# Gender differences in the renal changes induced by a prolonged high fat diet in rats with altered renal development.

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Running Head: Renal changes, high fat, renal development, sex-differences

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**Abstract**

The mechanisms involved in renal dysfunction induced by high fat diet (HFD) in subjects with altered renal development (ARDev) are understudied. The objective of this study is to examine whether there are sex-dependent differences in the mechanisms involved in the hypertension and deterioration of renal function in SD rats with prolonged HFD and ARDev. The role of angiotensin II (Ang II) in the arterial pressure (AP) increments, the renal hemodynamic sensitivity to Ang II, glomerular damage and changes in fat abdominal volume, plasma adipokines levels, renal NADPHp67phox expression and renal infiltration of immune cells were examined. Hypertension and deterioration of renal function were enhanced ($P<0.05$) in both sexes of rats with HFD and ARDev. The decrease ($P<0.05$) of AP elicited by candesartan in hypertensive rats was similar to that induced by the simultaneous administration of candesartan and apocynin. The greater ($P<0.05$) renal vasoconstriction induced by Ang II in both sexes of rats with HFD and ARDev, was accompanied by an enhanced ($P<0.05$) infiltration of CD-3 cells and macrophages in the renal cortex and renal medulla. The increments ($P<0.05$) in the renal expression of NADPHp67phox and glomeruloesclerosis were greater ($P<0.05$) in males than in females with HFD and ARDev. Our results suggest that the hypertension and deterioration of renal function induced by HFD in rats with ARDev are Ang II-dependent, and mediated by increments in oxidative stress and immune system activation. Sex-dependent increments in oxidative stress and glomerular damage may contribute to the deterioration of renal function in these rats.

**Key Words:** Angiotensin II, High fat diet, Immune system, Oxidative stress, Renal development, Hypertension, Renal damage.

58 **Key Points**

59 • An early and prolonged high fat diet induces a greater increment of arterial pressure in
60 male than in female rats with a reduced nephron endowment.
61 • Renal damage is enhanced by a high fat diet in both sexes of rats with altered renal
62 development.
63 • Angiotensin II is involved in the hypertension and deterioration of renal function in rats
64 with a prolonged high fat diet and reduced nephron endowment.
65 • The activation of oxidative stress and inflammatory pathways is greater in male than in
66 female rats with a prolonged high fat diet and reduced nephron endowment.
67

## INTRODUCTION

The mechanisms involved in the sex-dependent differences in cardiovascular and renal alterations, as a consequence of an altered renal development (ARDev), have been examined in several models of developmental programming [7, 31]. It has also been reported that a decreased renal reserve and hypertension enhances the possibility to have a further deterioration of renal function, when exposed to a second insult such as a prolonged increment of sodium [29] or proteins [26] in the diet. However, studies evaluating whether there is a sexual dimorphism in the renal function changes secondary to a prolonged exposure to a high fat diet (HFD) in subjects with an ARDev are limited [10, 12]. It is also unknown to what extent glomerular damage is enhanced in both sexes, and whether the mechanisms involved in the arterial pressure and renal alterations, as a consequence of the prolonged HFD and an ARDev, are sex-dependent.

The main objective of this study was to examine whether there is a sexual dimorphism in the mechanisms involved in the hypertension and deterioration of renal function observed in rats with ARDev and a prolonged HFD from an early age. Changes in fat abdominal volume (FAV), plasma adipokines levels and glomerular damage were evaluated since they may contribute to hypertension and renal dysfunction [8, 20, 22, 27, 28]. The involvement of angiotensin II (Ang II) was examined by evaluating both, the AP response to candesartan administration and the renal hemodynamic sensitivity to Ang II. It was also examined whether the Ang II effects are associated to increments in oxidative stress and in the renal infiltration of T lymphocytes and macrophages. To evaluate the contribution of these mechanisms is important considering that obesity precipitates the appearance of renal dysfunction among patients with a reduced nephron endowment [24], and that the progressive prevalence of overweight and obesity in children and adolescents is highly correlated with an increase of kidney disease [30].

## MATERIAL AND METHODS

Studies were performed in *Sprague-Dawley* (SD) rats purchased from the Laboratory Animals Service of the University of Murcia. Rats were housed in a temperature (23ºC) and humidity (45-60%) controlled room on a 12/12 light-dark cycle, with ad libitum access to water and food. Female SD rats (250 g b.w.) were placed with males, and day 1 of pregnancy was marked as the morning that sperm was found in the vaginal smear. On postnatal day 1, litter size was fixed (8-10 pups) to ensure similar nourishment during the suckling period. Newborn rats were treated from postnatal day 1 to postnatal day 14 with vehicle (isotonic saline) or an $AT_1$ receptor antagonist (candesartan, 7 mg·kg-1·day-1) by oral administration. Previous studies performed at four months of age have shown that this treatment induces a 36-38% reduction in nephron endowment [27], the development of hypertension and a progressive deterioration of renal function [25, 28, 29]. A reduced nephron endowment and a decrease in the renin-angiotensin system (RAS) activity are two common findings in several models of developmental programming of cardiovascular and renal diseases [7, 31].

After weaning, male and female rats were fed with either a normal (NFD) or a high (HFD) fat diet until the end of the experimental period. The calories in the NFD (Tekland 2014, Energy density: 2,9 Kcal·gr-1) are from proteins (20%), fat (soybean oil) (13%) and carbohydrate (67%). The calories in the HFD (Tekland TD.06414, Energy density: 5,1 Kcal·gr-1) are from proteins (18,4%), fat (lard + soybean oil) (60,3%) and carbohydrate (21,3%). NaCl content is similar in both diets (NFD: 0,3%; HFD: 0,4%). Therefore, four groups of male and female rats were included to perform each experiment: rats treated with vehicle during the first two weeks and fed a NFD (NFD group); rats treated with vehicle during the first two weeks and fed a HFD (HFD group); rats with ARDev and NFD (ARDev+NFD group); and rats with ARDev and exposed to a prolonged HFD from weaning (ARDev+HFD group). One male and one female rat from each dam were included in each group to perform the next experiments.

*Arterial pressure response to candesartan*. The SAP response to a 3-day oral administration of candesartan (7 mg·kg-1·day-1) was examined at 4 months of age in each group of male (NFD, n=6; HFD, n=8; ARDev+NFD, n=6; ARDev+HFD, n=6) and female (NFD, n=6; HFD, n=6; ARDev+NFD, n=6; ARDev+HFD, n=7) rats. Systolic AP was measured before treatment and the third day of candesartan administration

*Arterial pressure response to apocynin and candesartan*. The SAP response to a 3 weeks' oral administration of apocynin (60 mg·kg-1·day-1 in drinking water) was examined at 14 weeks of age in each group of male (NFD, n=8; HFD, n=8; ARDev+NFD, n=8; ARDev+HFD, n=7) and female (NFD, n=7; HFD, n=7; ARDev+NFD, n=9; ARDev+HFD, n=9) rats. During the last three days, apocynin was simultaneously administered with candesartan (7 mg·kg-1·day-1) to examine whether the Ang II effects on arterial pressure are mediated by changes in oxidative stress and/or in inflammatory mediators. Systolic AP was measured before apocynin administration (14 weeks of age), immediately before and the third day of candesartan treatment.

Systolic AP was measured in rats by plethysmography (CODA, Kent Scientific Corporation, CT) and under superficial anesthesia (isoflurane: 4% to induce; 2-2,5% to maintain) to avoid the stress during the inflation-deflation cycles in the tail. The average of at least 10 measurements was taken as SAP value. Previous studies showed that the SAP values obtained by plethysmography are highly correlated with those obtained in conscious freely moving rats with intra-arterial catheters [29].

*Renal hemodynamic response to acute Ang II infusion*. After overnight fasting, 4 months old rats were instrumented to measure arterial pressure (PowerLab, ADInstruments) and to perform renal function studies [25, 26, 28] in each group of male (NFD, n=8; HFD, n=8; ARDev+NFD, n=7; ARDev+HFD, n=6) and female (NFD, n=8; HFD, n=6; ARDev+NFD, n=6; ARDev+HFD, n=6) rats. Briefly, glomerular filtration rate (GFR) was calculated by the

143 [$^3$H] inulin clearance. A transit-time flow probe (Transonic Systems) was implanted on the
144 left renal artery for renal blood flow (RBF) measurement. A 70-min stabilization period was
145 allowed before experiments began. Two 20-min basal clearance periods were followed by an
146 i.v. captopril (10 ng·kg-1·min-1) infusion that does not modify GFR but leads to an increment
147 of RBF [24]. An i.v. Ang II infusion (30 ng·kg-1·min-1) was started 30 min after captopril
148 administration began. Fifteen minutes after initiating Ang II infusion, two more 20-min
149 clearances were obtained.
150 *Fat abdominal volume, leptin and adiponectin levels, renal NADPHp67phox expression and*
151 *immunohistopathology studies*. FAV was measured at 4 months of age as described [17], (n=8
152 in each experimental group). Rats were anesthetized by isoflurane (4% to induce and 2.5% to
153 maintain) for image acquisitions using the Albira CT system (Bruker Molecular Imaging,
154 Woodbridge, CT). Images were reconstructed using the filtered back projection algorithm via
155 the Albira Suite 5.0 Reconstructor using "standard" parameters. These settings produce a final
156 image with 125 μm isotropic voxels, deemed sufficient for abdominal cavity analysis.
157 Analysis was performed using the PMOD (PMOD Technologies LTD, Zurich, Switzerland)
158 software. Images were segmented in PMOD according to tissue density, first for total
159 abdominal volume and then for fat volume.
160 One day after FAV measurements, rats were again anesthetized (isoflurane) and blood
161 withdrawn by cardiac puncture. Plasma samples were stored at -80ºC until analysis of leptin
162 and adiponectin concentrations. Right kidney was removed, weighed and stored at -80ºC until
163 processed for Western blot analysis of NADPHp67phox expression. Left kidney was fixed in
164 10% neutral formalin (Panreac, Barcelona, Spain) for 24 hours, processed and paraffin-
165 embedded to examine the glomerular damage and for immune histopathologic analysis.
166 Plasma *leptin* and *adiponectin* concentrations were determined in duplicate by commercially
167 available ELISA kits (R&D System, MN, USA) in each group of male (NFD, n=7; HFD, n=7;

ARDev+NFD, n=7; ARDev+HFD, n=7) and female (NFD, n=8; HFD, n=7; ARDev+NFD, n=7; ARDev+HFD, n=7) rats.

*Renal NADPHp67phox expression*. Renal cortex and renal medulla were homogenized at 10% (w/v) using a Dounce tissue grinder (Sigma, USA) in a homogenization buffer [20 mM HEPES, pH 7.9, 1 mM MgCl2, 0.5 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 5 μg·ml-1 aprotinin, and 2.5 μg·ml-1 leupeptin] (n=5 rats per group). Homogenates were cleared by centrifugation at 1,000 g for 5 min at 4°C. Supernatants were removed and centrifuged at 15,000 g at 4°C for 40 min to obtain the cytosolic fraction. Protein content was determined using the Bradford assay and protein extracts were stored at −80°C until use. Briefly, 50 μg of protein was separated on 4–20% SDS-PAGE gels (Bio-Rad) and transferred to a nitrocellulose membrane (Hybond ECL, Amersham). A mouse monoclonal antibody against NADPHp67phox (Santa Cruz Biotechnology, sc-374510) and β-Actin (Sigma, A1978) were used. Bound antibody was detected using a horseradish peroxidase-conjugated antibody (Santa Cruz, sc-516102). Bands were visualized using an enhanced chemiluminescence system (ECL Plus, Amersham), and images were captured on autoradiography film, scanned, and quantified with the Image J program. Expression in male and female rats was examined on the same gels.

Glomerular damage. To examine the degree of glomerulosclerosis, 3 μm-thick sections from formalin-fixed and paraffin-embedded samples kidneys were stained with hematoxylin-eosin (HE) and Masson´s trichrome (Tric). Each glomerular profile was graded and assigned to 1 of 4 groups with respect to the degree of glomerular damage [27, 28] (6 rats per group). Approximately 500 glomerular profiles were examined for each experimental group.

*Immunohistopathology.* To establish the distribution of T-cells and macrophages on the renal cortex and renal medulla, an indirect immunohistochemical procedure was carried out on 3-μm-thick sections from formalin-fixed and paraffin-embedded samples (n=6 per group). After

deparaffination and rehydration, a demasking antigen procedure was performed (EDTA buffer pH 9.0 at 98ºC for 30 min for T-cells, and proteinase K, incubation at 37°C for 15 min for macrophages). After peroxidase blocking, sections were incubated (4ºC) overnight with the primary antibody (polyclonal rabbit anti-T CD3, dilution 1:500, Dako, A0452, Barcelona, Spain) or monoclonal rat anti-CD68 (dilution 1:100, Merck, MAB1435, Madrid, Spain) and after that, incubated with a secondary anti-rabbit (Dako) or anti-rat (Vector IMPRESS, Vector Labs., Madrid, Spain) biotynilated polymer for 20 min at 37ºC. Immunoreaction was finally revealed with 3,3´-diaminobencidine and counterstained with Harry´s hematoxylin (Thermo Scientific, Madrid, Spain). Positive reaction was identified by a dark-brown precipitated with membrane pattern. Average number of positive cells were determined by analysis of 10-random high power fields (X400) for each cell population. This analysis was performed by using a Zeiss Axio Scope A10 (Carls Zeiss, Jenna, Germany) light microscope with a digital camera and software system (AxioCam IcC3 and Axio Vision SE64, Zeiss).

**Statistical analysis**

Data in text, tables, and figures are given as means ± SE. Normal distributions were tested by Shapiro-Wilk. Differences between experimental periods within one group were evaluated using one way ANOVA for repeated measures. Differences between groups and sex were assessed by two way ANOVA with interaction terms between groups and sex. Bonferroni correction was used for multiple comparisons. A p value $< 0.05$ was considered significant. Data were analysed using SPSS software (v 24.0; Chicago, USA).

## RESULTS

*Body weight, FAV and plasma adipokines levels* (table 1). Body weight was similar in rats with NFD and in rats with ARDev+NFD, and enhanced ($P<0.05$) in both groups with a prolonged exposure to a HFD (table 1). FAV was similarly enhanced ($P<0.05$) in both sexes

and in both groups of rats exposed to a prolonged HFD, with respect to the FAV found in rats with NFD. Leptin levels were greater ($P<0.05$) in HFD and ARDev+HFD rats than in NFD and ARDev+NFD rats. Adiponectin concentration was similar among each group of HFD and ARDev+HFD rats and their respective groups with NFD. However, plasma adiponectin levels were greater ($P<0.05$) in female than in male rats with NFD, ARDev+NFD and ARDev+HFD.

*Systolic AP (SAP)*. Figure 1 shows that SAP was enhanced ($P<0.05$) in male rats with HFD (148 ± 2 mmHg), and in male rats with ARDev exposed to either a NFD (154 ± 2 mmHg), or a HFD (167 ± 2 mmHg), with respect to the SAP found in male rats with NFD (124 ± 1 mmHg). Similar changes in SAP were found in females since it was greater ($P<0.05$) in rats with HFD (136 ± 2 mmHg), ARDev+NFD (135 ± 2 mmHg), or ARDev+HFD (145 ± 2 mmHg) than in female rats with NFD (113 ± 1 mmHg). It also can be seen in figure 1 that SAP was greater ($P<0.05$) in both sexes of rats with ARDev+HFD than in those with only HFD or with ARDev+NFD. Sex-dependent differences in SAP were found in each group, being higher ($P<0.05$) in males than in females.

The administration of candesartan alone at four months of age, induced a reduction of SAP in each group of rats (Figure 2). The fall of SAP was greater ($P<0.05$) in male rats with ARDev+HFD (55 ± 3 mmHg) than in male rats with only HFD (39 ± 3 mmHg) or with ARDev+NFD (41 ± 4 mmHg). However, no significant differences in the reduction of SAP elicited by candesartan were found between females with ARDev+HFD (46 ± 6 mmHg) and female rats with only HFD (33 ± 5 mmHg) or with ARDev+NFD (38 ± 2 mmHg) (Figure 2). Apocynin administration only induced a decrease ($P<0.05$) of SAP in both sexes of rats with ARDev+NFD (males: 24 ± 1 mmHg; females: 18 ± 5 mmHg) and ARDev+HFD (males: 23 ± 5 mmHg; females: 21 ± 2 mmHg) (Figure 2). Blockade of $AT_1$ receptors in apocynin-treated rats induced a further decrease ($P<0.05$) of SAP in rats with only HFD, rats with

ARDev+NFD and rats with ARDev+HFD. The decrease in SAP elicited by the simultaneous administration of apocynin+candesartan in HFD rats (males: 28 ± 2 mmHg; females: 30 ± 7 mmHg), ARDev+NFD rats (males: 34 ± 4 mmHg; females: 31 ± 4 mmHg), and ARDev+HFD (males: 42 ± 3 mmHg; females: 34 ± 3 mmHg) was not significantly different to that induced by candesartan alone (Figure 2).

*Renal hemodynamic response to acute Ang II infusion*. Basal renal hemodynamic was significantly affected in male and female rats with ARDev+HFD, with respect to the GFR and RBF found in rats with NFD (Table 2). Acute Ang II infusion induced a decrease of RBF and GFR in each experimental group (Table 2). However, GFR decreased to lower levels in male and female rats with ARDev+HFD than in those with only HFD or in rats with ARDev+NFD. The Ang II-induced increment in RVR was greater ($P<0.05$) in both sexes of rats with ARDev+HFD (males: 212 ± 23%; females: 163 ± 31%) than in HFD (males: 106 ± 14%; females: 97 ± 15%) or ARDev+NFD (males: 104 ± 14%; females: 90 ± 7%) rats.

The expression of NADPHp67phox in the renal cortex and renal medulla from each group is shown in Figure 3, with respect to the expression found in male rats with NFD. The expression of NADPHp67phox was similar in rats with NFD and in those only exposed to a HFD from weaning. The ARDev led to an increment ($P<0.05$) of NADPHp67phox in the renal cortex and renal medulla in both sexes but its overexpression was greater ($P<0.05$) in rats with HFD than in those with NFD. A sex-dependent difference in the renal NADPHp67phox expression was found in rats with HFD and ARDev+NFD, being greater ($P<0.05$) in females than in males. However, the overexpression of NADPHp67phox in the renal cortex and renal medulla of rats with ARDev+HFD was greater ($P<0.05$) in males than in females (Figure 3).

Infiltration of T-cells and macrophages in the renal cortex and renal medulla. Figure 4 shows the count of CD3 T-cell in each group of rats. The number of CD3 T-cell was enhanced

(P<0.05) in the renal cortex of both sexes in rats with ARDev but the number of these cells was greater (P<0.05) in ARDev+HFD than in ARDev+NFD rats. The infiltration of CD3 T-cells in the renal cortex was greater (P<0.05) in male than in female rats with ARDev+HFD. The number of CD3 T-cell was significantly elevated in the renal medulla of male rats with ARDev but no differences were found between rats with ARDev+NFD or ARDev+HFD (Figure 4). However, the infiltration of CD3 T-cell in the renal medulla was similar in each group of female rats. Figure 5 shows representative images of the T-CD3 lymphocyte infiltrate in the renal cortex of rats with NFD and rats with ARDev+HFD.

Figure 6 shows the infiltration of macrophages in the renal cortex and renal medulla in each group. When compared to the number of macrophages in NFD rats, it was unchanged in the renal cortex and renal medulla in both sexes of HFD rats and only increased (P<0.05) in the renal medulla of male ARDev+NFD rats. The infiltration of macrophages was enhanced (P<0.05) in the renal cortex of both sexes in ARDev+HFD rats being greater (P<0.05) in male than in female rats. The number of macrophages was elevated (P<0.05) in the renal medulla of male but not of female ARDev+HFD rats (Figure 6).

Glomerulosclerosis index in each group of rats is shown in table 3. The degree of glomerular damage was slightly elevated in both sexes of rats exposed to a HFD from weaning. Glomerular damage was enhanced (P<0.05) in both sexes of rats with ARDev+NFD but the increment was greater (P<0.05) in males than in females. A further sex-dependent increase (P<0.05) of glomerular damage was found in both sexes of rats with ARDev+HFD, with respect to that found in ARDev+NFD or HFD rats. Figure 7 shows representative images of glomeruli with grade 0, 1, 2 and 3 of damage.

## DISCUSSION

This study reports new findings showing that the prolonged exposure to a HFD early in life induces a higher increase of arterial pressure and a further deterioration of renal function in

both sexes of rats with ARDev. The results obtained suggest that the hypertension and renal hemodynamic changes induced by HFD in both sexes of rats with an ARDev are secondary to the Ang II effects, to greater increases in oxidative stress and immune system activation and to an enhanced glomerular damage. The increments in arterial pressure, oxidative stress and glomerular damage are sex-dependent, being greater in males than in females.

Previous studies have examined the AP increments after a prolonged HFD in experimental models of developmental programming [10, 12, 18]. However, studies evaluating the renal changes in both sexes of subjects with an ARDev and a prolonged HFD are very limited. The mechanisms involved in the cardiovascular and renal function alterations in these subjects are also understudied. Our study shows that the prolonged exposure to a HFD induced an increment of SAP in both sexes of rats with an ARDev, but SAP was greater in male that in female rats (Figure 1). This different AP increment may be partly involved in the sex-dependent glomerular damage found in rats with ARDev+HFD. These results are in contrast with those reported in two different models of developmental programming with reduced nephron endowment [10, 12]. The differences in the AP increment elicited by the prolonged HFD may be related to the content of fat in the diet and/or to the degree of ARDev in the experimental models used. As also occur in infants born small for their gestational age [30], with smaller kidneys [21], the results of this study suggest that a faster body weight gain early in life leads to a further AP increment in subjects with an ARDev.

The increment in AP elicited by a prolonged HFD in rats with ARDev is mainly secondary to the Ang II effects because a three days' infusion of candesartan reduces SAP to the levels found in normotensive rats. The involvement of oxidative stress and inflammatory mediators in the systemic vascular Ang II effects was examined by evaluating the AP changes in response to apocynin administration since it has anti-oxidative and anti-inflammatory effects [15]. The increment in oxidative stress was confirmed by the sex-dependent elevation of

NADPHp67phox expression in the renal cortex and renal medulla of rats with ARDev+HFD. The activation of inflammatory pathways is suggested by the enhanced infiltration of T-CD3 cells and macrophages in the renal cortex of both sexes and the elevated infiltration of these inflammatory cells in the renal medulla of male but not female rats with ARDev and HFD. An increase in plasma and renal oxidized proteins has also been shown in rats with ARDev [25]. The results obtained show that apocynin induces a reduction in SAP in rats with ARDev, and that the administration of candesartan alone in these rats leads to a decrease in SAP that is similar to that elicited by the simultaneous apocynin and candesartan treatment. Collectively, these results suggest that vascular Ang II effects in both sexes of rats with ARDev and a HFD are partly mediated by the activation of oxidative and inflammatory pathways. The contribution of oxidative stress to the hypertension elicited by a HFD in rats with ARDev confirms the results obtained in male offspring of rats exposed to dexamethasone [16] during gestation period. However, this is the first study showing that the increment in oxidative stress elicited by a HFD in subjects with ARDev is greater in males than in females.

Prolonged HFD in our study induced an increase in FAV and in leptin levels that were similar in males and females, indicating that FAV may be contributing to the higher AP by increasing leptin levels [8]. A similar increment in plasma leptin in both sexes after a prolonged HFD has been reported to occur in rats with intrauterine growth restriction (IUGR) [12] and in Dahl SS rats [6]. Furthermore, similarly to female rats, women show greater adiponectin levels [20]. The SAP response to candesartan in rats with HFD may be related to the increments in FAV and leptin since RAS activity is enhanced in adipose tissue of rats with HFD [4], and an increase in leptin levels is associated with an elevated AT1 receptors expression [33].

This study examined to what extent renal function is more susceptible to a prolonged exposure to a HFD in rats with ARDev and whether the mechanisms involved in the greater renal susceptibility are sex-dependent. The hypothesis that renal function would be more

susceptible to a prolonged HFD was supported by studies performed in rats exposed to high sodium [29] or high protein [26] diets. The results obtained show that basal renal hemodynamic was deteriorated in both sexes of rats with ARDev and a prolonged HFD. Our results are in contrast with those showing that the exposure to a high fat high sugar diet induces a decrease of GFR only in males with IUGR secondary to a maternal placental insufficiency [12]. Differences in basal renal function between both studies may be explained by the degree of ARDev in the experimental models used and the % of fat in the diet (45% vs. 60%). Our results suggest that the prolonged HFD from an early age reduces even more renal functional reserve in subjects with ARDev and exposes the glomerulus to elevated renal perfusion pressures, with the consequent glomerular damage, the impairment of renal function and exacerbation of hypertension. A greater renal vulnerability to overweight and obesity has been reported in patients with a reduced nephron number [24].

The results obtained show that the Ang II-induced increment in RVR is significantly enhanced in rats with ARDev and a prolonged exposure to a HFD, when compared to the rise in RVR found in rats with only a HFD or in rats with ARDev+NFD. To the best of our knowledge, the important increment in the renal hemodynamic sensitivity to Ang II, as a consequence of the prolonged HFD from an early age in both sexes of subjects with ARDev, has not been previously reported. The renal hemodynamic changes before and during Ang II infusion could be explained by the coexistence of a progressive reduction in the number of undamaged nephrons, due to glomerular sclerosis, with significant increments in leptin, renal oxidative stress and pro-inflammatory cytokines derived from the elevated infiltration of lymphocytes and macrophages in the kidney. The enhanced renal sensitivity to Ang II may then contribute to a further increase of glomerular damage [19]. Elevated leptin levels may contribute to these hemodynamic changes because the kidney expresses abundant leptin receptors, and patients with high leptin levels are more prone to develop nephropathy [32].

The renal hemodynamic effects of leptin seem to be mediated by an increase in sympathetic activity [8] and reactive oxygen species (ROS) production [3].

An enhanced oxidative stress may contribute to the renal hemodynamic effects of Ang II because it stimulates NADPH oxidase [1] and the blockade of RAS activity delay the progression of renal disease by promoting anti-oxidative effects [2]. The increments of immune cells may be Ang II-dependent because their receptors are present on the surface of monocytes [11]. The elevated oxidative stress probably contributes to the generation of cytokines that would recruit additional inflammatory cells, and to the progressive elevations of RVR and AP in both sexes of rats with ARDev and HFD [9]. The greater increment in the infiltration of T-lymphocytes and macrophages in the kidney of these male rats may also contribute to the sex-dependent oxidative stress increment since an enhanced infiltration of immune cells create oxidative stress, and the prevention of their infiltration not only reduces oxidative stress but also ameliorates the hypertension [5]. Accumulation of ROS may provide the initial stimulus for attracting immune cells [14] and their secretion of pro-inflammatory cytokines would then lead to a further ROS production and a vicious circle with a progressive deterioration of renal function. The possible increase of cytokines secreted by the elevated infiltration of CD-3 cells and macrophages and the increment in oxidative stress may also be involved in the greater glomerular damage in rats with ARDev and a prolonged HFD [11].

Contrary to what was expected from those obtained in other experimental model [6], the results in this study suggest that there are no sex-dependent differences in the deterioration of renal function as a consequence of a prolonged exposure to a HFD in subjects with ARDev during nephrogenic period. The absence of sex-dependent differences in the renal hemodynamic changes occurs despite glomerular damage and the increments in oxidative stress and infiltration of immune cells are greater in male than in female rats. This difference in the renal T-cells infiltration has also been shown in rats with Ang II hypertension [23] and

394 may contribute to the sex-dependent differences in renal NADPHp67phox expression and AP
395 levels found in rats with ARDev and a prolonged HFD [13].
396 The new data reported in this study contribute to improve our understanding of the
397 mechanisms involved in the hypertension and renal dysfunction secondary to the prolonged
398 exposure to a HFD in both sexes of subjects with ARDev. These results strongly suggest that
399 high calorie diets should be especially prevented during childhood in patients with ARDev
400 since it may lead to a cardiovascular and renal dysfunction by increasing the vascular Ang II
401 effects and immune system activation, and inducing a sex-dependent elevation in oxidative
402 stress and glomerular damage. Considering that the intake of high calorie diets is frequent
403 during childhood, these results may help to improve the treatment of the cardiovascular and
404 renal consequences of a prolonged exposure to a HFD from an early age in both sexes of
405 subjects with ARDev.
406

## DECLARATIONS

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### Conflicts of interest/Competing interests

They are no disclosures.

### Ethics approval

The procuration of animals, the husbandry and the experiments conform to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. Approval was obtained from the ethics committee of the University of Murcia, Spain.

### Consent for publication

All authors revised the manuscript and approved the version to be published.

### Availability of data and material

All raw data that support the findings of this study will be available without restriction from the corresponding author on reasonable request.

### Authors' contributions

- Moreno JM, Salazar FJ and Llinas MT contributed to the conception and design of the work.
- All authors contributed to the acquisition, analysis or interpretation of data, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The authors declare that all data were generated in-house and that no paper mill was used.

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**Figure legends**.

**Figure 1**. Systolic arterial pressure in male and female rats with normal (NFD) or high (HFD) fat diet, and in rats with altered renal development and normal (ARDev+NFD) or high (ARDev+HFD) fat diet. Rats were exposed to NFD or HFD from weaning to the end of the experiment. Values are shown as mean ± SEM. Differences between groups and sex were assessed by two way ANOVA with Bonferroni's multiple comparisons analysis. * P<0.05 vs. NFD rats; # P<0.05 vs. HFD rats; ♦ P<0.05 vs. ARDev+NFD rats. + P<0.05 vs. respective male rats; ϕ P<0.05 sex-dependent differences.

**Figure 2**. Systolic arterial pressure changes elicited by 3-day oral administration of candesartan (7 mg·kg-1·day-1), or 3 weeks oral administration of apocynin (60 mg·kg-1·day-1), without or with simultaneous candesartan treatment. Arterial pressure changes were examined in rats with normal (NFD) or high (HFD) fat diet, and in rats with altered renal development and normal (ARDev+NFD) or high (ARDev+HFD) fat diet. Rats were exposed to NFD or HFD from weaning to the end of the experiment. Values are shown as mean ± SEM. Differences with respect to basal period and vs. apocynin treatment were assessed by one-way ANOVA for repeated measures. Differences between groups and sex were evaluated using two-way ANOVA with Bonferroni's multiple comparisons analysis. δ P<0.05 vs. basal period. # P<0.05 vs. HFD rats; ♦ P<0.05 vs. ARDev+NFD rats; λ P<0.05 vs apocynin treatment alone.

**Figure 3**. NADPHp67phox expression in the renal cortex and renal medulla from rats with normal (NFD) or a high (HFD) fat diet, and in rats with altered renal development and normal (ARDev+NFD) or high (ARDev+HFD) fat diet. Representative blots for NADPHp67phox and β-actin levels are shown. Values are shown as mean ± SEM. Differences between groups and sex were assessed by two way ANOVA with Bonferroni's multiple comparisons analysis. * P<0.05 vs. NFD rats; # P<0.05 vs. HFD rats; ♦ P<0.05 vs. ARDev+NFD rats. + P<0.05 vs. respective male rats; ϕ P<0.05 sex-dependent differences.

**Figure 4**. Number of T-CD3 lymphocytes cells per field in the renal cortex and renal medulla of male and female rats with normal (NFD) or a high (HFD) fat diet, and in rats with altered renal development and normal (ARDev+NFD) or high (ARDev+HFD) fat diet. Rats were exposed to NFD or HFD from weaning to the end of the experiment. Values are shown as mean ± SEM. Differences between groups and sex were evaluated using two-way ANOVA with Bonferroni's multiple comparisons analysis. * P<0.05 vs. NFD rats; # P<0.05 vs. HFD rats; ♦P<0.05 vs. ARDev+NFD rats. + P<0.05 vs. respective male rats.

**Figure 5**. Representative images of the T-CD3 lymphocyte infiltrate (arrowheads) in the renal cortex of male and female rats with normal fat diet (NFD) and male and female rats with altered renal development and high fat diet (ARDev+HFD). Indirect ABC-immunohistochemical anti T-CD3. Scale bar: 50 micrometers.

**Figure 6**. Number of macrophages cells per field in the renal cortex and renal medulla of rats with normal (NFD) or a high (HFD) fat diet, and of rats with altered renal development and normal (ARDev+NFD) or high (ARDev+HFD) fat diet. Rats were exposed to NFD or HFD from weaning to the end of the experiment. Values are shown as mean ± SEM. Differences between groups and sex were evaluated using two-way ANOVA with Bonferroni's multiple comparisons analysis. * P<0.05 vs. NFD rats; # P<0.05 vs. HFD rats; ♦P<0.05 vs. ARDev+NFD rats. + P<0.05 vs. respective male rats. ϕ P<0.05sex-dependent differences.

**Figure 7**. Representative images of different degrees of glomerular sclerosis. Profiles without pathological changes were assigned to grade 0; grade 1 (moderated changes) was composed of glomerular profiles with an increase of mesangial matrix and thickening of glomerular basement membrane (arrows); grade 2 (advanced changes) consisted of sclerotic glomerular profiles (arrows); and grade 3 was global sclerosis (*). Scale bar = 50 micrometers.

**Table 1**. Body weight (BW, grams), fat abdominal volume (FAV) ($cm^3 \cdot 100cm^3$-1) and plasma levels of leptin (ng·ml-1) and adiponectin (ng·ml-1) in rats treated with normal (NFD) or high (HFD) fat diet, and in rats with altered renal development and normal (ARDev+NFD) or high (ARDev+HFD) fat diet. Rats were exposed to NFD or HFD from weaning to 4 months of age.

| **MALES** | NFD | HFD | ARDev+NFD | ARDev+HFD |
|---|---|---|---|---|
| BW | 450 ± 7 | 538 ± 5* | 458 ± 15# | 510 ± 12*♦ |
| FAV | 7,9 ± 0,5 | 10,1 ± 0,4* | 5,7 ± 0,5*# | 11,1 ± 0,5*♦ |
| Leptin | 1,69 ± 0,37 | 4,89 ± 0,65* | 1,24 ± 0,16# | 3,52 ± 0,55*♦ |
| Adiponectin | 5,33 ± 0,45 | 5,79 ± 0,40 | 4,90 ± 0,52 | 5,64 ± 0,33 |
| | | | | |
| **FEMALES** | NFD | HFD | ARDev+NFD | ARDev+HFD |
| BW | 273 ± 6+ϕ | 298 ± 4*+ϕ | 262 ± 5#+ϕ | 296 ± 10♦+ϕ |
| FAV | 6,4 ± 0,6+ | 9,7 ± 0,4* | 4,5 ± 0,5* | 9,8 ± 0,6*♦ |
| Leptin | 2,11 ± 0,34 | 3,35 ± 0,29*+ | 0,80 ± 0,24# | 3,15 ± 0,69♦ |
| Adiponectin | 8,56 ± 0,44+ | 6,66 ± 0,58 | 8,77 ± 0,49+ | 8,16 ± 0,82+ |

Values are presented as mean ± SE. Differences between groups and sex were evaluated using two-way ANOVA with Bonferroni's multiple comparisons analysis. * P<0.05 vs. NFD group. # P<0.05 vs. HFD. ♦ P<0.05 vs. ARDev+NFD. + P<0.05 vs. respective male rats. ϕ P<0.05 sex-dependent differences.

**Table 2**. Changes in renal plasma flow (RPF) (ml·min-1·g kw-1) and glomerular filtration rate (GFR) (ml·min-1· g kw) in response to Ang II infusion, in rats with normal (NFD) or high (HFD) fat diet, and in rats with altered renal development and normal (ARDev+NFD) or high (ARDev+HFD) fat diet. Rats were exposed to NFD or HFD from weaning to 4 months of age.

| **MALES** | NFD | HFD | ARDev+NFD | ARDev+HFD |
|---|---|---|---|---|
| RBF | | | | |
| Basal | 3,55 ± 0,47 | 2,96 ± 0,27 | 2,55 ± 0,27 | 1,81 ± 0,17* |
| Ang II | 2,51 ± 0,32δ | 1,63 ± 0,15*δ | 1,43 ± 0,13*δ | 0,75 ± 0,15*δ |
| GFR | | | | |
| Basal | 1,10 ± 0,09 | 1,19 ± 0,08 | 0,80 ± 0,04*# | 0,53 ± 0,07*#♦ |
| Ang II | 0,86 ± 0,06δ | 0,72 ± 0,04δ | 0,55 ± 0,05*δ | 0,19 ± 0,05*δ#♦ |
| **FEMALES** | NFD | HFD | ARDev+NFD | ARDev+HFD |
| RBF | | | | |
| Basal | 4,03 ± 0,52 | 3,43 ± 0,14 | 4,67 ± 0,41+ | 1,92 ± 0,15*♦ |
| Ang II | 2,79 ± 0,35δ | 1,97 ± 0,16δ | 2,82 ± 0,28+δ | 0,93 ± 0,13*δ♦ |
| GFR | | | | |
| Basal | 1,16 ± 0,07 | 1,21 ± 0,11 | 1,09 ± 0,04+ | 0,62 ± 0,04*#♦ |
| Ang II | 0,88 ± 0,10δ | 0,83 ± 0,11δ | 0,69 ± 0,05δ | 0,32 ± 0,04*δ#♦ |

Values are presented as mean ± SEM. Differences with respect to basal period were assessed by one-way ANOVA for repeated measures. Differences between groups and sex were evaluated using two-way ANOVA with Bonferroni's multiple comparisons analysis.

δ P<0.05 vs. Basal; * P<0.05 vs. NFD rats; # P<0.05 vs. HFD rats; ♦ P<0.05 vs. ARDev+NFD rats. + P<0.05 vs. respective male rats.

**Table 3**. Percent of glomeruli with degrees of glomerulosclerosis Index

| **MALES** | % Grade 0 | % Grade 1 | % Grade 2 | % Grade 3 |
|---|---|---|---|---|
| NFD | 90,6 ± 0,7 | 9,0 ± 0,3 | 0,5 ± 0,5 | 0,0 ± 0,0 |
| HFD | 67,2 ± 9,3* | 30,2 ± 9,0 | 2,3 ± 0,8 | 0,3 ± 0,3 |
| ARDev+NFD | 23,8 ± 3,3*# | 62,0 ± 2,6*# | 12,8 ± 3,9 | 1,4 ± 0,6 |
| ARDev+HFD | 17,8 ± 5,2*# | 49,8 ± 8,5* | 22,8 ± 8,4*# | 9,6 ± 3,3*#♦ |
| | | | | |
| **FEMALES** | | | | |
| NFD | 93,8 ± 0,3 | 6,2 ± 0,3 | 0,0 ± 0,0 | 0,0 ± 0,0 |
| HFD | 65,3 ± 7,1* | 33,3 ± 6,7* | 1,2 ± 0,5 | 0,2 ± 0,2 |
| ARDev+NFD | 55,8 ± 6,7*+ϕ | 36,8 ± 5,6*+ | 4,8 ± 3,9*# | 2,6 ± 0,9 |
| ARDev+HFD | 45,0 ± 1,6*+ϕ | 39,0 ± 2,2* | 7,6 ± 0,8*#+ϕ | 8,4 ± 2,0*#♦ |

Grade 0 indicates normal; grade 1, moderated changes: grade 2, advanced changes; grade 3, global sclerosis. Data are expressed as percent of area. Values are presented as mean ± SE. Differences between groups and sex were evaluated using two-way ANOVA with Bonferroni's multiple comparisons analysis. * $P<0.05$ vs. NFD rats; # $P<0.05$ vs. HFD rats; ♦$P<0.05$ vs. ARDev+NFD rats. + $P<0.05$ vs. respective male rats; ϕ $P<0.05$ sex-dependent differences.