, to the increased growth of these pups after birth at a time when there is still active nephrogenesis occurring in the rat. This finding is similar to that we made in males although restoration was greater (24%) in males compared to females (11%). The apparent discrepancy may be due to the large range in glomerular number observed in the Control-on-Control females along with the slightly different growth profiles. Further studies are required in both males and females to explore the mechanisms through which an increase in nephrogenesis can occur. The possibility of increasing nephron formation postnatally has exciting therapeutic implications for infants born prematurely.

### Summary

In conclusion, we have shown that female offspring subjected to uteroplacental insufficiency have a significant nephron deficit, in the absence of hypertension. The latter finding differs from that in male offspring subjected to the same prenatal insult who were also hypertensive (Wlodek *et al.* 2007). Furthermore, we show that as female offspring subjected to perinatal restraint age, kidney MMP activity alters, which may directly translate into altered ECM turnover, leading to renal damage and a decline in renal function. The nephron deficit may be an underlying cause of the modest renal insufficiency and along with alterations in kidney MMP/TIMP expression and an upregulation of TGF-$\beta_1$ may have implications for susceptibility to renal disease in perinatally restricted females in adult life.

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Conception and design: Wlodek, Moritz, Tare, Owens; analysis and interpretation of data: all authors (Wlodek, Moritz, Mazzuca, Tare, Owens, Siebel, Mibus, Arena); Drafting the article: Wlodek, Moritz, Mazzuca; Revising the article: all authors (Wlodek, Moritz, Mazzuca, Tare, Owens, Siebel, Mibus, Arena); Final approval: all authors (Wlodek, Moritz, Mazzuca,Tare, Owens, Siebel, Mibus, Arena).

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