EFFECT OF SEX ON EXPERIMENTAL DIABETIC NEPHROPATHY AND THE RENIN-ANGIOTENSIN SYSTEM. ROLE OF ACE2

Thesis submitted by

Sergi Clotet Freixas

For the degree of Doctor of Biochemistry, Molecular Biology and Biomedicine

Directors

Dr. Julio Pascual Santos

Dr. Mª José Soler Romeo

Dr. Marta Riera Oliva

Tutor

Dr. Assumpció Bosch

PhD Student

Sergi Clotet Freixas

Department of Biochemistry and Molecular Biology
Universitat Autònoma de Barcelona

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6. DISCUSSION

6.A DISCUSSION OF THE *IN VIVO* FINDINGS

6.A.I Exploring the effect of sex in diabetic nephropathy

In our first *in vivo* study we evaluated the **effect of sex hormones** in diabetes and DN progression. For this purpose, we included and compared male versus female mice, and GDX versus non-GDX males, after **19 weeks of STZ-induced T1DM**. In our animal model, STZ diabetes induction was accompanied by many physiological, functional and histological lesions of DN, most of them observed in the human manifestation of the disease. STZ administration led to **hyperglycemia, body weight loss, decrease in heart rate and heart weight, renal hypertrophy, hyperfiltration, polyuria, albuminuria, and cortical fibrosis** in diabetic female and male mice. However, diabetes did not significantly alter blood pressure and did not promote an advanced glomerulopathy in terms of glomerulosclerosis. In this sense, the UAE values observed in diabetic females and males were approximately 10-15 times higher than the ones of their controls. Based on the guidelines from Breyer *et al*. to define the severity of DN in animal models, our mice developed the expected albuminuria in this STZ model[81].

The severity of the renal alterations observed **differed between sexes**. Diabetic males showed a more accentuated hyperglycemia, hyperfiltration and albuminuria, than diabetic females. Furthermore, kidneys from this group presented several alterations that were not observed in diabetic females, namely glomerular hypertrophy, mesangial matrix expansion, and tubular damage by means of cytoplasmic vacuolization and lipiduria. Overall, female sex was associated to a milder form of DN at the end of the study, especially in terms of functional and morphological glomerular alterations. However, opposing the general trend, the gene and protein expression of all the fibrotic markers analyzed was clearly higher in diabetic females than in males (Figure 94).

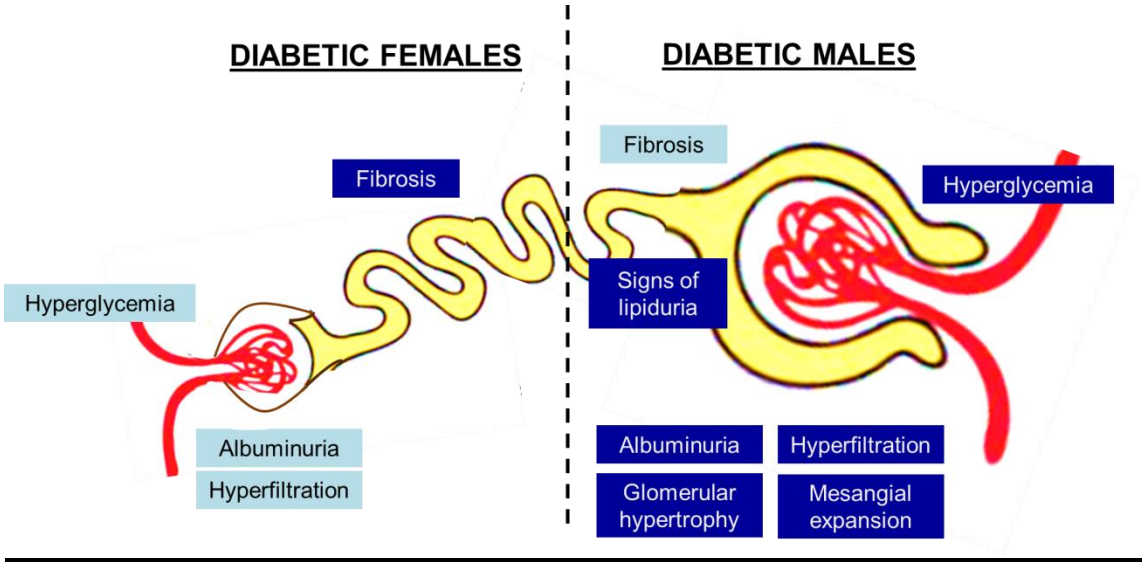

Figure 94. Sex effects on diabetic renal disease in experimental T1DM. For each sex, alterations associated to the renal microvasculature, the glomerulus, the tubule and the tubulointerstium are represented. Light blue indicates milder lesions, whereas dark blue indicates more severe alterations, compared with the other sex.

The association between **male sex** and the **increased susceptibility** to develop certain glomerular lesions in the context of T1DM has been observed previously[341]. In concordance to our data, Costa *et al*. reported a greater increase in UAE in STZ-diabetic male rats as compared to females, after 12 weeks of follow-up. At the clinical level, many authors have shown that men with T1DM have an increased risk of developing microalbuminuria and progressing to macroalbuminuria than women[349,350,352,392,524]. This robust evidence supports the employment of STZ animals as a valid choice for studies evaluating sex disparities in DN. In addition, several investigators have observed hyperfiltration, glomerular hypertrophy and mesangial matrix expansion in male mice of the STZ model[321,322,325]. Furthermore, the same alterations have been consistently described in males from another model of T1DM, the Akita mice[323,525–527]. These observations strengthen the idea that male sex contributes to a **more severe impairment in glomerular function and morphology in T1DM**, and that this effect was not only ascribed to a particular animal model. However, diabetic females were not evaluated in any of those studies. At present, little is known about how the presence T1DM can perturb the glomerular integrity in STZ-induced females. To our knowledge, the present project is the first effort to simultaneously and accurately study the effect of T1DM on glomerular functional and morphometric parameters in both sexes. In regard to the signs of lipiduria and proximal tubule vacuolization observed in our diabetic males, similar findings have been found in other experimental studies employing exclusively STZ-induced males[75–77].

In contrast to our results, it has been reported that STZ-diabetic males, but not females, showed increased renal collagen I and fibronectin mRNA levels compared with controls[341]. Of note that, in this study, Sprague-Dawley rats were employed instead of mice, and were followed for a shorter period of time (12 weeks instead of 19). Low dose of STZ (65 mg/kg) was enough to achieve a clear hyperglycemia (which they defined as blood glucose levels >270mg/dL). However, the authors did not report the exact blood glucose values at the end-point[341]. Despite our diabetic females showed lower blood glucose levels than males at week 12 of follow-up, the degree of hyperglycemia did not differ across sexes at the end of the study. This fact allowed us to compare renal alterations between sexes, with the certainty that the sex differences observed would not be ascribed to different blood glucose values at the end point. On the other hand, employing a **high dose of STZ** may have contributed to a higher **renal toxicity** of the drug, especially in females, which was reflected by an increase in the number of focal infiltrations of inflammatory cells in the subscapular region of the renal cortex (Figure 95). These secondary effects of STZ probably led to a higher activation of fibrotic and inflammatory processes beyond the classic development of fibrosis associated to the natural progression of DN.

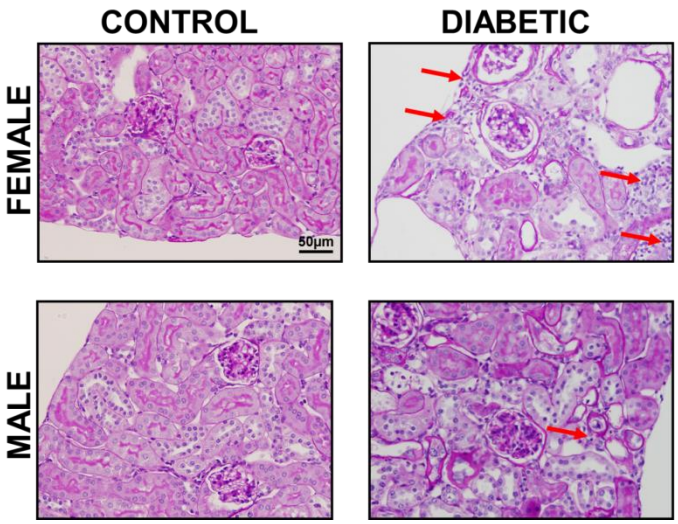

Figure 95. Secondary toxicity of streptozotocin(STZ) in the kidney. As indicated by the red arrows, STZ administration in the diabetic groups was accompanied by subcapsular inflammatory infiltrates and acute tubular injury, especially in females.

Another important finding that emerged from this study is that **surgical castration** after diabetes induction dramatically decreased the levels of glycemia in diabetic males. At the end of the study, **diabetic GDX males** showed blood glucose values that were still greater than 250mg/mL, and significantly higher than the ones observed in GDX controls. In addition, GDX attenuated, and in some cases almost reverted, the

renal alterations that appeared to be exacerbated in diabetic males. Specifically, GDX in diabetic males was accompanied by (1) decreased body and kidney weights; (2) attenuated renal hypertrophy; (3) prevention of hyperfiltration, albuminuria, glomerular hypertrophy, and mesangial matrix expansion; and (4) reduced proximal tubule vacuolization.

It is known that **male sex** and androgens stimulate growth and **hypertrophic processes** in several organs such as the pancreas, heart and kidney[528,529]. In addition, castration decreased heart and kidney weights in non-diabetic mice[530] and prevented renal hypertrophy in STZ rats[531]. In concordance to these observations, we observed that the heart and the kidneys from control and diabetic C57BL/6 male mice were larger and heavier than the ones from females. As expected, this sex effect was abolished in the GDX group. Little is known about the effects of GDX in glomerular function. Iwase et al. reported a **protective effect of GDX** against the development of albuminuria in STZ-diabetic rats[531]. Supporting these findings, chemical castration by flutamide administration also reduced the urinary excretion of albumin in diabetic STZ males[341], suggesting that the deleterious effects of androgens on the progression of albuminuria are mediated by the conversion of testosterone to DHT and subsequent binding to the AR.

In contrast to the other studied parameters, GDX in our diabetic males did not prevent or attenuate the development of **renal fibrosis** at the gene level. Thus, the mRNA levels of all the fibrotic markers were notably up-regulated in this group. Similar findings have been found previously in STZ-diabetic Sprague-Dawley rats[385,387]. Sun *et al*. reported a dramatic increase in renal TGF-β1 protein expression and CTGF mRNA levels in castrated male rats with adult onset of diabetes (induced at 16 weeks of age by STZ injection), after 6 weeks of follow-up[385]. This increase was also observed in non-diabetic rats. In the same work, testosterone treatment in diabetic prepuberal rats augmented TGF-β1 and CTGF gene expression in association with a more pronounced hyperglycemia[385]. When followed for 14 weeks, castrated STZ rats exhibited exacerbated albuminuria and fibrosis in the glomerular and the tubulointerstitial compartment, by means of higher TGF-β1, collagen I and collagen IV protein expression, with no alterations in the blood glucose levels[387]. This increase was accompanied by a more pronounced expression of the proinflammatory marker CD68 (indicating a higher abundance of macrophages in this group) as well as a more exaggerated decrease in the protein expression of the matrix metalloprotease MMP-9 (suggesting deficient degradation of ECM). In that model, a more advanced stage of diabetic nephropathy was achieved, as the authors described glomerulosclerosis in the diabetic group, which was accentuated by GDX[387]. In front of these data, one may

surmise that either a significant reduction of androgens or excessive levels of these hormones may be detrimental for the diabetic kidney, especially for the development of renal fibrosis. To deepen in the relationship between androgen levels and the severity of kidney disease and renal fibrosis, the same research group evaluated the effects of two different doses of DHT (low dose: 0.75 mg/day, high dose: 2.0 mg/day) in previously castrated STZ rats treated for 14 days. Interestingly, the low dose of DHT attenuated, whereas the high dose accentuated, the adverse effects of castration in diabetic male rats in terms of glomerular and tubulointerstitial fibrosis (TGF-β1, collagen IV) and inflammation (CD68, TNF-α, IL-6), and glomerular apoptosis[532].

Altogether, these data suggest that impaired androgen levels (either too low or too high) predispose to a more prominent renal fibrosis in the diabetic kidney. In our study, however, significant detrimental effects of sex hormone reduction on renal fibrosis were observed **only at the mRNA level**. The fact that the GDX-induced up-regulation of pro-fibrotic genes was also observed in our control mice indicates that androgen deficiency exerted a more predominant effect than diabetes in this perturbation at the mRNA level. In this regard, it is worthy to mention that our results differ from the ones reported from Xu *et al*. by the fact that we observed a dramatic reduction of blood glucose levels after GDX. Although this group was diabetic (as these mice presented blood glucose levels greater than 250mg/dL, and statistically higher than their controls), a more severe hyperglycemic environment in the other study (>430mg/dL) could have contributed to the decrease in MMP-9 expression and, in consequence, to the accumulation of ECM proteins in the glomerulus and the tubuluinterstitium[387]. Together with other possible mechanisms such as the modulation of renal RAS and sex hormone receptors expression (which will be explained in upcoming sections), we speculate that, in our diabetic GDX males, decreased glucose-dependent downregulation of MMP expression led to a **more efficient ECM degradation** and, in consequence, prevented the accumulation of fibrotic markers at the protein level, even when their gene expression was clearly increased (Figure 96).

6.A.II Sex differences under physiological conditions: what are the controls telling to us?

Female and GDX mice presented decreased **heart weight**, which was accompanied by a higher **heart rate** as compared to control intact males. This association was expectable, as it is widely known that cardiac weights are inversely proportional to heart rates[533]. Interestingly, this pattern was more evident and statistically significant in GDX mice than in females, suggesting that, despite “feminization” of GDX males by sex hormone reduction, the balance of sex hormones in both groups is not exactly the

same. Several factors such as the synthesis of endogenous testosterone in other organs a part from the testicles, like the ovary and the adrenal glands, as well as its conversion to estradiol by aromatase activity, may explain this effect. In addition, female and GDX controls presented higher values of **SPB** and **DPB** than intact males. In this sense, increased heart rate has been associated to hypertension in previous studies[534].

Regarding renal morphological studies, we found that **female glomeruli** showed a different morphology than **male glomeruli**, in terms of increased glomerular and mesangial area. Androgen levels probably played a role in this effect, as GDX in control males augmented these parameters to similar levels than females. We think that **sex-specific physiological characteristics** in this compartment should be considered when performing morphometric analyses in murine models of glomerular diseases. For example, we observed lower values of mesangial index in diabetic female and GDX mice as compared to their controls. Other alterations in the glomeruli associated to DN, such as congestions or microaneurysms, rather than a reduction of the mesangium itself, may have contributed to this decrease.

Another interesting finding related to sex and the healthy glomeruli is the presence of an extra layer of **tubular cell epithelization** under the parietal layer of males **Bowman's capsule**, which was absent, or clearly shorter, in the glomeruli from female mice. This effect was already observed in 1980. In that study, the authors described a cuboidal cell transformation of the outer layer of Bowman's capsule, which was observed in the 60% of male glomeruli and in the 5% of female glomeruli, and considered this feature as an indicator of hyperplasia of proximal tubular cells in males[535]. These observations suggest that, in male mice, the proximal tubule not only connects the urinary space with the other segments of the nephron, but also plays a structural role in the glomeruli. The direct relation between male sex and the degree of glomerular epithelialization was strengthened with our findings in GDX mice, which showed poor or null epithelialization of the capsule. To our knowledge, we are the first to observe a link between androgen reduction and this particular characteristic in the murine kidney.

6.A.III Sex-specific changes in DN

Interestingly, the morphologic differences between female and male Bowman's capsules were inverted under hyperglycemic conditions. In females, diabetes increased the thickness of the capsule, as PAS-stained sections from this group displayed a disorganized, hyperplasic accumulation of cells lining the inner side of the parietal layer

of the capsule. Under physiological conditions, this parietal layer is formed by a single layer of simple squamous epithelium.

In the setting of DN, EMT of renal epithelial cells has been proposed as the main source of **myofibroblasts**[536]. TGF-β1 and CTGF are key mediators driving EMT and renal fibrosis[537,538]. The expression of these molecules was stimulated in the cortex of our diabetic females, compared with males. Therefore, PEC hyperplasia in diabetic females may be ascribed to up-regulated TGF-β1 and CTGF levels, which presumably promoted **EMT** of these cells and consequent **periglomerular fibrosis**. It is conceivable that this process in the glomeruli mimics the progression of fibrosis in the **tubulointerstital** compartment, as female mice in our study presented higher cortical expression of **TGF-β1 and CTGF** (indicating a higher activation of EMT in the tubules), α-SMA (suggesting greater differentiation to myofribroblasts), fibronectin, and collagens I and IV (indicating accentuated ECM accumulation). As mentioned in previous sections, we surmise that diabetes decreased estrogen levels in our female mice, with the subsequent attenuation of TGF-β inhibition, leading to its overexpression and the activation of its downstream deleterious and fibrogenic mechanisms**.** The proposed mechanisms to explain up-regulation of fibrosis in female and GDX mice are shown in Figure 98.

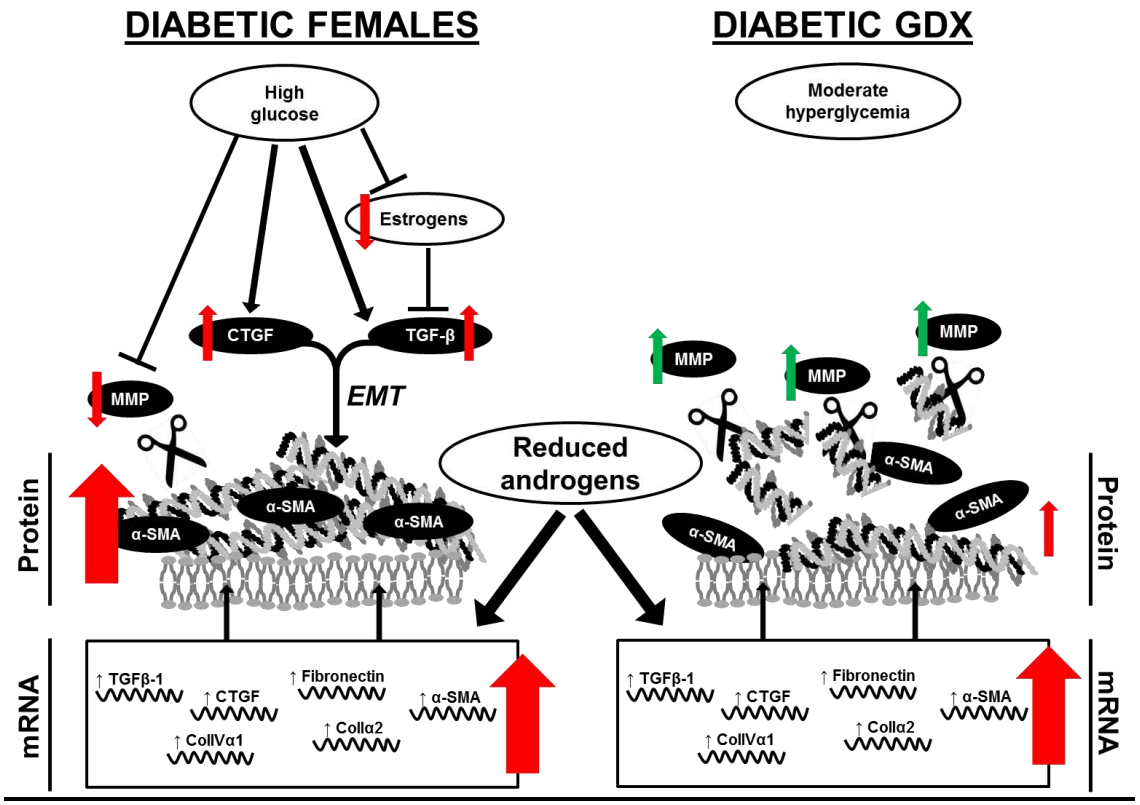

Figure 96. Different regulation of fibrosis in the renal cortex of female and GDX mice with T1DM. Reduced androgen levels were associated with increased expression of fibrotic and pro-fibrotic markers at mRNA level. In diabetic females, higher blood glucose and reduced estrogen levels may have contributed

to a more accentuated fibrosis at the protein level through several mechanisms, such as decreased MMP levels, or increased EMT driven by augmented CTGF and TGF-β protein expression.

In the tubular compartment, **intracytoplasmic vacuolization** was increased in control males and prevented by GDX. In the context of T1DM, the male group showed a certain degree of vacuolization in the lumen, probably indicating **lipiduria**, as well as more severe hyperglycemia, than the other groups. These observations suggest that male hormones may alter processes related to lipid metabolism in the renal tubule cells, which may confer a higher susceptibility to develop certain tubular alterations under pathologic hyperglycemic conditions.

In summary, we observed that **male sex** predisposes to a more severe diabetes and **worsened glomerular disease and tubular injury** in experimental T1DM, and that these alterations are attenuated by surgical castration after the onset of diabetes. Conversely, **female sex** and "feminization" of male mice by gonadectomy are associated to a **more exaggerated renal fibrosis** in the context of T1DM. The molecular studies of sex-specific RAS regulation and cortical expression of sex hormone receptors, along with the interpretation of previously published data, allowed us to propose two **possible mechanisms** implicated in this sexual dimorphism, which will be explained in the next two sections.

6.A.IV Sex-specific RAS modulation in T1DM. Implications for renoprotection

T1DM was accompanied by a considerable **dysregulation of circulating and renal RAS**. In females and intact males, diabetes was associated with increased *Agt*, *Ace2*, and *Nep*, as well as decreased *Ren* and *Ace* gene expression. In addition, increased ACE2 and decreased ACE in the context of T1DM were also observed at the protein level in both sexes. The imbalance in ACE and ACE2 levels in the renal cortex reflected an impaired activation of RAS in terms of increased ACE2/ACE ratio under hyperglycemia. Diabetes also augmented urinary ACE2 activity in all groups. In the circulation, diabetic females and intact males showed a significant increase in ACE and ACE2 activity as compared to their respective controls, which was also reflected in a higher serum ACE2/ACE ratio.

Supporting these results, RAS hyperactivation has been associated to **increased renal AGT and decreased renin expression** in other studies employing STZ-induced models of T1DM[341,539]. Interestingly, the diabetes-induced increase in renal AGT was already observed after 12 weeks[341] and even after only 4 weeks of diabetes, where

was accompanied by a clearly augmented AGT excretion into urine[539]. The increases in cortical, circulating, and urinary ACE2, as well as the dichotomy between ACE augmentation in serum and its reduction in the kidney, are also very consistent findings in the context of experimental diabetes[187,203,312,321,540]. Indeed, the increments in circulating ACE and ACE2 activities are detected even in early stages of DN[89,496,539].

The effect of T1DM on **renal NEP expression** has not been widely studied. The increase in *Nep* mRNA levels coupled with decreased renin expression in the kidneys of our diabetic mice may be interpreted as an ACE2-independent mechanism to avoid ANGII accumulation in front of a status of pathological RAS hyperactivation[541,542]. In this sense, recent studies support a key role of NEP on catalyzing ANG(1-7) formation in the murine and the human kidney[543,544]. Interestingly, it has been demonstrated that NEP inhibition attenuates renal damage in the diabetic kidney[541]. One may surmise that overactivation of this NEP-mediated compensatory mechanism may lead to an excessive imbalance towards the pro-ANG(1-7) axis of RAS.

A **sexual dimorphism** was observed in the majority of **RAS components** analyzed. Under physiological conditions, male sex was associated to higher *Apn*, as well as lower *Agt*, *Ren*, and *Nep* gene expression. Gonadectomy in control males changed the expression of these four genes to the direction of the female values. In concordance to our results, renal NEP activity was found to be higher in non-diabetic female rats as compared to males[539]. However, the fact that *Agt* and *Ren* are increased in the female and GDX groups contrasts with previous studies that reported a clear association between androgens/male sex and higher expression of these RAS components, accompanied by increased levels of AGT in urine[545–547]. Of mention that we assessed AGT expression only in the renal tissue, and not in serum and urine; moreover, we did not measure its expression at the protein and enzymatic activity level. Thus, we cannot state if decreased gene expression of AGT in males correlated with lower protein levels, or if they were ascribed to a higher turnover and greater protein synthesis in the normal kidney. As we have shown and discussed, female sex and castration were associated to higher cortical expression of fibrotic genes. In this regard, other possible explanation to the up-regulation of AGT and renin genes in our female and GDX control mice may consist on the participation of DHT-independent mechanisms of AR, which have been demonstrated in several cell types and are related to pro-fibrotic and proinflamattory pathways[444,448].

In regard to the main regulatory arms of RAS, the **effect of sex on ACE and ACE2 expression** depends on the place of cortex analyzed. In the **total cortex**, control females showed higher ACE protein expression and lower ACE2 gene, protein, and activity than males. Whereas the effect of sex on ACE2 expression was maintained in

the **outer cortex**, ACE expression presented an inverted pattern, as control males showed a clear up-regulation of ACE as compared to females. The effect of GDX in the cortical expression of ACE and ACE2 was very consistent, as castration reduced ACE and augmented ACE2 regardless of the area analyzed. In the serum, the activity of both, ACE and ACE2, was reduced by GDX. Overall, androgen reduction by GDX "feminized" the expression of RAS in the normal serum and kidney, excepting for renal ACE2 expression, which was further elevated, leading to a tremendous increase in the ACE2/ACE ratio in the kidney cortex. These data indicate that, while male hormones may play a strong effect on modulating the expression of AGT, renin, NEP, APN, and ACE, the regulation of ACE2 is probably under control of both, male and female sex hormones. In concordance, Lim *et al*. reported a **reduction in plasma ACE activity** after surgical castration of male mice[195]. Furthermore, it has been demonstrated that estrogen treatment reduces plasma ACE activity in ovariectomized cynomolgus monkeys[548] as well as in ovariectomized female rats[549]. These experimental data is comparable to clinical studies where estrogen replacement therapy has shown to decrease circulating ACE levels in postmenopausal women[550,551]. Therefore, it seems fairly clear that, while androgens upregulate, estrogens downregulate circulating ACE activity under non-diabetic conditions.

At kidney level, cortical and medullar activity of **ACE were greater in female** than in male healthy rats, although this increase was significantly different only in the cortex, a pattern that is similar to our findings in the total cortex. Accordingly, a reduction in cortical ACE mRNA and activity has been observed in ovariectomized Sprague-Dawley[552] and Wistar[194] rats after 17β-estradiol replacement therapy. However, contrary effects of sex hormone reduction on renal ACE have been reported, as castration promoted up-regulated ACE levels in the cortex, but not in the medulla, of female and male rats[553]. Together with other factors, such as the strain and the age of the animals, these discrepancies may be ascribed to the area of the cortex analyzed. Of note that testosterone can be metabolized to 17β-estradiol by aromatase activity[554], which has been demonstrated to be present in the glomeruli and the peritubular endothelium[555]. These observations suggest that **sex-specific ACE modulation** follows a **different pattern in the outer cortex**. In this project we provide robust and consistent data demonstrating that the effect of sex hormones on ACE expression is influenced by the portion of cortex analyzed. When studying the renal cortex, investigators do not specify if they analyze the entire cortex or only the outer part. From our perspective, this ambiguity may complicate the global interpretation of data and promote the inference of biased conclusions.

Regarding **ACE2**, its renal expression has been found to be increased in males from different murine models not only at enzymatic activity but also at protein and mRNA level[211,212], as compared to females, giving consistency to our findings. In contrast, Sampson *et al*. observed greater ACE2 gene expression in the kidney cortex of adult female rats than in age-matched males[556]. However, they also noticed that males had higher levels of ACE2 mRNA at younger ages, and that the renal expression of this enzyme significantly decreased with age in both sexes. It is therefore conceivable that sex hormone fluctuations with age influence the sex-specific pattern of ACE2 expression in the kidney.

Overall, **male sex** is related to **higher ACE2 expression** in the healthy kidney. This may explain that non-diabetic $ACE2^{-/y}$ male mice show greater development of renal damage than $ACE2^{-/-}$ females[319]. Since *Ace2* gene is located in the sexual X chromosome, several studies have been conducted to elucidate whether gender differences observed in ACE2 expression are exclusively due to the gonadal sex or they also depend on the sex chromosome complement (XX vs XY). Liu *et al*. studied the effect of 17β-estradiol on renal ACE2 activity in the four core genotypes (FCG) mice. In this model, GDX significantly increased renal ACE2 activity levels in both XX and XY females. Moreover, normal levels of ACE2 activity were reestablished after administration of EST in all groups. The major conclusion of this work is that sex differences in **renal ACE2 activity** are **EST-dependent** and sex chromosome-independent[342]. These findings suggest that physiological levels of estrogens maintain the lower levels of cortical ACE2 expression and activity in the renal cortex of our non-diabetic females. In this sense, one may expect that GDX in male mice would feminize the expression of the enzyme, but we observed an opposite effect. We hypothesize that, in GDX males, reduced levels of androgens may have contributed to lower conversion of testosterone to 17β-estradiol, with the consequent lack of estrogen-mediated inhibition of ACE2 expression.

In the context of **T1DM**, diabetic males and females showed similar mRNA levels of **AGT and renin in the kidney cortex**. However, diabetic males showed lower **NEP** and higher **APN** gene expression than diabetic females. In concordance to our data, decreased plasma renin activity and renal mRNA levels, as well as increased expression of NEP, have been reported in STZ-diabetic Sprague-Dawley rats[341,539]. In this model, renal AGT expression was increased in males (but not in females), showing strong association with albuminuria and renal fibrosis, after 12 weeks of T1DM[341]. In front of this controversial findings, we argue that, in our hands, the use of a high dose of STZ, and a prolonged period if diabetes, may have facilitate that diabetic females developed a more pronounced **hyperglycemia** and, in consequence, a major **RAS**

hyperactivation within the kidney. Surgical castration in diabetic males dramatically augmented AGT, renin, and NEP mRNA levels, and notably reduced APN gene expression. Whereas the NEP and APN values in diabetic GDX males resembled the ones observed in females, AGT and renin expression where considerably higher, indicating that the impaired hormonal status due to GDX promotes a more accentuated dysregulation of cortical RAS than female sex.

Diabetic males presented decreased ACE expression in both, the total and the outer cortex, as compared to females. In turn, the diabetes-induced increase in ACE2 expression was accentuated by male sex in the total cortex but not in the outer part, where this augmentation was observed exclusively in diabetic females. In consequence, although diabetic males showed a more elevated **ACE2/ACE ratio** than females, this trend was significant only in the total cortex but not in the outer area. The balance between ACE and ACE2 was also affected by sex and diabetes in the serum. Diabetic males showed a more accentuated increase in serum ACE activity than females. Conversely, diabetes induced a more pronounced increase in circulating ACE2 activity in females (2.4-fold) than in males (1.87-fold), in a similar fashion than previously reported[539]. Accordingly, diabetic females presented the highest serum ACE2/ACE ratio among all diabetic groups, reflecting a more pronounced hyperactivation of RAS in the circulation, which may be related to the elevated SBP and DPB observed in this group.

GDX clearly attenuated the increment in circulating ACE and ACE2 related to diabetes, which was reflected by showing a lower ACE2/ACE ratio than the other groups. In contrast to the pattern observed in controls, GDX in type 1 diabetic males did not modify cortical ACE expression (which was already very low), and augmented ACE2 activity only in the outer cortex, which was accompanied by a significant downregulation of active ACE2 in urine. These results suggest that, in diabetic GDX mice, a substantial number of ACE2 molecules are retained in the renal cortex by preventing its shedding and release to urine and circulation. It is known that a desintegrin and metalloproteinase 17 (ADAM17) can cause **shedding of ACE2** from the cell membrane[557]. Evidence for ACE2 shedding has been demonstrated in mouse proximal tubular cells[500,558], where this process is stimulated under conditions of high-glucose or ANGII[500], and mediated by PKC-δ subunit[558]. It is conceivable that, in our control and diabetic GDX mice, milder hyperglycemia attenuated the high-glucose- and ANGII- induced effect on ACE2 shedding.

As we have discussed, there are many factors that can alter the status of RAS in a particular tissue and in a certain time, such as age, diabetes onset, STZ dose, and sex hormone levels. In front of this complexity, we schematically analyzed the global effect

of T1DM on the regulation of renal, circulating and urinary RAS in each of our diabetic groups. As shown in Figure 98, four different **mechanisms** were activated to **counterbalance** the increase in AGT induced by diabetes: (1) downregulation of **renin**, (2) decrease in **ACE**, (3) increase in **ACE2**, and (4) increase in **NEP**. APN expression was not changed by diabetes in any group, indicating a predominant effect of the other ANGII escape pathways over the activity of the aminopeptidases. Interestingly, these mechanisms seem to be activated in a sex- and androgen-dependent manner.

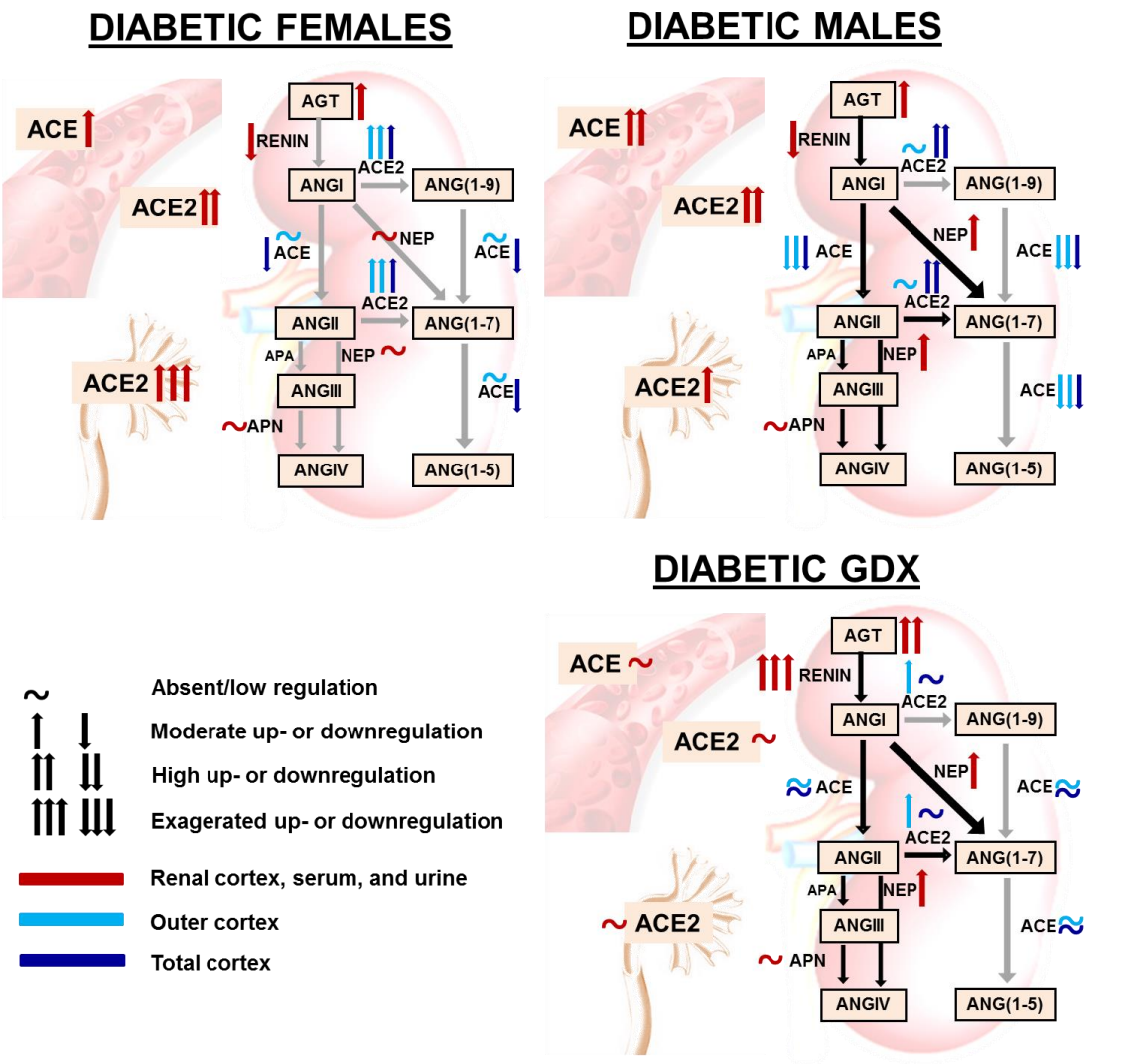

Figure 97. Sex-specific activation of compensatory mechanisms of RAS in experimental T1DM. The schemes represent the changes of the measured RAS components in the renal cortex, serum and urine of each diabetic group, in relation to the pertinent control group.

- In **diabetic females**, there is a predominance of the **ACE2-dependent renoprotection**, especially in the outer cortex, as well as a moderate downregulation of ACE in the total cortex. In this group, lower ACE downregulation and NEP up-regulation may first lead to increased accumulation ANGII, with the subsequent necessity for ACE2-mediated degradation of the peptide. The clear increase in ACE2 activity in the serum and urine strengthen the role of ACE2 as the predominant counteracting mechanism of RAS activated in females in the context of T1DM.
- In **diabetic males**, decreased ACE expression may be the principal compensatory mechanism in the outer cortex. Together with up-regulation of ACE2, this decrease is also present in the entire cortex. The synergic effect between **lower ACE and increased ACE2** actions may have provided a more efficient prevention of ANGII accumulation in the diabetic renal cortex. In the serum, ACE and ACE2 activities are highly augmented, probably reflecting the modulation of these two enzymes on the renal cortex.
- In **diabetic GDX**, the rate-limiting step of RAS is tremendously activated in terms of AGT and renin expression. As compared to their non-diabetic controls, GDX males do not present alterations in cortical ACE, and only a moderate overexpression of ACE2 in the outer cortex. These observations may explain the more stable ACE and ACE2 levels in serum and urine. Together with NEP up-regulation, these changes at renal level may indicate a hyperactivation of RAS towards both, the ANGII and the ANG(1-7) pathways.

6.A.V Sex-specific perturbation of sex hormone signaling in T1DM

In the present study, a clear sex effect was observed in the cortical expression of sex hormone receptors. Among the non-diabetic groups, **ERα** gene expression was significantly higher in males than in females, and modestly reduced by GDX. Androgen reduction in control males also caused a clear augment in the mRNA levels of **AR**.

The **ERα/AR ratio** in the kidneys from our control mice reflects a shift towards ERα signaling in males, and predominant AR in the female and the GDX groups. Androgens are substantially higher in male animals as compared to females[559], and Leydig cells in the testes are the primary source of testosterone, the main androgen[560]. We therefore assume that the lack of testicles in our female and GDX mice was associated to lower androgen levels in comparison to males. Based on this, it is conceivable that AR gene

expression was augmented in GDX males as a compensatory mechanism in front of reduced amounts of ligand.

Overall, **diabetes** exerted a more relevant effect in the expression of sex hormone receptors expression in the female and GDX groups, as compare to males. Specifically, diabetes significantly increased cortical estrogen receptors ERα and GPER30 in female and GDX, but not in male mice, whereas decreased AR in all groups. Increased renal expression of ERα has been previously reported in STZ females[416]. Among our diabetic mice, **GDX males** were the only group showing a **clear up-regulation of all the receptors** analyzed, reinforcing the idea that perturbed androgen levels due to GDX altered the status of not only male, but also female signaling pathways. As mentioned above, **detrimental effects of ERα and GPER** activation under conditions of diabetes and deregulated RAS have been demonstrated[417]. Specifically, aldosterone promotes GPER activation[561], and ANGII stimulates the synthesis of aldosterone in the adrenal gland[562,563]. These observations support the idea of a more hyperactivated cortical RAS and higher ANGII production in diabetic females and GDX males (Figure 102), which would help to explain the augmented **renal fibrosis** found in these groups.

Experimental and clinical studies have shown that **T1DM** is associated with reduced plasma levels of **17β-estradiol** and **testosterone**[416,425], and that restoring physiological levels of androgens and estrogens attenuates the development of renal disease. We have observed that GDX notably regulated the majority of the RAS components analyzed. In front of this clear effect, it is conceivable that reestablishing normal sex hormone levels would help to slow down DN progression through the normalization of RAS expression. Despite we have not assessed sex hormone levels in the serum of our study groups, it is presumable that they were downregulated after 19 weeks of T1DM. Considering the increased gene expression of ERα, AR and GPER in the renal cortex of diabetic GDX males, and the fact that reduced androgen levels probably contributed to diminished aromatase-mediated estrogen production, we speculate that the imbalance in sex hormone levels was more accentuated in diabetic GDX males. In this group, overactivation of DHT-independent mechanisms of AR could have contributed to the exaggerated dysregulation of RAS and the more exacerbated fibrosis at gene level.

6.A.VI The role of ACE2 in diabetic nephropathy is sex-specific

The effect of *Ace2* deletion on females has not been widely studied. Oudit *et al*. reported that non-diabetic ACE2KO female but not male mice were protected from the development of glomerulosclerosis at 1 year of age[319].

In our study we observed that loss of ACE2 promoted **glomerular hypertrophy** and accentuated **KW/BW** and **UAE** in type 1 diabetic females. Exaggerated albuminuria and renal and glomerular hypertrophy have been previously described in ACE2KO males with T1DM[322,527]. We now demonstrate that these alterations are also present in diabetic ACE2KO females. In addition, we observed increased **capillary dilatation** in the glomeruli of ACE2KO diabetic females, which probably contributed to the increment in the area of the glomerular tuft. Glomerular capillary aneurysms are associated with human diabetic glomerulopathies[564]. Although this alteration has not been described in the kidneys from diabetic female mice, it is known that ACE2 decreases the formation and severity of ANGII aortic aneurysms[565]. Thus, it is likely that this glomerular alteration was promoted by deficient ANGII degradation and intrarenal accumulation of the peptide in the kidneys of diabetic ACE2KO females.

Loss of ACE2 and diabetes were associated to increased **SBP**, **DBP**, and **GFR**. In consequence, ACE2KO diabetic females showed the highest values of these parameters across all groups. It is widely known that *Ace2* deletion increases blood pressure, and that this increase is probably attributed to the accumulation of ANGII[316], which causes hypertension primarily through effects on AT1 receptors in the kidney[317]. The hypertensive effects of *Ace2* deletion have also been observed in STZ-diabetic mice[311]. In turn, podocyte-specific overexpression of ACE2 attenuated hyperfiltration in STZ-induced mice[325]. We are the first to report these alterations in diabetic females, as those previous studies were performed exclusively in males.

ACE2 deficiency caused an increase in **cardiac weight**, which was significant in the diabetic group. This may explain that ACE2KO females presented the lowest values of **heart rate**. Crackower and colleagues were the first to report cardiac defects in ACE2KO males. They described a contractility defect, slight wall thinning of the left ventricle and increased chamber dimensions, but not cardiac hypertrophy or altered heart rate, in these mice[137]. Later studies using the mice generated by Yamamoto *et al*. reported cardiac hypertrophy in ACE2KO males[315,566]. In contrast, transthoracic echocardiography in ACE2KO Akita males revealed no significant changes in heart rate, ventricular dimensions, or myocardial contractility[527]. Our results suggest that, in the context of T1DM, ACE2 exerts a protective role against ANGII-induced cardiac hypertrophy in females.

Loss of ACE2 in females from our model of T1DM also exacerbated **renal fibrosis**. Similar findings have been previously reported in type 1 diabetic male mice[322,527]. In this sense, loss of ACE2 accelerated intrarenal ANGII-mediated fibrosis in obstructive nephropathy[567], whereas administration of recombinant human ACE2 prevented this ANGII-induced effect[568]. It is well documented that ANGII can activate several intracellular signaling pathways to mediate renal fibrosis and inflammation, including **TGF-β**/Smads[455,569]. However, it has been described that ANGII can also induce extracellular matrix production through TGF-β-independent mechanisms in tubular epithelial cells [570] and vascular smooth muscle cells[571]. Our results suggest that, among the different ANGII-related pro-fibrotic pathways, enhanced renal fibrosis in ACE2KO diabetic females was mostly ascribed to mechanisms related to ANGII/TGF-β signaling.

Of mention that diabetes induced a more severe **hyperglycemia** and **body weight** loss in ACE2KO females as compared to the WT during the follow-up. Supporting our results, other authors have observed a reduction in BW in ACE2KO male mice[311]. In addition, loss of ACE2 has been associated to impaired glucose homeostasis after intraperitoneal glucose injection. In that study, no significant differences were observed in fasting blood glucose levels in male ACE2KO mice as compared to those in male WT[572]. Similarly, at the end of our study we found that the more elevated blood glucose levels in diabetic ACE2KO females was observed under non-fasting conditions, but not after 3h of fasting, suggesting that lack of ACE2 expression predisposes to a worse glucose metabolism after ingestion. In this sense, several authors support a protective role of ACE2 within the pancreas. Chhabra *et al*., injected an adenovirus encoding human ACE2 to ANGII-infused mice, and observed that ACE2 overexpression counteracted the deleterious effects of ANGII on pancreatic β-cell function and oxidative stress[573]. Furthermore, ACE2 participates in the adaptive hyperinsulinemic response of β-cell to high-fat feeding, through regulation of β-cell proliferation and growth[574].

6.A.VII Loss of ACE2 alters circulating and renal RAS in type 1 diabetic females

The more relevant findings in terms of the expression of cortical RAS are that loss of ACE2 accentuated the diabetes-induced increase in **cortical AGT** and **circulating ACE** activity. As reported by Yamaleyeva *et al*., hypertension enhanced the effect of diabetes on increasing renal AGT expression in STZ females. Those animals also showed increased expression of IL-6 and other inflammatory cytokines, suggesting

increased ANGII levels within the kidney[539]. In fact, hypertension is generally associated with elevated intrarenal ANGII levels[575]. In addition, it has been shown that IL-6 contributes to ANG II-mediated angiotensinogen stimulation in renal proximal tubular cells[576]. Our ACE2KO diabetic females showed higher SBP and DBP than the others groups, indicating that up-regulation of cortical AGT expression in this group may be related to a synergistic effect of high glucose and endogenous ANGII. As observed in other studies, overexpression of renal AGT contributes to hyperactivated RAS in the kidney and circulation[539,540], which may explain the augmented ACE activity in the serum of diabetic ACE2KO females.

In agreement with Oudit and coauthors[319], no significant glomerular or tubular lesions were found in non-diabetic ACE2KO females. In present study we found that **renin gene expression** was lower in ACE2KO females than in WT. Several authors have provided evidence for a **negative feedback loop** regulation of RAS, consisting on ANGII-mediated inhibition of renin expression in the juxtaglomerular cells[577,578]. Therefore, it is conceivable that downregulation of the first limiting step of RAS confers renoprotection in the absence of ACE2.

6.A.VIII Gonadectomy prevents the increase in blood pressure and glomerular injury in *Ace2* knockout diabetic male mice. Effects on renin-angiotensin system

Gonadectomized ACE2KO diabetic mice showed lower blood pressure values and decreased nephropathy than male ACE2KO diabetic mice. These animals exhibited modulation of circulating and renal RAS favoring the 'pro-ANG(1–7)' axis; histological evidence of renal protection, namely, a reduction in mesangial expansion and attenuation of glomerular hypertrophy; attenuation of podocyte loss; and reduction in interstitial fibrosis and collagen deposition. To our knowledge, the present work is the first to simultaneously study the influence of ACE2 deficiency and GDX on hypertension and kidney damage in diabetic male mice. Our results contribute to the knowledge of sex differences in RAS in the pathological context of diabetes.

Studies in ACE2-deficient mice have demonstrated a role for ACE2 in the **regulation of BP**, identifying ACE2 as a functioning component of RAS *in vivo*[316]. Furthermore, induction of diabetes by STZ in ACE2-deficient mice either by pharmacologic inhibition or genetic ablation increased BP values in these animals[311,579]. In concordance, our study also demonstrated increased SBP and DBP in ACE2KO diabetic mice. Of note, this increase was not observed in gonadectomized

diabetic ACE2KO mice. Increased BP in hypertensive and diabetic ACE2KO mice has been ascribed to ANGII accumulation within the kidney and circulation[316,539,579].

We also found that circulating ACE activity was increased in ACE2KO and diabetic mice. It has been previously reported that STZ-induced diabetes is associated with an increase in circulating ACE activity[539]. To our knowledge, the effect of genetic *Ace2* deletion on serum ACE activity has not been previously studied. In this work, we demonstrated that ACE2 deficiency increased circulating ACE activity. Our study also shows that there is a dichotomy between circulating ACE and renal ACE expression. Specifically, in ACE2KO and diabetic mice circulating ACE was increased, whereas **cortical ACE was decreased** at gene and protein expression and at enzymatic activity levels. Similar results in terms of reduced kidney ACE expression were reported in STZ-treated rats, in the db/db model of type 2 diabetes, and in models of ACE2 downregulation[311,321,539].

Interestingly, we now find that **GDX decreases renal ACE** gene expression in control ACE2KO mice as well as circulating ACE activity in control and diabetic ACE2KO mice, suggesting a role of the absence of androgens on the transcriptional regulation of ACE that would lead to lower levels of renal and circulating ANGII. In agreement with our findings, Lim *et al*. demonstrated that plasma ACE activity in both male and female mice is reduced by GDX. This decrease was more severe in males than in females[195]. Considering that males have higher androgen levels than females, it is conceivable that androgens exert a stronger influence than estrogens on plasma ACE activity. Of mention that ACE also degrades ANG(1–7) to ANG(1–5) in the renal tubules and circulation[580]. Thus, it is conceivable that increased circulating ACE and decreased cortical ACE because of *Ace2* deletion and diabetes altered not only ANGII but also ANG(1–7)-related pathways in our mice. Therefore, increased SBP in diabetic ACE2KO mice may be ascribed not only to an ANGII accumulation in serum but also to an excessive ACE-dependent metabolism of ANG(1–7) and, in consequence, a downregulation of the vasodilatory effects promoted by this peptide.

In our work, GDX dramatically augmented renal *Agt* and *Ren* gene expression in diabetic ACE2KO mice. Furthermore, GDX was also accompanied by elevated *Nep* mRNA levels in control and diabetic ACE2KO mice. In concordance, Yamaleyeva *et al*. observed lower levels of renal NEP in male Lewis rats compared with females[539]. Together with cortical ACE and APN changes, our data suggest alterations of cortical RAS in ACE2KO-DB+GDX mice in which both, the AOGEN/REN/ANGI/NEP/ANG(1–7) and the AOGEN/REN/ACE/ANGII axes, are modulated.

Oudit *et al*. demonstrated that loss of ACE2 in male mice leads to age-dependent development of glomerular mesangial expansion and glomerulosclerosis[319]. In our

study, we showed an increase in the kidney weight to body weight ratio, GFR, and mesangial expansion in ACE2KO-CONT mice at 7 months of age. However, glomerulosclerosis and alterations in UAE were not observed, suggesting an **early stage of renal disease**. These differences may be related to the younger age of our animals. As expected, *Ace2* deletion exacerbated glomerular injury, namely, mesangial matrix expansion and podocyte loss in diabetic mice. In agreement, Soler *et al*. described a worsening of albuminuria and glomerular histological lesions after 4 weeks of ACE2 pharmacological inhibition in STZ-induced diabetic mice[321]. In Akita mice, *Ace2* deletion also exacerbated albuminuria in association with increased mesangial matrix deposition, glomerular basement membrane thickening, and glomerulosclerosis[527]. Our results of **hypercellularity**, **decreased podocyte number, and increased mesangial matrix** within the glomeruli suggest that the increase in glomerular cellularity in our diabetic ACE2KO model is mainly because of an expansion of the mesangial cell lineage. These features of diabetic glomerular disease were absent in gonadectomized ACE2KO mice. Different mechanisms such as modulation of renal RAS and decreased BP may play a role in this renoprotective effect at the glomerular level.

In regard to **renal fibrosis**, diabetic ACE2KO mice showed an increase in the myofibroblast pro-fibrotic marker **α-SMA** and accentuated **collagen deposition** compared with diabetic wild-type mice. Our findings are consistent with previous studies, where ACE2 deficiency either by pharmacological inhibition or gene deletion also increased fibronectin and/or α-SMA expression in kidneys from diabetic mice[209,322,527]. Loss of ACE2 markedly increased intrarenal ANGII in association with enhanced TGF-β/Smad-mediated renal fibrosis in a mouse model of obstructive nephropathy[567]. ANGII can activate several intracellular signaling pathways to mediate renal fibrosis and inflammation, including TGF-β/Smads[455,569] and PI3K/Akt[581,582]. GDX prevented the outcome of hypertension, glomerular alterations, and the accumulation of pro-fibrotic and fibrotic markers such as α-SMA and collagen in diabetic ACE2KO mice, suggesting a decrease in circulating and renal ANGII levels and a subsequent downregulation of these ANGII downstream pathways.

Akt is important in many cellular processes, including proliferation, migration, cell growth, and metabolism, and plays a critical role in the cardiovascular and renal system[583]. Akt activity can be transcriptionally modulated by its upstream regulatory pathways and also at posttranslational level by phosphorylation of the Thr308 and Ser473 residues[584]. Higher levels of Akt and pAkt have been described in animal models of STZ-induced type 1 diabetes[585–587], as well as in high glucose-treated renal cell lines[588,589]. In addition, it has been reported that pAkt is enhanced by ANGII and

ANG(1–7) *in vivo* and *in vitro*[590–592], as well as by the actions of testosterone[593] and androgen receptor[594,595]. In the present study, diabetes and loss of ACE2 were accompanied by increased **Akt expression and phosphorylation**, whereas GDX clearly reduced pAkt levels in control and diabetic ACE2KOmice. As expected, Akt expression and phosphorylation was not modified by GDX in diabetic wild-type mice. The decreased MasR gene expression and the absence of ACE2 in diabetic ACE2KO+GDX mice may explain lower activation of ANG(1–7)-dependent Akt stimulation, as compared with the wild-type mice. In diabetic wild-type and GDX mice, the presence of ACE2 and increased mRNA levels of MasR, may suggest a higher activation of the ANG(1–7)/MasR axis. Thus, the effects of GDX on reducing pAkt may be counterbalanced by an increase on ANG (1–7)-mediated pAkt in WT-DB+GDX mice.

We simultaneously evaluated the effect of *Ace2* deletion, diabetes, and GDX by **PCA of all experimental groups**. We found that the effects of *Ace2* deletion on diabetic nephropathy are influenced by the levels of male sex hormones. When evaluating the renal markers in a global fashion, we observed an opposite effect of ACE2 deficiency depending on the hormonal status of our mice (castrated vs. sham operated), suggesting that sex hormone reduction by GDX may be protective in diabetic ACE2KO but not diabetic wild-type mice. Additional PCA indicated that this global effect was mainly ascribed to the changes observed on tubulointerstitial fibrosis. In concordance with our results, Xu *et al*. also found that GDX increased fibrosis in STZ diabetic rats[387]. In addition, PCA for renal RAS gene expression suggested that modulation of renal RAS played a relevant role on these changes.

To sum up, **GDX attenuated diabetic nephropathy** by preventing hypertension, glomerular injury, and renal fibrosis in type 1 diabetic ACE2KO mice. Given our results, one hypothesizes that, under deficient ANGII degradation, GDX may confer a protective effect at kidney level by different mechanisms such as a decrease in BP, a decrease in pAkt, and RAS modulation (Figure 98). These positive effects were absent in wild-type mice, which showed enhanced MasR expression. As previously demonstrated, excessive activation of the NEP/ACE2/ANG(1–7)/MasR axis may stimulate fibrotic and inflammatory processes that contribute to kidney injury[596]. In this sense, future experimental studies in diabetic models evaluating the effects of RAS blockade or/and recombinant ACE2 administration, either in the setting of reduced male sex hormones or in combination with androgen replacement therapy, will shed new light on the molecular mechanisms involved on androgen-mediated effects on RAS expression and, in consequence, the progression and severity of diabetic nephropathy.

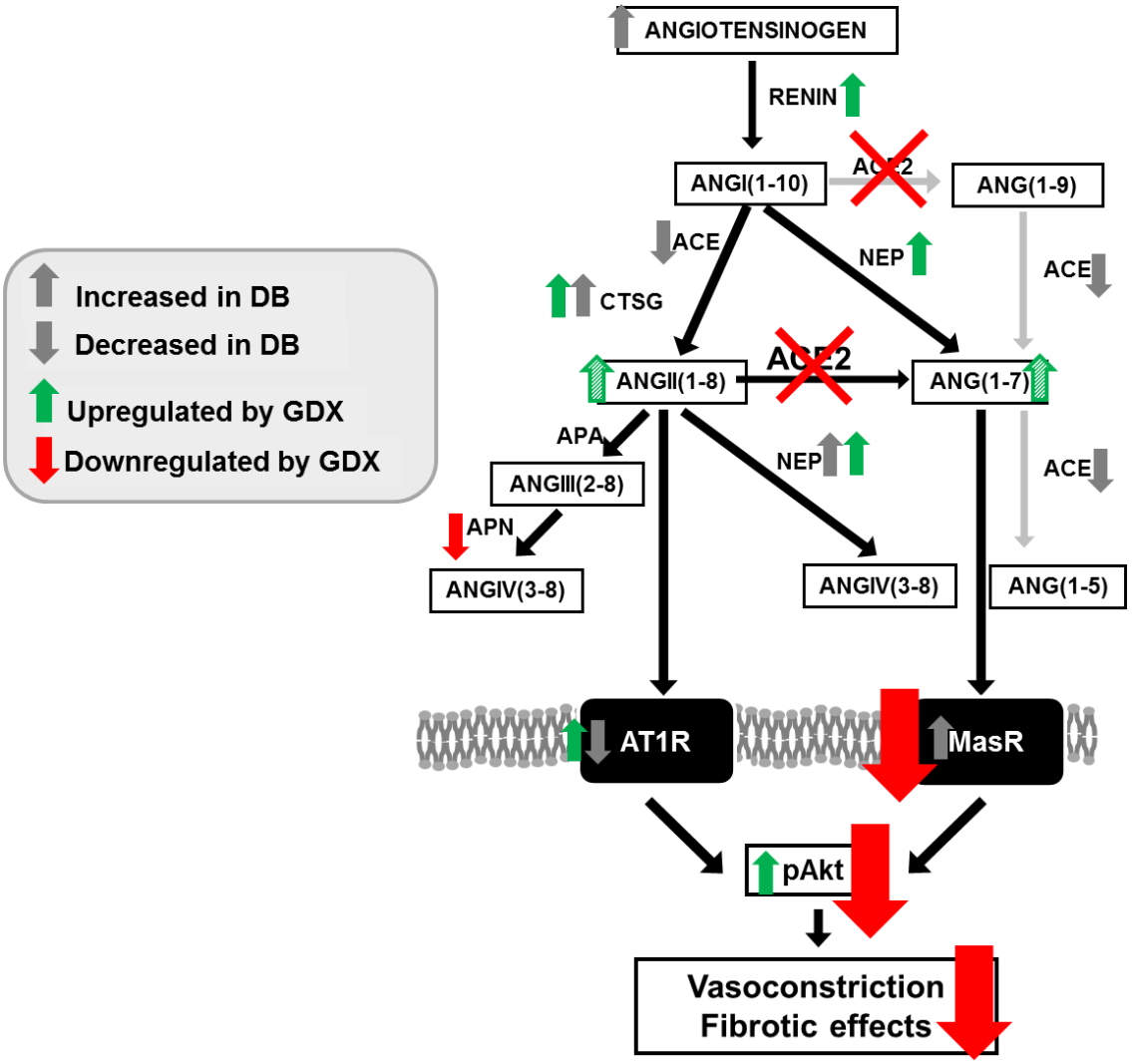

Figure 98. Protective effects of gonadectomy by modulating renal RAS and Akt phosphorylation in ACE2KO diabetic mice. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; ACE2KO, angiotensin-converting enzyme 2 knockout; ANG, angiotensin; APA, aminopeptidase A; APN, aminopeptidase N; AT1R, Ang-II type 1 receptor; CSTG, cathepsin G; GDX, gonadectomy; MasR, Mas receptor; NEP, neprylisin; STZ, streptozotocin.

6.A.IX Sexual dimorphism on the effect of *Ace2* deletion diabetic and ANGII-infused mice

ANGII infusion promoted cardiac hypertrophy, hypertension, albuminuria, and GFR decrease, as previously published[317,597–599]. In the groups expressing ACE2, ANGII induced a greater increase in blood pressure, UAE, and HW/BW in males than in females. Interestingly, we observed an **opposite sex effect in the absence of ACE2**. The main finding of this study is that loss of ACE2 induced renal hypertrophy, and accentuated ANGII-mediated cardiac hypertrophy, hypertension, albuminuria, and glomerular hypertrophy in diabetic females. In addition, the effect of ANGII on **glomerular histology and morphometry** was also sex-specific. Again, this effect

presented a dichotomy between the wild-type and the ACE2KO groups. Among the wild-type groups, WT-DB+ANGII males presented significantly higher glomerular and mesangial area; in turn, ANGII exacerbated podocyte loss exclusively in WT-DB females. In agreement with our results, it has been demonstrated that estrogens attenuate proliferation and collagen synthesis of cultured mesangial cells, and this effect is thought to be protective in the context of hypertension[600,601].

In **ACE2KO females, ANGII**, diabetes and loss of ACE2 exerted a synergic effect on increasing glomerular hypertrophy, without significant changes in the mesangium. **In ACE2KO males, ANGII** promoted glomerular hypertrophy, mesangial matrix expansion, and podocyte loss, only in the diabetic ACE2KO group. *In vitro* studies have demonstrated that ANGII induces hypertrophy of mesangial cells. *In vivo*, expansion of the mesangium after ANGII administration has been observed in animal models of hypertension[602] and diabetes[603]. To our knowledge, we are the first to report a **sex effect on glomerular and mesangial area** in diabetic and ANGII-infused mice.

Sex differences were also observed when evaluating the **tubular compartment**. ANGII administration was associated to increased **tubular dilatation** and **acute necrosis**. The presence of these lesions was enhanced by diabetes and loss of ACE2 only in females. Interestingly, ANGII alone was able to increase nuclear glycogen accumulation in control ACE2KO females and males; among the ACE2KO diabetic groups, this alteration was more abundant in males. Regarding renal fibrosis, ANGII infusion stimulated cortical TGF-β1 levels in both, female and male diabetic mice, but did not modify the diabetes-induced increase in *col1α2* expression. The progression of fibrosis in experimental hypertension has been explored by other groups. It is known that ANGII upregulates gene expression and production of active protein TGF-β1 in the kidney[457] and in renal cells[604]. In addition, when ANGII was infused to male Sprague-Dawley rats receiving high-salt diet, they showed exacerbated renal interstitial and glomerular fibrosis[602]. In this sense, the pattern of TGF-β1 expression observed in our wild-type groups was expectable. Surprisingly, a different pattern was observed in the absence of ACE2, where ANGII reduced the expression of TGF-β1 and *col1α2* in the diabetic ACE2KO groups, especially in males. We surmise that ACE2 deficiency promotes a series of events in the kidney (including a clear dysregulation of RAS expression), which differ from the effects caused by high salt diet, and may partially attenuate ANGII-mediated fibrosis.

It is known that the activation of the immune system activation contributes to the pathogenesis of hypertension, resulting in progression of CKD. This process involves the participation of many cytokines and chemokines. Among them, **monocyte chemoattractant protein 1 (MCP1)** is a small cytokine that belongs to the CC

chemokine family, and recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation in the injured tissues[605,606]. A **proinflammatory effect of ANGII** was observed in our study, as cortical expression of MCP1 was increased in the majority of ANGII-infused groups. Interestingly, WT and ACE2KO diabetic females presented higher MCP1 expression than their corresponding male groups. When receiving ANGII, diabetic ACE2KO females showed the more elevated mRNA levels of *mcp1* among all the experimental groups. These data suggest that the higher expression of pro-fibrotic genes in diabetic ACE2KO females was accompanied by more severe inflammation processes, as compared to males. The positive association between female sex and renal inflammation in hypertensive DN has been observed previously, as STZ-diabetic mRen2.Lewis hypertensive female rats exhibited a more marked increase in serum C-reactive protein and tubulointerstitial $CD68^+$ cells, compared with males[539].

Overall, loss of ACE2 in diabetic females increased their susceptibility to several ANGII-induced hemodynamic and tubular alterations (Figure 99). However, ANGII effects on glomerular histological alterations, namely glomerular hypertrophy, mesangial matrix expansion, and podocyte loss, were more evident in diabetic ACE2KO males. We next analyzed if a sexual dimorphism on RAS expression could help to explain this dual effect in the diabetic and hypertensive kidney.

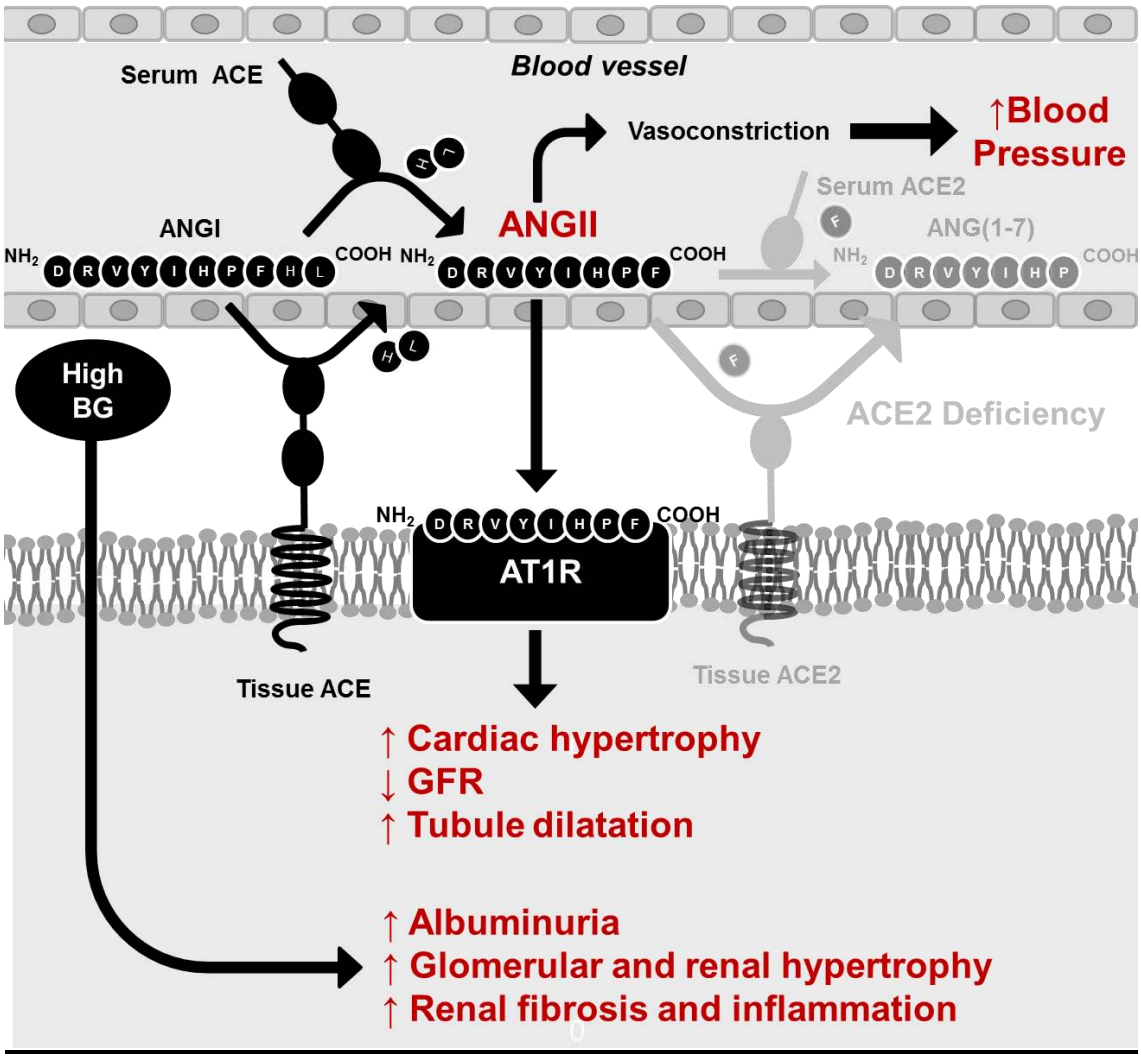

Figure 99. Effects of diabetes, ACE2 deficiency, ANGII infusion in female mice. In females, loss of ACE2 and ANGII infusion promoted cardiac hypertrophy, GFR decrease, and tubular dilatation, as well as accentuated the diabetes-induced albuminuria, glomerular and renal hypertrophy. In addition, ACE2KO-DB + ANGII infused females showed increased cortical mRNA levels of markers of fibrosis and inflammation, as compared to males.

Among the wild-type groups, ANGII infusion attenuated the diabetes-induced increase in **ACE2 activity** in the female outer cortex, which was associated with a more pronounced increase in the circulation. Interestingly, the opposite effect was observed in diabetic males. As we mentioned above, in contrast to the ACE2KO groups, diabetes and ANGII infusion were accompanied by a more accentuated increase in SBP, DBP, UAE, and glomerular hypertrophy in wild-type males, compared with females. Again, the inverted pattern between wild-type and ACE2KO mice suggests that, when attempting to counteract the deleterious effects of high glucose and ANGII, female compensatory mechanisms strongly depend on ACE2 regulation.

Similarly to our first study, the **modulation of ACE** in the circulation and the outer cortex was **more relevant in males**. In this sense, ACE2KO-DB + ANGII males

presented significantly lower levels of cortical AGT and NEP, a modest decrease in AT1R, and a more pronounced ANGII-induced decrease in renin and ACE gene expression, as compared to ACE2KO-DB + ANGII females. These data suggest a direct correlation between male sex and the negative short loop **feedback regulation** of cortical RAS under pathological conditions of diabetes, loss of ACE2 and ANGII-induced hypertension, which may be protective for the kidney. In concordance with our findings, hypertensive mRen2.Lewis males showed a more pronounced decrease in renal renin concentration than females, when compared to the normotensive Lewis rats[211]. In addition, PCA analysis in our experimental groups revealed a clearly predominant effect of sex on ACE expression, with male mice showing lower renal ACE content than the females. In this sense, mice lacking renal ACE proofed to be more resistant to ANGII-hypertension[607]. Taken together, these observations indicate that, in diabetic and hypertensive ACE2KO males, downregulation of endogenous ANGII production is promoted in an attempt to counterbalance the harmful effects of exogenous ANGII. Despite showing attenuated renal alterations. Despite showing milder hypertension, cardiac hypertrophy, albuminuria, fibrosis and inflammation than females, our data suggest that this mechanism was not sufficient to prevent glomerular hypertrophy, mesangial expansion and podocyte loss in diabetic and ANGII-infused ACE2KO males.

In turn, higher levels of AGT, ACE and renin in the renal cortex of diabetic and hypertensive ACE2KO females indicate **greater hyperactivation of the AGT/renin/ACE axis of RAS**, and may explain the worsened hypertension and renal alterations in this group. Supporting these data, STZ-diabetic and hypertensive mRen2.Lewis female rats showed increased AGT content in the kidney than the corresponding male group[539]. Furthermore, elevated blood pressure values have been associated to increased AGT expression in other models of hypertension, such as the 2-kidney 1-clip[608] and the Dahl salt-sensitive rats[545]. It is believed that an increased blood pressure synergizes with higher intrarenal ANGII to stimulate AGT production and exert greater renal injury[608]. In turn, transgenic animals overexpressing AGT exhibit increased blood pressure and manifest hypertensive sequel[160], whereas administration of AGT antisense mRNA to hypertensive rats induces a profound reduction in blood pressure[162]. Therefore, it is reasonable that ACE2KO-DB + ANGII females entered in the **vicious circle of AGT→ANGII→SBP→AGT**, in which AGT stimulated hypertension, and vice versa. In the clinics, urinary AGT has been proposed as a possible biomarker of hypertension. In this sense, it has been reported that increased levels of AGT in urine are associated to intrarenal RAS activation in experimental T1DM[166], and precede higher blood pressure in normoalbuminuric

children with T1DM[165]. We now suggest that, in type 1 diabetic and hypertensive women, higher levels of urinary AGT may be indicative of ANGII-mediated alterations taking place in the kidney.

PCA analysis in the sixteen study groups revealed that, in females, loss of ACE2 and ANGII infusion, accentuate the predominant effects of diabetes in the renal injury markers analyzed and the cortical expression of RAS. In males, the WT-DB + ANGII (and not the ACE2KO-DB + ANGII) mice appeared to be the most separated group from the healthy controls when evaluating renal injury markers, but not RAS expression, indicating the activation of RAS compensatory mechanisms in the ACE2KO-DB + ANGII group. As shown in Figure 100, we propose two sex-specific mechanisms of RAS modulation in the kidney cortex of diabetic and ANGII-infused ACE2KO mice, which may help to explain, at least in part, the different susceptibility to ANGII-mediated renal injury observed across sexes.

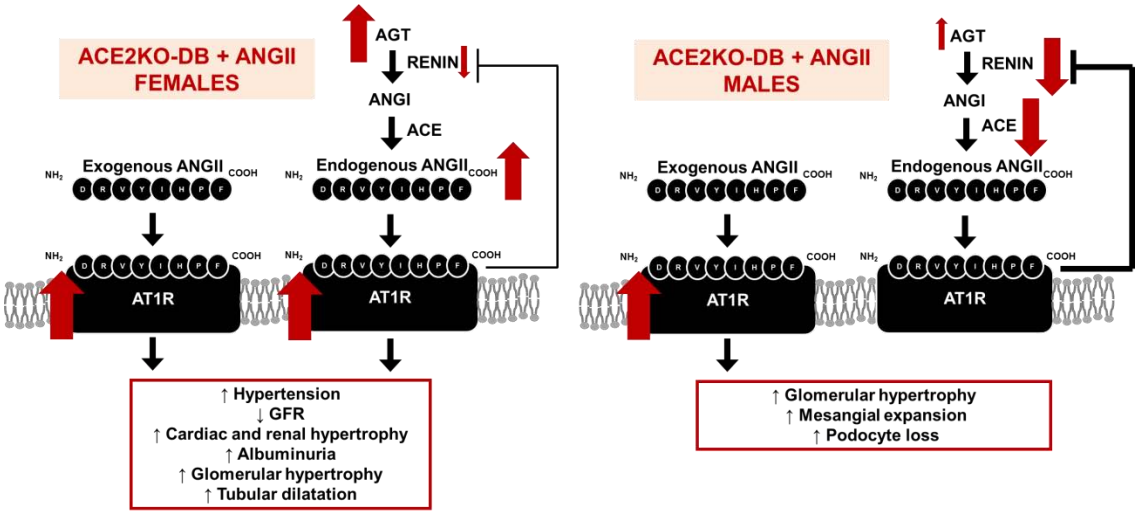

Figure 100. Sex-specific modulation of RAS in the renal cortex of diabetic and ANGII-infused ACE2KO mice. In ACE2KO-DB + ANGII females, overexpression of cortical AGT may contribute to higher activation of the endogenous RAS and, in consequence, increased production of intrarenal ANGII. Therefore, detrimental effects of exogenous ANGII may be enhanced by endogenous ANGII activity. In ACE2KO-DB + ANGII males, glomerular alterations may be mostly ascribed to the actions of exogenous ANGII, as they presented lower cortical AGT levels than females, as well as a more significant negative feedback loop regulation, suggesting decreased formation of endogenous ANGII.

With these findings, we demonstrate a link between the effect of sex on the crosstalk between the different components of RAS and the sexual dimorphism in glomerular injury and renal fibrosis in the context of T1DM, and we confirm that ANGII plays a crucial role on these sex differences. A better understanding of these connections may help to design more efficient therapies to slow down the progression of DN through pharmacologic modulation of RAS.

6.B DISCUSSION OF THE *IN VITRO* FINDINGS

The *in vitro* study was designed to shed new light on the biological processes and cellular compartments involved in the **deleterious effects of androgens in CKD**, by studying proteins regulated by male and female sex hormones in the kidney. Our specific goal was to capture a DTH and EST "protein signature" *in vitro* that reflected an early and consistent **response of the PTEC to sex hormones**.

We identified over 5000 proteins, most of which were also quantified, thus providing a unique depth of insight into PTEC responses to sex hormones. The strengths of our approach include good proteome coverage, use of instruments with high sensitivity and accuracy, consideration of the effect of cell passage on protein expression, and control experiments to minimize false-positive hits. To the best of our knowledge, this is the first effort to date to characterize proteomic responses of human kidney cells to DHT and EST stimulation.

We first noticed that among our **DHT-differentially regulated proteins**, many were enzymes that have been shown to play a role in CKD and DN. As an example, in cultured fibroblasts from type 1 diabetic patients with and without nephropathy, mRNA levels of GPI and other enzymes of the glycolytic pathway were found to be increased in the context of DN[609]. In turn, HADHA and HADHB, as subunits of the mitochondrial trifunctional protein, are involved not only in FAO but also in molecular events relevant to kidney disease, such as lysine acetylation[610], and modulation of renin expression[611], respectively. In addition, GNPNAT1 (which catalyzes a reaction critical for HBP activation) participates in proliferative and hypertrophic processes associated with diabetes[612].

Our next aim was to use **systems biology** to demonstrate **biological processes** and **molecular pathways** fundamental in PTEC response to DHT and EST. Three of the dominant biological processes identified among our 72 DHT/CONT differentially regulated proteins were glucose catabolic process, N-acetylglucosamine metabolic process, and FAO, and one of the most enriched cellular compartments was the mitochondrion. In concordance with our results from SILAC proteomics and MS, validation experiments by Western Blot confirmed that GPI and HADHA protein levels were augmented in DHT-treated renal cells and in kidneys from C57BL/6 males, supporting the idea that androgens impair **carbohydrate and fatty acid metabolism** in the kidney through the **action of DHT**.

It is known that alterations in the processing of carbohydrates and fatty acids may ultimately compromise the **mitochondrial function** and the **oxidative stress** status within the cell[3]. Thus, the increase in glycolytic enzymes and proteins responsible for

FAO and glutamine metabolism after DHT treatment may explain the up-regulation of several TCA cycle enzymes such as malate dehydrogenase, as well as the increased expression of many proteins related to the mitochondrial compartment. Furthermore, in this work, we demonstrate that perturbed energy metabolism and up-regulation of mitochondrial proteins by male sex hormones are associated with increased oxidative stress levels in male kidneys under non-pathological conditions. Therefore, our findings suggest that these processes may play a critical role in mediating the deleterious effects of androgens in the physiology of the renal cells. In this sense, DHT has been shown to alter glycolytic metabolism and impair mitochondrial function in other cell types[613,614]. As recently reported by Wang *et al*., pancreatic islets from DHT-treated rats showed significant changes in the levels of several key genes involved in mitochondrial biogenesis, mitochondrial oxygen consumption rate, and ATP production. The authors also demonstrated that androgens can directly impair beta cell function by inducing mitochondrial dysfunction *in vitro* in an AR-dependent manner[613]. In a different context, DHT enhanced glucose consumption and lactate export in prostate cancer cells[614].

We speculate that increased metabolism of sugar, lipid and amino acids in DHT-treated PTEC induced an **imbalance** on the levels of several **metabolites** such as pyruvate, α-ketoglutarate and acetyl-CoA. Thus, the increase in the protein expression of pyruvate dehydrogenase and other enzymes involved in N-acetylglucosamine metabolism and TCA cycle may be explained, at least in part, as an attempt of the tubular cell to counterbalance the abnormal accumulation of these metabolites. For example, GNPNAT1, one of the key enzymes of the HBP that was found to be significantly increased by DHT in both SILAC and Western Blot experiments, uses acetyl-CoA as the acetyl group donor for the conversion of glucosamine-6-phosphate to N-acetylglucosamine-6-phosphate.

The enrichment in energy metabolism processes after DHT stimulation, together with the association between male sex and renal oxidative stress levels, pointed out a possible activation of the classic and well defined **mitochondrial-dependent apoptotic** pathways[615,616]. In DHT-treated cells, apoptosis was represented by increases in TOP2A and CAD, which were specifically related to apoptotic chromosome condensation. Chromatin condensation is one of the first morphological changes in cells undergoing apoptosis[615,617]. It has been reported that long exposure to testosterone (24h to 48h) promoted apoptosis in HK-2 cells and PTEC[380,382]. We decided to treat PTEC for only 8h, a time that we considered long enough to observe both genomic and non-genomic actions of sex hormones, but still prevented us from the interference of androgen-induced apoptosis and cell death in our cell culture. Thus, the lack of a more significant number of apoptotic players among our DHT proteomic

signature despite the increase in proteins related to energy metabolism may be indicative of an early, adaptive response of the cell to DHT signaling, previous to the gradual development of pathological consequences due to a continuous impaired metabolism and subsequent accumulation of ROS. In this sense, protein expression of the antioxidant enzyme SOD2 was also found to be up-regulated by DHT, together with CASP6 and PARK7 as part of response to hydrogen peroxide. SOD2 is responsible for the conversion of superoxide anion to hydrogen peroxide[618]. Decreases in SOD2 expression and activity have been associated with exacerbated levels of oxidative stress and mitochondrial dependent apoptosis in HK-2 cells and murine kidneys after long exposures to high glucose, ANGII[619] or nephrotoxic reagents such as cisplatin[620]. From this point of view, evidence for not decreased but increased SOD2 after 8h of DHT treatment in PTEC suggests that oxidative stress and apoptosis are partially blunted by a physiological early response of the tubular cell. Furthermore, several proteins related to mitochondrial p53-mediated apoptosis, namely BAX, BCLAF1, BCL2L13, TP53BP1, and TP53BP2, were detected and quantified in all four experiments but were not affected by DHT treatment (data available online in the Proteome Xchange data repository).

GSL metabolism emerged as the second significant functional group altered by DHT in PTEC. Supporting our **functional category enrichment** analysis, we found that HEXB, a key protein in GSL metabolism that was up-regulated by DHT, was more expressed in male kidneys than in females. In addition, the vacuolar compartment was connected to GSL metabolism and also enriched among our DHT-regulated proteins. In concordance, control and diabetic male mice showed a clear increase in tubular vacuolization as compared to females. Indeed, the presence of vacuoles was minimal in the tubules of female mice. Given the link between impaired GSL metabolism and proximal tubular vacuolization in the context of diabetes[621], we surmise that higher vacuolization and impaired GSL metabolism in males predispose to a more marked dysregulation of lipid metabolism in the context of diabetes, probably resulting on increased lipiduria, compared to females.

Glycosphyngolipids are molecules derived from the addition of sugar-moieties to the sphingolipid ceramide, and are highly abundant in the kidney[622]. Alternatively, they may be considered as a subtype of glycolipids containing the amino alcohol sphingosine[623]. GSL play crucial roles in cellular membranes. Together with protein receptors and sterols, GSL constitute the microdomains termed **lipid rafts**[624]. From the plasma membrane, GSL may be recirculated through vesicular transport. Thereby, several enzymatic reactions of GSL metabolism take place in the lysosomal compartment[625]. This may confer a reasonable explanation for the enrichment of this

organelle in our gene ontology analysis of DHT signature proteins. Furthermore, evidence for altered androgen signaling in terms of renal AR mRNA and AKT phosphorylation was found in a mouse model of Fabry disease, a rare genetic lysosomal storage disorder[626].

Ganglosides are a subgroup of GSL with one or more sialic acids linked on the sugar chain[627]. **HEXB** is a key protein in GSL metabolism that emerged from the functional enrichment analysis and was validated *in vivo*. HEXB is the beta subunit of the lysosomal enzymes hexosaminidases A and B, which participate in the catabolism of globosides to ceramide and, together with the cofactor GM2 activator protein, catalyzes the degradation of ganglioside GM2 to GM3[628–630]. Increased tubular and glomerular GM3 content has been described in animal models of type 1 and type 2 diabetes, suggesting a role of GM3 in the early pathogenesis of DKD 112. Since hexosaminidases cleave N-acetylglucosamine groups from glycoconjugates, these enzymes are involved in the degradation of the N-acetylglucosamine-6-phosphate generated by GNPNAT1, conferring a link between HBP and GSL metabolism. In addition, fatty acyl-CoA derived from FAO is required for the synthesis of ceramide and subsequent GSL[627]. One may surmise that DHT treatment enhanced sugar, fatty acid and amino acid metabolism and, as a by-product, induced hyperactivation of HBP and GSL metabolism in PTEC. In concordance with our results, testosterone has been associated to increased urinary excretion of mouse kidney lysosomal GSL[631]. Previous data also indicate that GSL play a role in mediating the increment in **kidney size** induced by testosterone[632].

Cumulative evidence has linked altered energy metabolism and mitochondrial modification to the pathogenesis of **diabetes** and its complications[633]. In this sense, proteomic efforts in different tissues from mouse models of T1DM detected increased expression of several mitochondrial proteins related to FAO, TCA cycle and oxidative stress[634,635]. Within the diabetic kidney, label-free quantification of the mitochondrial proteome revealed increased FAO protein content and induction of TCA cycle enzymes[634]. In this sense, renal mitochondrial complex III of the respiratory chain, one of the major sites for ROS generation[618], was found to be altered in the early stage of STZ-induced T1DM in rats[636]. We provide strong evidence supporting that these metabolic processes relevant to diabetes are impaired by male sex hormones and increased in diabetic males, compared to females. In addition, the increase in renal oxidative stress levels in diabetic males indicates that these alterations may ultimately lead to a more rapid kidney disease progression in males. In accordance with our results, male sex has been associated with higher urinary and kidney levels of oxidative stress in animal models of hypertension 4 and renal ischemia[637], respectively.

To our knowledge, we are the first to report a **sexual dimorphism in renal nitrotyrosine levels** in the diabetic kidney.

As defended by Forbes and coauthors, it is important for the cells to maintain **glucose homeostasis** when exposed to hyperglycemic conditions by reducing the transport of glucose inside the cells. However, certain cell groups are unable to decrease glucose concentration, and are thus susceptible to damage[638]. From this perspective, it is conceivable that several molecular pathways related to energy metabolism are activated in the tubular cell as a mechanism to compensate the tremendous increase in glucose influx under diabetic conditions. Among these pathways, the role of HBP in the pathophysiology of diabetic cardiorenal disease has been extensively studied[639–641]. UDP-GlcNAc, the donor sugar for O-GlcNAcylation of proteins, is synthesized from glucose, glutamine, and UTP via the HBP. In turn, GNPNAT1 requires acetyl-CoA, which can be generated through FAO or transformation of citrate from TCA cycle by ATP-citrate lyase activity. Thus, it is generally accepted that HBP sits at the nexus of glucose, nitrogen, fatty acid and nucleic acid metabolism, which are altered in the context of diabetes[642]. According to our SILAC data, these metabolic pathways were enhanced in DHT- but not EST-treated PTEC. This may explain the fact that renal GNPNAT1 appeared to be higher in our diabetic but not control male mice as compared to females.

It is of importance that diabetic male mice developed **renal hypertrophy** and accentuated **hyperglycemia** in comparison to diabetic females. In addition, significant correlations between renal HADHA and GPI protein expression and KW were found in the present study, even under non-diabetic conditions. In this sense, metabolic remodeling has been extensively associated to maladaptive hypertrophic processes in other organs such as the heart[643], the pancreas[644] and the adipose tissue[644]. In particular, remodeling of glucose[645] and fatty acid[646] metabolisms promoted ventricular hypertrophy in experimental models of heart dysfunction. When accompanied by increased oxidative stress levels, these hypertrophic changes are more likely to become maladaptive and lead to organ failure[647]. In the endocrine pancreas of mice exposed to a high-fat diet, metabolic changes associated to hypertrophy were found to be more accentuated in males[528]. Alterations in processes related to mitochondrial biogenesis also play a role in hypertrophy[648]. Interestingly, these processes can be differentially regulated by female and male sex hormones[649]. In the kidney, increased amino acid delivery to the proximal tubule cells has been associated with renal growth and hypertrophy; regulation of molecular mechanisms such as AKT-pathways, that are also regulated by sex hormones, play a role in these renal alterations[650]. It has been proposed that HBP activation leading to O-GlcNAcylation is also involved in

hypertrophy, especially under hyperglycemic conditions[612]. Therefore, we presume that glucose influx into HBP was augmented in the kidneys of diabetic males, probably contributing to the increase in protein expression of renal GNPNAT1 and the accentuated renal hypertrophy. Overall, strong experimental evidence demonstrating a link between these hypertrophic processes and perturbations in bioenergetics in the kidney is still lacking. With our data, we reinforce the idea that the **maladaptive hypertrophy** as a consequence of **metabolic remodeling** observed in other organs under certain pathological conditions also occurs in the kidney, and in a sex-specific manner.

GSL have also drawn attention as bioactive signaling molecules in diabetes. These lipids are involved in the regulation of renal oxidative stress and apoptosis, especially in the lipid rafts localized in the **brush border of renal tubules**. Elevated renal GSL have been found in early DN in association with mesangial and glomerular hypertrophy[651], and proximal tubular vacuolization[621,652,653]. Interestingly, these glomerular alterations and the vacuolization of renal tubules have been observed in the STZ-diabetic males from our project.

To date, little is known about the effect of sex on energy metabolism within the kidney. In this work, we are the first to provide strong evidence that, under physiologic conditions, **DHT and male sex** promote higher activation of **energy metabolism** in the tubular cell and in the renal cortex. We also demonstrate that renal expression of the three candidates representing glycolysis, N-acetylglucosamine metabolism and FAO was increased in males in the context of T1DM. Further bioinformatics analyses and validation experiments allowed us to link this effect of male sex hormones in renal energy metabolism with accentuated **renal hypertrophy** and **oxidative stress** levels. For the first time, we suggest that altered metabolic activity in male kidneys under physiologic conditions confer a major susceptibility to develop renal complications, especially in the context of diabetes.

The strengths of our work include 1) the use of a solid, robust and accurate quantitative proteomic approach that was not previously used in renal cells; 2) good proteome coverage by employing a mass spectrometer with high sensitivity and accuracy, 3) control experiments to minimize false-positive hits, 4) the use of two different models of DN for validation; 5) the inclusion of publically available data on renal transcriptomics in our systems biology analyses, and 6) the discovery of a new, biologically relevant link between male sex and perturbed renal energy metabolism.

DHT alone led to dysregulated metabolic processes that are also altered in the diabetic kidney. These processes, including **glucose metabolism, HBP and FAO**, are associated with diabetes and may represent the link to understand the **more rapid**

progression of CKD in males. Top candidate proteins representative of each of these processes were verified and validated in sex hormone-treated PTEC and in male and female control and diabetic mice. Specifically, sex-specific regulation of GPI, HADHA and GNPNAT1 was demonstrated *in vitro* and *in vivo*. To the best of our knowledge, we are the first to demonstrate that GPI, HADHA and GNPNAT1 are responsive to androgens. Our results suggest that detrimental effects of androgens in diabetic nephropathy and other kidney diseases are mediated, at least in part, by altered energy metabolism within the tubular cell (Figure 101).

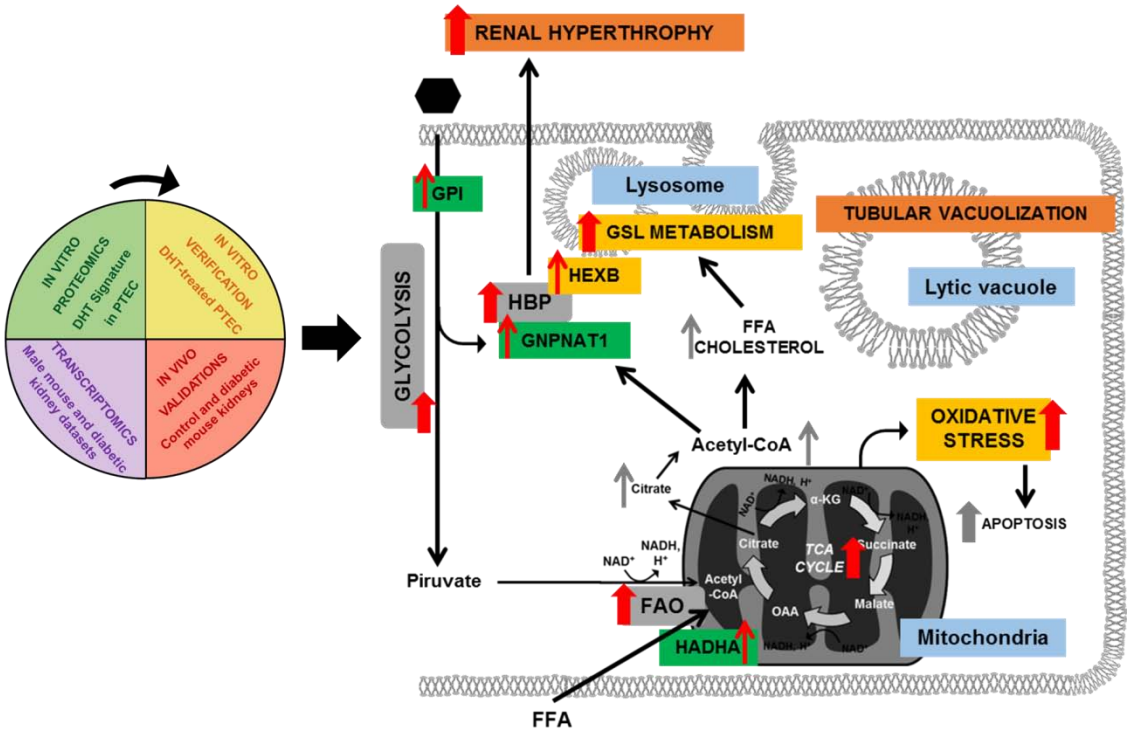

Figure 101. Proposed scheme for the role of male sex on energy metabolism in the diabetic kidney. By integrating our proteomic findings with previous transcriptomics data using bioinformatics tools, and supported by the corresponding *in vitro* and *in vivo* validation studies, we propose a model of interconnected enzymes, metabolites, biological processes, and pathophysiological events increased by male sex hormones and diabetes within the renal tubule. Red arrows indicate effects of DHT or male sex relevant to diabetes and demonstrated in the present study. Grey arrows depict hypothetical connections that have not been investigated in the present work. Green boxes indicate validated proteins in our study. Grey and blue boxes indicate significantly enriched biological processes and cellular components in our gene ontology analysis, respectively. Yellow boxes indicate processes that emerged from our functional enrichment analysis. Orange boxes indicate physiological events triggered by impaired energy metabolism observed in DN and accentuated by androgens in the present study. FFA, free fatty acids; α-KG, alpha-ketoglutarate; OAA, oxaloacetate; HBP, hexosamine biosynthetic pathway; FAO, fatty acid beta-oxidation; GSL, glycosphyingolipid.

6.C GENERAL DISCUSSION

- In the ***in vivo*** part of this project, we have demonstrated a **sexual dimorphism in DN progression**. Wild-type diabetic males presented a more important worsening in all the glomerular hallmarks of DN studied, including albuminuria, hyperfiltration, glomerular hypertrophy, and mesangial matrix expansion, than females. Male sex hormones play a direct role in the accentuated glomerular injury observed in males, as GDX prevented all these alterations. Such sex effect has been accompanied by a **sex-specific regulation of RAS**. Studies in ACE2KO mice have revealed that ACE2-dependent mechanisms in females are critical for their renoprotection in the context of diabetes and ANGII-induced hypertension, as loss of ACE2 led to a huge increase in BP and tubular lesions. In males, ACE2 deficiency mechanisms favored BP increase and glomerular alterations such as podocyte loss and mesangial area expansion.

- In the ***in vitro*** part, we have demonstrated a link between **DHT** and impaired **glucose and lipid metabolism** in human renal proximal tubular cells. Further validation studies of top candidate proteins have confirmed that male sex perturbs energy metabolism in the renal cortex, and that these alterations are associated to increased **renal hypertrophy and oxidative stress** levels in experimental T1DM.

In front of these findings, our last challenge as part of this doctoral thesis was to discuss how our *in vivo* and *in vitro* findings could be connected. We have shown that androgens and blood glucose are related to higher renal hypertrophy and oxidative stress by altering processes related to energy metabolism in the proximal tubular cell and in the renal cortex. Among these processes, the **hexosamine pathway** has been associated to **increased AGT levels** in several studies. Augmented AGT levels in the kidney usually imply higher activation of renal RAS and, in consequence, **augmented ANGII production**. In this sense, advanced oxidation protein products (which are accumulated in DN) have also shown to activate intrarenal renin-angiotensin by upregulating AGT, ACE, ANGII and AT1R renal expression[654], suggesting a positive feedback between oxidative stress and ANGII.

It is known that the oxidant effects of ANGII in the renal cortex involve impaired metabolism and mitochondrial activity, which may ultimately lead to ANGII-related apoptosis. Our results suggest that, physiologically, androgens promote a perturbed

metabolic status in the male **tubules**, which confer increased susceptibility to **oxidative stress** and **tubular injury** in the setting of hyperglycemia and hyperactivated RAS. Furthermore, impaired GSL metabolism in the brush border of male tubules may play a role in DN and enhance the ANGII-mediated up-regulation of oxidative stress. In this regard, ACE2 (which also localizes in the brush border), has been associated to the prevention of lipid accumulation in the liver[655], ANGII-induced mitochondrial damage and perturbations on cardiac energy metabolism[656], β-cell defects in the pancreas[657], atherosclerotic plaques progression due to lipid deposition[658], and metabolic disorders in the adipose tissue[659]. In addition, studies in the pancreas and the skeletal muscle have demonstrated a protective role of ACE2 on counterbalancing the deleterious effects of ANGII on glucose homeostasis[572–574,657]. Given these observations, it is conceivable that ACE2 also improves the metabolic profile in the kidney. Here we suggest a link between male sex, diabetes, impaired lipid and glucose metabolism, and increased ACE2 expression in the renal cortex.

As we and others have demonstrated[660,661], ANGII also exerts detrimental effects in the **glomerular compartment**. Our observations from ACE2KO and ANGII-infused males clearly indicate that ACE2 plays a renoprotective role on attenuating glomerular hypertrophy, mesangial matrix expansion and podocyte loss in the context of T1DM, and that these protective effects are mostly ascribed to the ACE2 capability of degrading ANGII. Of mention that, in the glomeruli of our control male mice, positive ACE2 staining was observed not only in the podocytes, but also in the apical membrane of the epithelial cells lining the capsule (Figure 109). Decreased glomerular ACE2 has been previously reported in experimental diabetes[209]. In turn, here we report the glomerular epithelialization is totally or partially lost in the male diabetic glomeruli. In this sense, it is conceivable that androgens also predispose these “tubule-like” cells to worsened metabolic alterations. We presume that, in diabetic males, the reduction in glomerular ACE2 expression is due not only to the lower number of podocytes, but also to the damage and loss of this epithelial layer (Figure 102). Together with increased circulating and glomerular ACE by diabetes[183,502], the reduction on glomerular ACE2 may lead to higher intraglomerular ANGII accumulation, which may explain the accentuated glomerular injury in males, compared to females.

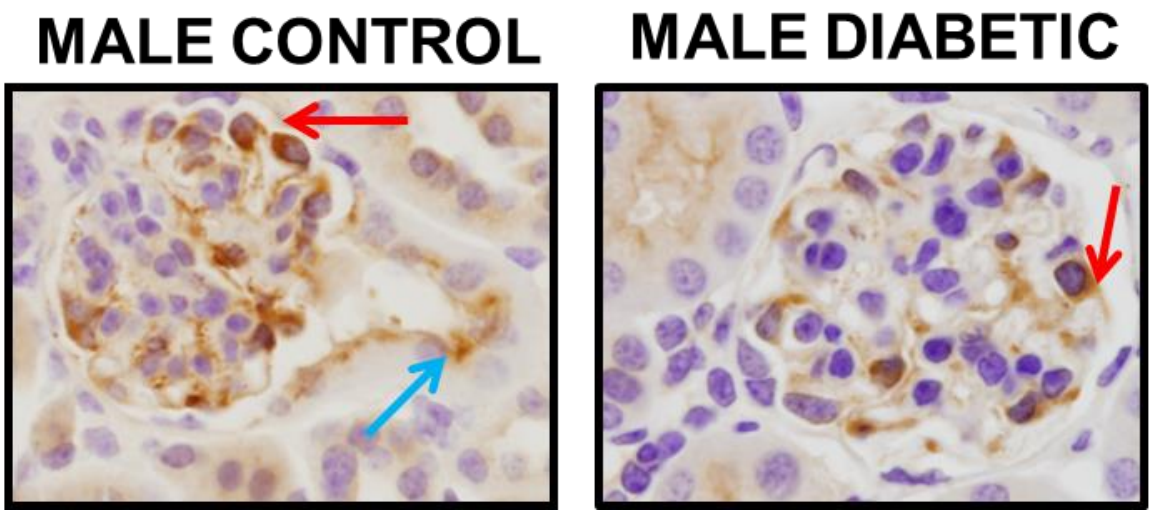

Figure 102. Glomerular ACE2 expression in control and diabetic males. Red arrows indicate positive ACE2 staining in the glomerular podocytes. The blue arrow points out ACE2 positive staining in the apical membrane of the epithelial cells lining the Bowman's capsule. The size of the pictures has been digitally modified for clarity. The original photomicrographs were taken at 40x magnification before being cropped.

In the **tubulointerstitium**, loss of ACE2 accentuated renal fibrosis in diabetic males. However, this increase was less exaggerated than the observed in ACE2KO diabetic females, and was not enhanced by ANGII administration. Evaluation of cortical RAS expression in diabetic males revealed that ACE downregulation may act as a compensatory mechanism, which may be still more activated in the context of ACE2 deficiency and ANGII-hypertension.

In this **final discussion** we have proposed **RAS hyperactivity as a link** between impaired energy metabolism, glomerular lesions, and cortical fibrosis in the male diabetic kidney. We also suggest that compartment-specific compensatory mechanisms of RAS play a role on defining the severity of these alterations (Figure 103).

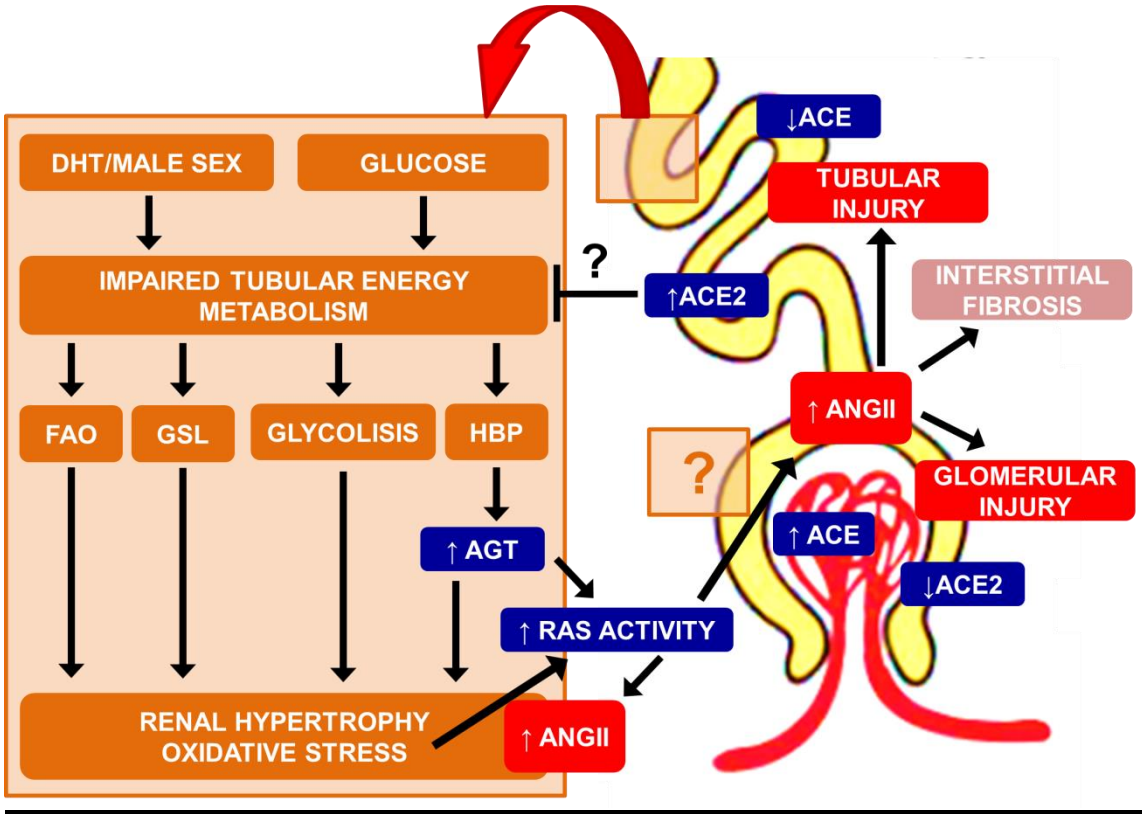

Figure 103. Proposed connection between impaired energy metabolism, RAS modulation and glomerular, tubular and tubulointerstitial alterations in type 1 diabetic males. Orange boxes contain factors and impaired metabolic processes playing a role in the tubular cell. Severe ANGII-related injury is depicted in red, whereas partially attenuated alterations are shown in pink. Changes related to RAS are colored in blue. DHT, dihydrotestosterone; FAO, fatty acid β-oxidation; GSL, glycosphingolipid; HBP, hexosamine biosynthetic pathway; AGT, angiotensinogen; RAS, renin-angiotensin system; ANGII, angiotensin II; ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2.

Our work is a perfect example on how exploring sex differences in a particular disease can be critical for a better understanding of the mechanisms involved on its progression. In this regard, we expect to contribute to a greater awareness of how important it is to consider the effect of sex when designing a biomedical research study. Our researches reinforce the current trend postulating that females and males should be simultaneously studied in biomedical studies, especially when investigating cardiovascular and metabolic disorders, or other diseases with an important hormonal component. At present, there is still a long way to go, as the majority of published works are focused exclusively in a unique sex; moreover, some authors do not even report the sex of the animals employed. In this sense, we appeal to researchers to specify the sex of the animals used in each study, and encourage them to justify the reasons of their choice.

This PhD project demonstrates a strong sex effect on DN progression. With our findings, we aim to improve the understanding of the sexual dimorphism in diabetic kidney disease, and shed light on new sex-specific therapeutic approaches targeting

RAS. We also hope that our observations regarding sex-specific alterations in circulating RAS and renal energy metabolism will lead to the consideration and design of new studies in the fields of biomarker discovery, metabolomics, and personalized medicine.

7. CONCLUSIONS

7. CONCLUSIONS

1. The severity and progression of DN is strongly influenced by sex, and is related to sex-specific changes in circulating and renal RAS.

2. In the STZ model of T1DM, male sex is associated to worsened alterations in glomerular function and structure, which are attenuated by gonadectomy.

3. In this model, cortical RAS is hyperactivated by diabetes and shifted towards the pro-ANG(1-7) axis, indicating the activation of compensatory mechanisms.

4. Male sex hormones exert a strong influence on the expression of the components of RAS in the kidney and the circulation.

5. ACE and ACE2 expression are modulated by sex hormones and T1DM. While increasing cortical ACE2 expression seems to be the predominant compensatory mechanism activated in STZ females, downregulation of cortical ACE play a more relevant role in STZ males.

6. Loss of ACE2 increases blood pressure and accentuates hyperfiltration, mesangial matrix expansion, podocyte loss, and renal fibrosis in male mice with T1DM. These alterations are prevented by gonadectomy.

7. Loss of ACE2 modifies the effects of gonadectomy on cortical fibrosis and RAS expression, indicating a crosstalk between androgen levels, ACE2 and the other RAS components.

8. Loss of ACE2 accentuates renal hypertrophy and fibrosis, as well as ANGII-induced hypertension, albuminuria, and glomerular hypertrophy in female mice with T1DM. This effect is not observed in males, probably due to a greater up-regulation of the negative feedback loop of RAS.

9. DHT predispose to impaired energy metabolism in human proximal tubular cells, resulting on increased oxidative stress levels in the male diabetic kidney.

10. We are the first to demonstrate that applying the spike-in SILAC methodology employing two different renal cell lines is a valid approach in the field of quantitative proteomics.

8. LIMITATIONS AND FUTURE PERSPECTIVES

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8.A LIMITATIONS

In the *in vivo* studies, we demonstrated sex-specific regulation of the pro-ANGII and pro-ANG(1-7) axes of RAS under pathological conditions of diabetes, ACE2 deficiency and hypertension. However, we did not assess ANGII and ANG(1-7) concentration in our biological samples. In this regard, we were not able to elucidate if the changes in RAS were reflected in altered levels of angiotensin peptides in the kidney and circulation. These data would have provided a direct link between the expression of RAS components and the downstream pathways and harmful effects related to ANGII.

The study was not designed to perform adequate separation of tubules and glomeruli in our renal tissue. Efficient glomerular and/or tubular isolation would have allowed us to obtain a clearer and picture of the sex-specific regulation of RAS in these two compartments altered in DN progression.

Another limitation of this project is that we did not measure sex hormone levels in our experimental groups. As we have discussed, sex hormone levels were probably altered not only by surgical castration but also by diabetes, among other factors. In addition, androgens can be converted to estrogens and play a critical role in RAS regulation. Having a clear picture of the circulating sex hormone levels, as well as the status of androgen and estrogen downstream mechanisms, in each experimental group, would have probably unmasked other interconnections between sex hormones, diabetes, and RAS.

Despite the novelty of our findings, the *in vitro* study also had several limitations. Most significantly, we studied PTEC responses to sex hormones at a single time point, and the expression levels of proteins are likely to be dynamic rather than static. Although we studied a single time point, the variety of identified processes has been implicated to CKD and DN processes. In turn, the intrinsic variability of PTEC proteome across passages may have probably contributed to the relatively low number of differentially expressed proteins between conditions. The MS analysis of EST-treated PTEC in passage 4 was limited to a single experiment and thus could contribute only marginally to the overall findings.

8.B FUTURE PERSPECTIVES

8.B.I. Sex-directed therapies in DN

In our *in vivo* studies, we demonstrated that male sex reduction by gonadectomy exerted protective effects in diabetic ACE2KO mice, but exacerbated renal fibrosis in wild-type males. Thus, the effect of gonadectomy is mainly observed in the absence of *Ace2* deletion. One may surmise that the effect of male hormones is clearly linked to the RAS system and that RAS imbalance alters the evolution of the disease. To assess this issue, ACE2 and androgen levels should be monitored in future clinical studies in male patients with diabetic nephropathy at different stages of the disease. In this sense, future experimental studies in diabetic models evaluating the effects of RAS blockade or recombinant ACE2 administration, either in the setting of reduced male sex hormones or in combination with androgen replacement therapy, will probably shed new light on the molecular mechanisms involved on androgen-mediated effects on RAS expression and, in consequence, the progression and severity of diabetic nephropathy.

8.B.II. Sex-specific markers of DN progression

More recently, high throughput “omics” technologies came on stage in the attempt of unmasking novel diabetes-related biological signals and biomarker discovery in cardiovascular disease. In our *in vitro* work we employed bioinformatics to integrate our DHT proteomic signature with previous data at the level of transcriptomics. This approach has unveiled a relationship between male sex and renal carbohydrate, fatty acid and GSL metabolism, and oxidative stress in the context of diabetes. Future studies focused on evaluating the influence of male sex hormones on the connection between these metabolic processes, together with other “omics” efforts in DN approached from a “sex-specific perspective”, may potentially shed new light on the identification of new serum and urine biomarkers for sex-directed therapies in renal disease.

8.B.III. DHT-dependent and –independent mechanisms of AR signaling

In this project, we observed a strong role of male sex hormones *in vivo* and *in vitro*. In the murine kidney, androgen reduction by gonadectomy markedly alters the expression of several RAS and renal fibrosis markers. In cultured renal cells, DHT alters the bioenergetics status of the cell by perturbing several metabolic processes. However, we have not deepened in the mechanisms of androgen signaling involved in such changes. Published works suggest that DHT binding to AR, but also DHT-independent transcriptional activity of AR, may play a role in the regulation of these genes. In this regard, future studies exploring the downstream AR mechanisms in experimental models of sex hormone supplementation or deprivation will shed new light on the complexity of sex hormone effects in the kidney. We also consider that chromatin immunoprecipitation experiments should be conducted with the aim to elucidate if the regulation of “androgen-dependent proteins” is directly mediated by AR binding to their promoter, or implies the participation of other players.

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9. BIBLIOGRAPHY

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GENERAL ANNEXES

Original Article

Gonadectomy prevents the increase in blood pressure and glomerular injury in angiotensin-converting enzyme 2 knockout diabetic male mice. Effects on renin–angiotensin system

Sergi Clotet[a,*], Maria Jose Soler[a,d,*], Marta Rebull[a], Javier Gimeno[b], Susan B. Gurley[c], Julio Pascual[a,d,†], and Marta Riera[a,d,†]

Background: Angiotensin-converting enzyme 2 (ACE2) deletion worsens kidney injury, and its amplification ameliorates diabetic nephropathy. Male sex increases the incidence, prevalence, and progression of chronic kidney disease in our environment.

Method: Here, we studied the effect of ACE2 deficiency and gonadectomy (GDX) on diabetic nephropathy and its relationship with fibrosis, protein kinase B (Akt) activation, and the expression of several components of the renin–angiotensin system (RAS).
Mice were injected with streptozotocin to induce diabetes and followed for 19 weeks. Physiological and renal parameters were studied in wild-type and ACE2 knockout (ACE2KO) male mice with and without GDX.

Results: Diabetic ACE2KO showed increased blood pressure (BP), glomerular injury, and renal fibrosis compared with diabetic wild type. Gonadectomized diabetic ACE2KO presented a decrease in BP. In the absence of ACE2, GDX attenuated albuminuria and renal lesions, such as mesangial matrix expansion and podocyte loss. Both, α-smooth muscle actin accumulation and collagen deposition were significantly decreased in renal cortex of gonadectomized diabetic ACE2KO but not diabetic wild-type mice. GDX also reduced circulating ACE activity in ACE2KO mice. Loss of ACE2 modified the effect of GDX on cortical gene expression of RAS in diabetic mice. Akt phosphorylation in renal cortex was increased by diabetes and loss of ACE2 and decreased by GDX in control and diabetic ACE2KO but not in wild-type mice.

Conclusions: Our results suggest that GDX may exert a protective effect within the kidney under pathological conditions of diabetes and ACE2 deficiency. This renoprotection may be ascribed to different mechanisms such as decrease in BP, modulation of RAS, and downregulation of Akt-related pathways.

Keywords: blood pressure, diabetic nephropathy, gonadectomy, renin–angiotensin system, streptozotocin

Abbreviations: ACE, angiotensin-converting enzyme; Akt, protein kinase B; Ang, angiotensin; AOGEN, angiotensinogen; APN, aminopeptidase N; CTSG, cathepsin G; GDX, gonadectomy; GFR, glomerular filtration rate; KO, knock-out; PAS, periodic acid–Schiff; rACE, renal angiotensin-converting enzyme; RAS, renin–angiotensin system; sACE, serum angiotensin-converting enzyme; STZ, streptozotocin; UAE, urinary albumin excretion; WT, wild type; WT-1, Wilm's tumour 1

INTRODUCTION

Angiotensin-converting enzyme (ACE) 2 is an homolog of ACE that degrades angiotensin (Ang)-II to Ang-(1–7), and Ang-I to Ang-(1–9) in renin–angiotensin system (RAS) [1,2]. It is generally accepted that Ang-II promotes vasoconstriction, fibrosis, inflammation, and apoptosis [3], whereas Ang-(1–7) is associated with the opposite beneficial effects [4]. Ye *et al*. showed a decrease in ACE2 glomerular expression in diabetic db/db mice, which was accompanied by an increase in intraglomerular expression of ACE. These studies suggest that the imbalance in intrarenal expression of ACE and ACE2 leads to increased accumulation of intrarenal Ang-II with its consequent adverse effects [5]. Furthermore, it has been demonstrated that deletion or chronic inhibition of ACE2 worsens renal damage in experimental diabetic nephropathy [6–8].

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[a]Department of Nephrology, [b]Department of Pathology, Hospital del Mar – Institut Hospital del Mar d'Investigacions Mèdiques, Barcelona, Spain, [c]Division of Nephrology, Department of Medicine, Duke University and Durham VA Medical Centers, Durham, North Carolina, USA and [d]Red de Investigación Renal (REDINREN), Instituto Carlos III-FEDER, Madrid, Spain

Correspondence to María José Soler, MD, PhD, Department of Nephrology, Hospital del Mar – Institut Hospital del Mar d'Investigacions Mèdiques, Passeig Maritim 25-29, 08003 Barcelona, Spain. Tel: +34932483162; fax: +34932483373; e-mail: msoler@parcdesalutmar.cat

*Sergib Clotet and Maria Jose Soler contributed equally to this work.

†Julio Pascual and Marta Riera share credit for senior authorship.

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Male sex increases the incidence, prevalence, and progression of chronic kidney disease (CKD) [9]. Thus, most of the patients that need renal replacement therapy are men [10]. Although these phenomena have always been attributed to the protective effects of estrogens, recently it has been suggested that male hormones may play a critical role in these differences [11]. In CKD patients without previous history of cardiovascular disease, our group recently reported that ACE2 activity from human EDTA-plasma samples is significantly increased in men compared with women [12]. In agreement, in an experimental model, Liu *et al.* [13] found that, under normal conditions, males have higher renal ACE2 activity than females. Oudit *et al.* studied the effect of ACE2 deletion in kidneys from male and female mice. Their data showed that loss of ACE2 in males (but not females) is associated with the development of age and Ang-II-dependent glomerular damage [2]. Later studies have shown that the expression of several components of the RAS, including ACE2, can be affected by sex and modulated by sex steroids under physiologic and pathological conditions [13–16].

We hypothesized that ACE2 deletion and male sex hormones exert a harmful effect on the diabetic nephropathy progression, and renal lesions can be diminished in the absence of such hormones. Thus, we studied the influence of ACE2 deficiency and gonadectomy (GDX) on hypertension and kidney damage in diabetic C57BL/6 male mice after streptozotocin (STZ) injection. We also analyzed the effect of ACE2 deficiency, diabetes, and GDX on renal RAS modifications. In addition, we assessed the levels of protein kinase B (Akt) phosphorylation as a downstream signaling effector of both, Ang-II and Ang-(1–7)-related axis.

METHODS

Animal model and experimental groups

Experiments were performed in wild-type and ACE2 knockout (ACE2KO) male mice with the C57BL/6 background. The generation of ACE2KO mice has been previously described by Gurley *et al.* [17]. Mice were housed in ventilated cages with full access to chow and water. The Ethical Committee of Animal Experimentation of the Barcelona Biomedical Research Park approved this study. Diabetes was induced to 10-week-old mice following the High-Dose STZ Induction Protocol from the Animal Models of Diabetic Complications Consortium with slight modifications. The 4-h-fasted mice were given two intraperitoneal injections of 150 mg/kg STZ (Sigma, St Louis, Missouri, USA) in 2 consecutive weeks as previously reported [6]. Citrate buffer was used as vehicle. GDX or sham operation was performed 1 week prior to diabetes induction. (See Supplementary Information, http://links.lww.com/HJH/A639).

The study included eight to 12 animals per group that were followed for 19 weeks after diabetes induction. During this period, body weight and blood glucose were measured every 2 weeks. For glucose level determination, fasting blood samples from the saphenous vein were obtained for measurements with the ACCU-CHEK Compact meter system (Roche Diabetes Care, Spain). Mice were considered diabetic if blood glucose levels higher than 250 mg/dl were detected during the first 4 weeks after STZ administration.

At the end of the follow-up mice were sacrificed by terminal surgery. Blood was extracted by cardiac puncture and serum was obtained by centrifugation at 8000*g* for 10 min. Mice were perfused with cold phosphate buffer solution prior to kidneys removal and weighting. The left kidney and half of the right kidney were snap frozen with liquid nitrogen and kept at −80°C for further analysis. Half of the right kidney was maintained in formalin solution, neutral buffer 10% (Sigma) and paraffin embedded for histological studies.

Blood pressure

SBP and DBP were measured during the last week of follow-up using the CODA mouse tail-cuff system (Kent Scientific Corporation, Torrington, Connecticut, USA). Values were obtained from conscious, trained mice on five consecutive morning sessions. Results are expressed in mmHg.

Urinary albumin excretion

Urinary albumin excretion (UAE) was determined using the albumin-to-creatinine ratio on morning spot urine collections. Urinary albumin and creatinine levels were measured by ELISA (Albuwell M; Exocell, Philadelphia, Pennsylvania, USA) and a colorimetric assay (Creatinine Companion; Exocell), respectively. Albumin-to-creatinine ratio was calculated and expressed as μgAlb/mgCrea [18].

Glomerular filtration rate

Mice were anesthetized at the end of the study through a single intraperitoneal injection of sodium pentobarbital (45 mg/kg). Glomerular filtration rate (GFR) was estimated in three to seven animals per group using clearance kinetics of plasma fluorescein isothiocyanate-inulin after a single intravenous bolus injection as previously described [19]. GFR values were expressed as μl/min per g of body weight as previously published [20].

Enzymatic activity assay of angiotensin-converting enzyme

The ACE fluorometric enzymatic assay was performed in serum and kidney protein extracts as previously described with modifications [21]. (See Supplementary Information, http://links.lww.com/HJH/A639).

Reverse transcriptase real-time–PCR assays

RNA was isolated from renal cortex and real-time PCR was performed as previously described [22]. Primer sequences were synthesized by Sigma and are described in Supplementary Table 1, http://links.lww.com/HJH/A639. (See Supplementary Information, http://links.lww.com/HJH/A639).

Kidney histology

Paraffin blocks were cut into 3-μm sections, deparaffined in xylene, and rehydrated through graded alcohols. Sections were stained with periodic acid–Schiff for measurement of mesangial index [19]. Immunohistochemistry staining was

TABLE 1. Physiologic parameters at 4 weeks of diabetes and at the end of the study

	CONT-WT	CONT-ACE2KO	CONT-ACE2KO + GDX	DB-WT	DB-WT + GDX	DB-ACE2KO	DB-ACE2KO + GDX
BG(mg/dl) 4 weeks	191.88 ± 5.31	192.26 ± 4.05	182.67 ± 7.04	442.25 ± 31.29*	303.91 ± 27.36§	493.43 ± 28.02*	268.93 ± 21.53*,§
BG(mg/dl) 19 weeks	207.29 ± 5.49	194.00 ± 6.66	208.50 ± 10.05	538.33 ± 25.35*	294.30 ± 21.59§	545.57 ± 22.25*	242.14 ± 10.11*,†,§
BW(g) 4 weeks	31.44 ± 0.54	27.31 ± 0.60†	25.62 ± 0.80	28.18 ± 0.61*	24.24 ± 0.23§	25.95 ± 0.48†	24.60 ± 0.49
BW(g) 19 weeks	36.55 ± 0.89	29.98 ± 0.75†	26.47 ± 1.24§	27.35 ± 0.73*	23.92 ± 0.43§	25.09 ± 0.67*,†	24.74 ± 0.73
KW(g) 19 weeks	0.37 ± 0.01	0.33 ± 0.01†	0.25 ± 0.01§	0.35 ± 0.02	0.22 ± 0.02§	0.35 ± 0.01	0.20 ± 0.01*,§
KW/BW(%) 19 weeks	0.98 ± 0.02	1.12 ± 0.03†	0.95 ± 0.07§	1.28 ± 0.07*	0.93 ± 0.08§	1.43 ± 0.05*	0.79 ± 0.02*,†,§

Blood glucose and body weight were measured after 4 weeks of diabetes and after 19 weeks, the end-point. At the end of the study, KW was also recorded in all the experimental groups. Eight to 12 animals were analyzed in each group. Values are expressed as mean ± SEM. ACE2KO, angiotensin-converting enzyme 2 knockout; BG, blood glucose; BW, body weight; CONT, control; DB, diabetic; GDX, gonadectomized; KW, kidney weight; WT, wild type.
*$P < 0.05$ compared with nondiabetic controls.
†$P < 0.05$ compared with wild type.
§$P < 0.05$ compared with non-GDX.

performed for ACE [5], α-smooth muscle actin (α-SMA) [23] and the podocyte marker Wilms tumor 1 (WT-1) [19]. In addition, Sirius red staining was performed on 4.5-μm kidney sections and cortical collagen accumulation was semiquantitatively evaluated (0–4 score) [24]. All analyses were performed in a double-blinded fashion. (See Supplementary Information, http://links.lww.com/HJH/A639).

Immunoblotting

Kidney cortical tissue was prepared for immunoblot analysis with antibodies to ACE and phosphorylated and total Akt. (See Supplementary Information, http://links.lww.com/HJH/A639).

Principal component analysis

To evaluate the predominant effects of ACE2 deletion, diabetes and GDX in the physiological and renal parameters, tubulointerstitial fibrosis, hemodynamics and glomerular injury markers, and RAS components gene expression, principal component analysis (PCA) was performed using Perseus software (version 1.5.1.6; Max Planck Institute of Biochemistry, Martinsried, Germany). The mean value for each variable in each experimental group was used for the PCA. Category enrichment was calculated two principal components, and Benjamini–Hochberg false discovery rate was used as a cut-off method with a threshold value of 0.05.

Statistical analysis

Statistical analyses were performed using SPSS 18.0 statistical software (SPSS Inc. Chicago, Illinois, USA). Because the sample size was small, non-parametric tests were conducted. Kruskal–Wallis tests were performed for multiple comparisons in the study. In addition, Mann–Whitney *U*-tests were used for comparison between two groups. Significance was defined as $P < 0.05$, and data are expressed as means ± SE.

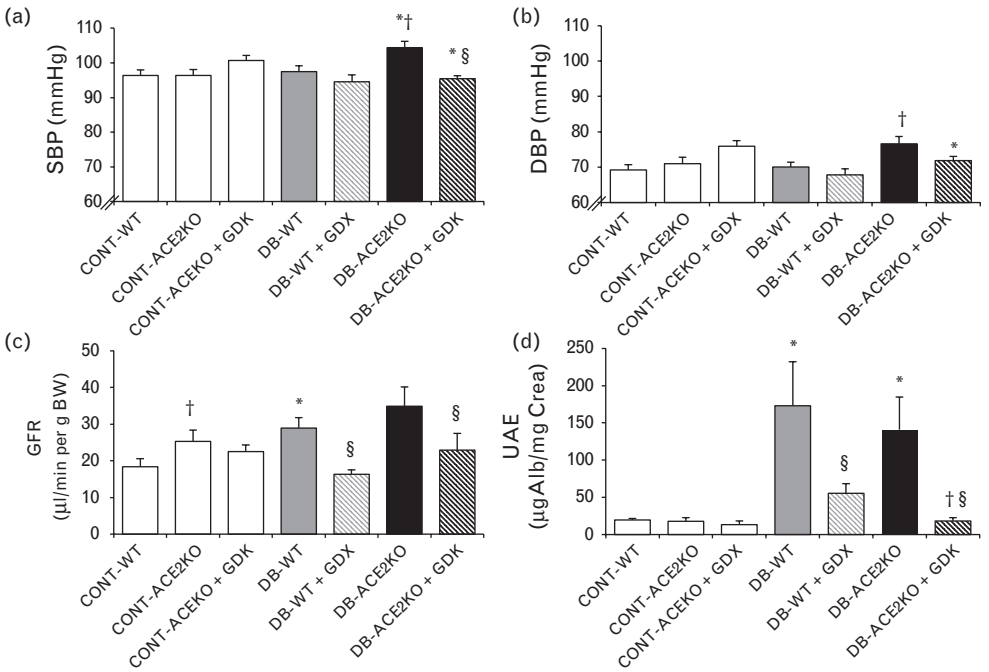

FIGURE 1 Influence of diabetes, ACE2 deletion, and gonadectomy on hemodynamics and glomerular function. SBP and DBP (8 to 12 animals per group), glomerular filtration rate, and UAE (8 to 12 animals per group) were evaluated in all the experimental groups. Data are expressed as mean ± SEM. ACE2KO, angiotensin-converting enzyme 2 knockout; CONT, control; DB, diabetic; GDX, gonadectomized; UAE, urinary albumin excretion; WT, wild type. *$P < 0.05$ compared with nondiabetic controls. †$P < 0.05$ compared with WT. §$P < 0.05$ compared with non-GDX.

RESULTS

Diabetes, blood pressure, and renal functional parameters

Blood glucose levels were significantly elevated in all STZ-treated groups compared with their controls (Table 1). Throughout the follow-up, diabetic animals showed lower body weight than their controls (Table 1). In this model, ACE2 deletion was accompanied by lower body weight. In addition, GDX also diminished the body weight.

After 19 weeks of study, renal hypertrophy was evaluated by calculating the kidney weight/body weight ratio. ACE2 deletion significantly increased the ratio compared with the wild-type animals. In turn, GDX in wild-type and ACE2KO mice clearly reduced the ratio (Table 1). Diabetes was accompanied by an increased ratio in intact wild-type and ACE2KO animals, indicating the presence of renal hypertrophy associated to diabetes in these groups. Interestingly, diabetic ACE2KO + GDX mice showed significantly lower kidney weight/body weight ratio than the diabetic wild-type + GDX group.

ACE2 deletion significantly increased SBP and DBP in intact diabetic mice (Fig. 1a and b). This increase was not observed in diabetic ACE2KO + GDX animals. GFR was increased in diabetic wild-type mice compared with their controls (Fig. 1c). ACE2 deletion accentuated hyperfiltration by increasing GFR in controls. GDX significantly reduced GFR in diabetic wild-type and ACE2KO mice. Diabetes significantly increased UAE in wild-type and ACE2KO mice (Fig. 1d). Surgical castration significantly decreased UAE in diabetic wild-type and ACE2KO mice. Among these groups, diabetic ACE2KO + GDX mice presented significantly lower UAE than their respective diabetic wild-type + GDX mice.

Histological analysis

Glomerular tuft area was increased in diabetic wild-type and diabetic ACE2KO compared with controls (Fig. 2a and c). In concordance to the low renal weight observed in these groups, glomerular area was decreased in gonadectomized wild-type and ACE2KO diabetic mice. However, this reduction was only significant in the ACE2KO. Diabetic wild-type and diabetic ACE2KO mice showed an elevated

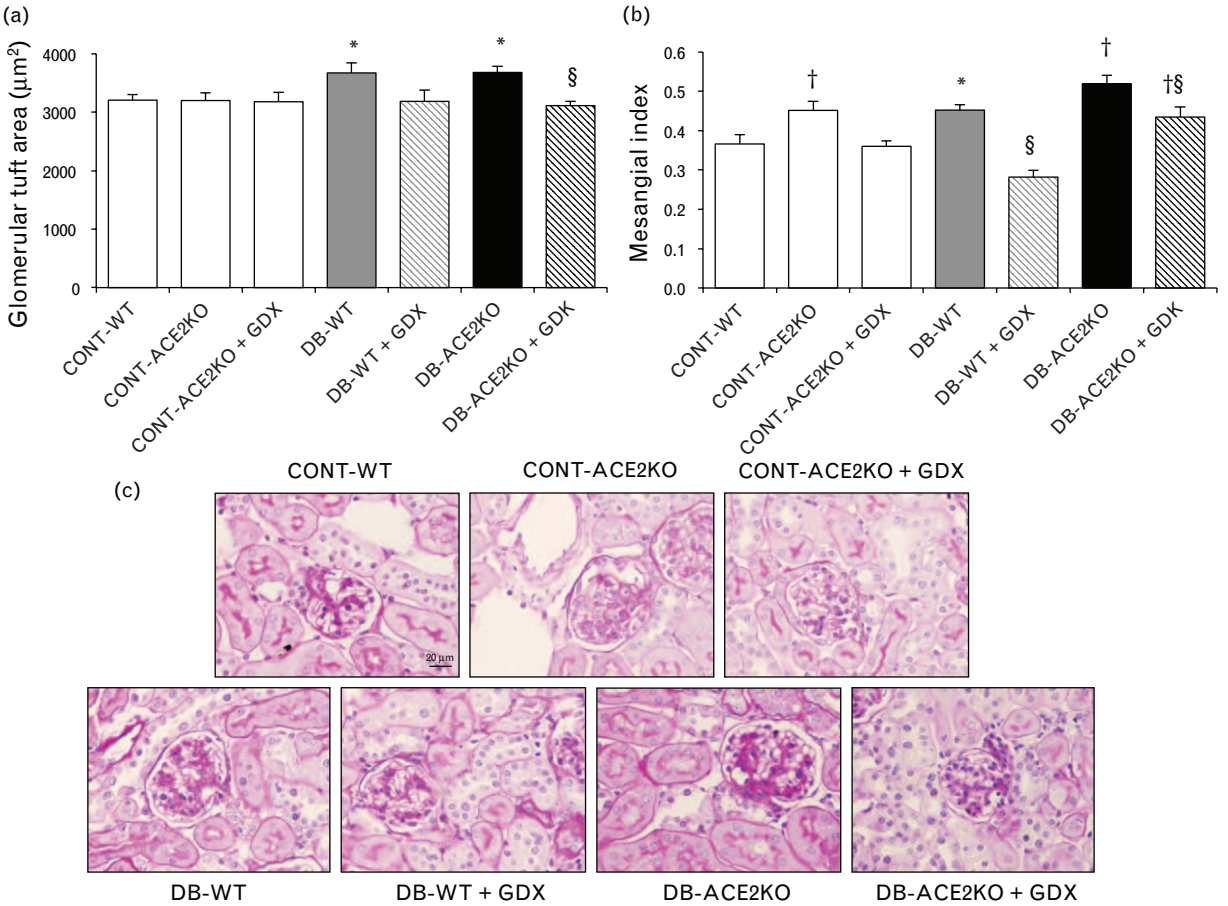

FIGURE 2 Influence of diabetes, ACE2 deletion, and gonadectomy on glomerular structural alterations. PAS was performed on 3-μm kidney sections from all the experimental groups. Glomerular tuft area (a) and mesangial index (b) were calculated by ImageJ software (National Institute of Mental Health, Bethesda, Maryland, USA). Representative PAS sections from all the experimental groups are shown in (c). For these experiments, seven to 10 animals were analyzed in each group. Data are expressed as mean ± SEM. ACE2KO, angiotensin-converting enzyme 2 knockout; CONT, control; DB, diabetic; GDX, gonadectomized; PAS, periodic acid-Schiff; WT, wild type. $^{*}P < 0.05$ compared with non-diabetic controls. $^{\dagger}P < 0.05$ compared with WT. $^{\S}P < 0.05$ compared with non-GDX. Scale bar = 20 μm. Original magnification ×40.

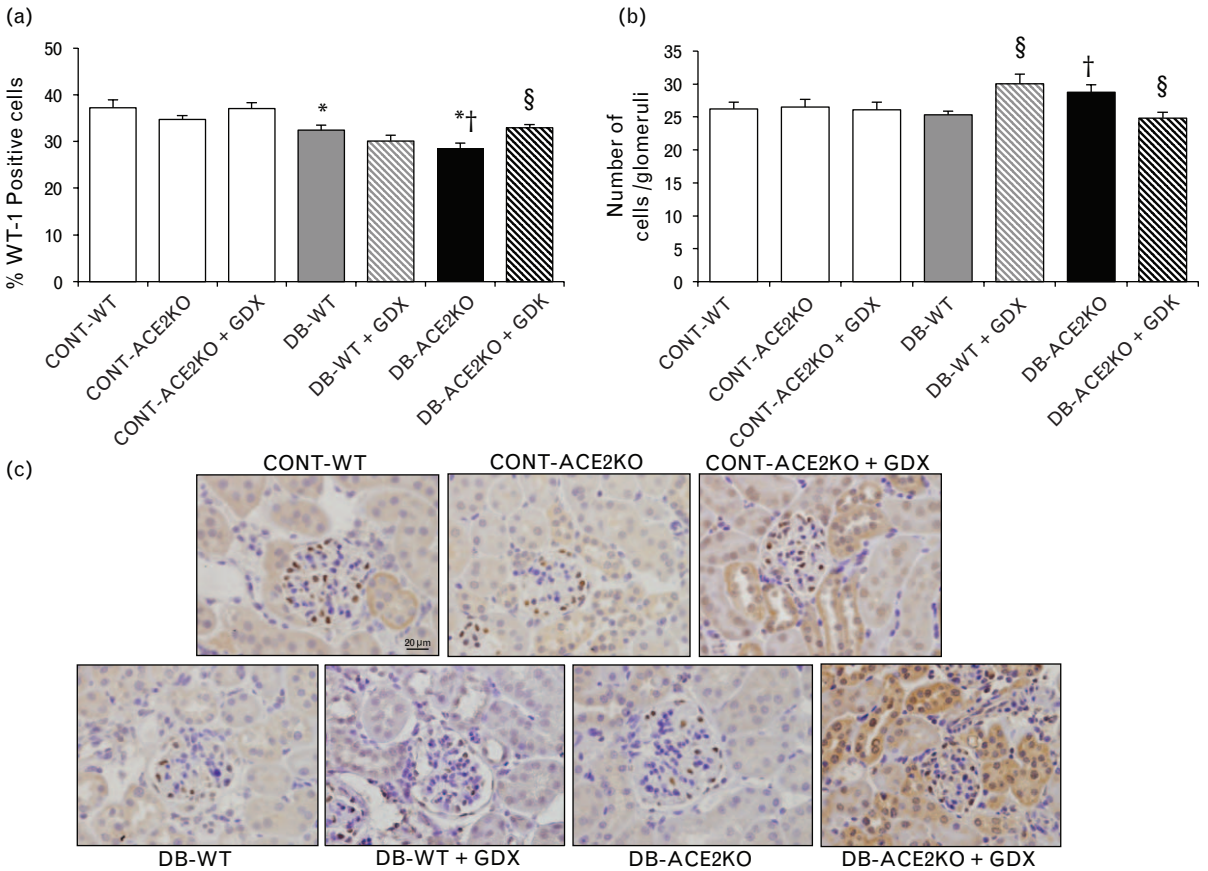

FIGURE 3 Influence of diabetes, ACE2 deletion, and gonadectomy on podocyte loss. Podocyte number is represented as the percent of brown positive cells after WT-1 immunostaining (a). Representative photomicrographs depicting glomerular WT-1 staining from all the experimental groups are shown in (b). Total cell number was also assessed in the same photomicrographs (c). For these experiments, six to 10 animals were analyzed in each group. Data are expressed as mean ± SEM. ACE2KO, angiotensin-converting enzyme 2 knockout; CONT, control; DB, diabetic; GDX, gonadectomized; WT, wild type. $^{*}P < 0.05$ compared with non-diabetic controls. Scale bar = 20 µm. Original magnification ×40.

mesangial index in comparison to the non-diabetic groups (Fig. 2b and c). In ACE2KO mice, the mesangial index was significantly higher compared with wild type. GDX prevented the increase in the mesangial index in both, diabetic wild-type and ACE2KO mice. Interestingly, diabetic ACE2KO + GDX mice showed a significantly higher mesangial index than their respective diabetic wild-type + GDX mice.

The proportion of cells that were identified as podocytes in glomerulus was significantly decreased in the diabetic groups compared with controls (Fig. 3a and c). This decrease was significantly accentuated in diabetic ACE2KO mice compared with diabetic wild type. Diabetic ACE2KO mice also showed a significantly higher number of cells per glomeruli compared with the diabetic wild type (Fig. 3b and c). In contrast, gonadectomized ACE2KO diabetic mice showed decreased cell number and increased podocytes within the glomeruli. These changes were not observed in the diabetic wild-type + GDX group.

Evaluation of renal fibrosis

Interstitial fibrosis was evaluated by two different approaches. To test the progression of fibrogenesis, the presence of interstitial myofibroblasts in renal cortex was evaluated by α-SMA immunostaining. This actin isoform predominates within vascular smooth muscle cells and plays an important role in fibrogenesis. As expected, α-SMA staining was detected in the media of renal arteries and arterioles and diabetes significantly increased its interstitial expression in wild-type and ACE2KO mice compared with their non-diabetic controls (Fig. 4). The second approach was the evaluation of Sirius red staining as collagen type I and III fibril markers. The technique revealed increased collagen deposition in diabetic mice compared with controls (Fig. 5). Interestingly, collagen accumulation was enhanced in diabetic ACE2KO mice compared with diabetic wild type. Surgical castration significantly reduced both α-SMA staining and collagen deposition in ACE2KO diabetic mice. In contrast, diabetic wild-type + GDX mice exhibited a significant increase in α-SMA expression and collagen deposition, being these markers of fibrosis significantly higher in comparison to the diabetic ACE2KO + GDX group (Fig. 4 and Fig. 5).

Protein kinase B expression and activation in kidney cortex

To determine whether the status of Akt Ser473 phosphorylation in diabetes was related to ACE2 deletion and the effect of surgical castration, western blot analysis was performed. Akt phosphorylation (pAkt) was increased in wild-type diabetic mice compared with their controls (Fig. 6a and b).

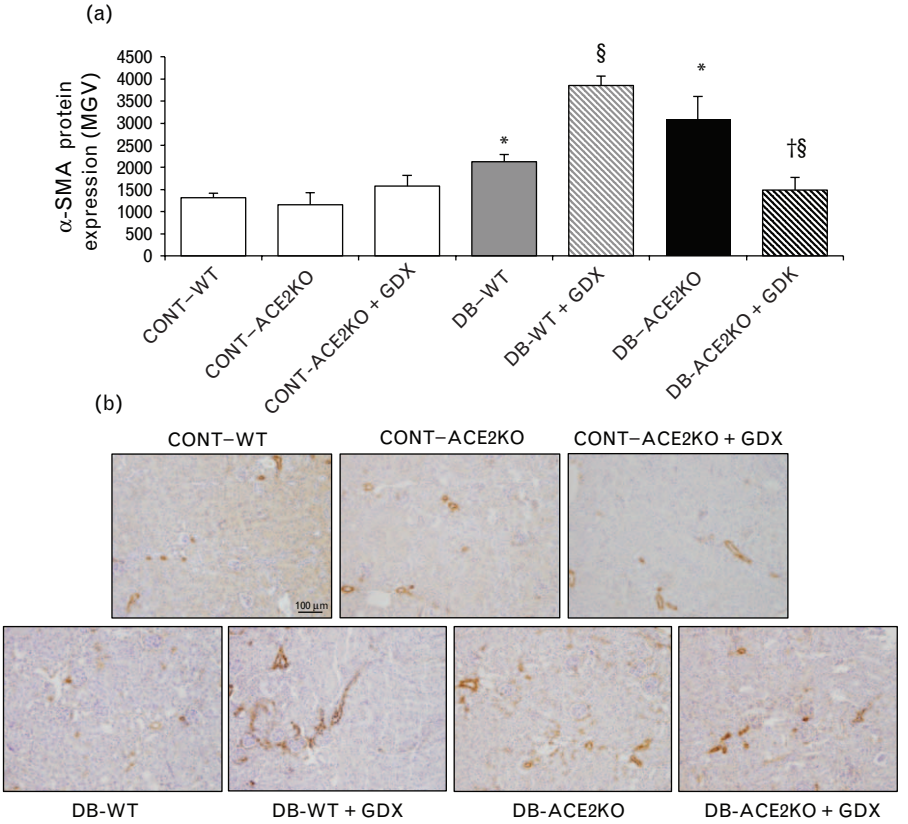

FIGURE 4 Influence of diabetes, ACE2 deletion, and gonadectomy on cortical α-SMA expression. The degree of brown staining as α-SMA-positive area was quantified by ImageJ software and represented as mean grey value (a). (b) It shows representative sections for tubulointerstitial α-SMA immunostaining from all the experimental groups. Scale bar = 100 μm. Original magnification ×10. For these experiments, six to nine animals were analyzed in each group. Data are expressed as mean ± SEM. ACE2KO, angiotensin-converting enzyme 2 knockout; α-SMA, α-smooth muscle actin; CONT, control; DB, diabetic; GDX, gonadectomized; MGV, mean grey value; WT, wild type. $^{*}P < 0.05$ compared with non-diabetic controls. $^{\dagger}P < 0.05$ compared with WT. $^{\S}P < 0.05$ compared with non-GDX.

In addition, ACE2 deletion was accompanied by an increase of pAkt and the phospo/Akt ratio in both, control and diabetic mice. In turn, GDX clearly decreased pAkt and the phospo/Akt ratio in these groups. GDX did not modify pAkt and phospo/Akt ratio in wild-type diabetic mice (Fig. 6a–d).

Angiotensin-converting enzyme expression in serum and kidney cortex

Serum ACE (sACE) activity was significantly increased in wild-type and ACE2KO diabetic mice compared with controls (Fig. 7a). sACE was also enhanced in ACE2KO control (ACE2KO-CONT) compared with wild-type CONT. Gonadectomized mice markedly displayed low sACE.

Renal ACE (rACE) was significantly decreased in diabetic mice in terms of enzymatic activity (Fig. 7b) and protein levels (Fig. 7c) in comparison to controls. ACE2 deletion was accompanied by lower rACE enzymatic activity and protein expression. As shown in Fig. 7d, the reduction of rACE levels by ACE2 deletion or diabetes was observed in the renal cortex by immunohistochemistry.

Renin–angiotensin system expression in kidney cortex

With the aim to describe the deregulation of RAS in our model, gene expression of its components was studied. Diabetes was accompanied by higher cortical angiotensinogen (AOGEN) gene expression in all experimental groups (Fig. 8a). Mas receptor (MasR) mRNA levels were also augmented, whereas ACE gene expression was decreased in both diabetic wild-type and diabetic ACE2KO mice compared with their controls (Fig. 8c and i). Interestingly, only ACE2KO diabetic mice showed a significant decrease in renin (REN) and Ang-II type 1 receptor (AT1R) expression, as well as higher cathepsin G (CTSG) mRNA levels (Fig. 8b, d and h). In turn, neprilysin (NEP) gene expression was augmented in diabetic wild-type mice (Fig. 8g). Gonadectomized animals depicted a hyperactivated RAS by means of gene expression analysis. In control gonadectomized ACE2KO, cortical gene expression of AOGEN, REN, and NEP was significantly increased compared with CONT-ACE2KO mice (Fig. 8a, b, and g). In contrast, ACE, aminopeptidase N (APN), and MasR mRNA levels were significantly decreased in this group (Fig. 8c, f, and i). In diabetic mice, GDX significantly enhanced REN, NEP, and AT1R cortical mRNA levels (Fig. 8b, g, and h), whereas diminished APN and MasR gene expression in both wild-type and ACE2KO animals (Fig. 8f and i). Among the diabetic gonadectomized groups, MasR mRNA levels were significantly increased in diabetic wild-type + GDX compared with diabetic ACE2KO + GDX mice (Fig. 8i). Interestingly, GDX dramatically increased AOGEN gene expression in diabetic wild type but not ACE2KO

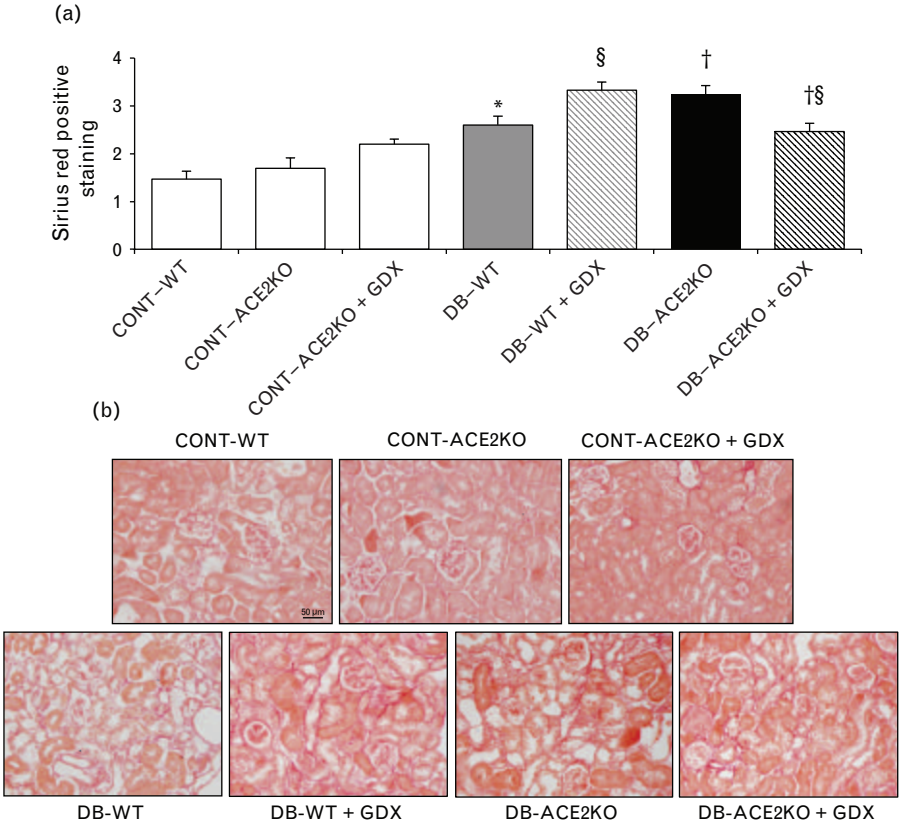

FIGURE 5 Influence of diabetes, ACE2 deletion, and gonadectomy on collagen deposition. Cortical collagen was analyzed in a semiquantitative manner (scale 0–4) on Sirius red-stained tissue sections (a). (b) It shows representative photomicrographs for Sirius red-positive staining (intense red) from all the experimental groups. Scale bar = 50 μm. Original magnification ×20. For these experiments, six to eight animals were analyzed in each group. Data are expressed as mean ± SEM. ACE2KO, angiotensin-converting enzyme 2 knockout; CONT, control; DB, diabetic; GDX, gonadectomized; WT, wild type. $^{*}P < 0.05$ compared with non-diabetic controls. $^{\dagger}P < 0.05$ compared with WT. $^{\S}P < 0.05$ compared with non-GDX.

(Fig. 8a). In addition, GDX induced a significant increase in CTSG only in ACE2KO mice (Fig. 8d).

Principal component analysis

In this study, we simultaneously evaluated the effect of ACE2 deletion, diabetes, and GDX in several hallmarks of diabetic nephropathy in the glomeruli and the tubulointerstitial compartment, as well as its relationship with changes in renal RAS. To have a better understanding of the predominant effects of each of these three factors on renal injury and renal RAS expression, we undertook PCA of all experimental groups. Distribution of variances for all the analyzed renal parameters showed that the effect of diabetes was more pronounced in ACE2KO mice compared with wild type. In diabetic and gonadectomized mice, a different effect was observed between the wild-type and the ACE2KO groups (Fig. 9a). Interestingly, additional PCA revealed a similar distribution when considering only the tubulointerstitial fibrosis markers as determinants of sample variation (Fig. 9b). In contrast, independent analysis for the hemodynamic parameters and glomerular injury markers indicated a predominant effect of diabetes over GDX in both, wild-type and ACE2KO mice (Fig. 9c). When evaluating the distribution of our study groups according to RAS components gene expression, PCA reflected that both GDX and diabetes alone exerted a clear effect on changing the levels of these genes. This effect was notably accentuated when these factors were combined in diabetic and gonadectomized animals, and even more pronounced in mice expressing ACE2 (Fig. 9d).

DISCUSSION

We studied the effect of GDX on a mouse model of diabetes without the expression of ACE2 protein. Overall, gonadectomized ACE2KO diabetic mice showed lower blood pressure (BP) values and decreased nephropathy than male ACE2KO diabetic mice. These animals exhibited modulation of circulating and renal RAS favoring the 'pro-Ang(1–7)' axis; histological evidence of renal protection, namely, a reduction in mesangial expansion and attenuation of glomerular hypertrophy; attenuation of podocyte loss; and reduction in interstitial fibrosis and collagen deposition. To our knowledge, the present work is the first to simultaneously study the influence of ACE2 deficiency and GDX on hypertension and kidney damage in diabetic male mice. Our results contribute to the knowledge of sex differences in RAS in the pathological context of diabetes.

Studies in ACE2-deficient mice have demonstrated a role for ACE2 in the regulation of BP, identifying ACE2 as a functioning component of RAS *in vivo* [17]. Furthermore, induction of diabetes by STZ in ACE2-deficient mice either

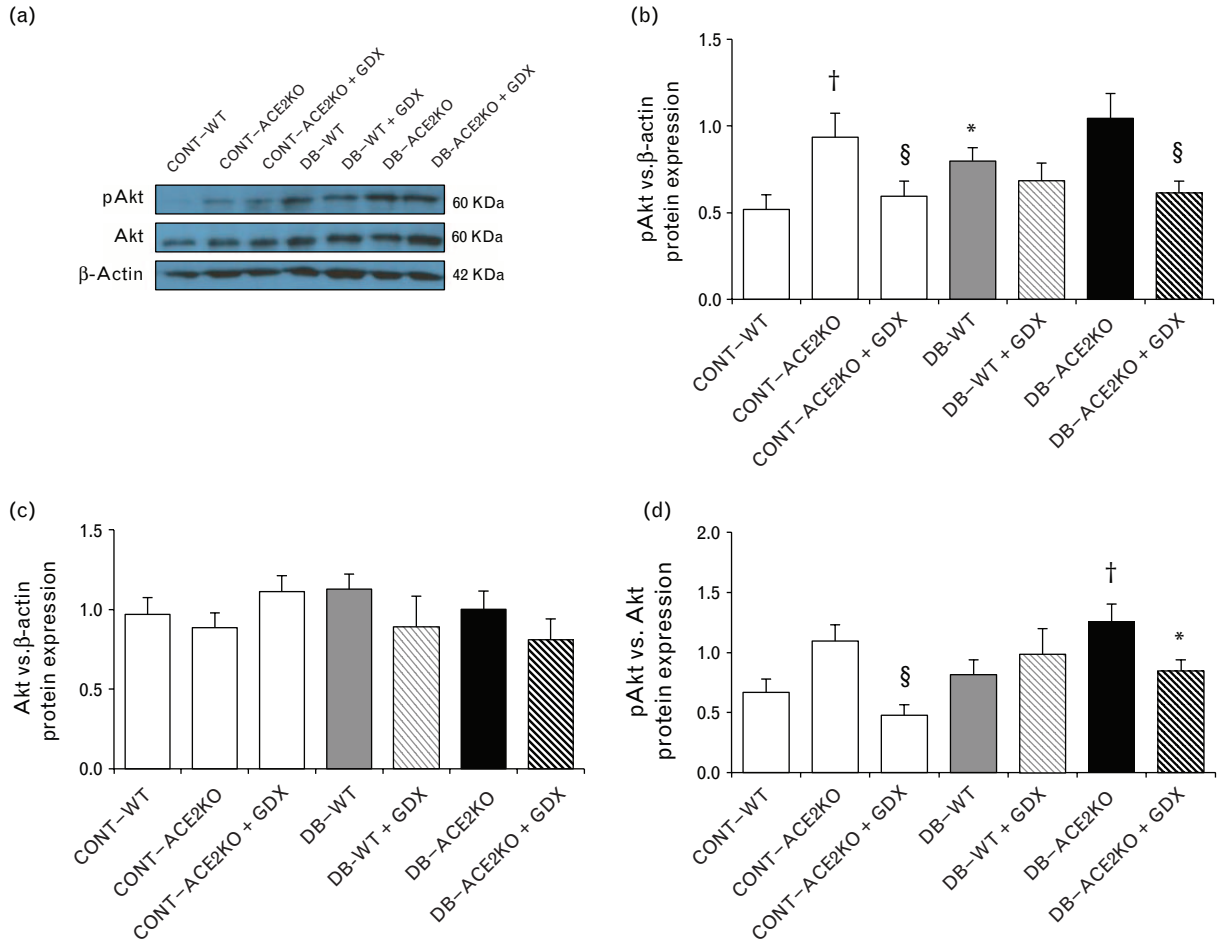

FIGURE 6 Influence of diabetes, ACE2 deletion, and gonadectomy on phosphorylated and total Akt in kidney cortex. (a) Representative immunoblot depicting cortical pAkt, Akt, and β-actin in kidneys from all the experimental groups. Densitometry analysis of each band was performed using ImageJ. Intensities for pAkt and Akt were normalized to β-actin (b and c), and pAkt/Akt ratio was also calculated (d). For these experiments, six to eight animals were analyzed in each group. Data are expressed as mean ± SEM. ACE2KO, angiotensin-converting enzyme 2 knockout; Akt, protein kinase B; CONT, control; DB, diabetic; GDX, gonadectomized; pAkt, Akt phosphorylation; WT, wild type. $^{*}P < 0.05$ compared with non-diabetic controls. $^{\dagger}P < 0.05$ compared with WT. $^{\S}P < 0.05$ compared with non-GDX.

by pharmacologic inhibition or genetic ablation increased BP values in these animals [25,26]. In concordance, our study also demonstrated increased SBP and DBP in ACE2KO diabetic mice. Of note, this increase was not observed in gonadectomized diabetic ACE2KO mice. Increased BP in hypertensive and diabetic ACE2KO mice has been ascribed to Ang-II accumulation within the kidney and circulation [15,17,26]. We also found that circulating ACE activity was increased in ACE2KO and diabetic mice. It has been previously reported that STZ-induced diabetes is associated with an increase in circulating ACE activity [15]. To our knowledge, the effect of ACE2 deletion or inhibition on sACE activity has not been previously studied. In this work, we demonstrated that ACE2 deficiency increased circulating ACE activity. Our study also shows that there is a dichotomy between circulating ACE and rACE expression. Specifically, in ACE2KO and diabetic mice circulating ACE was increased, whereas cortical ACE was decreased at gene and protein expression and at enzymatic activity levels. Similar results in terms of reduced rACE expression were found in STZ-treated rats, in the db/db model of type 2 diabetes, and in models of ACE2 downregulation [6,15,25]. Proteolytic release of membrane-bound ACE was first described by Ehlers *et al.* [27] in Chinese hamster ovary cells transfected with human ACE cDNA. Although the identity of the secretase that sheds ACE remains unknown, it has the properties of a member of a disintegrin and metalloproteinase (ADAM) family of membrane-bound zinc metalloproteases [28]. In this context, expression and sheddase activity of ADAM10 and ADAM17 metalloproteases have been found to be increased in type 1 [29] and type 2 [30] diabetes. In addition, high glucose and Ang-II have been associated with ADAM17 upregulation in mesangial [31] and proximal tubular [32] cells. Thus, augmented circulating levels of ACE in our diabetic and ACE2KO male mice may be ascribed to Ang-II accumulation. We therefore hypothesize that, under pathological conditions of hyperglycemia and ACE2 downregulation, there is an increase of ACE shedding accompanied by a downregulation of ACE expression within the renal cortex. Interestingly, we now find that GDX decreases ACE gene expression in control ACE2KO mice as well as circulating ACE activity in control and diabetic ACE2KO mice, suggesting a role of the absence of androgens on the

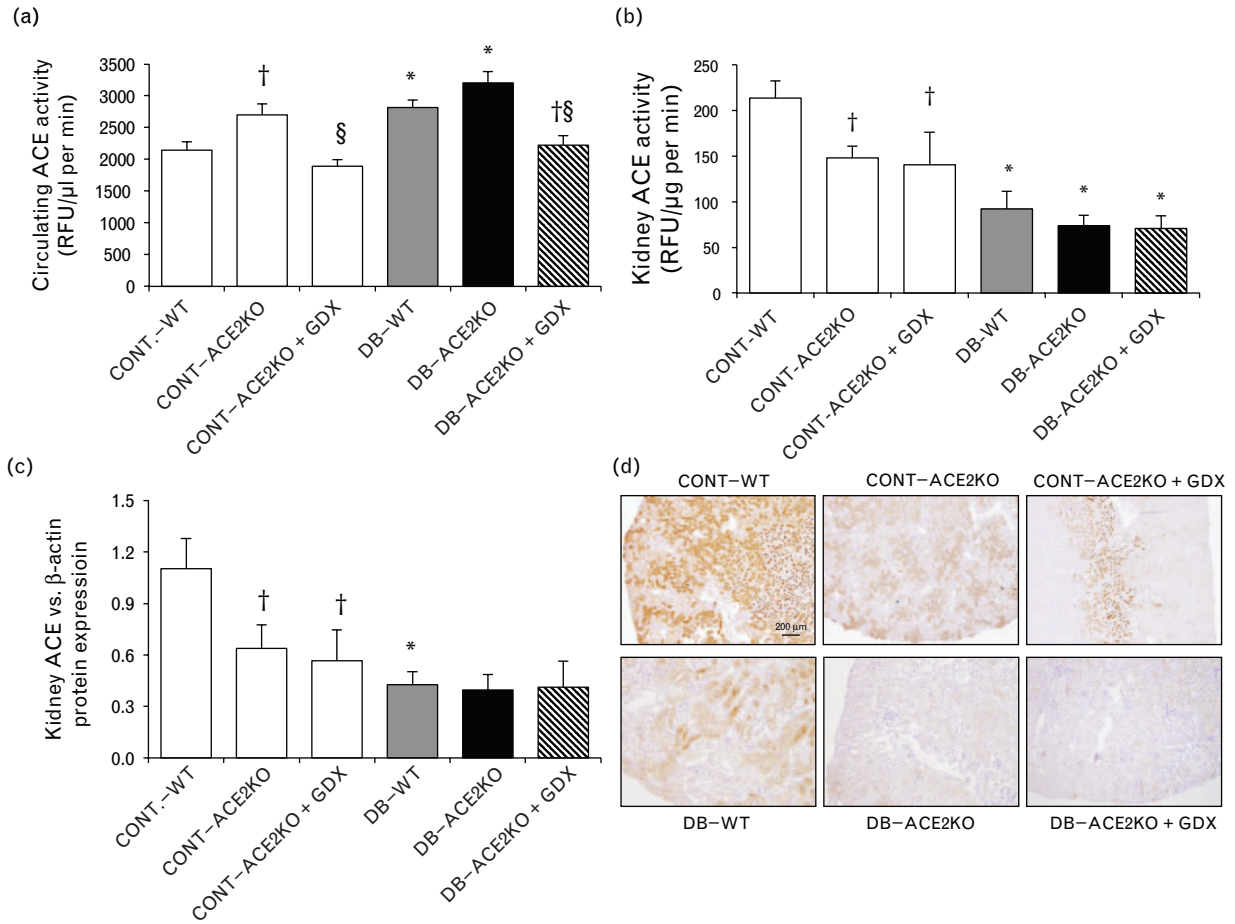

FIGURE 7 Influence of diabetes, ACE2 deletion, and gonadectomy on ACE expression in serum and kidney cortex. ACE activity in serum (a) and kidney cortex (b) from all the experimental groups. (c) Immunoblot of cortical ACE protein expression normalized to β-actin from all the experimental groups. (d) It shows representative photomicrographs depicting ACE protein localization in the renal cortex from all the experimental groups. Scale bar = 200 µm. Original magnification ×4. For these experiments, six to 10 animals were analyzed in each group. Data are expressed as mean ± SEM. ACE2KO, angiotensin-converting enzyme 2 knockout; CONT, control; DB, diabetic; GDX, gonadectomized; WT, wild type. $^{*}P < 0.05$ compared with non-diabetic controls. $^{\dagger}P < 0.05$ compared with WT. $^{\S}P < 0.05$ compared with non-GDX.

transcriptional regulation of ACE that would lead to lower levels of renal and circulating Ang-II. In agreement with our findings, Lim *et al.* [33] demonstrated that plasma ACE activity in both male and female mice is reduced by GDX. This decrease was more severe in males than in females. Considering that males have higher androgen levels than females, it is conceivable that androgens have a stronger influence than estrogens on plasma ACE activity [33]. Of mention that ACE also degrades Ang-(1–7) to Ang-(1–5) in the renal tubules and circulation [34]. Thus, it is conceivable that increased circulating ACE and decreased rACE because of ACE2 deletion and diabetes altered not only Ang-II but also Ang-(1–7)-related pathways in our mice. Therefore, increased SBP in diabetic ACE2KO mice may be ascribed not only to an Ang-II accumulation in serum but also to an excessive ACE-dependent metabolism of Ang-(1–7) and, in consequence, a downregulation of the vasodilatory effects promoted by this peptide.

To further elucidate the mechanisms responsible for the attenuated diabetic nephropathy in the context of reduced male sex hormone levels, alterations in several RAS components within the kidney were evaluated. During the last years, other RAS components have been described as ACE and ACE2-independent mechanisms producing and degrading Ang-II and Ang-(1–7), such as neprilisysin, CTSG, APN, and aminopeptidase A. NEP degrades Ang-II to Ang-(1–4), as well as forms Ang-(1–7) from Ang-I [34]. In our work, GDX dramatically augmented renal AOGEN and REN gene expression in diabetic ACE2KO mice. Furthermore, GDX was also accompanied by elevated NEP mRNA levels in control and diabetic ACE2KO mice. In concordance, Yamaleyeva *et al.* [15] observed lower levels of renal NEP in male Lewis rats compared with females. Together with cortical ACE and APN changes, our data suggest alteration of cortical RAS in ACE2KO + GDX mice in which both AOGEN/REN/Ang-I/NEP/Ang-(1–7) and AOGEN/REN/ACE/Ang-II axis are modulated.

Oudit *et al.* [2] demonstrated that loss of ACE2 in male mice leads to age-dependent development of glomerular mesangial expansion and glomerulosclerosis. In our study, we showed an increase in kidney weight to body weight ratio, GFR, and mesangial expansion in ACE2KO-CONT mice at 7 months of age. However, glomerulosclerosis and alterations in UAE were not observed, suggesting an early stage of renal disease. These differences may be related to the younger age of our animals.

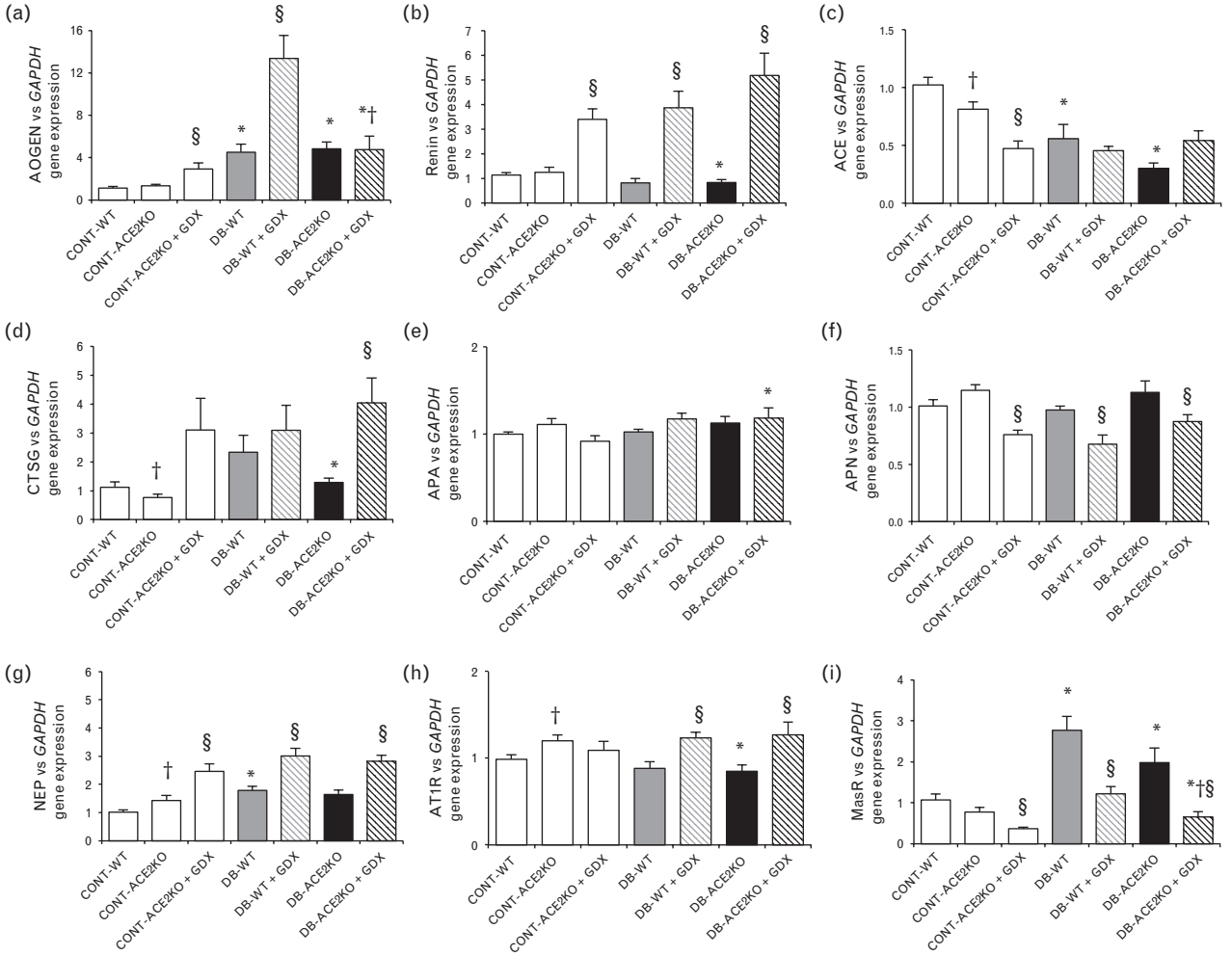

FIGURE 8 Cortical gene expression of RAS components in renal tissue. Renal cortex gene expression of angiotensinogen (a), renin (b), ACE (c), cathepsin G (d), aminopeptidases A (e) and N (f), neprylisin (g), AT1R (h), and Mas receptor (i) was determined by real-time quantitative PCR and normalized to *GAPDH* in all the experimental groups. For these experiments, six to 10 animals were analyzed in each group. Data are expressed as mean ± SEM. ACE2KO, angiotensin-converting enzyme 2 knockout; CONT, control; DB, diabetic; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDX, gonadectomized; WT, wild type. $^{*}P < 0.05$ compared with nondiabetic controls. $^{\dagger}P < 0.05$ compared with WT. $^{\S}P < 0.05$ compared with non-GDX.

As expected, ACE2 deletion exacerbated glomerular injury, namely, mesangial matrix expansion and podocyte loss in diabetic mice. In agreement, Soler *et al.* [6] described worsening of albuminuria and glomerular histological lesions after 4 weeks of ACE2 pharmacological inhibition in STZ-induced diabetic mice. In Akita mice, ACE2 deletion also exacerbated albuminuria in association with increased mesangial matrix deposition, glomerular basement membrane thickening, and glomerulosclerosis [35]. Our results of hypercellularity, decreased podocyte number, and increased mesangial matrix within the glomeruli suggest that the increase in glomerular cellularity in our diabetic ACE2KO model is mainly because of an expansion of the mesangial cell lineage. These features of diabetic glomerular disease were absent in gonadectomized ACE2KO mice. Different mechanisms such as modulation of renal RAS and decreased BP may play a role in this renoprotective effect at the glomerular level.

Diabetic ACE2KO mice showed an increase in the myofibroblast profibrotic marker α-SMA and accentuated collagen deposition compared with diabetic wild-type mice. Our findings are consistent with previous studies where ACE2 deficiency either by pharmacological inhibition or gene deletion also increased fibronectin and/or α-SMA expression in kidneys from diabetic mice [5,8,35]. Loss of ACE2 markedly increased intrarenal Ang-II in association with enhanced transforming growth factor (TGF)-β/mothers against decapentaplegic (Smad)-mediated renal fibrosis in a mouse model of obstructive nephropathy [36]. Ang-II can activate several intracellular signaling pathways to mediate renal fibrosis and inflammation, including TGF-β/Smads [37,38] and PI3K/Akt [39,40]. GDX prevented the outcome of hypertension, glomerular alterations, and the accumulation of profibrotic and fibrotic markers such as α-SMA and collagen in diabetic ACE2KO mice, suggesting a decrease in circulating and renal Ang-II levels and a subsequent downregulation of these Ang-II downstream pathways.

Akt is important in many cellular processes, including proliferation, migration, cell growth, and metabolism, and plays a critical role in the cardiovascular and renal system [41]. Akt activity can be transcriptionally modulated by its

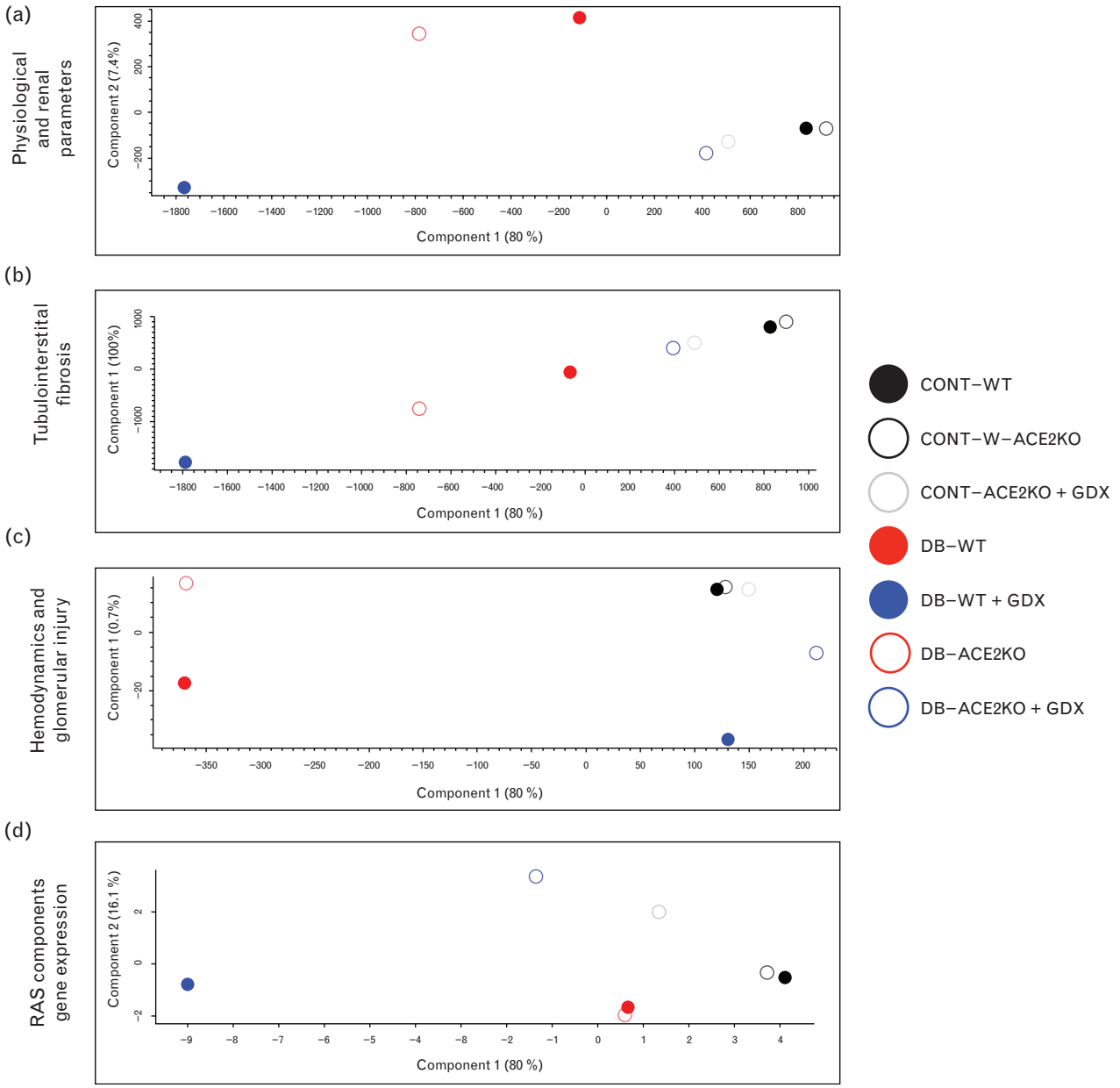

FIGURE 9 PCA plot of the experimental groups. PCA was performed using Perseus software for different groups of variables to evaluate the predominant effects of ACE2 deletion, diabetes, and gonadectomy in the physiological and renal parameters, tubulointerstitial fibrosis, hemodynamics and glomerular injury markers, and RAS components gene expression. (a) It depicts PCA for all the assessed physiological and renal parameters, namely, blood glucose, body weight, KW/BW, SBP, UAE, GFR, glomerular area, mesangial index, WT-1-positive cells, total cell number, Sirius red score, and α-SMA protein expression. PCA was also performed independently for the hemodynamic and glomerular parameters, namely, SBP, UAE, GFR, glomerular area, mesangial index, WT-1-positive cells, and total cell number (b), as for the markers of tubulointerstitial fibrosis, namely, Sirius red score and α-SMA protein expression (c). In addition, predominant effects of ACE2 deletion, diabetes, and gonadectomy on the expression of the nine analyzed RAS genes were assessed (d). ACE2, angiotensin-converting enzyme 2; α-SMA, α-smooth muscle actin; BW, body weight; GFR, glomerular filtration rate; KW, kidney weight; PCA, principal component analysis; RAS, renin–angiotensin system; UAE, urinary albumin excretion; WT-1, Wilms tumor 1.

upstream regulatory pathways and also at posttranslational level by phosphorylation of the Thr308 and Ser473 residues [31]. Higher levels of Akt and pAkt have been described in animal models of STZ-induced type 1 diabetes [42–44], as well as in high glucose-treated renal cell lines [45,46]. In addition, it has been reported that pAkt is enhanced by Ang-II and Ang-(1–7) *in vivo* [47,48] and *in vitro* [40,49,50], as well as by the actions of testosterone [51] and androgen receptor [52,53]. In the present study, diabetes and loss of ACE2 were accompanied by increased Akt expression and phosphorylation, whereas GDX clearly reduced pAkt levels in control and diabetic ACE2KO mice. As expected, Akt expression and phosphorylation was not modified by GDX in diabetic wild-type mice. The decreased MasR gene expression and the absence of ACE2 in diabetic ACE2-KO + GDX may explain lower activation of Ang-(1–7)-dependent Akt stimulation compared with the wild-type mice. In diabetic wild-type + GDX mice, the presence of ACE2 and increased mRNA levels of MasR, may suggest a higher activation of the Ang-(1–7)/MasR axis. Thus, the

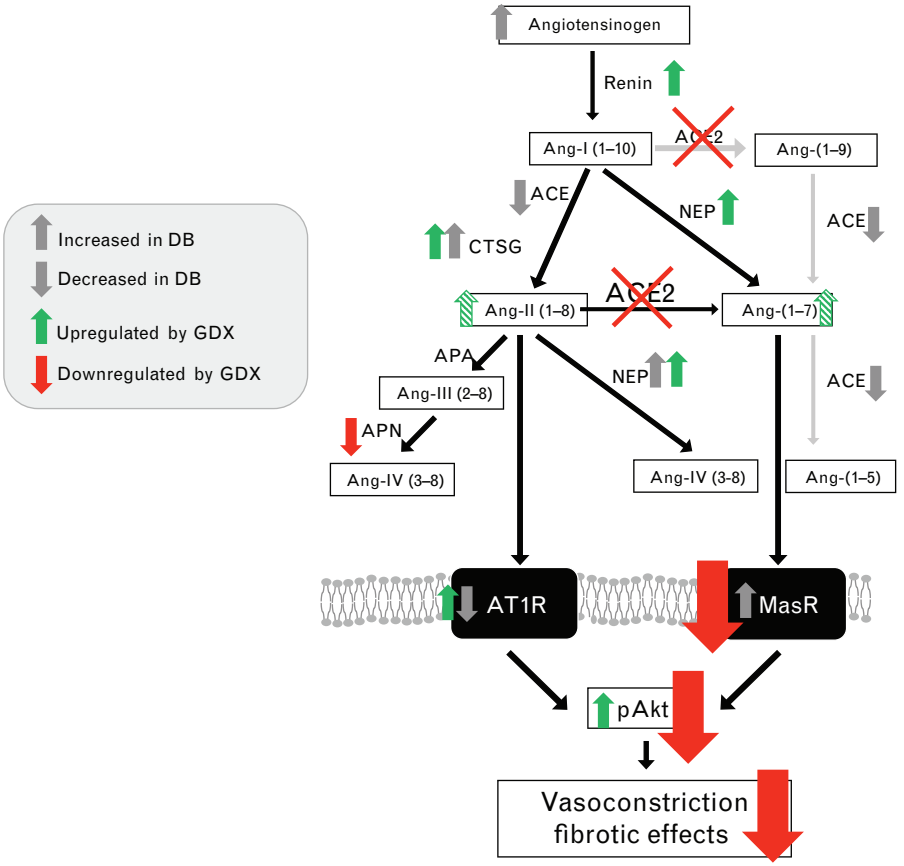

FIGURE 10 Protective effects of gonadectomy by modulating renal RAS and Akt phosphorylation in ACE2KO diabetic mice. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; ACE2KO, angiotensin-converting enzyme 2 knockout; Ang, angiotensin; APA, aminopeptidase A; APN, aminopeptidase N; AT1R, Ang-II type 1 receptor; CTSG, cathepsin G; DB, diabetes; GDX, gonadectomy; MasR, Mas receptor; NEP, neprylisin.

effects of GDX on reducing pAkt may be counterbalanced by an increase on Ang-(1–7)-mediated pAkt in diabetic wild-type + GDX mice.

In this study, we simultaneously evaluated the effect of ACE2 deletion, diabetes, and GDX by PCA of all experimental groups. We found that the effects of ACE2 deletion on diabetic nephropathy are influenced by the levels of male sex hormones. When evaluating the renal markers in a global fashion, we observed an opposite effect of ACE2 deficiency depending on the hormonal status of our mice (castrated vs. sham operated), suggesting that sex hormone reduction by GDX may be protective in diabetic ACE2KO but not diabetic wild-type mice. Additional PCA indicated that this global effect was mainly ascribed to the changes observed on tubulointerstitial fibrosis. In concordance with our results, Xu *et al.* [54] also found that GDX increased fibrosis in STZ diabetic rats. In addition, PCA for renal RAS gene expression suggested that modulation of renal RAS played a relevant role on these changes. In conclusion, GDX attenuated diabetic nephropathy by preventing hypertension, glomerular injury, and renal fibrosis in type 1 diabetic ACE2KO mice. Given our results, one hypothesizes that, under deficient Ang-II degradation, GDX may confer a protective effect at kidney level by different mechanisms such as a decrease in BP, a decrease in pAkt, and RAS modulation (Fig. 10). These positive effects were absent in ACE2 intact mice. Under these conditions of enhanced MasR expression, excessive activation of the NEP/ACE2/Ang-(1–7)/MasR axis may activate profibrotic pathways that contribute to kidney injury. In this sense, future experimental studies in diabetic models evaluating the effects of RAS blockade or/and recombinant ACE2 administration, either in the setting of reduced male sex hormones or in combination with androgen replacement therapy, will shed new light on the molecular mechanisms involved on androgen-mediated effects on RAS expression and, in consequence, the progression and severity of diabetic nephropathy.

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This work has not been presented before in whole or in part.

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Conflicts of interest

There are no conflicts of interest.

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Reviewers' Summary Evaluations

Referee 1

The current study elucidated the role of androgens and the expression of the ACE homolog ACE2 in an experimental model of diabetic nephropathy. Overall, the results suggest that androgens exhibit a negative influence on renal injury. However, the findings that androgen depletion exacerbate renal fibrosis which may reflect an overstimulation of the neprilysin-Ang-(1–7)-AT7/Mas receptor axis are particularly novel. Additional studies that block this particular system are necessary to completely establish the deleterious actions of this pathway in the diabetic kidney following androgen depletion.

Referee 2

This study indicates that androgen effects on diabetes-induced kidney injury partly depend on angiotensin converting enzyme 2 (ACE2). Gonadectomy mitigated renal fibrosis in diabetic ACE2-deficient mice but enhanced it in diabetic wild-type animals. Furthermore, gonadectomy differentially affected renal cortical angiotensinogen as well as mas receptor mRNA abundance in these animals. While a comprehensive data set is presented, the link between the androgen-dependence of renal fibrosis in ACE2-knockout mice and the respective activity pattern of renal RAS components remains to be established.

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RAS and sex differences in diabetic nephropathy

Sergi Clotet, Marta Riera, Julio Pascual, and María José Soler

Department of Nephrology, Hospital del Mar-Institut Hospital del Mar d'Investigacions Mèdiques, Barcelona, Spain

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Clotet S, Riera M, Pascual J, Soler MJ. RAS and sex differences in diabetic nephropathy. *Am J Physiol Renal Physiol* 310: F945–F957, 2016. First published March 9, 2016; doi:10.1152/ajprenal.00292.2015.—The incidence and progression of kidney diseases are influenced by sex. The renin-angiotensin system (RAS) is an important regulator of cardiovascular and renal function. Sex differences in the renal response to RAS blockade have been demonstrated. Circulating and renal RAS has been shown to be altered in type 1 and type 2 diabetes; this enzymatic cascade plays a critical role in the development of diabetic nephropathy (DN). Angiotensin-converting enzyme (ACE) and ACE2 are differentially regulated depending on its localization within the diabetic kidney. Furthermore, clinical and experimental studies have shown that circulating levels of sex hormones are clearly modulated in the context of diabetes, suggesting that sex-dependent RAS regulation may also be affected in these individuals. The effect of sex hormones on circulating and renal RAS may be involved in the sex differences observed in DN progression. In this paper we will review the influence of sex hormones on RAS expression and its relation to diabetic kidney disease. A better understanding of the sex dimorphism on RAS might provide a new approach for diabetic kidney disease treatment.

angiotensin-converting enzyme 2; angiotensin-converting enzyme; gender

DIABETES IS THE LEADING CAUSE of end-stage renal disease (ESRD) in the Western world, responsible for nearly one-half of all new ESRD cases in the United States (21). In addition, hypertension is a major risk factor for the development and progression of diabetic nephropathy (DN) (21). The renin-angiotensin system (RAS) is an important regulator of cardiovascular and renal function (100), and its blockade using angiotensin-converting enzyme inhibitors (ACEi) or angiotensin-receptor blockers (ARB) is the cornerstone in the treatment of DN (100). Interestingly, sex differences in the renal response to RAS blockade have been demonstrated (115). Clinical and experimental studies have also shown sex differences in RAS components in several tissues and in the circulation (33, 94, 100, 140). In addition, chemical and surgical castration modulate the expression of different RAS components such as angiotensinogen (AOGEN), renin, angiotensin-converting enzyme (ACE), and ACE2 (24, 57, 59).

Sexual dimorphism on the progression of renal disease has become an area of active investigation (83, 110). The mechanisms responsible for sexual dimorphism in diabetic pathology represent an area of investigation (81). In diabetic people, men are at higher risk than premenopausal women for microvascular complications, such as nephropathy (2). The relationship between RAS and the progression of diabetic renal disease has been widely studied. However, the specific mechanisms in which sex hormones such as dihydrotestosterone (DHT) and 17β-estradiol (E_2) modulate RAS expression in DN remain unclear. For this reason, we aimed to review the more relevant clinical and experimental studies focused on the sex differences in DN, the influence of sex hormones in RAS regulation, and the effect of diabetes on androgen and estrogen levels.

SEX DIFFERENCES IN DIABETIC NEPHROPATHY

Clinical Studies

Sex differences in diabetic nephropathy progression in type 1 diabetic patients. Several studies suggest that males with type 1 diabetes mellitus (T1DM) have significantly higher rates of decline in glomerular filtration rate (GFR), and an increased risk of developing microalbuminuria and progressing to macroalbuminuria than women (84). In particular, a large nationalwide prospective study from Germany in 27,805 type 1 diabetic patients reported that the male sex was associated with the development of macroalbuminuria. This study was conducted in children, adolescents, and adults with a follow-up time of 2.5 yr. Interestingly, childhood diabetes onset was found to be protective against the development of micro- or macroalbuminuria while the majority of the patients showing macroalbuminuria or ESRD at the last visit were adults (mean age 37.2 yr). These observations suggest that high male sex hormone levels in the onset of diabetes predispose the patients to a worsened outcome in terms of renal disease (97). In concordance, Orchard et al., in a large epidemiological study of 657 type 1 diabetic subjects diagnosed in childhood, observed a higher risk of nephropathy in men coupled with increased progression from microalbuminuria to macroalbuminuria compared with female subjects (86). Subsequently, in their study of predictors of microalbuminuria in 340 normotensive patients with T1DM, Villar et al. (129) found male sex to be a predictor of progression to microalbuminuria, independent of glycated hemoglobin levels. In a population of subjects with insulin-requiring diabetes, some of whom had T1DM, men had sig-

Address for reprint requests and other correspondence: M. J. Soler, Dept. of Nephrology, Hospital del Mar, Passeig Maritim 25-29, 08003 Barcelona, Spain (e-mail: msoler@parcdesalutmar.cat).

nificantly more microalbuminuria than women. This study found that hypertension and obesity were associated with an increase in albumin excretion rate (UAE) (91). In addition, the incidence of ESRD in people with diabetes in the United States was studied (47). Among Caucasians younger than 45 yr old, the progression to ESRD was significantly increased in men compared with women (7.3/100,000 vs. 2.8/100,000, average annual increments in risk of ESRD) (47). However, the protective effect of the female sex with T1DM in the progression to ESRD was lost after menopause (47). One may surmise that, when women lose female hormones, the positive effect disappears, and the progression of diabetic kidney disease is not favorable (Fig. 1).

Sex differences in diabetic nephropathy progression in type 2 diabetic patients. Male sex has also been associated with higher rates of albuminuria compared with females in the context of type 2 diabetes mellitus (T2DM) (90, 98, 107). A prospective and cross-sectional study of the prevalence and causes of persistent albuminuria (>300 mg/24 h) conducted in 224 males and 139 females with T2DM, age <66 yr, revealed a higher prevalence of albuminuria in males (19%) than in females (5%) (90). To further examine the risk factors associ-

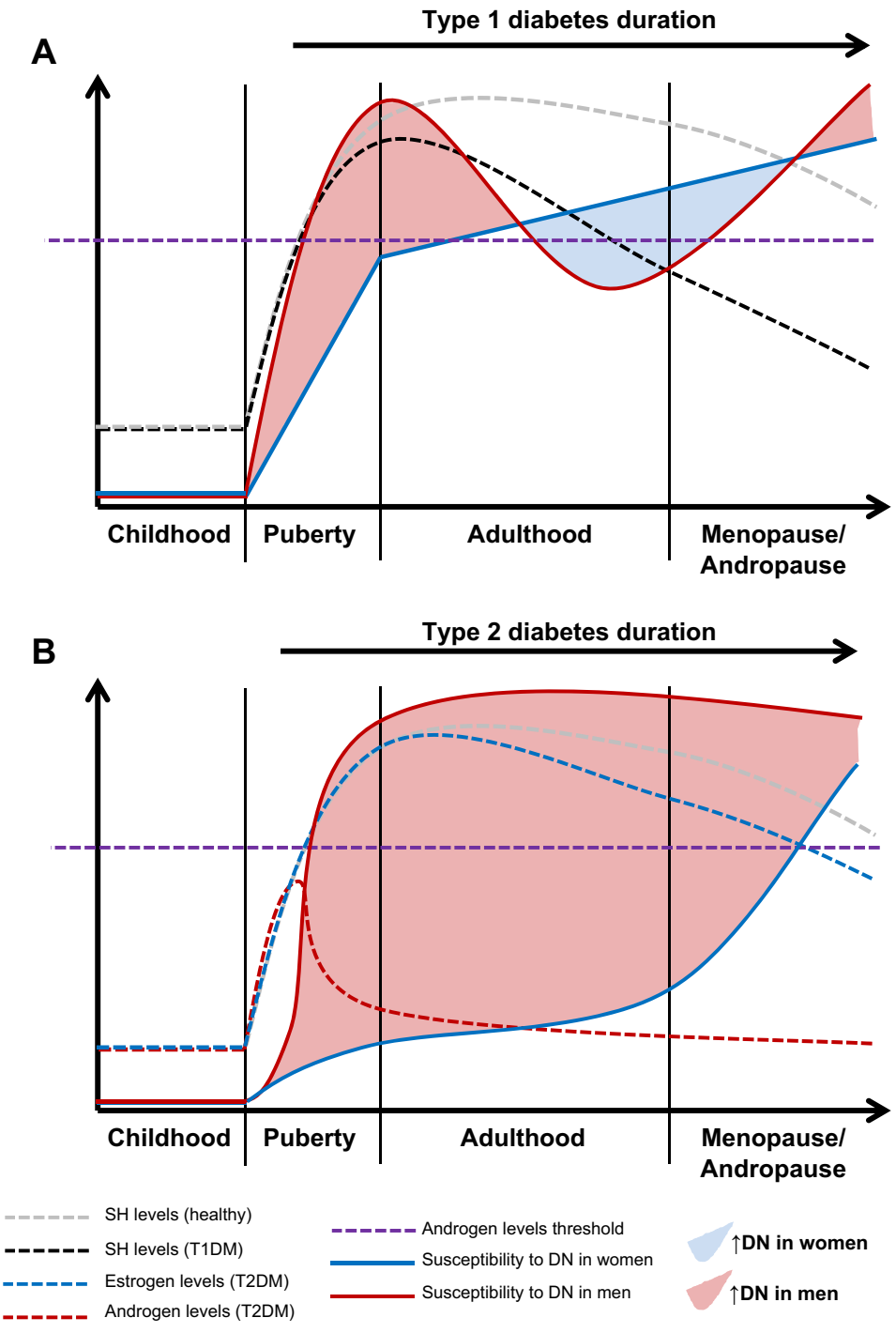

Fig. 1. Schematic representation depicting the evolution of sex hormone levels throughout a lifespan under type 1 (*A*) or type 2 (*B*) diabetic conditions, and its corresponding effect on sex-specific susceptibility to develop diabetic nephropathy (DN). Under physiological conditions, male and female sex hormones dramatically increase at puberty, remain elevated during adulthood, and slightly decrease until the onset of andropause or menopause, when such decrease is clearly accentuated. During puberty, high levels of estrogens in female adolescents are protective against DN while high androgen levels predispose male adolescents to albuminuria (area depicted in red). *A*: after the onset of type 1 diabetes mellitus (T1DM), sex hormone levels are reduced, increasing the susceptibility of women to develop DN compared with men (area depicted in blue). When androgen levels are diminished below a certain threshold due to age and diabetes progression, susceptibility to DN in men increases again. *B*: type 2 diabetes mellitus (T2DM) is characterized by very low levels of androgens and higher levels of estrogens compared with T1DM. As a consequence, the incidence and severity of DN is clearly augmented in men throughout life. After menopause, the decrease of estrogen levels in type 2 diabetic women is accompanied by a rise in the progression of DN, attenuating the difference between the sexes at later stages in life. SH, sex hormone.

ated with UAE in T2DM, Savage et al. recruited 933 patients with T2DM from the appropriate blood pressure control in diabetes trial and classified them according to urinary albumin excretion (UAE) status: normoalbuminuria (<20 pg/min), microalbuminuria (20–200 pg/min), and macroalbuminuria (>200 pg/min). Using univariate analyses, it was found that the male sex significantly correlated with microalbuminuria and macroalbuminuria, together with Hispanic ethnicity, African-American race, poor glycemic control, insulin use, long duration of diabetes, dyslipidemia, diastolic and systolic hypertension, smoking, and obesity. However, sex differences were lost in the multivariate analysis. It is worth noting that in this study the mean age was 59 yr (107). Another prospective long-term follow-up study conducted on 574 patients, aged 40–60 yr, with recent onset of T2DM showed that male sex was associated with DN according to the final value of UAE, together with low levels of high-density lipoprotein, body mass index, cigarette smoking, and low socioeconomic status (98). Altogether these results suggest that the deleterious effect of the male gender in the development and progression of DN are related to the patient age and subsequently the hormonal changes that are observed with aging (Fig. 1). In a study of national US and United Kingdom heart disease mortality for three birth cohorts (1916-25, 1926-35, and 1936-45), all birth cohort's linear heart disease mortality rates peaked in men around age 45, with slower age-related increases thereafter. Conversely, in women there was no accelerated increase in heart disease mortality rate at age 50 (menopause). In both sexes, proportional increases fit the data better than absolute increases, presumably reflecting competing risks with aging. The authors concluded that deceleration of the age-related increase in male heart disease mortality in midlife explained sex differences in cardiovascular mortality better than postmenopausal estrogen deficiency in women. Thus, at a younger age, diabetic men have an increased risk in cardiovascular diseases and DN compared with women, but once the disease is present and progresses over the years, it seems that renal- and cardiovascular-related mortality tend to equilibrate (8).

Effect of estrogen replacement therapy and oral contraceptives in DN. Estrogen replacement therapy (ERT) in postmenopausal women has shown to exert beneficial effects by attenuating diabetic complications within the kidney. Short-term administration of estrogen alone or together with norgestrel (a synthetic progestin) has been shown to reduce proteinuria and improve creatinine clearance in diabetic and hypertensive postmenopausal women (117). In addition, short-term treatment with raloxifene [a selective estrogen receptor (ER) modulator] limited the progression of albuminuria in 39 postmenopausal women with T2DM included in a 6-mo double-blind placebo-controlled trial (40). Interestingly, ERT has shown to improve not only renal function but also metabolic control in postmenopausal women with diabetes mellitus. In a systemic review and meta-analysis of 16 studies comprised of 17,971 cases, postmenopausal women taking low-dose combined ERT (estrogen and progesterone) showed a decreased risk of developing diabetes and better diabetic control. Specifically, ERT significantly reduced the incidence of diabetes and the levels of fasting plasma glucose, hemoglobin A_{1c} (HbA_{1c}), total cholesterol, and low-density lipoprotein (139). In contrast, ERT had no significant effect on microalbuminuria, glucose levels, or lipid profile in 60 healthy postmenopausal patients receiving E_2 in a prospective randomized double-blind placebo-controlled study (64). In summary, the protective role of estrogens within the kidney seems to be more clinically relevant in the context of diabetes, suggesting that restoring female sex hormone levels in diabetic women may attenuate the effect of profibrotic and proinflammatory factors such as high glucose and ANG II (49, 56).

Hormonal contraceptives treatments, in particular combined oral contraceptives (OC), are well known to increase the cardiovascular risk and affect the metabolic system by inducing changes in lipids, lipoproteins, carbohydrate metabolism, and hemodinamic factors (108). In concordance, several authors have reported that the use of OC at a high dose is associated with elevated glucose and insulin levels, higher rates of impaired glucose tolerance, and adverse effects on lipid profile and blood pressure (36, 43, 130, 135). As a consequence, OC can alter RAS and exert a detrimental effect in diabetic kidneys. Ahmed et al. reported in a prospective observational study with type 1 diabetic women a strong association between OC use and the angiotensin-dependent control of renal circulation in addition to the development of macroalbuminuria, highlighting OC use as a risk factor for DN (3). These adverse effects, however, are almost absent in clinical trials using progestin-only (27) and low-dose contraceptives (124). The use of high-dose OC for a prolonged period may cause hyperestrogenicity and lead to an adaptation state in which the beneficial actions of estrogens are downregulated. In addition, high doses of OC may overactivate estrogen-regulated cellular pathways in a detrimental manner (72). The differences observed between the estrogen hormone replacement regarding DN development and progression may be related to the baseline hormonal status of the women. When given in a deficient status (as it is in menopause), their effects are clearly beneficial, whereas in fertile women as anticonceptive therapy, they exert a deleterious effect. Thus, it seems that a hormonal balance is needed to maintain a decreased DN progression.

Effect of androgen replacement therapy in DN. Testosterone replacement therapy (TRT) in men with T2DM improved some of the key parameters associated with metabolic syndrome in several observational, retrospective, and prospective trials (38, 55, 79, 119). In particular, body weight, waist circumference, blood pressure, heart rate, fasting blood glucose and insulin sensitivity, as well as HbA_{1c}, triglycerides, cholesterol, and low-density lipoprotein levels were significantly improved in type 2 diabetic men receiving TRT for at least 24 mo (34, 123, 141). In concordance, TRT in nine men with T1DM, erectile dysfunction, and hypogonadism improved in glycemic control, lipid profiles, and erectile function (104). Note that some observational studies have reported that TRT increases cardiovascular events in patients with metabolic syndrome (119). However, the Food and Drug Administration in the US has reviewed these reports and found them to be seriously flawed (22). Although the beneficial effects of TRT in diabetic men have been widely demonstrated in terms of metabolic parameters, whether they are accompanied by an improvement in kidney function in patients with renal complications remains unclear. To our knowledge, interventional clinical trials evaluating the influence of TRT in albuminuria and other typical alterations of DN have not been conducted.

Experimental Studies

Sex differences in experimental type 1 diabetes. Different studies in experimental models of diabetes have been performed to analyze the role of sex hormones on DN (6, 116, 138). Sex differences in several hallmarks of diabetic kidney disease have been assessed in the streptozotocin (STZ) model. In STZ-induced 6-wk-old Sprague-Dawley rats, 12 wk of T1DM led to significantly higher albuminuria and systolic blood pressure in diabetic males compared with females. In addition, diabetic males, but not females, showed increased renal collagen I and fibronectin mRNA levels compared with controls (24). In contrast, when STZ was administrated to 11-wk-old mRen2.Lewis hypertensive rats, diabetic females exhibited a marked increase in the inflammatory marker C-reactive protein that was not evident in the diabetic males. This alteration observed in females was associated with an increase in proteinuria and albuminuria after 4 wk of follow-up. Diabetic and hypertensive females also exhibited greater glomerular vascular endothelial growth factor staining and higher levels of inflammation in terms of tubulointerstitial $CD68^+$ cells within the kidney (140). Of note, the onset and duration of diabetes in these studies were clearly different (24, 140), which probably determined a different hormonal status at the end of each follow-up. These data suggest that sex-specific susceptibility to develop certain features of DN can vary according to different factors, such as age, diabetes duration, and the presence of hypertension.

Sex differences in experimental type 2 diabetes. Few studies have been focused on the study of sex differences in experimental T2DM. Slyvka et al. demonstrated that female obese Zucker rats (*fa*/*fa*) showed better renal function than males at 13 wk of age. In addition, males exerted higher levels of eNOS and nNOS mRNA (cortex) and higher protein levels of eNOS (cortex and medulla), nNOS (medulla), and iNOS (cortex) than females. These differences observed may indicate upregulation of NOS isoforms in males compared with females in an attempt to increase NO levels and vasodilation (111). In another murine model of T2DM, the high-fat diet model, males showed increased blood glucose, UAE, and kidney weight compared with females. However, GFR was unchanged (85). To our knowledge, no other studies on DN and sex differences have been performed in models of T2DM.

Experimental studies with androgen supplementation or deprivation. Testosterone administration promotes tubular damage in STZ-induced rats. Sun et al. demonstrated that testosterone worsens tubular damage in diabetic rats in terms of increased fibrotic markers, such as α-smooth muscle actin and fibroblast-specific protein, two markers of cell damage and potential epithelial mesenchymal transition (116). In concordance, Xu et al. demonstrated that the administration of a high dose of DHT also exacerbated the development of albuminuria, index of glomerulosclerosis, and tubulointerstitial fibrosis associated with diabetes. However, a lower dose of DHT attenuated renal injury in castrated diabetic rats. DHT may play an important role in the pathophysiology of diabetic renal disease, and these effects are dose-dependent (138). Thus, a dual and dose-specific effect of DHT in the diabetic kidney has been observed; while the administration of low doses of DHT is renoprotective, higher doses are damaging. The determinants of these seemingly opposing effects of DHT remain unclear. There are several potential explanations for this apparent paradox, including dose-dependent expression and activation of androgen receptor (AR) interacting with transcriptional coactivator proteins; however, the most likely explanation is that of the indirect effect of E_2 rather than the direct effects of DHT. Interestingly, while diabetic male rats previously presented a reduction in the ratio of AR to ER protein expression in the renal cortex compared with nondiabetic animals (67, 96), the dual treatment with DHT and anastrozole restored this ratio (66). Considering that changes in sex hormone receptor expression in the diabetic kidney may reflect altered levels of circulating androgens and estrogens, it is conceivable that the diminished renal alterations observed after this dual treatment were achieved by restoring relative balance between sex hormones.

The effect of surgical androgen depletion by castration is controversial. While Xu et al. showed that castration worsens albuminuria as a marker of renal function in type 1 diabetic rats (138), in the Otsuka-Long Evans-Tokushima fatty (OLETF) rat, a model of T2DM (121), and the Cohen diabetic rat, a genetically selected sucrose-fed rat, castration attenuated proteinuria (20). In addition, in the STZ model of T1DM, castration was shown to have neither a detrimental nor a protective effect on the progression of diabetic renal disease (116). These apparent discrepancies in the effects of castration on diabetic renal disease may be ascribed to the duration and model of diabetic renal disease.

Experimental studies with estrogen supplementation or deprivation. Supplementation with E_2 has shown to exert a protective effect on the development of functional and structural kidney damage by reducing albuminuria, glomeruloesclerosis, and tubulointerstitial fibrosis after several weeks of untreated diabetes (26, 68, 69). These effects have been attributed to different cellular mechanisms, including reduction of TGF-β synthesis, decreased accumulation of collagen type IV, laminin, and fibronectin, and increased production of matrix metalloproteinases (MMP) (31, 82, 95). In this sense, studies in knockout of estrogen receptor-α ($ER\alpha^{-/-}$) in nondiabetic mice and ovariectomy in diabetic rats were associated with increased renal expression of TGF-β, and E_2 supplementation to ovariectomized rats normalized TGF-β expression. Similarly, treatment of intact diabetic (*db*/*db*) mice with E_2 decreased TGF-β expression in podocytes compared with *db*/*db* mice not treated with E_2. Furthermore, in female mice overexpressing TGF-β, treatment with E_2 ameliorated TGF-β-induced progressive kidney disease without decreasing TGF-β expression; in fact, E_2-treated mice had higher levels of TGF-β than untreated mice. This suggests that the most important effect of E_2 is to disrupt TGF-β signaling, rather than to regulate its expression (25). Raloxifene, a selective ER modulator, has also been shown to diminish these renal alterations via similar mechanisms (19, 26).

The protective effects of estrogens have also been described in podocytes. E_2 treatment protected nondiabetic podocytes from apoptosis induced in vitro by TGF-β and TNF-α (29). Such effect may be mediated by activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-protein kinase B (AKT) signaling cascade, since podocytes isolated from E_2-treated *db*/*db* mice presented increased levels of AKT phosphorylation. Activation of extracellular signal-regulated kinases (ERKs), another downstream path-

way of TGF-β signaling, was decreased in these E_2-treated podocytes. Lower activation of ERK may lead to increased expression of MMP2 and MMP9, which could explain the amelioration of extracellular matrix (ECM) accumulation and glomerular basement membrane thickening observed in these mice. In this work, tamoxifen also modulated podocyte signaling pathways via upregulation of ERβ (15). In addition, estrogens are thought to potentially decrease reactive oxygen species (ROS)-induced events by regulating podocyte antioxidant markers, such as Mn-superoxide dismutase and glutathione (17).

In contrast, ovariectomy caused a significant decrease in the incidence of nephropathy (20) while E_2 administration exacerbated renal disease (101) in Cohen sucrose-fed diabetic rats. In the diabetic OLETF rat, treatment with E_2 had no effect on albuminuria, although it diminished mesangial expansion and glomerulosclerosis (121). Once again, different hormone circulating levels and ER expression may represent a reasonable explanation for these discrepancies. In this sense, it has been demonstrated that, after experimental induction of menopause by selectively killing the small primordial and primary ovarian follicles through 4-vinylcyclohexene diepoxide (VCD) administration, renal damage develops more rapidly and severely in diabetic postovarian failure female mice compared with cycling females (50). In this study, cortical mRNA abundance of MMP9 was decreased after menopause, strengthening the results from previous in vivo studies in which MMP9 protein expression and activity level were decreased in ovariectomized diabetic (69) and Dahl salt-sensitive (71) rats. In concordance, in vitro experiments in mesangial cells in nonhyperglycemic conditions found increased MMP9 expression after estrogen treatment (95). Thus, loss of estrogens after menopause may explain the observed decrease in MMP9 expression, which can possibly lead to pathological accumulation of ECM.

SEX DIFFERENCES ON RAS IN DIABETIC NEPHROPATHY

Clinical studies have shown that inhibiting the ACE-ANG II-ANG II type 1 receptor (AT_1R) axis through the action of ACEi or ARB slows the progression of chronic kidney disease (CKD), especially when the renal disease is associated with proteinuria (11, 56, 118, 142). Thus, clinical guidelines recommend RAS blockade in patients with diabetic kidney disease (4). Interestingly, it has been demonstrated that renal and peripheral hemodynamic responses to RAS activation may vary according to sex (18, 76). To design more specific and efficient treatments for DN, understanding this sex effect has become a critical issue during the last decades. For this purpose, a large number of studies assessing sex differences at different levels of renal and circulating RAS in the context of diabetes have been performed.

Sex Differences on RAS Activation: Angiotensinogen and Renin

Renal AOGEN expression was increased in males (but not in females), showing strong association with albuminuria and renal fibrosis in STZ diabetic Sprague-Dawley rats. On the contrary, the plasma renin activity and renal renin mRNA levels were decreased in both diabetic males and females. Androgen blockade by flutamide administration decreased UAE only in diabetic males without affecting the endocrine or renal RAS (24). In concordance to cortical data, diabetes increased the medullary AOGEN content in male STZ Wistar rats. In addition, a diabetes-induced increase of urinary AOGEN was also found (92) (Fig. 2). Thus, the increase of renal AOGEN in the setting of diabetes is mainly observed in males, whereas the AOGEN decrease in plasma from diabetic animals is observed in both males and females. Again, these results suggest that there is a difference in the regulation of intrarenal and circulating RAS depending on the sex. These differences between the tissue and circulating RAS combined with the tissue sex divergences add complexity to the system (see Fig. 2). In rodent models (13, 46) and in patients with diabetes (48), it has been reported that ROS are important for intrarenal AOGEN augmentation in the progression of DN, highlighting the importance of the activated oxidative stress-AOGEN-RAS axis in the pathogenesis of DN. In addition, the redox-responsive transition of AOGEN to a form that preferentially interacts with receptor-bound renin has been demonstrated by crystallography and kinetic analysis (143). Clinical and experimental studies have provided evidence that oxidative damage parameters in renal tissue may vary according to sex (23, 126). It is conceivable that sex differences on renal RAS hyperactivation are due not only to genomic actions of sex hormones directly on AOGEN and renin genes, but also to sex-specific modulation of the oxidative stress status within the diabetic kidney (Fig. 2).

In contrast, in the context of diabetes and hypertension, circulating AOGEN is decreased also in both males and females. Interestingly, diabetic males showed higher plasma AOGEN compared with females (Fig. 2). Surprisingly, hyperglycemia was also associated with increased renal AOGEN and renin expression only in females, whereas the urinary excretion of AOGEN was similarly increased in both sexes (140). In this study, T1DM was accompanied with renal inflammation. It has been demonstrated that inflammatory cytokines, IL-1 and IL-6, can inhibit renin promoter activity via ERKs and signal transducer and activator of transcription 3 (STAT3) (60, 89). Both ERK and STAT3 are involved in DHT-independent AR activation and translocation to the nucleus (62, 93). Briefly, activation of the PI3K/Akt pathway results in phosphorylation and activation of AR (58). Activated STAT3 can form a heterologous complex with the phosphorylated AR (STAT3-AR) by interacting directly with amino acids 234–558 in the NH_2-terminal domain of the receptor (125). This interaction takes place whenever both STAT3 and AR are activated, for example, as a response to epidermal growth factor or IL-6 (1), resulting in enhanced AR-mediated transcriptional activity (74) (Fig. 3). Diabetes is associated with lower levels of androgens (53, 70); thus, decreased male sex hormones in the context of diabetes and hypertension may favor the ERK/STAT3-mediated inhibition of renin expression and explain the lack of renin upregulation within the male diabetic kidney. Taken together, these results suggest that lower levels of renal AOGEN in diabetic hypertensive males may be ascribed, at least in part, to a higher ANG II-mediated turnover of renal AOGEN compared with diabetic females (140). In summary, sex-dependent effect of diabetes on renal AOGEN and renin expression may vary in the context of hypertension.

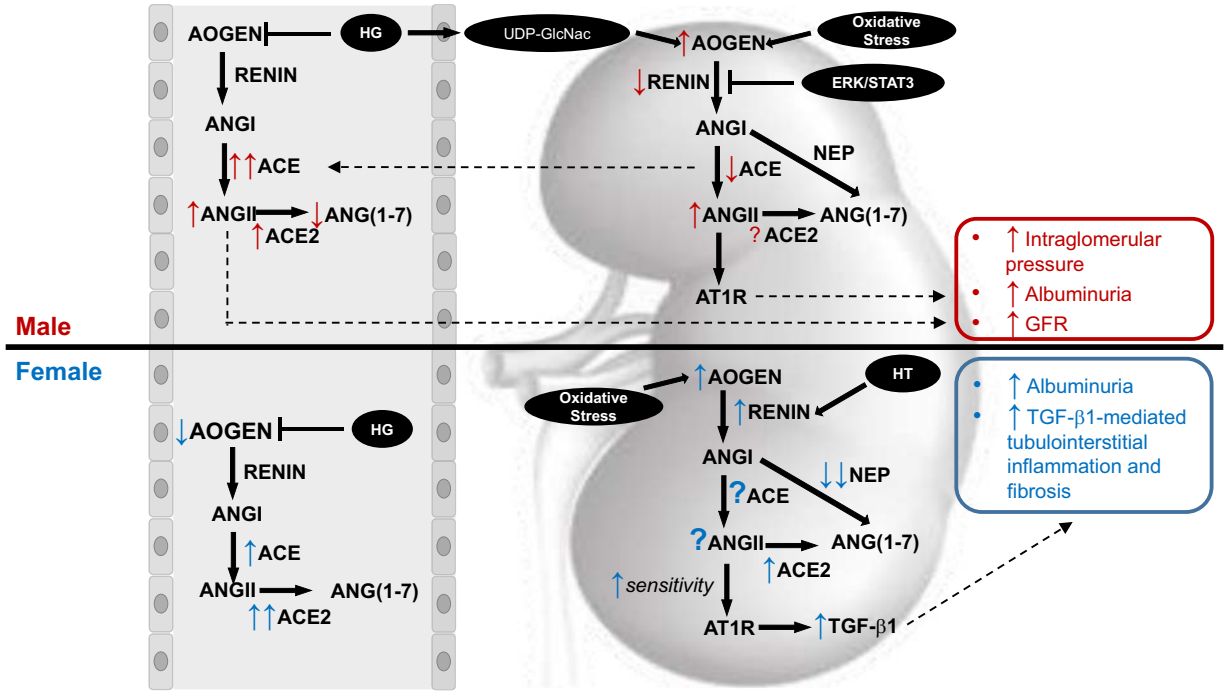

Fig. 2. Sex differences in diabetic nephropathy in circulating and renal renin-angiotensin system (RAS). Red arrows indicate effects associated with androgens or male sex, whereas blue arrows correspond to estrogens/female sex. In diabetic males, hyperglycemia [via the uridine diphosphate *N*-acetylglucosamine (UDP-GlcNac) pathway] and oxidative stress provoke augmentation of renal angiotensinogen (AOGEN). Inhibition of renin synthesis via extracellular signal-regulated kinase (ERK)/signal transducer and activator of transcription 3 (STAT3) and reduced renal angiotensin-converting enzyme (ACE) may partially prevent ANG II accumulation. However, despite that the dramatic increases in circulating ACE and consequently ANG II are not always accompanied by elevated blood pressure, they seem to lead to hemodynamic alterations reflected in glomerular hypelfiltration and albuminuria. In diabetic females, the more pronounced increase in circulating ACE2 may result in protection against hypertension. At the kidney level, however, diabetes-associated decrease in estrogen levels results in RAS hyperactivation and stimulation of the most relevant ANG II-mediated effector mechanisms such as TGF-β1. HG, hyperglycemia; HT; hypertension; GFR, glomerular filtration rate.

Sex Differences on RAS Regulatory Arms: ACE and ACE2

Circulating ACE activity is increased in diabetic mRen2.Lewis rats. Interestingly, this increase was more pronounced in males compared with females (140) (Fig. 2). In contrast, a decrease in renal ACE expression has been described in both male and female mice (88, 134, 136). However, studies focused on the assessment of renal ACE expression in the context of diabetes and sex differences are lacking.

Different groups have studied sex differences regarding ACE2 activity. Soro-Paavonen et al. found that males had significantly higher ACE2 activity than females, both among patients with T1DM and healthy individuals (113). For this reason, the analysis of their study was performed separately for males and females. In concordance, in CKD patients without previous history of cardiovascular disease, our group recently reported that ACE2 activity from human EDTA-plasma samples is significantly increased in males compared with females. In addition, in a multivariate analysis, circulating ACE2 activity directly correlated with the classical cardiovascular risk factors, namely older age, diabetes, and male sex (7). Increased activity of circulating ACE2 has also been detected in males compared with females after kidney transplant (112) and in hemodialysis patients (99). Mizuiri et al. did not find sexual dimorphism in urinary ACE2 activity or protein levels in patients with CKD (78). Surprisingly, when analyzing urine, ACE2 mRNA levels were significantly increased in females compared with males in renal transplant patients with diabetes (137). These results may be ascribed to an increased ACE2 shedding from female diabetic proximal tubules. Taken together, these data reinforce the idea that separate analyses for males and females should be considered when measuring ACE2 activity in future studies. The increase of circulating ACE2 activity in males may be an early marker of increased risk of cardiovascular disease in CKD patients. Oudit et al. studied the effect of ACE2 deletion in nondiabetic kidneys from male and female mice. Their data showed that loss of ACE2 in male (but not female) C57BL/6 mice is associated with the development of age- and ANG II-dependent glomerular damage (87). Gupte et al. also used ACE2-deficient mice to investigate the mechanistic role of ACE2 on the development of obesity-associated hypertension in males vs. females. They observed that male high-fat-fed $ACE2^{-/y}$ mice had significantly greater systolic blood pressure compared with high-fat-fed $ACE2^{-/-}$ females (39). These data suggest that males have a higher dependence on ACE2-mediated renoprotection.

In experimental studies with hypertensive and diabetic animals, kidney ACE2 activity did not change in females but showed a 30% reduction in the diabetic males compared with their controls (140). In addition, circulating ACE2 activity was significantly increased in both male (3-fold) and female (9-fold) diabetic mice. Despite the marked increase in circulating ACE2 and the maintenance of renal ACE2 activity, female

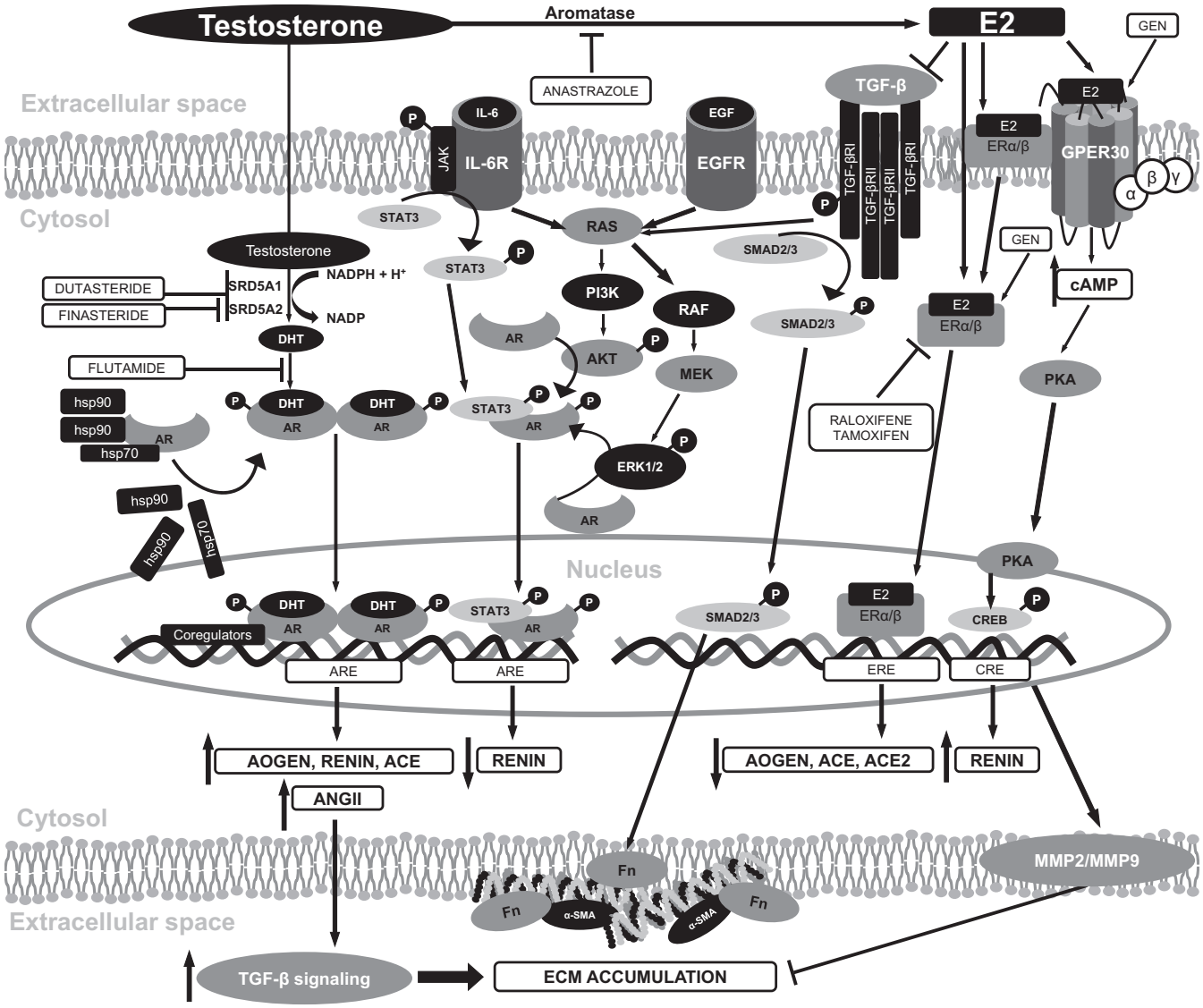

Fig. 3. Molecular mechanisms of androgens and estrogens and their putative relationship to RAS and other relevant pathways within the kidney. Classical genomic androgen actions involve conversion of testosterone to dihydrotestosterone (DHT) by 5α-reductase, binding of DHT to androgen receptor (AR), and dimerization and phosphorylation of the receptor, resulting in a higher activation of RAS and increased production of extracellular matrix (ECM). However, AR can also exert genomic effects and downregulate renin expression through a DHT-independent mechanism that involves AR phosphorylation by phospho (p)-AKT or pERK1/2 and the formation of a complex with pSTAT3. Testosterone can also be metabolized to estradiol (E_2) by aromatase. Genomic actions of estrogens involve their binding to ERα or ERβ in the cell membrane or in the cytosol, followed by translocation to the nucleus, and have been attributed to downregulation of several RAS components, such as AOGEN, ACE, and ACE2. Estrogens can also exert nongenomic actions by binding to G protein-coupled estrogen receptor (GPER) 30 receptor in the cell membrane, leading to renin upregulation and increased matrix metalloproteinase (MMP) 2 and MMP9, which are associated with ECM degradation. Thus, changes in sex hormone levels under diabetic conditions may lead to impaired ECM metabolism and RAS alterations that ultimately contribute to the development of nephropathy. GEN, genistein; ER, estrogen receptor; SRD5A1, 5α-reductase type 1; SRD5A2, 5α-reductase type 2; hsp, heat shock protein; IL-6, interleukin 6; JAK, janus kinase; AKT, protein kinase B; MEK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; EGF, epidermal growth factor; TGF, transforming growth factor; PKA, protein kinase A; ARE, androgen response element; ERE, estrogen response element; CRE, cAMP-response element; CREB, cAMP-response element-binding protein.

mRen2.Lewis diabetic rats were not protected from vascular damage, renal inflammation, and kidney injury in this model of early STZ-induced diabetes.

Sex Differences on RAS Effector Mechanisms: Angiotensin Peptides and Their Receptors

Experimental studies demonstrated that males have greater expression of "classical" components of the RAS, including ANG II and AT_1R, whereas females have greater expression of "non-classical" components of the RAS, including ANG II type 2 receptor (AT_2R) and ANG-(1–7) (12, 122). To our knowledge, only one experimental study has assessed sex differences on renal and circulating ANG II levels in DN. In this work, STZ-induced mRen2.Lewis rats presented augmented plasma levels of angiotensin peptides compared with controls, with no significant variations within the kidney. Diabetic and hypertensive males showed increased circulating and renal ANG II as well as decreased ANG-(1–7) in plasma compared with females (140) (Fig. 2). It is well accepted that ANG II mediates progressive diabetic kidney injury by enhancing renal fibrosis and inflammation (102, 103) via

stimulation of growth factor TGF-β expression (9, 133). Interestingly, in vitro experiments revealed that E_2 is capable of inhibiting TGF-β-mediated upregulation of α_1 type IV collagen gene transcription in murine mesangial cells (109). Thus, decreased estrogen levels in the context of aging or diabetes progression might be responsible, at least in part, for the increased susceptibility to ANG II-induced renal alterations in postmenopausal or diabetic women. Further investigations are needed to confirm if the sex differences on angiotensin peptide levels observed under physiological and hypertensive conditions are changed or maintained in the presence of diabetes.

Sex Differences on RAS Blockade in Diabetic Nephropathy

Clinical studies. Renal hemodynamic responses to RAS blockade differ between men and women. Miller et al. evaluated the effects of 8 wk of ARB irbesartan administration on the pressor response to ANG II ($3\ ng{\cdot}kg^{-1}{\cdot}min^{-1}$) in young healthy men ($n = 15$; mean age = 27 yr) and women ($n = 15$; mean age = 28 yr). In this study, lower dosages of irbesartan in women achieved significantly reduced ANG II sensitivity compared with men. Interestingly, after 8 wk of irbesartan administration, AT_1R gene expression was decreased in women but not in men skin biopsies compared with baseline levels, indicating that blocking the ANG II-AT_1R interaction can result in decreased expression of the receptor, enhancing the favorable effects of the compound in a sex- and dose-dependent manner (76). In turn, Cherney et al. studied sex differences in the renal response to hyperglycemia and ACE inhibition after 21 days of treatment with enalapril in young adolescents with uncomplicated T1DM. During clamped hyperglycemia, only females exhibited reductions in renal plasma and blood flow, as well as increased renal vascular resistance and filtration fraction. After ACE inhibition treatment, both sexes exhibited significant declines in arterial pressure, but only females displayed a reduction in GFR and filtration fraction (18). In both studies, the female sex was associated with a major sensitivity to RAS blockade, suggesting that renal alterations due to RAS dysregulation in T1DM may be more relevant in women. Accordingly, clinical trials studying the effects of irbesartan (56) or losartan (49) on the progression of nephropathy in T2DM found a protective role of male sex, with albuminuria progressing more rapidly in women. Once again, the inclusion in these studies of many postmenopausal women could have played a significant role in the outcome of these results.

Experimental studies. At an experimental level, both ACE inhibitors and ARBs have been shown to block the development of renal injury in both type 1 (61) and type 2 (42, 77, 144) diabetic male rodents. However, less is known regarding the effects of RAS inhibition on kidney disease in females, especially in the context of DN. To our knowledge, only Kelly et al. compared the effect of valsartan treatment with the endothelin receptor blockade in the STZ-induced diabetic female Ren-2 rat (51). They found that the administration of the AT_1R antagonist valsartan reduced systolic blood pressure, ameliorated kidney lesions, and improved the renal function in female rats overexpressing ANG II.

In recent years, many authors have examined the impact of compound 21 (C21), a selective AT_2R agonist, on DN. In STZ-induced type 1 diabetic male mice, C21 treatment showed a renoprotective effect by significantly attenuating renal hypertrophy and levels of cystatin C, albuminuria, mesangial expansion, and glomerulosclerosis, in association with inhibited expression of various proteins implicated in oxidative stress, inflammation, and fibrosis (54). In concordance, C21 improved albuminuria through the prevention of renal inflammation and production of NO and cGMP in STZ-induced male Sprague-Dawley rats (73). In T2DM, C21 treatment in combination with losartan showed an additive effect on reducing albuminuria and slowing the progression of nephropathy in male Zucker diabetic fatty rats (14). Acute C21 administration improved renal function in female, but not in male, spontaneously hypertensive (SHR) rats. Considering that AT_2R are expressed to a greater extent in the kidney of female SHR rats (44), this effect of C21 in DN prevention seems to be more effective in female rats than in males.

From our perspective, there is a lack of clear evidence regarding sex differences in experimental RAS blockade or modulation in DN. For this reason, male and female animals should be simultaneously included in future studies evaluating the potential effect of a specific compound on RAS activation and DN. Despite the increasing number of publications demonstrating sex differences in RAS expression in DN, the complexity of this pathology and the fact that sex hormones are altered in the setting of hyperglycemia make it more challenging to elucidate the specific molecular mechanisms by which androgens and estrogens modulate RAS expression.

EFFECT OF DIABETES ON ANDROGEN AND ESTROGEN LEVELS

Effect of Type 1 Diabetes on Androgen and Estrogen Levels

The relationship between T1DM and serum androgen levels is controversial. In some studies, men with T1DM do not appear to have a high prevalence of androgen deficiency (45, 52). However, Maric et al. demonstrated that diabetes without renal disease was associated with decreased testosterone and estrogen levels compared with healthy nondiabetic adult men. In this study, progression of renal disease from micro- to macroalbuminuria accentuated the decrease in serum total testosterone (70). Renal complications derived from T1DM are rarely observed before puberty (75). Interestingly, when studying females, Amin et al. found that testosterone levels were increased in T1DM patients with microalbuminuria compared with the normoalbuminuric ones (5). These results suggest that high androgen levels predispose T1DM females to the development of microvascular disease such as DN (Fig. 1).

In experimental models of T1DM, assessment of the effect of hyperglycemia on male fertility in rats revealed that animals injected with STZ also showed significant decrease in serum testosterone levels, which were accompanied by diminished testicular and epididymal weight (80). Interestingly, elevation of circulating testosterone by arecoline in rats with established T1DM was associated with increased levels of serum insulin and upregulation of critical genes related to β-cell regeneration, such as glucose transporter 2 (105). In experimental models, castration in T1DM male rats worsened renal injury accompanied by a reduction of serum testosterone and kidney AR expression. Interestingly, circulating E_2 levels and kidney aromatase activity remained increased after removal of male sex hormones, providing stronger evidence for extratesticular

sources of E_2, while the expression of kidney ERα was not altered (96, 138). These results may suggest that testosterone-E_2-ERα, rather than the testosterone-DHT-AR axis, plays a role in the development of nephropathy in T1DM males. Of note, proinflammatory cytokines are known to upregulate the activity of aromatase, to effectively reduce testosterone levels, and increase the intracellular concentration of E_2 (132). Thus, the particular inflammatory status in type 1 diabetic males might explain the divergences in E_2 patterns observed in different studies.

Whether the estrogen signaling pathway is also detrimental for diabetic females is controversial. Several authors have reported that T1DM is associated with decreased E_2 levels in human (28, 106, 114) and animal female subjects (63, 131). In the context of type 1 DN, decreased E_2 levels have been associated with an imbalance in the expression of renal ERs. Specifically, female diabetic kidney exhibited increased protein expression of ERα, but not ERβ (96, 131) (Fig. 3). While ovariectomy increased renal ERα and reduced ERβ expression in these diabetic females, E_2 administration caused the opposite effect (131). In this sense, it has been reported that the deletion of ERα in STZ-induced females attenuated the development of albuminuria and glomerular hypertrophy, suggesting a role of ERα on promoting harmful events in the kidney (63). Interestingly, the absence of ERα in nondiabetic mice was not protective and led to the development of glomerulosclerosis, probably due to accumulation of endogenous testosterone (30). Thus, despite that ERα-mediated actions may be beneficial under physiological conditions, it is presumable that decreased estrogen levels in females with T1DM promote pathological overexpression and hyperactivation of renal ERα that, together with a downregulation of the protective effects of ERβ, may contribute to a more severe progression of DN.

Effect of Type 2 Diabetes on Androgen and Estrogen Levels

Grossmann et al. demonstrated in a cross-sectional survey that testosterone deficiency is common in men with diabetes, regardless of the type (38). However, clinical evidence supports that low testosterone levels are more strongly associated with T2DM rather than T1DM. This tendency has been observed when studying either young or old patients with diabetes (16, 120). Poor glycemic control in Korean men with T2DM resulted in increased levels of fasting plasma glucose and HbA_{1c} values, the major markers of diabetes, which appeared to be associated with testosterone deficiency (53). In addition, diabetic men had also lower levels of sex hormone-binding globulin (SHBG) compared with nondiabetic men (10, 52). In fact, several prospective studies have shown that diabetes and metabolic syndrome are more strongly predicted by low SHBG than by low testosterone (41, 55, 128).

T2DM is associated with augmented E_2 levels in men (123, 124). These increased E_2 levels are associated with complications, such as atherosclerosis, in men with T2DM and metabolic syndrome (35, 65). The activation of G protein-coupled estrogen receptor (GPER) in isolated rat Leydig cells and adult human testis downregulates testosterone production (127). It is conceivable that these E_2-mediated mechanisms exacerbate the reduction on circulating testosterone levels in T2DM, conferring a reasonable explanation to the fact that type 2, but not type 1, diabetic men show a clearly increased susceptibility to develop DN than women (Fig. 1). These findings suggest that in T2DM males there is an imbalance between sex hormones that exacerbates DN.

When studying ERs, Doublier et al. found that the beneficial effects of E_2 treatment on attenuating DN in type 2 diabetic female mice were accompanied by increased ERβ but not ERα protein expression within the podocyte (29). While interaction between E_2 and ERα seems to be detrimental in T2DM, activation of ERβ can be considered renoprotective. Interestingly, it has been found that aldosterone activates GPER30 and induces rapid vascular effects (Fig. 3). Under physiological conditions, these GPER-mediated nongenomic effects are considered beneficial in the vasculature (37). However, T2DM in female *db*/*db* mice increased expression of GPER30 in mesenteric resistance arteries and impaired the vascular effects of aldosterone (32). Thus, hyperactivation of GPER30 also plays a role in the pathophysiology of type 2 DN, at least at the vascular level. These vascular alterations can be attributed to the hyperactivation of circulating RAS in diabetes, which may lead to higher ANG II levels and, in consequence, increased stimulation of aldosterone secretion by adrenal glands and further GPER30 activation.

CONCLUDING REMARKS

RAS and sex differences in DN progression are observed and seem to be related to differences in the hormone levels through the development of the disease. While the progression of DN is accentuated in young males, with aging, and the subsequent estrogen deprivation in women, these sex differences are lost. As a consequence, ERT has been shown to exert beneficial effects by attenuating diabetic complications within the kidney. Therefore, testosterone administration worsens tubular damage in diabetic rats in terms of increased fibrotic markers and cell damage. RAS modulation is crucial in the development and progression of diabetic kidney disease. Interestingly, sex differences on RAS in DN have been observed in terms of expression in RAS compounds and RAS blockade response. In fact, diabetes per se alters sex hormone levels in males and females. These deregulations of sex hormones may lead to sex-dependent imbalance of ACE/ACE2 that, in turn, can vary between the different compartments and segments within the kidney, adding more complexity to the understanding of DN. To assess this issue, further studies focused on the sexual dimorphism in tubular and glomerular ACE and ACE2 localization and expression at the gene, protein, and activity level are required. Sex-specific modulation of RAS cascade can lead to different degrees of intrarenal ANG II accumulation according to the sex and the etiology of the disease. In conclusion, understanding the hormonal alterations coupled with RAS differences that take place at every stage of DN and sex dimorphisms may be helpful in designing a specific therapeutic approach for delaying the progression of DN.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.C. and M.J.S. conception and design of research; S.C. and M.J.S. prepared figures; S.C., M.R., and M.J.S. drafted manuscript; S.C., M.R., J.P., and M.J.S. edited and revised manuscript; S.C., M.R., J.P., and M.J.S. approved final version of manuscript.

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