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GONADECTOMY PREVENTS THE INCREASE IN BLOOD PRESSURE AND GLOMERULAR INJURY IN ACE2 KNOCKOUT DIABETIC MALE MICE. EFFECTS ON RENIN-ANGIOTENSIN SYSTEM

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Abstract:	Angiotensin Converting Enzyme 2(ACE2) deletion worsens kidney injury, and its amplification ameliorates diabetic nephropathy(DN). Male sex increases the incidence, prevalence and progression of chronic kidney disease in our environment. Here, we studied the effect of ACE2 deficiency and gonadectomy on DN and its relationship with fibrosis, 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 ACE2KO male mice with and without gonadectomy. Diabetic ACE2KO showed increased blood pressure, glomerular injury and renal fibrosis as compared to diabetic wild-type. Gonadectomized diabetic ACE2KO presented a decrease in systolic and diastolic blood pressure. In the absence of ACE2, gonadectomy 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. Gonadectomy also reduced circulating ACE activity in ACE2KO mice. Loss of ACE2 modified the effect of gonadectomy 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 gonadectomy in control and diabetic ACE2KO but not in wild-type mice. Our results suggest that gonadectomy 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 blood pressure, modulation of RAS, and downregulation of Akt-related pathways.

ABBREVIATIONS

ACE: Angiotensin Converting Enzyme

ACR: Albumin-to-Creatinine Ratio

Akt: Protein Kinase B

Ang: Angiotensin

AOGEN: Angiotensinogen

DBP: Diastolic Blood Pressure

DN: Diabetic Nephropathy

GDX: Gonadectomy

GFR: Glomerular Filtration Rate

PAS: Periodic Acid Schiff

rACE: Renal ACE

RAS: Renin Angiotensin System

ROS: Reactive Oxygen Species

sACE: Serum ACE

SBP: Systolic Blood Pressure

STZ: Streptozotocin

UAE: Urinary Albumin Excretion

CONDENSED ABSTRACT

We first evaluated the effect of gonadectomy on ACE2KO diabetic male mice. Our work shows that gonadectomy prevents the increase in blood pressure and attenuates kidney injury (namely albuminuria, mesangial matrix expansion and podocyte loss) in ACE2KO diabetic mice. We also demonstrated a decrease in circulating ACE activity and modification in renal RAS expression in gonadectomized mice. Our results suggest that androgen-mediated modulations of renal RAS in context of diabetes and hypertension may be the objective of further studies to focus on personalized treatments to control diabetic nephropathy progression in males.

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Short title: GONADECTOMY IN DIABETIC ACE2KO AND RAS

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ABSTRACT

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Mice were injected with streptozotocin to induce diabetes and followed for 19 weeks. Physiological and renal parameters were studied in wild-type and ACE2KO male mice with and without gonadectomy.

Diabetic ACE2KO showed increased blood pressure, glomerular injury and renal fibrosis as compared to diabetic wild-type. Gonadectomized diabetic ACE2KO presented a decrease in blood pressure. In the absence of ACE2, gonadectomy 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. Gonadectomy also reduced circulating ACE activity in ACE2KO mice. Loss of ACE2 modified the effect of gonadectomy 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 gonadectomy in control and diabetic ACE2KO but not in wild-type mice.

Our results suggest that gonadectomy 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 blood pressure, modulation of RAS, and downregulation of Akt-related pathways.

KEYWORDS

Diabetic Nephropathy, Blood Pressure, Gonadectomy, Renin-Angiotensin System, Streptozotocin

INTRODUCTION

Angiotensin converting enzyme 2 (ACE2) is an homologue 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], while 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 (DN) [6-8].

Male sex increases the incidence, prevalence and progression of chronic kidney disease [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 males compared to females [12]. In agreement, in an experimental model, Liu et al. found that, under normal conditions, males have higher renal ACE2 activity than females [13]. 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 gender 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 DN 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 (DB) C57BL/6 male mice after streptozotocin (STZ) injection. We also analysed the effect of ACE2 deficiency, diabetes and gonadectomy on renal RAS modifications. In addition, we assessed the levels of Akt phospohorylation as a downstream signalling effector of both, Ang-II- and Ang-(1-7)-related axis.

METHODS

Animal model and experimental groups

Experiments were performed in wild-type (WT) and 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. 4-hour-fasted mice were given two intraperitoneal injections of 150mg/Kg STZ (Sigma) in two consecutive weeks as previously reported[6]. Citrate buffer was used as vehicle. GDX or sham-operation was performed one week prior to diabetes induction. (See Supplementary Information).

The study included 8-12 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). Mice were considered diabetic if blood glucose levels higher than 250mg/dl were detected during the 4 first 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 8000g for 10min. Mice were perfused with cold phosphate buffer solution (PBS)

prior to kidneys removal and weighting. Left kidney and half of the right kidney were snap frozen with liquid nitrogen and kept at -80ºC for further analysis. Half of the right kidney was maintained in 10% formalin solution and paraffin embedded for histological studies.

Blood pressure and heart rate measurements

Systolic and diastolic blood pressure (SBP and DBP) were measured during the last week of follow-up using the CODA™ mouse tail-cuff system (Kent Scientific Corporation). 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 (ACR) on morning spot urine collections. Urinary albumin and creatinine levels were measured by ELISA (Albuwell M, Exocell) and a colorimetric assay (Creatinine Companion, Exocell), respectively. ACR 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 (45mg/Kg). GFR was estimated in 3-7 animals per group using clearance kinetics of plasma FITC-inulin after a single intravenous bolus injection as previously described [19]. GFR values were expressed as µL/min/gr of body weight as previously published [20].

ACE enzymatic activity assay

The ACE fluorometric enzymatic assay was performed in serum and kidney protein extracts as previously described with modifications [21]. (See Supplementary Information).

Real-time reverse transcriptase–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. (See Supplementary Information).

Kidney histology

Paraffin blocks were cut into 3µm sections, deparaffined in xylene and rehydrated through graded alcohols. Sections were stained with periodic acid–Schiff (PAS) for measurement of mesangial index [19]**.** Immunohistochemistry staining was performed for ACE [5], alpha-smooth muscle actin (α-SMA) [23] and the podocyte marker WT-1 [19]. In addition, Sirius Red staining was performed on 4.5µm kidney sections and cortical collagen accumulation was semiquantitatively evaluated (0-4 score) [24]. All analyses were performed in a blinded fashion.(See Supplementary Information).

Immunoblotting

Kidney cortical tissue was prepared for immunoblot analysis with antibodies to ACE and phosphorylated and total Akt. (See Supplementary Information).

Principal component analysis

To evaluate the predominant effects of ACE2 deletion, diabetes and gonadectomy in the physiologycal and renal parameters, tubulointerstitial fibrosis, hemodynamics and glomerular Injury markers, and RAS components gene expression**,** principal component analysis (PCA) were performed using Perseus software (version 1.5.1.6). The mean value for each variable in each experimental group was used for the PCA. Category enrichment was in two principal components, and Benjamini-Hochberg FDR was used as a cutoff method with a threshold value of 0.05.

Statistical analysis

Statistical analyses were performed using SPSS 18.0 statistical software. 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.

RESULTS

Diabetes, blood pressure and renal functional parameters

Blood glucose levels were significantly elevated in all STZ-treated groups as compared to 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 as compared to WT. In addition, gonadectomy 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 as compared to the WT animals. In turn, gonadectomy in WT and ACE2KO mice clearly reduced the ratio (Table 1). Diabetes was accompanied by an increased ratio in intact WT and ACE2KO animals, indicating the presence of renal hypertrophy in these groups due to diabetes. Interestingly, DB-ACE2KO + GDX mice showed significantly lower kidney weight/body weight ratio than the DB-WT + GDX group.

ACE2 deletion significantly increased SBP and DBP in intact diabetic mice (Figure 1A and B). This increase was not observed in DB-ACE2KO + GDX animals. GFR was increased in DB-WT mice as compared to their controls (Figure 1C). ACE2 deletion accentuated hyperfiltration by increasing GFR in controls. Gonadectomy significantly reduced GFR in diabetic WT and ACE2KO mice. Diabetes significantly increased UAE in WT and ACE2KO mice (Figure 1D). Surgical castration significantly decreased UAE in diabetic WT and ACE2KO mice. Among these groups, DB-ACE2KO + GDX mice presented significantly lower UAE than their respective DB-WT + GDX mice.

Histological analysis

Glomerular tuft area was increased in DB-WT and DB-ACE2KO as compared to controls (Figure 2A and C). In concordance to the low renal weight observed in these groups, glomerular area was decreased in gonadectomized WT and ACE2KO diabetic mice. However, this reduction was only significant in the

ACE2KO. DB-WT and DB-ACE2KO mice showed an elevated mesangial index in comparison to the non-diabetic groups (Figure 2B and C). In ACE2KO mice, the mesangial index was significantly higher as compared to WT. Gonadectomy prevented the increase in the mesangial index in both, diabetic WT and ACE2KO mice. Interestingly, DB-ACE2KO + GDX mice showed a significantly higher mesangial index than their respective DB-WT + GDX mice.

The proportion of cells that were identified as podocytes in glomerulus was significantly decreased in the diabetic groups as compared to controls (Figure 3A and B). This decrease was significantly accentuated in DB-ACE2KO mice as compared to DB-WT. DB-ACE2KO mice also showed a significantly higher number of cells per glomeruli as compared to the DB-WT (Figure 3C). In contrast, gonadectomized ACE2KO diabetic mice showed decreased cell number and increased podocytes within glomeruli. These changes were not observed in the DB-WT + GDX group.

Evaluation of renal fibrosis

Interstitial fibrosis was evaluated by two different approaches. To test the progression of fibrogenesis, the presence of interstitial myofibroblast 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 WT and ACE2KO mice as compared to their non-diabetic controls

(Figure 4). The second approach was the evaluation of Sirius Red staining as collagen type I and III fibril marker. The technique revealed increased collagen deposition in diabetic mice as compared to controls (Figure 5). Interestingly, collagen accumulation was enhanced in DB-ACE2KO mice as compared to DB-WT. Surgical castration significantly reduced both, α-SMA staining and collagen deposition, in ACE2KO diabetic mice. In contrast, DB-WT + GDX mice exhibited a significant increase in α-SMA expression and collagen deposition, being these markers of fibrosis significantly higher in comparison to the DB-ACE2KO + GDX group (Figure 4-5).

Akt 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 were performed. Akt phosphorylation (pAkt) was increased in WT diabetic mice as compared to their controls (Figure 6A-B). In addition, ACE2 deletion was accompanied by an increase of pAkt and the phospo/Akt ratio in both, control and diabetic mice. In turn, gonadectomy clearly decreased pAkt and the phospo/Akt ratio in these groups. Gonadectomy did not modify pAkt and phospo/Akt ratio in WT diabetic mice **(**Figure 6A-D).

ACE expression in serum and kidney cortex

Serum ACE (sACE) activity was significantly increased in WT and ACE2KO diabetic mice as compared to controls (Figure 7A). sACE was also enhanced in

ACE2KO-CONT as compared to WT-CONT. Gonadectomized mice markedly displayed low sACE.

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

RAS 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 (Figure 8A). MasR mRNA levels were also augmented, whereas ACE gene expression was decreased in both, DB-WT and DB-ACE2KO mice, as compared to their controls (Figure 8C,I). Interestingly, only ACE2KO diabetic mice showed a significant decrease in renin (REN) and AT1R expression, as well as higher cathepsin G (CTSG) mRNA levels (Figure 8D,H). In turn, neprilysin (NEP) gene expression was augmented in DB-WT mice (Figure 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 as compared to CONT-ACE2KO mice (Figure 8A,B,G). In contrast, renal ACE, APN and MasR mRNA levels were

significantly decreased in this group (C,F,I). In diabetic mice, gonadectomy significantly enhanced REN, NEP and AT1R cortical mRNA levels (Figure 8B,G,H), whereas diminished APN and MasR gene expression, in both WT and ACE2KO animals (Figure 8F,I). Among the diabetic gonadectomized groups, MasR mRNA levels were significantly increased in DB-WT + GDX as compared to ACE2KO-DB + GDX mice (Figure 8I). Interestingly, gonadectomy dramatically increased AOGEN gene expression in diabetic WT but not ACE2KO (Figure 8A). In addition, gonadectomy induced a significant increase in CTSG only in ACE2KO mice (Figure 8D).

Principal component analysis

In this study, we simultaneously evaluated the effect of ACE2 deletion, diabetes and gonadectomy 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 as compared to WT. In diabetic and gonadectomized mice, a different effect was observed between the WT and the ACE2KO groups (Figure 9A). Interestingly, additional PCA revealed a similar distribution when considering only the tubulointerstitial fibrosis markers as determinants of sample variation (Figure 9B). In contrast, independent analysis for the hemodynamic parameters and glomerular injury markers indicated a

predominant effect of diabetes over gonadectomy in both, WT and ACE2KO mice (Figure 9C). When evaluating the distribution of our study groups according to RAS components gene expression, PCA reflected that both, gonadectomy 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 (Figure 9D).

DISCUSSION

We studied the effect of gonadectomy on a mouse model of diabetes without the expression of ACE2 protein. Overall, gonadectomized ACE2KO diabetic mice showed lower blood pressure values and decreased nephropathy than male ACE2KO diabetic mice. These animals exhibited (1) modulation of circulating and renal RAS favoring the “pro-Ang(1-7)” axis; (2) histological evidence of renal protection, namely a reduction in mesangial expansion and attenuation of glomerular hypertrophy; (3) attenuation of podocyte loss; and (4) 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 gonadectomy 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 identified a role for ACE2 in the regulation of blood pressure, identifying ACE2 as a functioning component of RAS in vivo

[17]. Furthermore, induction of diabetes by STZ in ACE2-deficient mice either by pharmacologic inhibition or genetic ablation increased blood pressure 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 blood pressure 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 serum ACE activity has not been previously studied. In this work we demonstrated that ACE2 deficiency increased circulating ACE activity. Our study also confirms 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 renal ACE 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. in chinese hamster ovary (CHO) cells transfected with human ACE cDNA [27]. Although the identity of the secretase that sheds ACE remains unknown, it has the properties of a member of the 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 gonadectomy 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 Ang-II. In agreement with our findings, Lim et al. demonstrated that plasma ACE activity in both male and female mice is reduced by gonadectomy [33]. 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 renal ACE due to ACE2 deletion and diabetes altered not only Ang-II- but also Ang-(1-7)-related pathways in our mice. Therefore, increased SBP in DB-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, cathepsin G, aminopeptidase N and aminopeptidase A. Neprilysin degrades Ang-II to Ang-(1-4), as well as forms Ang-(1-7) from Ang-I [34]. In our work, gonadectomy dramatically augmented renal AOGEN and REN gene expression in diabetic ACE2KO mice. Furthermore, gonadectomy 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 as compared to females [15]. 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. demonstrated that loss of ACE2 in male mice leads to age-dependent development of glomerular mesangial expansion and glomerulosclerosis [2]. 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.

As expected, ACE2 deletion exacerbated glomerular injury, namely mesangial matrix expansion and podocyte loss in diabetic mice. In agreement, Soler et al. described worsening of albuminuria and glomerular histological lesions after 4 weeks of ACE2 pharmacological inhibition in STZ-induced diabetic mice[6]. 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 due to 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 blood pressure may play a role in this renoprotective effect at the glomerular level.

ACE2KO-DB mice showed an increase in the myofibroblast profibrotic marker α-SMA and accentuated collagen deposition as compared to WT-DB 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 TGF-β/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]. Gonadectomy 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, also known as protein kinase B, is important in many cellular processes including proliferation, migration, cell growth and metabolism, and play a critical role in the cardiovascular and renal system [41]. Akt activity can be transcriptionally modulated by its upstream regulatory pathways and also at post-translational 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 Akt phosphorylation 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, while gonadectomy clearly reduced pAkt levels in control and diabetic ACE2KO mice. As expected, Akt expression and phosphorylation was not modified by gonadectomy in diabetic WT mice. The decreased Mas receptor gene expression and the absence of ACE2 in DB-ACE2KO + GDX may explain lower activation of Ang-(1-7)-dependent Akt stimulation as compared to the WT mice. In DB-WT + GDX mice, the presence of ACE2 and increased mRNA levels of Mas receptor, may suggest a higher

activation of the Ang-(1-7)/Mas receptor axis. Thus, the effects of gonadectomy on reducing Akt phosphorylation may be counterbalanced by an increase on Ang-(1-7)-mediated Akt phosphorylation in DB-WT + GDX mice.

In this study, we simultaneously evaluated the effect of ACE2 deletion, diabetes and gonadectomy 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 gonadectomy may be protective in diabetic ACE2KO but not diabetic WT 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 gonadectomy increased fibrosis in STZ diabetic rats [54]. In addition, PCA for renal RAS gene expression suggested that modulation of renal RAS played a relevant role on these changes. In conclusion, gonadectomy attenuated diabetic nephropathy by preventing hypertension, glomerular injury and renal fibrosis in type 1 diabetic ACE2KO mice. Given our results one hypothesize that, under deficient Ang-II degradation, gonadectomy may confer a protective effect at kidney level by different mechanisms such as a decrease in blood pressure, a decrease in Akt phosphorylation and RAS modulation (Figure 10). These positive effects were absent in ACE2 intact mice. Under these conditions of enhanced Mas expression, excessive activation of the NEP/ACE2/Ang-1-7/MasR axis may

activate pro-fibrotic 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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FIGURE LEGENDS

Figure 1. Influence of diabetes, ACE2 deletion and gonadectomy on hemodynamics and glomerular function. SBP and DBP (8-12 animals per group), glomerular filtration rate and urinary albumin excretion (UAE, 8-12 animals per group) were evaluated in all the experimental groups. CONT, Control; DB, Diabetic; WT, wild-type; ACE2KO, ACE2 knockout; GDX, gonadectomized. Data are expressed as means±SEM. $^{*}P<0.05$ compared to non-diabetic controls. $^{\dagger}P<0.05$ compared to WT. $^{\S}P<0.05$ compared to non-GDX.

Figure 2. Influence of diabetes, ACE2 deletion and gonadectomy on glomerular structural alterations. Periodic Acid Staining (PAS) was performed on 3µm kidney sections from all the experimental groups. Glomerular tuft area (A) and mesangial index (B) were calculated by ImageJ software. Representative PAS sections from all the experimental groups are shown in panel C. For these experiments, 7-10 animals were analyzed in each group. CONT, Control; DB, Diabetic; WT, wild-type; ACE2KO, ACE2 knockout; GDX, gonadectomized. Data are expressed as means±SEM. $^{*}P<0.05$ compared to non-diabetic controls. $^{\dagger}P<0.05$ compared to WT. $^{\S}P<0.05$ compared to non-GDX. Scale Bar = 20µm. Original magnification x40.

Figure 3. Influence of diabetes, ACE2 deletion and gonadectomy on podocyte loss. Podocyte number is represented as the % of brown positive cells after WT-1 immunostaining (A). Representative photomicrographs depicting glomerular WT-1 staining from all the experimental groups are shown in panel B. Total cell number was also assessed in the same photomicrographs (C). For

these experiments, 6-10 animals were analyzed in each group. CONT, Control; DB, Diabetic; WT, wild-type; ACE2KO, ACE2 knockout; GDX, gonadectomized. Data are expressed as means±SEM. *P<0.05 compared to non-diabetic controls. Scale Bar = 20μm. Original magnification x40.

Figure 4. Influence of diabetes, ACE2 deletion and gonadectomy on cortical α-SMA expression. The degree of brown staining as α-SMA-positive areas was quantified by ImageJ software and represented as Mean Grey Value (A). Panel B shows representative sections for tubulointerstitial α-SMA immunostaining from all the experimental groups. Scale Bar = 100μm. Original magnification x10. For these experiments, 6-9 animals were analyzed in each group. CONT, Control; DB, Diabetic; WT, wild-type; ACE2KO, ACE2 knockout; GDX, gonadectomized. Data are expressed as means±SEM. *P<0.05 compared to non-diabetic controls. †P<0.05 compared to WT. §P<0.05 compared to non-GDX.

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). Panel B shows representative photomicrographs for Sirius Red-positive staining (intense red) from all the experimental groups. Scale Bar=50μm. Original magnification x20. For these experiments, 6-8 animals were analyzed in each group. CONT, Control; DB, Diabetic; WT, wild-type; ACE2KO, ACE2 knockout; GDX, gonadectomized. Data are expressed as means±SEM. *P<0.05 compared to non-diabetic controls. †P<0.05 compared to WT. §P<0.05 compared to non-GDX.

Figure 6. Influence of diabetes, ACE2 deletion and gonadectomy on phosphorylated and total Akt in kidney cortex. Panel 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 Image J. Intensities for pAkt and Akt were normalized to β-actin (panels B and C), and pAkt/Akt ratio was also calculated (Panel D). For these experiments, 6-8 animals were analyzed in each group. CONT, Control; DB, Diabetic; WT, wild-type; ACE2KO, ACE2 knockout ; GDX, gonadectomized. Data are expressed as means±SEM. *P<0.05 compared to non-diabetic controls. †P<0.05 compared to WT. §P<0.05 compared to non-GDX.

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. Panel C shows representative photomicrographs depicting ACE protein localization in the renal cortex from all the experimental groups. Scale Bar = 200μm. Original magnification x4. Panel D: immunoblot of cortical ACE protein expression normalized to β-actin from all the experimental groups. For these experiments, 6-10 animals were analyzed in each group. CONT, Control; DB, Diabetic; WT, wild-type; ACE2KO, ACE2 knockout; GDX, gonadectomized. Data are expressed as means±SEM. *P<0.05 compared to non-diabetic controls. †P<0.05 compared to WT. §P<0.05 compared to non-GDX.

Figure 8. Cortical gene expression of RAS components in renal tissue. Renal cortex of angiotensinogen (A), renin (B), neprylisin (C) and aminopeptidase N (D) mRNA levels were determined by Real Time quantitative PCR and normalized to

GAPDH in all the experimental groups. CONT, Control; DB, Diabetic; WT, wild-type; ACE2KO, ACE2 knockout; GDX, gonadectomized. For these experiments, 6-10 animals were analyzed in each group. Data are expressed as means±SEM. *P<0.05 compared to non-diabetic controls. †P<0.05 compared to WT. §P<0.05 compared to non-GDX.

Figure 9. Principal component analysis (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 physiologycal and renal parameters, tubulointerstitial fibrosis, hemodynamics and glomerular injury markers, and RAS components gene expression. Panel A depicts PCA for all the assessed physiologycal 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).

Figure 10. Protective effects of gonadectomy by modulating renal RAS and Akt phosphorylation in ACE2KO diabetic mice. GDX, Gonadectomy; STZ, streptozotocin; ACE, Angiotensin Converting Enzyme; ACE2 Angiotensin Converting Enzyme 2; Ang, Angiotensin; CTGS, cathepsin G; NEP, neprylisin;

APA, aminopeptidase A, APN, aminopeptidase N; AT1R, Ang-II type 1 receptor; MasR, Mas receptor.

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Response to reviewers and editor:

We thank the editor and the reviewers for their comments and suggestions. We believe that the revised version of the manuscript has improved substantially. The original number of the manuscript was JH-D-16-00280. Following there is a detailed description of the changes made to the manuscript according to the suggestions of the reviewers.

Reviewer #1:
The revised study has addressed previous concerns of the reviewer. The investigators have added additional analysis (PCA) as well as the additional GDX groups to account for the effects of castration alone. The paper remains quite complex particularly with the additional groups but remains a worthy study on the effects of androgens and the renin angiotensin system.

Minor concerns:
Is there any data that diabetes is associated with reduced androgens either clinically or in experimental models that would be relevant to the GDX model?

We thank the reviewer for his/her constructive comments. Regarding this question, we have also asked the same ourselves. For this reason, we recently published a paper entitled "RAS and sex differences in diabetic nephropathy" which is mentioned in the manuscript (REF 16); where we review the level of androgens in clinical and experimental models of diabetes.

Pg 12 (line 19). "…supposed…' should be exhibit or show. **As suggested by the reviewer, we have changed "supposed" by "exhibited". (See page 13, line 6 in the REDLINED version).**
Pg 14 (line 2). "anincrease" replace with higher. **As commented by the reviewer, we have changed "anincrease" by "higher". (See page 14, line 10).**

Pg 14 (line 20). Change to two separate sentences. **As suggested by the reviewer, we have separated these two sentences. (See page 15, line 5).**

Pg 17 (line 9). Add ref #15. **As commented by the reviewer, we have added ref#15. (See page 17, line 14).**

Pg 18 (line 4). Add ref #33. **As suggested by the reviewer, we have added ref#33. (See page 18, line 10).**

Pg 18 (line 23). Correct to forms. **As commented by the reviewer, we have corrected this word. (See page 19, line 4).**

Pg 19 (line 4). Correct to male Lewis rats. **As pointed by the reviewer, we have corrected the name of the animal model used in the study from Yamalayeva et al. (See page 19, line 9).**

Pg 19 (line 6). Confused over the statement that ACE2KO and GDX hyperactivate the Aogen/Renin/ACE/Ang II axis as the ACE2KO/GDX showed lower ACE expression? **We thank the reviewer for pointing out this issue. In the new version of the manuscript it is depicted that there is a modulation and alteration of the RAS system. (See page 19, line 11).**

Pg 20 (line 5). Correct to "…at the glomerular level". **As suggested by the reviewer, we have added the word "the" before "glomerular level". (See page 20, line 10).**

Pg 20 (line 10). Replace "largely" with markedly. **As indicated by the reviewer, we have replaced these two words (See page 20, line 17).**

Pg 22 (line 5). Replace "protected from" with attenuated diabetic nephropathy. **As suggested by the reviewer, we have replaced these two expressions (See page 22, line 13).**

Pg 22 (line 7). Remove "In summary," Begin sentence with "Our results…". **As commented by the reviewer, we have made modifications in this line. (See page 22, line 15).**

Pg 22 (line 10). Change the double negative sentence to "…these positive effects were absent in ACE2 intact mice. Under these conditions of enhanced Mas expression, excessive activation of the NEP/ACE2/Ang-1-7/MasR axis may activate pro-fibrotic pathways that contribute to kidney injury." These data also show an increase in renal NEP. **We agree with the reviewer and think that modifying the sentence contributes to a better understanding and clarifies the results of the manuscript. Thus, we have changed the sentence in accordance with his/her suggestion. (See page 22, line 19).**

Reviewer #2:
The present manuscript has been extensively revised. Several points still need attention:
1. It is still not clear way a Mann-Whitney test was used for between-group comparisons. This test is a non-parametric test to compare two samples (that are not normally distributed or that have unequal variances) and not a post-hoc test that accounts for multiple comparisons. **We thank the reviewer for his/her comment and now we realized that our statistical studies were not clear. For this reason, we now contacted to the statistics department in our institution (see Acknowledgements section page 24, line 2). After analyzing the studied variables he recommended the use of the following non-parametric test: Kruskal-Wallis for multiple comparisons and Mann-Whitney U-test for two group comparisons. We are sorry, but due to the small sample size we are not able to use a post-hoc test. Although the majority of our variables follow a non-parametric distribution we decided to present our data as means ± SEM for clarity. This approach has been previously performed by different groups (PMID: 25806942/ PMID: 26880802). (See page 10, line 8).**

2. Page 11, 1st paragraph: What do the authors mean with "ACE2 deletion…accentuated hyperfiltration in diabetic mice…"? Fig. 1 C does not indicate significant differences between control ACE2 k.o. and diabetic ACE2 k.o. mice. **We thank the reviewer for his/her exquisite revision of the manuscript. We are sorry for our mistake that is now corrected in the new version of the manuscript. (See page 11, line 12).**

3. Legend of Fig. 7 does not match the actual figure. There are only 4 panels (A-D) no panel E. Please revise.
We again thank the reviewer for pointing out this mistake. Accordingly, we have removed information regarding panel E in the legend of figure 7. (See page 27, line 17).

4. The part on principle component analysis is hard to follow. The figure legend to Fig. 9 hardly explains the meaning of the graphs. Why is blood glucose a renal injury marker? Chronically elevated blood glucose clearly predisposes to renal injury but it is not a marker of renal injury. Why is body weight a renal injury marker? Why is kidney weight to body weight ratio a hemodynamic and glomerular parameter? Please explain or revise.
We agree with the reviewer and in the new version of the manuscript we clarified the PCA. As he/she mentioned blood glucose and body weight are not a renal injury marker, for this reason, now we entitled this graph as: "physiologycal and renal parameters" (see Figure 9, legend and methods). In addition, we now omited the kidney weight/body weight ratio from the hemodynamic and glomerular PCA analysis. We think that with these changes the PCA analysis in the new version is easier to follow.

5. The scheme in Fig. 10 is rather hypothetical than supported by the data presented. This should be more clearly indicated. **We thank the reviewer for his/her comment and in agreement we made the changes in the manuscript indicating that our scheme is more hypothetical than supported by the data. (See page 22, line 15).**

Spelling/language style:
Page 6, line 11: phosphorylation. **As commented by the reviewer "phosphorylation" has been changed. (See page 6, line 11).**
Page 12, line 14: fibril marker. **As commented by the reviewer "fibril marker" has been changed. (See page 13, line 14).**
Page 14, line 2: an increase (add space). **As commented by the reviewer this has been modified. (See page 13, line 10).**

We thank the reviewers for all the considerations given to our work. We think that the changes performed have clearly improved the new version of the manuscript.

Sincerely,

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Journal of Hypertension,
Editor-in-Chief
Dear Dr. Alberto Zanchetti,

We are submitting a revision of the following manuscript for consideration in the Journal of Hypertension: **Gonadectomy prevents the increase in blood pressure and glomerular injury in ACE2KO diabetic mice. Effects on Renin-Angiotensin System.** The original number of the manuscript is JH-D-16-00280.

We have revised the manuscript and performed changes according to the suggestions of the referees. We think that we have been able to address their comments as outlined in our responses, which are attached. Thus, we hope that the suggested changes had clearly improved our new version of the manuscript.

All authors have read and approved the submission of the manuscript; the manuscript has not been published and is not being considered for publication elsewhere, in whole or in part, in any language, except as an abstract.
Thank you in advance for giving us the opportunity to perform a de novo submission of our work.

Sincerely,
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Passeig Marítim 25-29. 08003 Barcelona- Spain.
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Figure 1

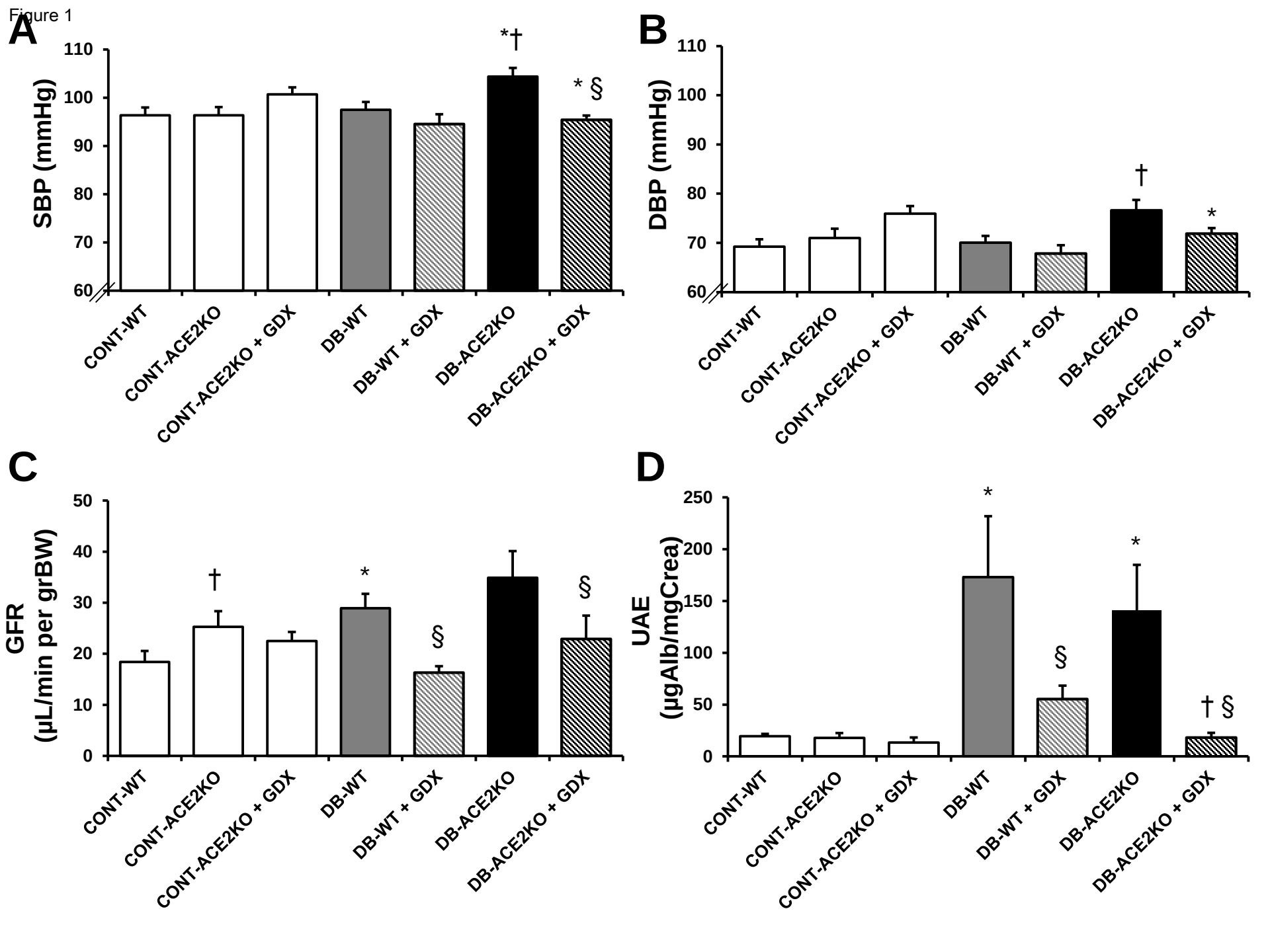
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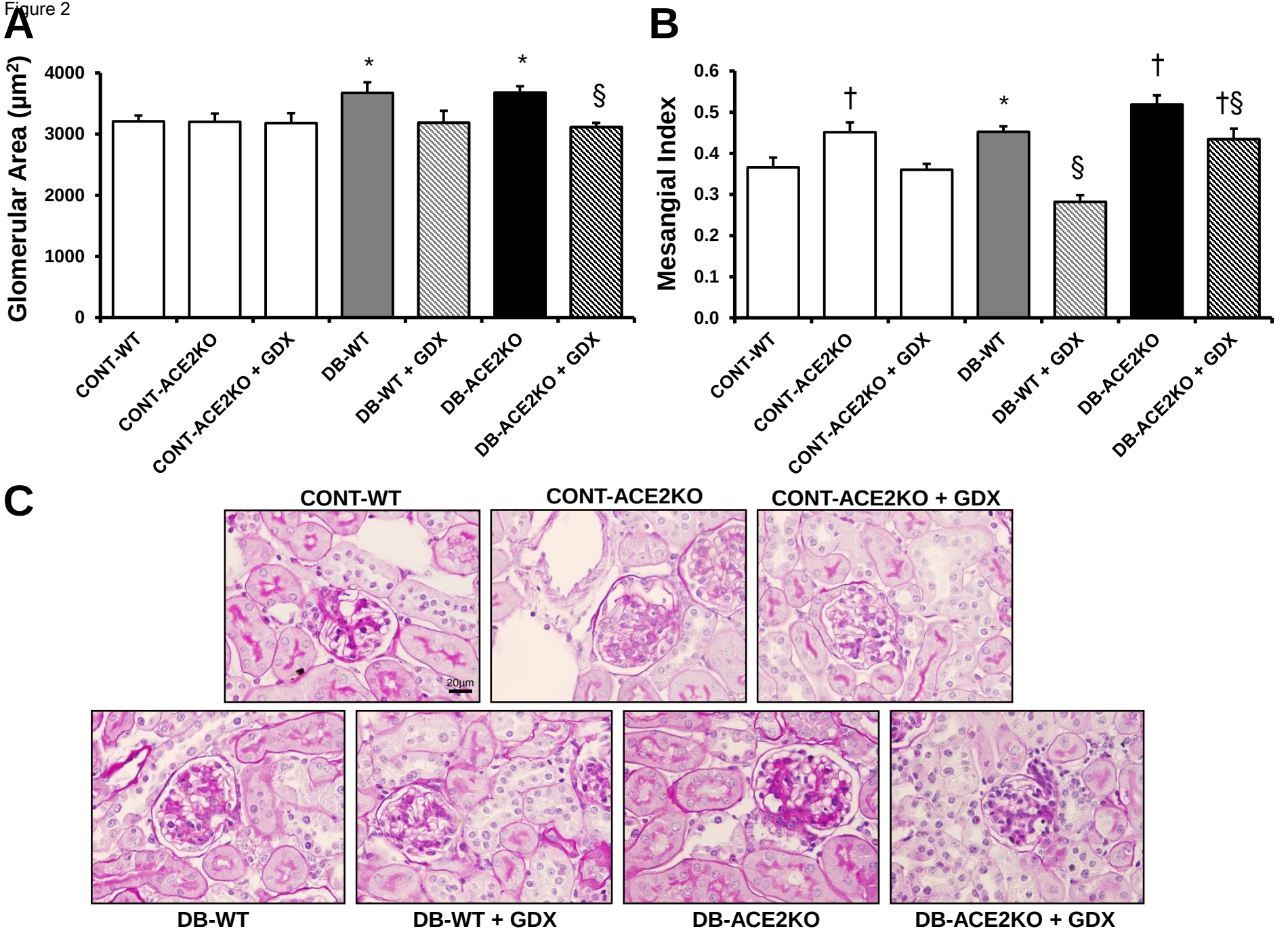
Figure 2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DB-ACE2KO + GDX
B
Mesangial Index
0.6
0.5
0.4
0.3
0.2
0.1
0.0
†
*
§
†
†§
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX
C
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
20μm
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX

Figure 3

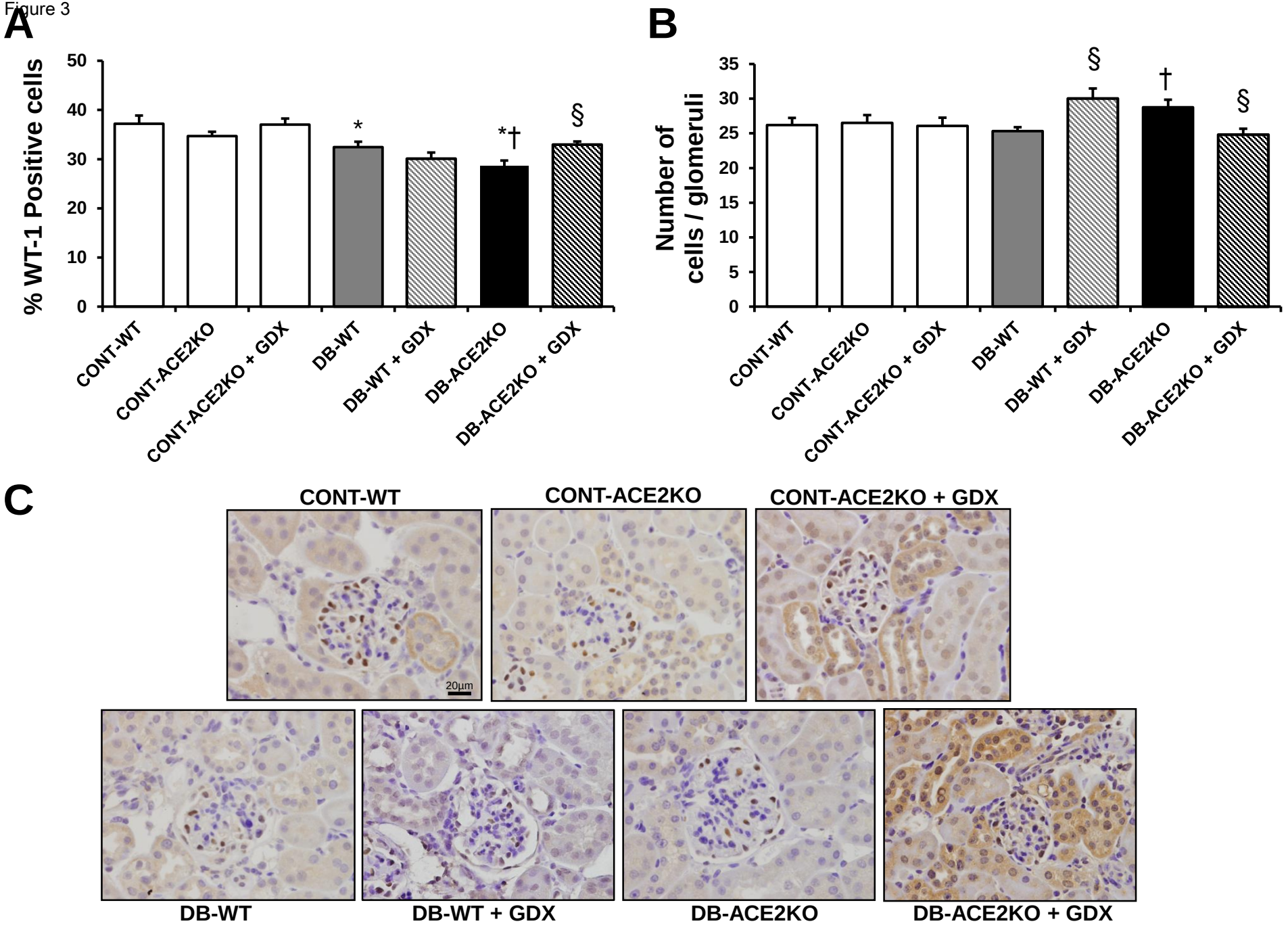
A
% WT-1 Positive cells
50
40
30
20
10
0
*
*†
§
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX
B
Number of cells / glomeruli
35
30
25
20
15
10
5
0
§
†
§
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX
C
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
20μm
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX

Figure 4

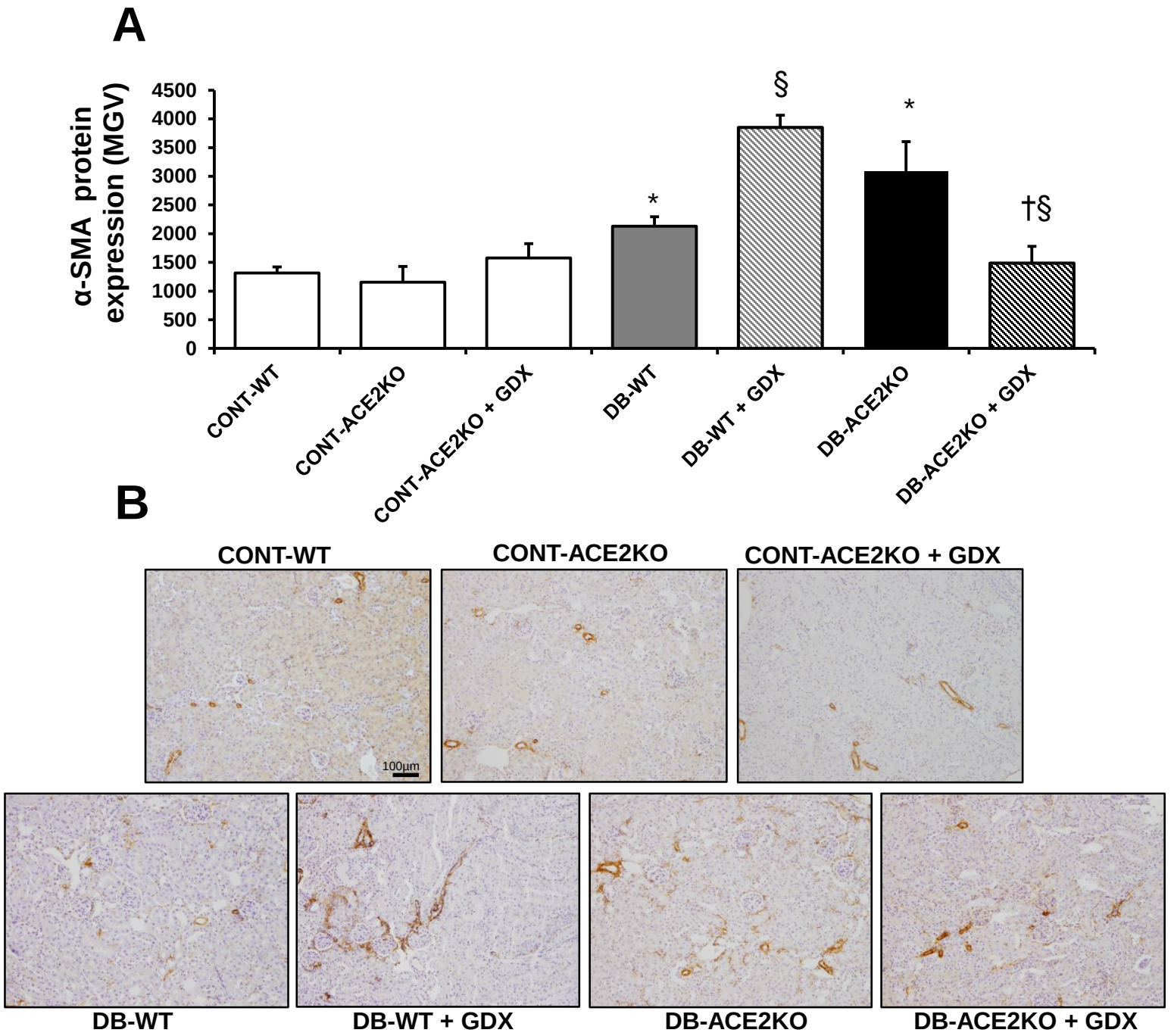
A
α-SMA protein expression (MGV)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
§
*
†§
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX
B
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
100μm
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX

Figure 5

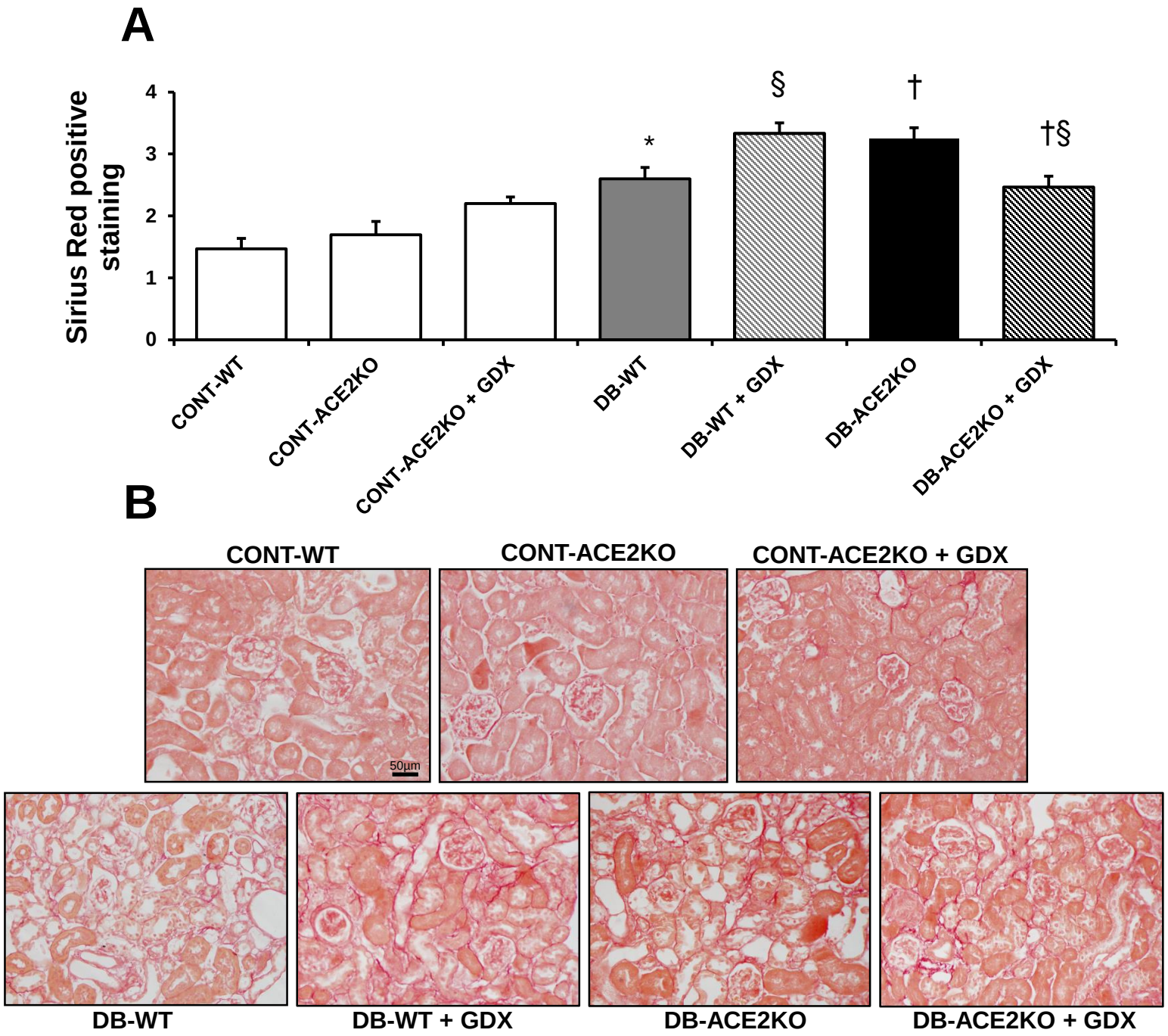
A
Sirius Red positive staining
0
1
2
3
4
*
§
†
†§
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX
B
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
50μm
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX

Figure 6

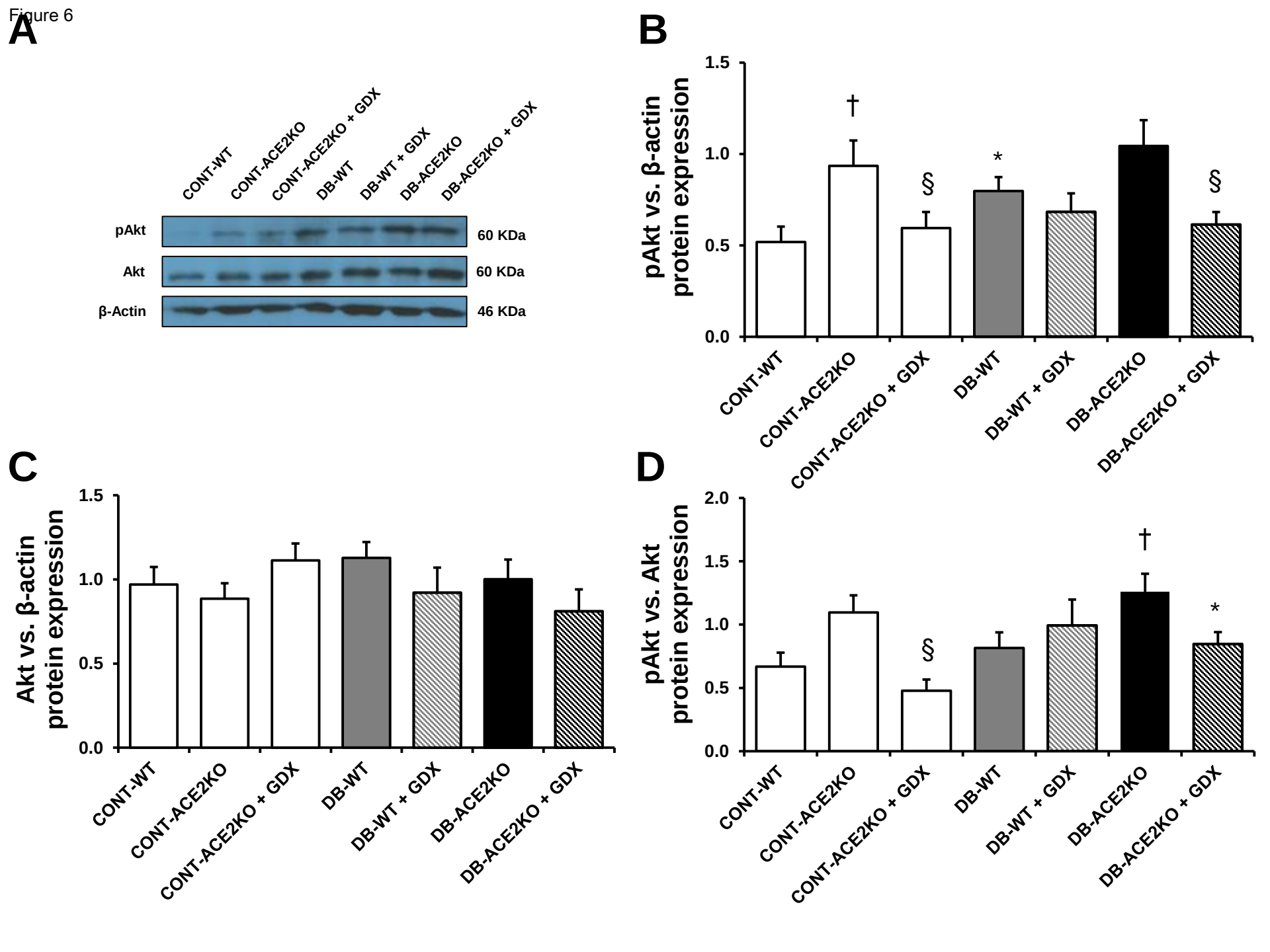
A
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX
pAkt
Akt
β-Actin
60 KDa
60 KDa
46 KDa
B
pAkt vs. β-actin protein expression
1.5
1.0
0.5
0.0
†
§
*
§
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX
C
Akt vs. β-actin protein expression
1.5
1.0
0.5
0.0
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX
D
pAkt vs. Akt protein expression
2.0
1.5
1.0
0.5
0.0
§
†
*
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX

Figure 7

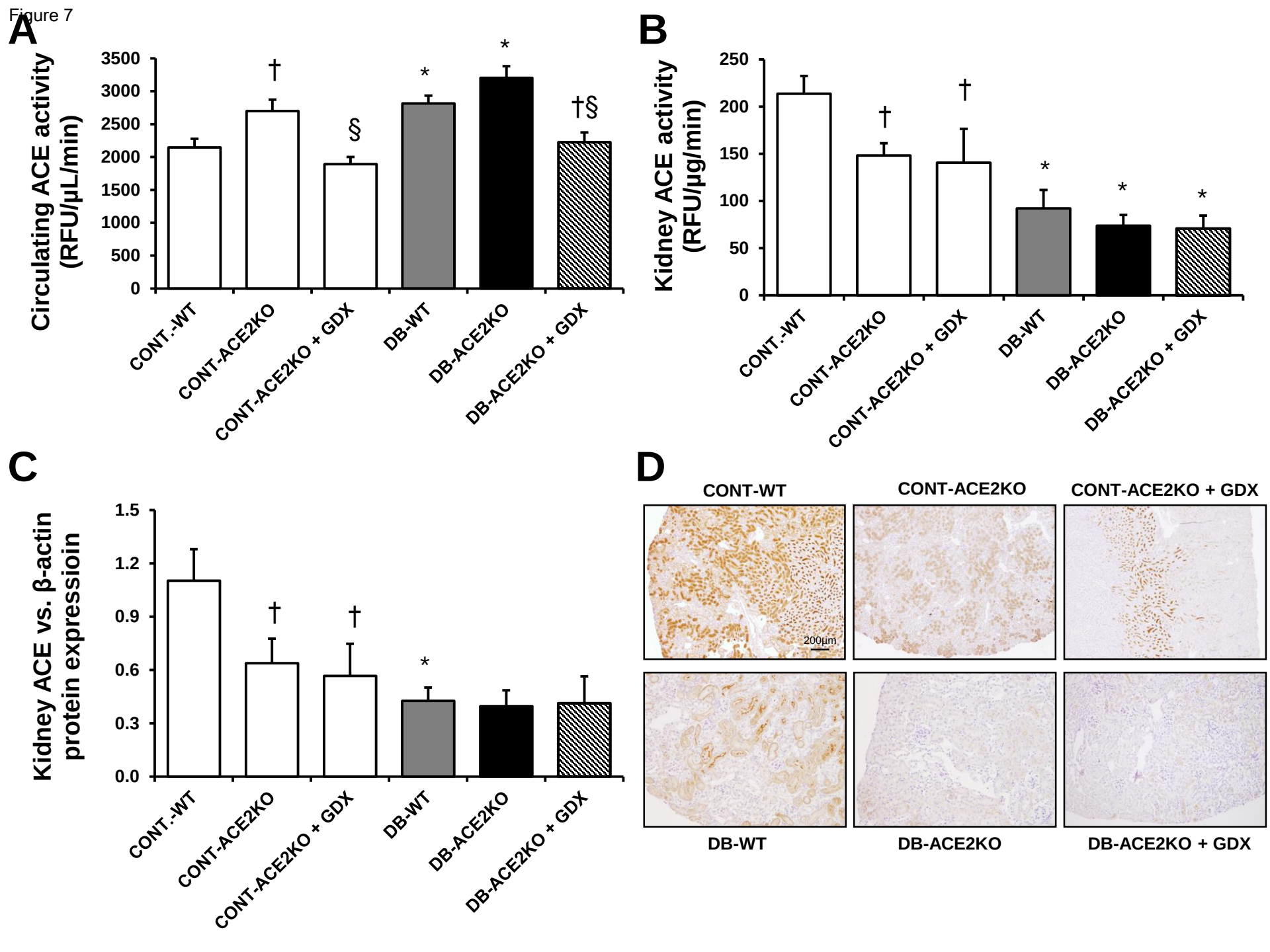
A
Circulating ACE activity (RFU/μL/min)
3500
3000
2500
2000
1500
1000
500
0
†
*
§
*
†§
CONT.-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-ACE2KO
DB-ACE2KO + GDX
B
Kidney ACE activity (RFU/μg/min)
250
200
150
100
50
0
†
†
*
*
*
CONT.-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-ACE2KO
DB-ACE2KO + GDX
C
Kidney ACE vs. β-actin protein expressioin
1.5
1.2
0.9
0.6
0.3
0.0
†
†
*
CONT.-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-ACE2KO
DB-ACE2KO + GDX
D
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
200μm
DB-WT
DB-ACE2KO
DB-ACE2KO + GDX

Figure 8

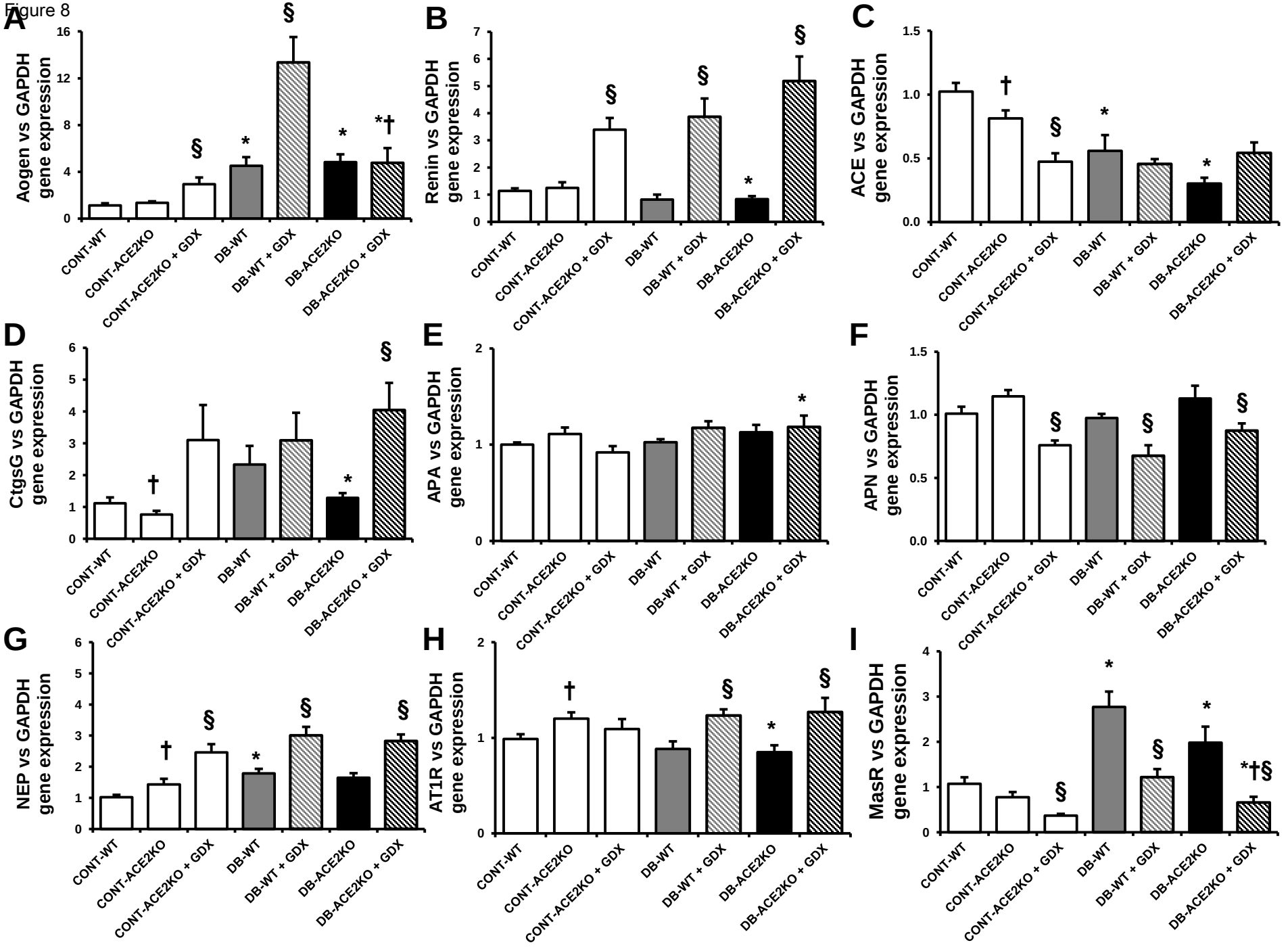
A
Aogen vs GAPDH gene expression
B
Renin vs GAPDH gene expression
C
ACE vs GAPDH gene expression
D
CtgsG vs GAPDH gene expression
E
APA vs GAPDH gene expression
F
APN vs GAPDH gene expression
G
NEP vs GAPDH gene expression
H
AT1R vs GAPDH gene expression
I
MasR vs GAPDH gene expression
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX

Figure 9

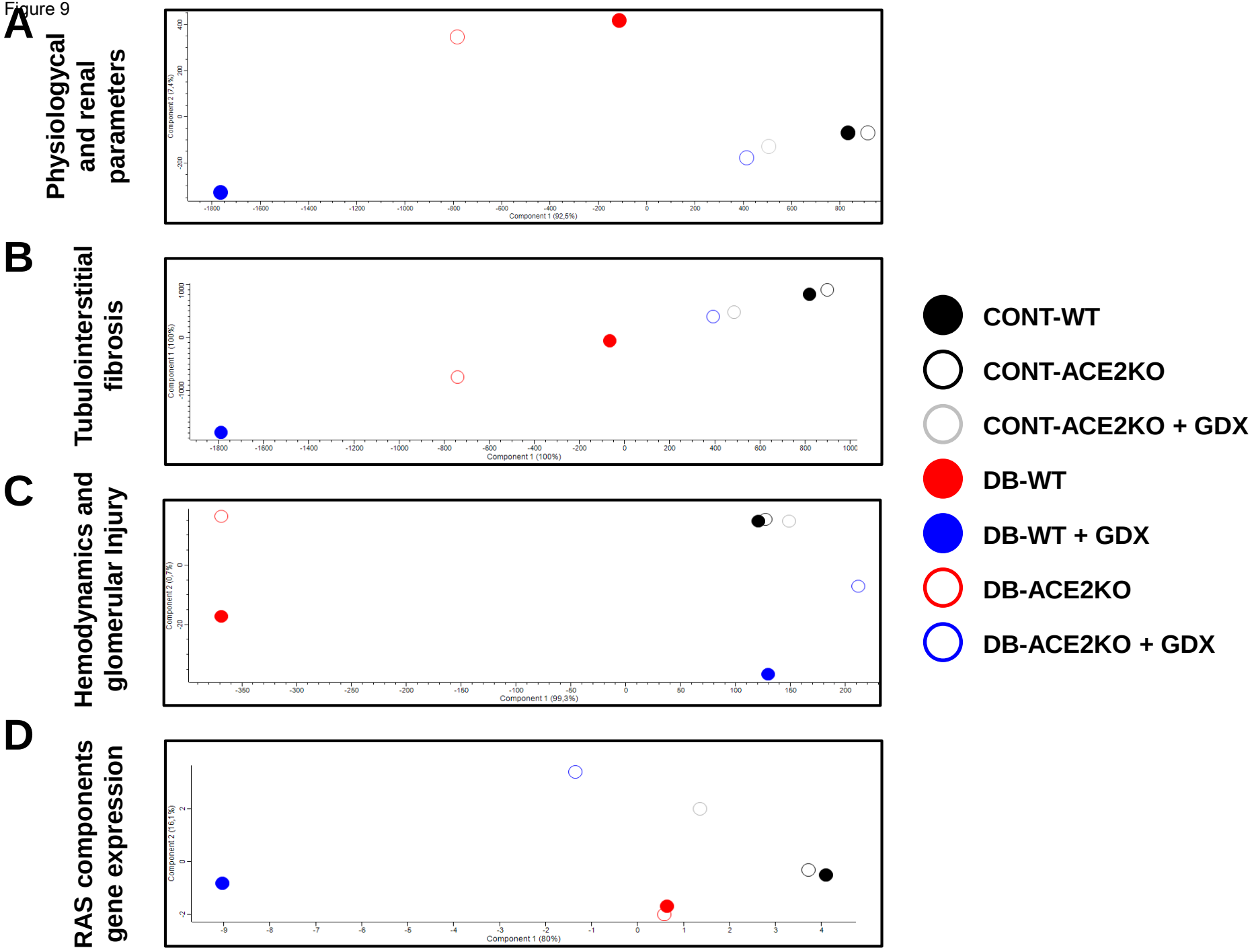
A
Physiologycal and renal parameters
Component 2 (7,4%)
Component 1 (92,5%)
B
Tubulointerstitial fibrosis
Component 1 (100%)
Component 1 (100%)
C
Hemodynamics and glomerular Injury
Component 2 (0,7%)
Component 1 (99,3%)
D
RAS components gene expression
Component 2 (16,1%)
Component 1 (80%)
CONT-WT
CONT-ACE2KO
CONT-ACE2KO + GDX
DB-WT
DB-WT + GDX
DB-ACE2KO
DB-ACE2KO + GDX

Figure 10

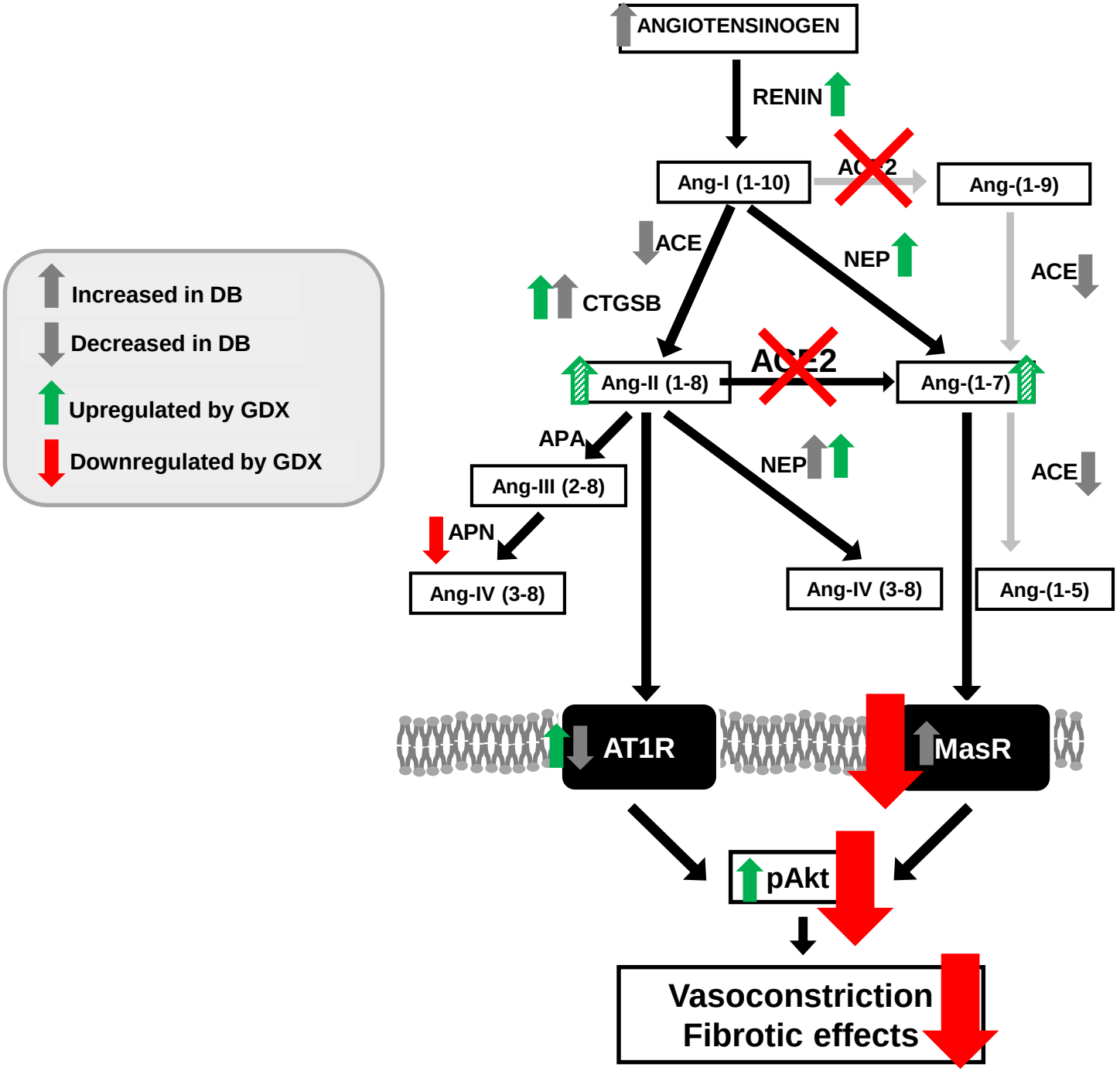
Increased in DB
Decreased in DB
Upregulated by GDX
Downregulated by GDX
ANGIOTENSINOGEN
RENIN
Ang-I (1-10)
ACE2
Ang-(1-9)
ACE
NEP
ACE
CTGSB
Ang-II (1-8)
ACE2
Ang-(1-7)
APA
NEP
ACE
Ang-III (2-8)
APN
Ang-IV (3-8)
Ang-IV (3-8)
Ang-(1-5)
AT1R
MasR
pAkt
Vasoconstriction
Fibrotic effects

Table 1. Physiologic parameters at one month 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) 4wks	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) 19wks	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 wks	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 wks	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 wks	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(%) 19wks	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 (BG) and body weight (BW) at two time-points of the study, after 4 weeks of diabetes induction and after 19 weeks of study, the end-point. At the end of the study, kidney weight (KW) was also recorded in all the experimental groups. 8-12 animals were analyzed in each group. CONT, Control; DB, Diabetic; WT, wild-type; ACE2KO, ACE2 knockout ; GDX, gonadectomized . Values are expressed as means ± SEM; (n = 8-12 per group). $^{*}P<0.05$ compared to non-diabetic controls. $^{\dagger}P<0.05$ compared to WT. $^{\S}P<0.05$ compared to non-GDX.