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The sex influence in chronic kidney disease. Teachings from a genomic approach to fibrosis in experimental obstructive nephropathy

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ABSTRACT

The unilateral obstruction model has been described as an excellent way to study the morphological and molecular mechanisms that characterise kidney fibrosis. The main aim of this study was to evaluate molecular matrix turnover in the progression of kidney fibrosis, and to what extent sex influences molecular behaviour. With this aim the authors developed a unilateral obstruction model in three groups of Wistar rats of both sexes, with 4, 8 and 21 days of obstruction, respectively. After the sacrifice both kidneys of all the groups were removed, and sagitally sectioned to perform histological observations using hematoxylin-eosin and Masson's trichrome and mRNA genomic analysis expression of Col1a2, Col5a1, Tgfβ1, Mmp2, Mmp7, Mmp9, Mmp12 and Mmp14, plus androgen and oestrogen receptors. Major variations were observed for collagen I, V mRNA and MMPs in obstructed kidneys, namely an overexpression of Mmp2 and Mmp7 in females. In contralateral kidneys collagen mRNA showed a downregulation trend in both sexes, but MMP genes profile exhibited an increased expression namely in females. Oestrogen and androgen receptors gene expression exhibited a complex and variable pattern, with a downregulation trend for oestrogen in both obstructed and contralateral kidneys from male rats. Our observations highlight the role metalloproteinases plays in the extracellular matrix turnover and developing fibrosis, the biological activity of which could be influenced by sex, conditioning the development of kidney disease.

Key-Words:
Fibrosis; sex; matrix turnover; unilateral ureteral obstruction model (UUO).

INTRODUCTION

Many studies in animal and clinical research have been performed to contribute to a better understanding of the cellular and molecular physiopathological mechanisms involved in the beginning and progression of kidney disease, namely in the development of fibrosis.

The obstruction of urinary flow, depending on the time of duration, is a major cause for acute and/or chronic nephropathy, leading to early but progressive morphological changes, characterised by tubular epithelial atrophy, inflammation and interstitial fibrosis, with a later involvement of the glomerula.

The unilateral ureteral obstruction model (UUO) quickly became an excellent method for studying the fibrosis phenomena, as it made possible a chronological analysis of the intervention of cells and molecules responsible for the altered extracellular matrix turnover in both the ligated and the contralateral kidney[1-3].

The fibrosis pathogenesis involves a complex molecular and cellular network including fibroblast proliferation, macrophage infiltration, cytokines, chemokines growth factors and multiple proinflammatory mediators, which culminates in an imbalance between extracellular matrix deposition and degradation[4].

The influence of sex hormones in the evolution of chronic nephropathy, and to what extent the balance between androgen and oestrogen can be more harmful or beneficial in the regulation of the extracellular matrix turnover, is the subject of debate. Some authors claim testosterone is more noxious than oestrogen in the progression of nephropathy[5]. Others state that it is oestrogen, while others assume that there is no sex influence[6-8]. Despite all this controversy and in accordance with most of the literature, sex hormones, *per se,* seem to determine a higher susceptibility of male sex to renal disease progression. They also seem to influence renal disease progression through different mechanisms, such as cell proliferation, molecular haemodynamics, cell oxidation, extracellular matrix turnover, several cytokine and growth factors release, as well as cell death[5,9-11]. According to some authors, the pathway for sex hormones' influence on renal disease progression is essentially made by activation of their receptors[9,12].

The aim of our study was to quantify the mRNA from molecules implicated in the fibrosis pathogenesis (Collagen: Col1a2, Col5a1; Transforming growth factor β: Tgfβ1; Metalloproteinase: Mmp2, Mmp7, Mmp9, Mmp12 and Mmp14), in a UUO model with 4, 8 and 21 days of duration. The study was performed in female and male Wistar rats, obstructed and contralateral kidneys, analysing differences in molecular mRNA expression possibly related to sex. Additionally, expression of Androgen (Ar) and Oestrogen (Esr1) receptors mRNA was quantified in the same kidneys to evaluate any relationship between sex and the mRNA fibrosis related molecules.

SUBJECTS AND METHODS

Animal experiment

In this study, 36 Wistar rats (from Charles River Laboratories, Barcelona, Spain), male and female, eight weeks of age, were used. All the procedures with animals were in concordance with Portuguese legislation / EU guidelines on ethical use of experimental animals (86/609/EEC). During the experimental period all animals had free access to water and food, and adequate conditions of light, humidity and temperature.

There were three experimental groups and a Control group: animals underwent UUO, through ligation of the left ureter with silk 4-0, and were sacrificed by anaesthetic excess, at 4th (LD4- ureter ligation four days; n=5 males, n=5 females), 8th (LD8- ureter ligation 8 days; n=5 males; n=5 females) and 21st (LD21 – ureter ligation 21 days; n=4 males, n=4 females) days after ureter ligation. The animals from the Control group were sham operated (n=4 males, n=4 females). Surgical procedures (UUO and sham operation) were performed via general anaesthesia with 10mg/100g ketamine chlorohydrate IM. In the necropsy, both kidneys (obstructed and contralateral) were removed, weighed and sagittally sectioned. One hemisection was stored in RNA later^{TM} solution (Ambion, Austin, USA), and the other was fixed in buffered formaldehyde at 10%, for molecular and histological analysis, respectively.

Histological analysis

Histological analysis of the kidneys (ligated and contralateral) was made essentially to confirm the existence of an obstructive nephropathy in evolution.

After fixation in buffered formaldehyde at 10%, the kidney hemisection was subsequently embedded in paraffin. For the histological analysis, 5μm sections were made for performing the current staining techniques in light microscopy (Hematoxylin–Eosin (H&E) and Masson's trichrome).

Total RNA extraction

The samples were removed from RNA later^{TM} solution (Ambion, Austin, USA) to proceed to disruption and homogenisation, which were performed

using a rotor-stator Ystral X10/25 (Ystral, Ballrechten-Dottingen, Germany). Tissue lysates were processed according to the protocol from RNeasy® Mini Kit for Isolation of Total RNA extraction from animal tissues (Qiagen, Hilden, Germany) and total RNA was eluted with 50µl of RNase-free water.

In order to quantify the amount of total RNA extracted, final RNA solutions were read at different optical densities in a GeneQuant pro spectrophotometer (Biochrom, Cambridge, England) to determine their concentration and purity. RNA integrity was verified with RNA 6000 Nano Chip® kit, Agilent 2100 bioanalyzer (Agilent Technologies, Walbronn, Germany) and 2100 expert software.

Reverse Transcription

One µg total RNA was reverse transcribed in 50µl using 1.25 U/µl of MuLV RT – TaqMan® Gold RT-PCR kit (Applied Biosytems, Foster City, USA) and a reaction mixture consisting of 1X Buffer RT, 5.5mM $MgCl_2$, 500µM of each dNTP, 2.5µM of random hexamers and 0.4 U/µl RNase Inhibitor. The reactions were performed in a thermocycler Gene Amp PCR System 9600 (Perkin Elmer, Norwalk, USA), 10 minutes at 25°C, 30 minutes at 48°C and 5 minutes at 95°C, and were finally stored at -20°C.

Relative quantification of gene expression

Relative quantification of gene expression by real-time PCR was performed in the 7900 HT Sequence Detection System (Applied Biosystems, Foster City, USA). A normalisation step preceded the gene expression quantification, using geNorm Housekeeping Gene Selection kit for *Rattus norvegicus* (Primer Design, Southampton, United Kingdom) to select optimal housekeeping genes for this study and geNorm software (Ghent University Hospital-Center for Medical Genetics, Ghent, Belgium).

Assays-on-Demand™ Gene Expression (Applied Biosystems, Foster City, USA): 20x Mixture of primers and probes TaqMan® MGB (FAM) for Ar, Col1a2, Col5a1, Esr1, Mmp2, Mmp7, Mmp9, Mmp12, Mmp14 and Tgfβ1 (assays reference in Table I), together with 100 ng cDNA sample and 1XTaqMan® Universal PCR Master Mix, No Amperase UNG (Applied Biosystems, Foster City, USA). TaqMan® Ribosomal RNA Control Reagents (Applied Biosystems, Foster City, USA) were used to detect the 18S ribosomal RNA (rRNA) gene as housekeeping gene. This 20 µl total volume reactions were performed with the following thermal profile: 10 minutes at 95°C plus 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Table I

Real time PCR assays references

Gene symbol	Gene name	Genebank Access Number	Assay ID
Ar	Androgene receptor	NM_012502	Rn01424244_m1
Col1a2	Procollagen, type I, alpha 2	NM_053356	Rn01526723_ml
Col5a1	Procollagen, type V, alpha 1	NM_134452	Rn00593170_m1
Esr1	Estrogen receptor 1	NM_012689	Rn01430443_m1
Mmp2	Matrix metalloproteinase 2	NM_031054	Rn01538176_m1
Mmp7	Matrix metalloproteinase 7	NM_012864	Rn01487001_m1
Mmp9	Matrix metalloproteinase 9	NM_031055	Rn00675898_m1
Mmp12	Matrix metalloproteinase 12	NM_053963	Rn01486489_m1
Mmp14	Matrix metalloproteinase 14	NM_031056	Rn01489223_m1
Tgfβ1	Transforming growth factor, beta 1	NM_021578	Rn01475962_m1

Gene symbol and name; Genebank Access Number; assay (primer and TaqMan® Probe mix) identification reference for studied genes.

Real-time PCR results were analysed with SDS 2.1 software (Applied Biosystems, Foster City, USA) and quantification used the 2-ΔΔCt method[13]. Statistical analysis used the Mann-Whitney to test differences between male and female kidneys from different groups ($p<0.05$) and was performed using SPSS software (SPSS Inc, Chicago, USA).

RESULTS

Renal weight and histological changes

Obstructed and contralateral kidneys did not present different wet weight in the Control and LD4 groups; however at the 8th (LD8) and 21st days (LD21) after UUO, obstructed kidneys' weight was superior to contralateral (Table II). No relevant histological changes were seen in kidneys from the Control group. In obstructed kidneys, a progressive tubular dilation with epithelial atrophy as well as infiltration by mononuclear cells and progressive

Table II

Renal weight of different groups (grams)

Sex	Male		Female	
Kidney	Obstructed	Contralateral	Obstructed	Contralateral
Control	0.50±0.04		0.49±0.03	
LD4	0.33±0.06	0.32±0.1	0.22±0.03	0.18±0.03
LD8	0.51±0.15	0.28±0.08	0.22±0.05	0.15±0.01
LD21	0.79±0.2	0.69±0.1	1.01±0.5	0.63±0.05

Average±SD of renal wet weight (grams) by groups. Male and female groups; Control – Sham-operated animals; LD4 – animals with 4 day ureter ligation; LD8 – animals with 8 day ureter ligation; LD21 – animals with 21 day ureter ligation

interstitial fibrosis was seen from the 4th to the 21st day of obstruction (Fig. 1A), while no changes were found at the contralateral kidney (Fig. 1B).

Gene expression

mRNA relative quantification in obstructed and contralateral kidneys was performed calculating mRNA expression in groups LD4 (ureter ligation at 4th day), LD8 (ureter ligation at 8th day) and LD21 (ureter ligation at 21st day) as compared to Control group (sham operation). The analysis of gene expression exhibited variation in obstructed and contralateral kidneys compared to Controls, and statistical analysis found differences between sexes.

Obstructed kidneys

Collagen I mRNA was upregulated (1 log) in obstructed kidneys; collagen V mRNA was also upregulated (1-2 logs) in LD4 and LD8 obstructed kidneys, but no significant differences were found between male and female kidneys (Fig. 2A).

The major variations in MMP gene expression were found in Mmp2 and Mmp7 mRNA. Mmp2 gene was overexpressed (1-3 logs) during all the UUO periods (Fig. 2B), although females from LD8 group presented more significant Mmp2 mRNA than males (p=0.028). Mmp7 mRNA showed a similar upregulated profile, with LD8 female obstructed kidneys (3 logs) higher than in males (1 log, p=0.006), but no differences were found for the other groups. Mmp9, Mmp12 and Mmp14 presented lower variations of mRNA (1-2 logs), while

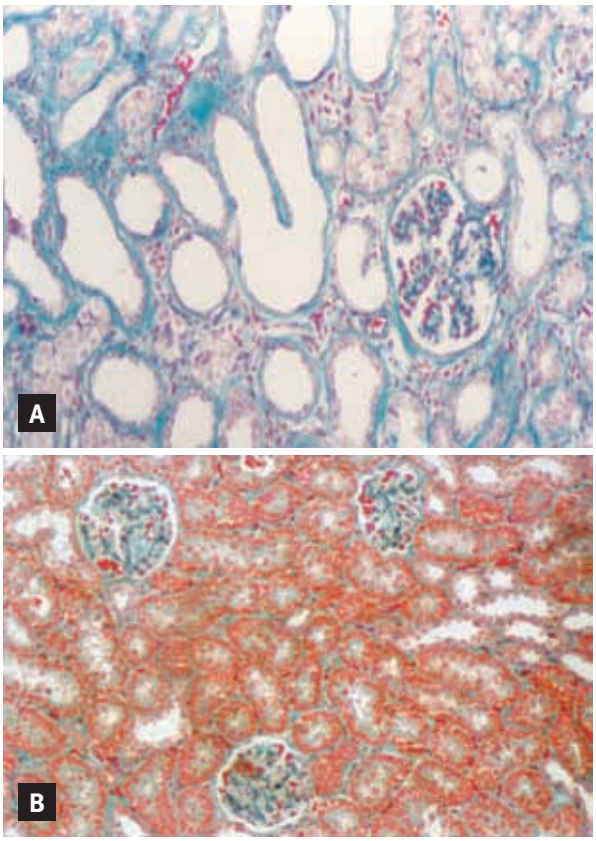


Figure 1

Histological renal obstruction analysis.

1A: Masson's trichrome staining (×200 magnification). Obstructed kidney, 21st day, exhibiting tubular dilation with epithelial atrophy and interstitial fibrosis

1B: Masson's trichrome staining (×200 magnification). Contralateral kidney, 21st day, exhibiting no histological changes

the LD8 group registered higher levels in female obstructed kidneys than males (p=0.018 for Mmp9; p=0.045 for Mmp12). In LD4 and LD21 groups, variations found in Tgfβ1 mRNA levels were low (1 log) but were higher in males than in females, in LD4 and LD21 groups.

Oestrogen receptor gene expression did not present significant variation in obstructed kidneys, except for the LD8 group where males showed a downregulation close to -2 logs (Fig. 2A) and females were slight upregulated (p=0.008). The androgen receptor mRNA expression showed distinct levels of expression between periods of UUO (0-3 logs in LD21 group), but no differences were found for sex.

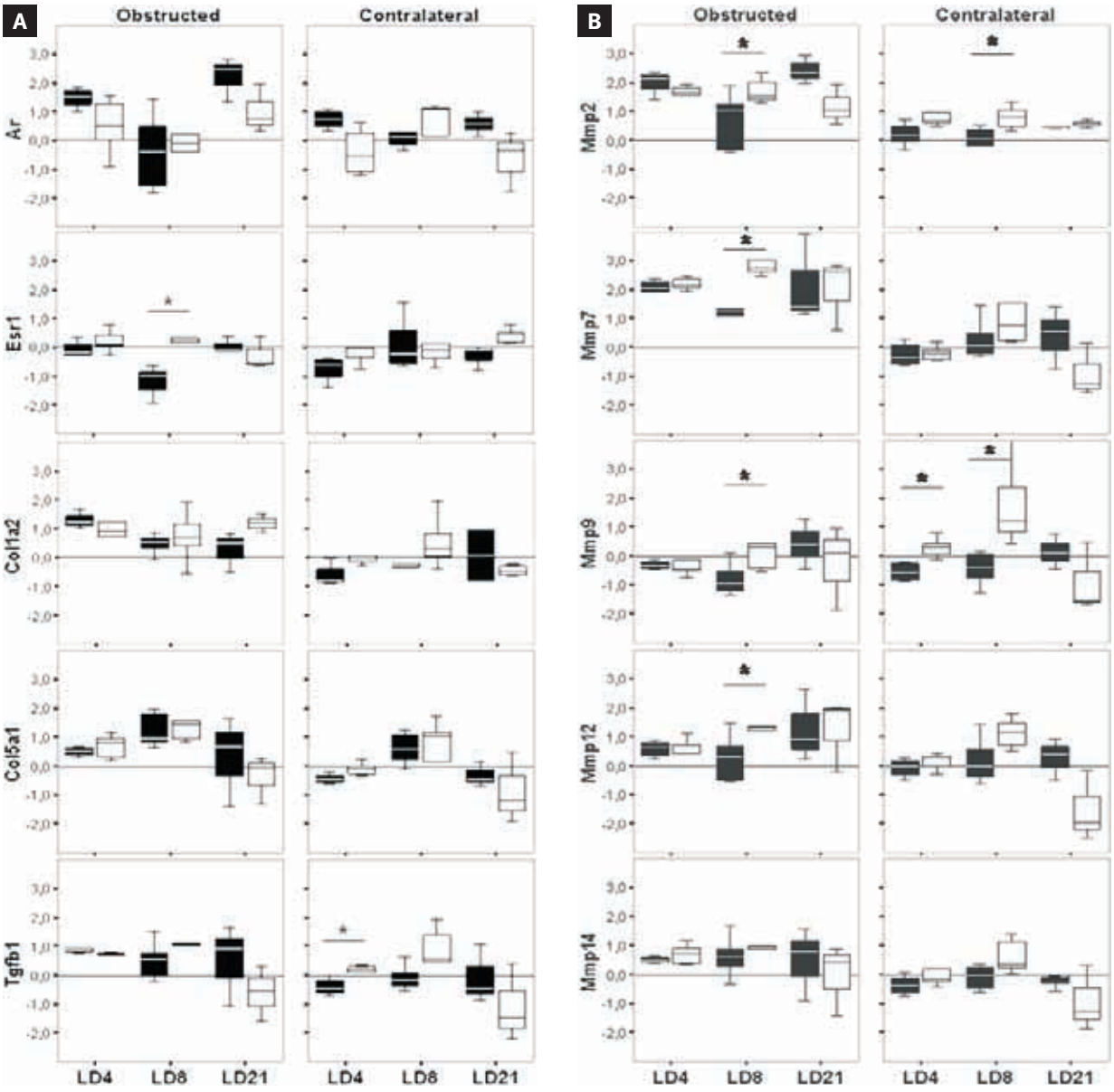


Figure 2

mRNA renal relative expression of fibrosis associated markers

mRNA relative expression levels, in logarithmic scale. **A:** Ar (Androgen receptor); Esr1 (Oestrogen receptor 1); Col1a2 (Procollagen type I α2); Col5a1 (Procollagen type V α1); Tgfβ1 (Transforming growth factor β 1); **B:** Mmp2 (Matrix metalloproteinase 2); Mmp7 (Matrix metalloproteinase 7); Mmp9 (Matrix metalloproteinase 9); Mmp12 (Matrix metalloproteinase 12); Mmp14 (Matrix metalloproteinase 14). ■ Males, □ females; * $p<0.05$ for male *vs.* female.

■ Contralateral kidneys

Collagen mRNA (Col1a2, Col5a1) in contralateral kidneys showed a downregulatory trend in both sexes. Col5a1 reached levels about -2 logs at 21st day, whereas it was upregulated 1 log and in LD8 group (Fig. 2A).

Contralateral kidneys exhibited a gene expression profile for all MMP genes with female levels greater than males'. Mmp2 mRNA was upregulated 1 log in females and males were similar to Controls (p=0.028 for LD8) (Fig. 2B). Mmp9 also exhibited great differences between upregulated levels of females and

downregulated for males (p=0.014 for LD4, p= 0.006 for LD8) (Fig. 2B). Contralateral kidneys from LD21 group expressed MMPs mRNA levels close to zero in males and decreased in females (maximum of -2 logs), and although distinct, no statistical significance was found. Tgfβ1 mRNA registered an expression profile similar to that of MMP; female contralateral kidneys from LD4 and LD8 group showed a slight upregulation, whereas in males they were downregulated and close to 0, respectively (p=0.027 for LD4); in female, LD21 downregulation reached -2 logs (Fig. 2B).

Esr1 mRNA variation was not significant in contralateral kidneys with only a slight downregulation in male from LD4 and upregulation in female from LD21 group (Fig. 2A). Androgen receptor mRNA expression in male and female contralateral kidneys were distinct for all groups; upregulations to 1 log were detected in male LD4 and LD21 in opposition to downregulation in female, on the other hand in LD8 group 1 log overexpression was found for female, and in male were close to zero (Fig. 2A).

DISCUSSION

To our knowledge, and compared to other studies on this matter, the most interesting finding was a sex difference in the expression of the main molecules involved in the progression of kidney fibrosis. Those differences were characterised by different mRNA levels in collagens I, V, MMPs and Tgfβ1, additionally, other variations in androgen and oestrogen receptors mRNA were also found.

Tgfβ1 signalling plays a pivotal role in renal disease progression; thus Tgfβ1 mRNA overexpression observed in obstructed kidneys, signals an increment of profibrotic activity with progressive development of renal fibrosis[14-17]. Moreover, as a consequence of profibrotic signalling, several types of collagen may contribute to the fibrotic process[18]; the increment of Collagen I and V mRNA at LD4 and LD8 in obstructed kidneys are in concordance with other studies and support fibrosis development suggested by Tgβ1 mRNA levels[15,16,19]. As a result of fibrosis undergoing, MMPs become crucial elements for extracellular matrix turnover. Since MMPs are not stored normally in the tissues and are present at a minimal constitutive expression, their transcription regulation depends on growth factors, cytokines and extracellular matrix molecules[20] and its mRNA expression gives a prognostic of antifibrotic activity.

It has been described that sex hormones can influence the synthesis/degradation of collagen[21]. Oestrogen downregulates collagen genes transcription and extracellular components synthesis[22,23] and androgen can stimulate collagen synthesis[24,12]. However, sex differences are not usually explored in studies using the UUO model.

This sex dimorphism in gene regulation is supported by studies giving evidence of positive regulation of MMP transcription by oestrogen, mainly the Mmp2[9,17,25]. Oestrogen's regulatory effects in kidney disease are mediated by its receptor and it is known that female oestrogen receptor (Esr1) renal expression is higher in females than in males[26]. In fact, Esr1 mRNA sex difference observed in LD8 group accompanies the distinct profile observed in Mmp2, Mmp7, Mmp9 and Mmp12 between male and female LD8 obstructed kidneys. The Mmp2 mRNA upregulation observed by us in female obstructed kidneys show an apparent greater capacity to collagen degradation; it was reported by others that enhancement of renal fibrosis following inhibition of Mmp2 activity during a certain period after UUO is due to decreased degradation of ECM[27].

Other studies have demonstrated that Mmp7 expression lacks in normal kidney, but it is present in the injured kidney where mRNA expression increases as fibrosis progresses[28]. The Mmp7 mRNA overexpression observed by us may signal its potential to activate other MMPs in latent state. MMP7 has also a role in cell death process, through activation of Tnfα and FasL, contributing to the decline of inflammatory cells[29]. Therefore the increment of Mmp7 mRNA may show the beneficial repairing activity of the matrylisin originated.

Regarding other MMPs, a slight downregulation of Mmp9 mRNA in undergoing obstruction has been reported[30], showing the controversial role of metalloproteases in renal disease and an ambiguous indicator for the disease stage. Due to its multifunction in the renal system, Mmp9 interferes

in apoptotic and inflammatory phenomena in addition to interstitial fibrosis[31,32]. In addition, decreased Mmp9 expression was correlated with the development of tubulointerstitial fibrosis and glomerulosclerosis in rats[17,33], but like other MMPs this was lower in males.

There are few studies into the role of Mmp12 in kidney diseases. For this reason we analysed Mmp12 relative gene expression. Mmp12 mRNA upregulation in female LD8 obstructed kidneys suggest the degradation of basement membrane components, such as collagen and also facilitates macrophages mobility through elastin digestion[29,34,35].

Interestingly, contralateral kidneys variations are in accordance with the hypothesis that UUO also affects the non-obstructed kidneys[36], probably in consequence of the systemic spread of inflammatory molecules arising from the obstructed kidney. This feature is absent from almost every study using the UUO model, because the contralateral kidneys are considered a control group. However, consequences of UUO on the contralateral kidney have already been reported, with blood pressure variations and compensatory hypertrophy based on an increased interstitial volume[37-39]. Some authors report changes in contralateral kidneys, both at the mRNA and protein level[40-42], and found that the severity of these changes depends on the degree and duration of the obstruction[43].

Previous reports of Tgfβ1 mRNA evaluation observed no variation in contralateral kidney compared with the controls, at five[44] and 14 days[45] post-UUO, respectively. On the other hand, an upregulation of Tgfβ1 receptors has been associated with its contribution to the fibrotic response in the obstructed kidney and the hypertrophic response of the contralateral kidney[2]. Although several collagen types have been measured in previous studies using the UUO model[18,46], those investigators usually select male animals, in which the contralateral kidney is used only as a control, and so variations are not considered. In our observations we found an increment of Col5a1 and different upregulation for Tgfβ1 mRNA at 4 and 8 days post-UUO in female contralateral kidneys, followed by a severe downregulations of both collagens and Tgfβ1 mRNA at the 21st day. This may be associated with hypertrophic response of the contralateral kidney, furthermore, female animals showed more variations, but seem to solve more easily the profibrotic condition at 21st day after UUO.

In our observations contralateral kidneys expressed variations of MMPs mRNA, generally in females. Considering the hypothesis of profibrotic signaling in contralateral kidney due to a systemic influence, female kidneys seem more susceptible; however, the observed levels of Mmp2 mRNA suggest a good prognosis, because Mmp2 potentiates collagens degradation. In addition, another study had also found some modest changes in Mmp2 activity in the unobstructed kidneys in male mice. Mmp-9 overexpression observed for females is in accordance with its increased activity shown by others[42]. This could be related to the compensatory mechanisms after UUO, which moderates matrix turnover, as was already observed for other molecules in progressive renal fibrosis[47]. However, variations in oestrogen receptor mRNA at the renal level was not observed in contralateral kidneys, also supporting the hypothesis of a systemic regulation.

In this study, we performed a basic approach to genomic variations in kidney fibrosis. The molecular analysis in obstructed and nonobstructed kidney proved to be an excellent method for capturing and evaluating the most subtle and ongoing progressive molecular changes. The renal expression of mRNA fibrosis markers evaluated in our UUO model provides useful information on the dynamics of extracellular matrix turnover, suggesting sex differences. Upregulated MMPs mRNA levels observed in obstructed kidneys, with female changes preceding male sex, suggests an increased potential to matrix remodelling and counteracting fibrosis, namely in females.

Our observations highlight the role of sex hormones in extracellular matrix turnover, namely in MMP, and suggest the interest of a sex influence in the progression of chronic kidney disease. On the other hand, further investigation into this mater, namely the development of new treatment drugs, should take into consideration the sex physiological specificities and influence on the apparently naive mechanisms of kidney fibrogenesis.

Conflict of interest statement. None declared.

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