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# DBA2J db/db mice are susceptible to early albuminuria and glomerulosclerosis that correlates with systemic insulin resistance

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**Running head:** Diabetic nephropathy in the db/db DBA/2J mouse

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**Abstract**

*Introduction:* Diabetic nephropathy (DN) is the leading cause of kidney failure in the world. To understand important mechanisms underlying this condition, and to develop new therapies, good animal models are required. In mouse models of type-1 diabetes, the DBA/2J strain has been shown to be more susceptible to develop kidney disease than other common strains. We hypothesized this would also be the case in type-2 diabetes. *Methods:* We studied db/db and wt DBA/2J mice and compared these with the db/db BLKS/J mouse, which is currently the most widely used type-2 DN model. Mice were analyzed from age 6 to 12 weeks for systemic insulin resistance, albuminuria and glomerular histopathological and ultra-structural changes. *Results:* Body weight and non-fasted blood glucose were increased by 8-weeks in both genders, while systemic insulin resistance commenced by 6-weeks in female and 8-weeks in male db/db DBA/2J mice. The urinary albumin-to-creatinine ratio (ACR) was closely linked to systemic insulin resistance in both sexes and was increased ~50-fold by 12 weeks age in the db/db DBA/2J cohort. Glomerulosclerosis, foot process effacement and glomerular basement membrane thickening were observed at 12-weeks of age in db/db DBA/2J mice. Compared to db/db BLKS/J mice, db/db DBA/2J mice had significantly increased levels of urinary ACR, but similar glomerular histopathological and ultrastructural changes. *Conclusion:* The db/db DBA/2J mouse is a robust model of early stage albuminuric DN and its levels of albuminuria correlate closely with systemic insulin resistance. This mouse model will be helpful in defining early mechanisms of DN and ultimately the development of novel therapies.

## Introduction

Diabetic nephropathy (DN) is the leading cause of kidney failure in the world with over half of patients in the United States entering the end stage renal failure (ESRF) program for this reason. It is increasing at alarming rates throughout both the developed and developing worlds predominantly due to the global epidemic increase in type-2 diabetes (33) caused by sedentary lifestyles, diet and obesity (16). DN generally has a well-defined natural history and initially affects the glomerulus manifesting as hyperfiltration and microalbuminuria (30-300 mg albumin/24 hours) before progressing to macroalbuminuria (more than 300mg/albumin/ 24 hours). Macroalbuminuria usually heralds the start of declining glomerular filtration and associated tubulointerstitial fibrosis. The best current biomarker for kidney involvement in DN is the presence of micro or macroalbuminuria. These may be helpful in identifying affected kidneys and allow therapies to be started early before established fibrotic kidney disease is present. It is now clear that systemic insulin resistance is closely linked to DN progression in both type-1 (25) and type-2 DN (12) so finding models that mimic this would be beneficial in understanding mechanisms and ultimately identifying therapeutic targets to stop early DN from progressing. To understand the mechanisms underlying DN it is vital to have good cellular and animal models of DN, but these are currently suboptimal.

In diabetic patients, multiple genetic factors modulate the risk of developing DN (11, 26). Similarly in mice, the susceptibility to kidney injury is influenced by the inbred mouse strain used as have been illustrated in models of type-1 diabetes. At one extreme, the C57BL/6J strain is resistant to DN in the STZ-induced and the *Ins2*$^{+/C96Y}$ (Akita) models of diabetes as diabetic mice develop only subtle albuminuria with slow advancement of mesangial matrix expansion (13, 14). At the other extreme, the DBA/2J strain displays enhanced susceptible to DN and develop exaggerated urinary albumin excretion compared with other common inbred mouse strains including C57BL/6J, A/J and Sv129 in the STZ-induced (13, 28) and the Akita (5, 14) models.

The genetic background also influences the susceptibility to DN in the db/db mouse model, where an autosomal recessive mutation in the *Lepr* gene (i.e. the diabetogenic *db* allele) renders mice obese, insulin resistant and diabetic (7). The phenotypical manifestations of the *db* allele were first described in the C57BLKS/J (BLKS/J) strain (17), which is a genetic composite between the DN resistant C57BL/6J and DN susceptible DBA/2J strains in addition to alleles from at least three other strains including SV129 (22). The db/db BLKS/J mouse is susceptible to DN and develops significant albuminuria by 8 weeks of age (2, 6, 23, 30) along with histopathological features of early DN by 12-weeks of age including glomerular enlargement (21, 23) and glomerulosclerosis (6, 21, 23). Despite having more than 70% of its genome derived from the DN resistant C57BL/6J strain, the db/db BLKS/J mouse constitutes a robust model of the early changes in human DN (1) and DBA/2J-derived genetic components may be responsible for the susceptibility to DN in this strain. Although the *db* allele has been introduced in the DBA/2J strain (19), the susceptibility to albuminuric kidney disease and development of DN in the db/db DBA/2J mouse remains to be explored in detail.

Here we describe the development of early DN in the db/db DBA/2J mouse to investigate the influence of genetic background on the susceptibility to kidney injury during the transition from prediabetes to diabetes in the db/db mouse model. We hypothesize that the DBA/2J strain display augmented albuminuria as well as histopathological and ultrastructural changes compared to the commonly used BLKS/J strain. Using female and male db/db DBA/2J mice alongside age- and gender-matched lean controls, we characterized the development of metabolic parameters (i.e. body weight, blood glucose and systemic insulin sensitivity) and the development of kidney injury (i.e. urinary albumin-to-creatinine ratio, glomerulosclerosis score and ultrastructural features). We investigated the effects of genetic background on DN by comparing our findings to a cohort of db/db BLKS/J mice.

## Materials & Methods

*Mouse breeding and housing*

Heterozygous db/wt DBA/2J (D2.BKS(D)-*Lepr*$^{db}$/J) breeders were purchased from The Jackson Laboratory (Bar Harbor, ME, US) to breed female and male db/db mice and lean wild type offspring *in house*. To obtain adequate numbers of lean control mice, both db/wt and wt/wt mice were included in the experiment - hereinafter referred to as wt mice. Data on outcome of breeding are presented in Table 1. Male db/db and heterozygous db/wt BLKS/J (BKS.Cg-*Dock7m* +/+ *Lepr*$^{db}$/J) mice were purchased from Charles River (Calco, Italy).

All mice were housed in a 12/12 h light/dark cycle with free access to standard chow (EURodent Diet 22%, percentage of energy: protein 25.9%, fat 9.3%, carbohydrate 64.8%; LabDiet, St. Louis, MO, US) and water. All animal procedures were carried out according to the Guidance of the Operation of the Animals (Scientific Procedures) Act 1986 and all protocols were approved by the Home Office (London, UK).

*Characterization of metabolic parameters*

To characterize the early metabolic phenotype of the db/db model, a total of 24 db/db (12 females, 12 males) and 15 wt (8 females, 7 males) DBA/2J mice were kept from 6 and up to 12 weeks of age, whereas 6 male db/db and 6 wt BLKS/J mice were kept from 8 to 12 weeks of age. Body weight and non-fasted blood glucose were measured biweekly. Blood glucose was measured in tail vein blood using an Accu-Chek Aviva Nano portable glucometer (Roche, Indianapolis, IN, US).

Systemic insulin sensitivity was assessed by insulin tolerance tests (ITT) at 6, 8 and 12 weeks in DBA/2J mice, and at 8 and 12 weeks in BLKS/J mice. Briefly, mice were fasted for 6 h with free access to water. Human insulin (Novo Nordisk, Måløv, Denmark) was injected intraperitoneally (i.p.) at a dose of 0.20 IU/kg in females and 0.50 IU/kg in males, respectively. Blood glucose was

measured in tail vein blood before (T=0 min) and 15, 30, 45, 60 and 90 min after insulin injection using an Accu-Chek Aviva Nano portable glucometer. The applied doses of insulin were empirically determined prior to ITTs as the dose required to reduce blood glucose by 20-40% from baseline 15 min after i.p. insulin injection in 8 week old wt DBA/2J mice.

By 12 weeks of age, mice were fasted for 6 h with free access to water and euthanized by an i.p. injection of pentobarbital (200 mg/kg). Blood was collected by cardiac puncture, transferred to heparinized tube, spun and plasma store at -80°C. Plasma insulin and IGF-1 were quantified using the Ultrasensitive Mouse Insulin ELISA kit and Mouse IGF-1 ELISA kit (both Crystal Chem, both Crystal Chem, Downers Grove, IL, US), respectively, according to manufacturer's instructions. Plasma creatinine was measured using the creatinine enzymatic assay as previously described (18). Finally, kidneys were dissected and either snap-frozen in liquid nitrogen or fixed for histological and ultrastructural evaluation as described below.

*Urine collection and urinary albumin excretion*

To assess urinary albumin excretion, spot urine samples were collected biweekly and stored at -20°C. The urinary albumin-to-creatinine ratio (ACR) was quantified using the Mouse Albumin ELISA Quantification Set (Bethyl Laboratories, Montgomery, TX) and the Creatinine Companion kit (Exocell, Philadelphia, PA, US) according to manufacturer's instructions. Urinary albumin excretion was also assessed by gel electrophoresis. Briefly, urine samples (5 µl per well) and a BSA control (Sigma-Aldrich, Gillingham, UK) of 10 µg per well were separated in 12% Mini-PROTEAN TGX gels and proteins visualized by coomassie stain using Bio-Safe Coomassie G-250 Stain (both Bio-Rad, Hemel Hempstead, UK).

*Glomerular histopathology and ultrastructure*

For histopathological evaluation, dissected kidneys were fixed in 4% formalin at 4°C, dehydrated, embedded in paraffin and cut at 3 µm before staining with Periodic acid–Schiff (PAS) and Masson's Trichrome (TRI) stains using kits (both Sigma-Aldrich) according to manufacturer's instructions. Glomerulosclerosis was scored semi-quantitatively according to the percentage of the glomerular tuft occupied with PAS-positive and nuclei-free matrix. From each mouse, a minimum of 18 glomeruli were scored according to a 5 point scale using the following criteria: 0) normal glomerulus; 1) up to 25% matrix area; 2) 25-50% matrix area; 3) 50-75% matrix area – focal; 4) 75-100% matrix area – global.

For immunofluorescence staining, frozen kidneys were cut at ~8 µm using a freezing microtome and sections blocked with 5% normal goat serum in 0.3% Triton X-100/PBS before incubation with primary antibodies against nephrin (Acris, Hereford, Germany) and collagen IV (Abcam, Cambridge, UK). All sections were incubated with Alexa Fluor® 405 and 488-conjugated secondary antibodies in 3% BSA, 0.3% Triton X-100/PBS and mounted with Vectashield mounting medium (Vector Laboratories, Peterborough, UK) before imaging using a Leica SP5II confocal laser scanning microscope attached to a Leica DMI 6000 inverted epifluorescence microscope with a 63x oil immersion objective lens.

For evaluation of glomerular ultrastructure including foot process effacement and glomerular basement membrane thickening, ~1 $mm^3$ cubes from the renal cortex were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C. Samples were post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.4) and en bloc stained in 3% aqueous uranyl acetate followed by dehydrated through a graded series of alcohol. Samples were embedded in Epon Resin and thin-sectioned at 70 nm. Digital micrographs were taken on a FEI Tecnai Spirit T12 (120KV) Transmission Electron Microscope (TEM).

*Statistical analyses*

Data were modelled and groups compared using linear mixed-effects models in R (version 3.2.1; open source software available at www.cran.r.project.org). Fixed effects (group, age, gender and strain) and random effects (mouse and litter) were included in the models as found appropriate. Longitudinal variables were analyzed as repeated measurements using the *lme* function to test for the effect of age and to compare groups, while variables that were independent of age and variables that were measured at one time-point only were analyzed using the *lmer* function. Model residuals and fitted values were tested for normality. In cases of non-normality, data were $\log_{10}$-transformed prior to modelling and group comparison. Linear correlation analyses were conducted in GraphPad Prism (version 6.05; GraphPad Software, La Jolla, CA). Unless stated otherwise, data are presented as means ± standard error of the mean (SEM). Resulting P values are evaluated at a 5% significance level.

**Results**

*Metabolic phenotype of the db/db DBA/2J mouse*

Body weight and non-fasted blood glucose were monitored from 6-12 weeks of age in female and male db/db and wt DBA/2J mice to characterize the early development of obesity and hyperglycemia. The body weight was significantly higher in db/db vs wt females from 6 through 12 weeks of age (all P<0.001, Fig. 1A) and reached a 96% increase by the end of the study period. Male db/db mice had increased body weight compared to wt mice at 8-12 weeks of age (P<0.001 at 8 and 10 weeks, P<0.05 at 12 weeks) reaching a 37% increase by 10 weeks after which the db/db males experienced a significant drop in body weight through to 12 weeks of age (P<0.05).

Non-fasted blood glucose was similar in all groups at 6-weeks of age but became significantly higher in db/db vs wt mice from 8 through to 12-weeks of age in both genders (all P<0.001, Fig. 1B). Blood glucose did not increase significantly beyond 8-weeks in female and 10-weeks in male

db/db mice. Plasma insulin was measured only at 12 weeks of age and was significantly increased in female db/db vs wt mice (7.15 ± 0.64 vs 0.29 ± 0.03 ng/ml, P<0.001, Fig. 1C), while no significant difference was observed between male groups (2.25 ± 0.98 vs 1.36 ± 0.44 ng/ml).

ITTs were conducted at 6, 8 and 12 weeks to assess systemic insulin resistance, which was quantified by the AUC from ITT curves in females (Fig. 2A) and males (Figs 2C). Systemic insulin resistance was significantly increased in female db/db vs wt mice by 6 weeks of age (P<0.01, Fig. 2B) and by 8 weeks of age in males (P<0.001, Fig. 2D). In both genders, insulin resistance worsened significantly from 6 through to 12 weeks of age (both P<0.001).

*Albuminuria correlates with systemic insulin resistance in the db/db DBA/2J mouse*

The development of albuminuria was explored in db/db DBA/2J mice from 6 to 12 weeks of age and quantified by the urinary ACR. Female db/db mice had significantly higher urinary ACR compared to wt controls through 6-12 weeks of age (P<0.01 by 6 weeks, P<0.001 by 8-12 weeks, Fig. 3A), while ACR was increased from 8 through to 12 weeks of age in male db/db vs wt mice (all P<0.001, Fig. 3B). This correlates with the onset of systemic insulin resistance as presented in figure 2B and 2D, respectively. The statistical analyses showed no significant effect of gender on the ACR fold change between db/db and wt controls, but a significant effect of age was observed as ACR fold change increased from 5-fold by 6 weeks to 50-fold by 12 weeks of age (P<0.001, Fig. 3C). To explore the association between albuminuria and metabolic parameters, we performed correlation analyses between urinary ACR and body weight, blood glucose and systemic insulin resistance, respectively. We observed significant positive correlations between ACR and all three metabolic parameters in both female and male mice (all P<0.001, see $R^2$ in Fig. 3D-F).

*Glomerular histopathology and ultrastructure in the db/db DBA/2J mouse*

To evaluate the glomerular histopathological changes in the db/db DBA/2J mouse, kidney sections were PAS, TRI and collagen-IV stained. Representative micrographs from 12-week old db/db and wt DBA/2J mice are presented in Fig. 4A. PAS stained sections showed glomerulosclerosis in the db/db mice, but not controls, at high magnification (second row - arrowed). Evaluation of TRI stained sections revealed that glomerular fibrosis was present when studying high magnification TRI stained micrographs (fourth row - arrowed). Finally, we observed type IV collagen accumulation in db/db, but not wild-type glomeruli (bottom row).

Semi-quantitative analysis was performed using glomerulosclerosis score (GS) in PAS stained kidney sections showed an increase in the percentage of glomeruli with GS ≥1 in db/db vs wt mice by 8 weeks of age (Fig. 4B). The mean GS was significantly increased in db/db vs wt DBA/2J mice at 8 and 12 weeks of age (both $P<0.001$, Fig. 4C), whereas no significant worsening in GS was observed from 8 to 12 weeks in db/db DBA/2J mice. The mean GS correlated positively with urinary ACR in the DBA/2J cohort ($P<0.001$, $R^2=0.7686$, Fig. 4D).

Ultrastructural changes between 12-week old db/db and wild-type controls were evaluated by TEM and representative images are displayed in Fig. 5A (top row). Significant increases in podocyte foot process width ($P<0.05$, Fig. 5B) and glomerular basement membrane (GBM) thickness ($P<0.001$, Fig. 5C) were detected in db/db vs wt DBA/2J mice.

*Effects of genetic background on the metabolic phenotype in the db/db mouse model*

The metabolic phenotype of male db/db DBA/2J and BLKS/J mice was compared to explore the effects of genetic background on the phenotypical manifestation of the *db* allele. The body weight of db/db DBA/2J mice was 16-20% lower than db/db BLKS/J mice throughout the study period ($P<0.001$, Table 2). Unlike db/db DBA/2J mice, db/db BLKS/J males did not demonstrate any weight loss during the study period although their body weight was unchanged from 10 weeks of

age. Non-fasted blood glucose were significantly lower in db/db DBA/2J mice at 8 weeks ($P<0.001$, Table 2), but reached similar levels to that of db/db BLKS/J mice by 12 weeks age. Systemic insulin resistance was significantly lower in db/db DBA/2J vs BLKS/J males at 8 and 12 weeks of age ($P<0.001$ and $P<0.05$, respectively, Table 2). In wt mice, genetic background did not significantly affect the metabolic parameters presented in Table 2. Finally, comparisons of plasma insulin levels showed no significant differences between male DBA/2J and BLKS/J cohorts in either db/db (2.25±0.98 vs 4.64±2.45 ng/ml) or wt mice (1.36±0.44 vs 1.11±0.2 ng/ml).

*Effects of genetic background on kidney injury in the db/db mouse model*

To evaluate the effects of genetic background on the susceptibility to kidney injury, we compared albuminuria as well as renal structural and histopathological changes between male db/db DBA/2J and db/db BLKS/J mice. Qualitative analysis of urine samples by SDS-PAGE indicated increased urinary albumin concentrations in db/db DBA/2J vs db/db BLKS/J mice at 8 and 12 weeks of age (Fig. 6A). In addition, urinary ACR was significantly higher in db/db DBA/2J vs db/db BLKS/J mice ($P<0.05$, Fig. 6B) with ACR fold changes in db/db vs wt mice within the male DBA/2J cohort ranging from 32- to 44-fold compared with 16- to 18-fold in the BLKS/J2 cohort. Urinary concentrations of albumin were also significantly higher in DBA/2J vs BLKS/J db/db males ($P<0.01$, Fig. 6C). Furthermore, the urinary creatinine concentrations were similar in db/db mice between strains, but significantly higher in BLKS/J vs DBA/2J wt mice ($P<0.05$, Fig. 6D). Finally, we assessed serum creatinine levels in a subset of DBA2J and BLKS/J mice at 12 weeks of age. This revealed no significant differences between db/db and their wild-type controls on either the DBA2J or BLKS/J backgrounds (Fig. 6E).

The impact of genetic background on kidney weights and glomerular histological changes was also investigated. Firstly, relative kidney weight was significantly reduced in db/db vs wt BLKS/J mice ($P<0.001$, Table 3), while no significant differences in kidney weight were observed

between DBA/2J groups. Between strains, relative, but not absolute kidney weight was significantly higher in db/db DBA/2J vs BLKS/J mice ($P<0.01$, Table 3).

Semi-quantitative histological evaluation showed increased GS in db/db vs wt mice by 12 weeks of age in both strains (both $P<0.001$, Table 3), whereas the glomerular area was significantly enlarged in db/db vs wt in the DBA/2J strain ($P<0.001$, Table 3), but not BLKS/J. Finally, comparison of the DBA/2J and BLKS/J cohorts showed no significant effect of genetic background on GS and glomerular area in db/db and in wt mice.

The effect of background strain on ultrastructural changes in glomeruli was evaluated by TEM and representative micrographs presented in Fig. 5A. In both strains, podocyte foot process width and GBM thickness was significantly increased in db/db vs wt mice (for both strains $P<0.05$, Fig. 5B and $P<0.001$, Fig. 5C, respectively). Furthermore, the width of foot processes was significantly higher in db/db BLKS/J vs DBA/2J mice ($P<0.001$, Fig.5B), while the GBM thickness did not differ between strains (Fig. 5C). No significant differences were observed between DBA/2J and BLKS/J wild-type mice.

**Discussion**

DN research and drug development is challenged by the shortcomings of current animal models. Considerable resources are therefore invested in the development of novel animal models that reproduce features of human disease. In this study, we followed the advancement of early DN in the db/db DBA/2J mouse and found that it developed robust albuminuria starting between 6 and 8 weeks of age that correlated closely with systemic insulin resistance, body weight and blood glucose levels. We then compared male db/db DBA/2J mice with an age-matched cohort of male db/db BLKS/J mice, which is the major strain used in type-2 DN research, and found a significant effect of genetic background on the severity of urinary albumin excretion despite similar levels of hyperglycemia and systemic insulin resistance in both strains. This study underlines the impact of

289 genetic background on the propensity to renal injury in mouse models of diabetes and DN, and
290 confirms the susceptibility of the DBA/2J strain to albuminuric kidney disease.

291 Like BLKS/J, the DBA/2J strain is susceptible to the diabetogenic actions of the *db* allele. This
292 causes these mouse strains to develop overt type-2 diabetes with pancreatic exhaustion, beta-cell
293 depletion and insulinopenia after a preceding period of systemic insulin resistance leading to
294 pancreatic hypertrophy and hyperinsulinemia as in human type-2 diabetes patients. The duration
295 of the gradual transition from systemic insulin resistance to overt diabetes-2 depend varies
296 between mouse strains and gender (20). Here, we observed an early onset of hyperglycemia and
297 systemic insulin resistance in female and male db/db DBA/2J mice at 6 and 8 weeks of age,
298 respectively. As seen in various mouse models of diabetes (10, 20), males displayed accelerated
299 advancement of the diabetic phenotype relative to females and developed severe hyperglycemia
300 and insulinopenia resulting in weight loss starting at 10 weeks of age possibly due to glycosuria. In
301 contrast, female db/db DBA/2J mice displayed sustained hyperinsulinemia by 12 weeks of age
302 explaining the stabilized, although elevated, blood glucose levels through 8-12 weeks of age.
303 Furthermore, the metabolic manifestations of the *db* allele - in terms of hyperglycemia and
304 systemic insulin resistance - were similar in the male db/db DBA/2J and BLKS/J cohorts by 12
305 weeks of age. These data are consistent with previous studies by Leiter *et al*. who studied the
306 metabolic parameters in DBA/2J and BLKS/J db/db mice and classified them “diabetes prone” in
307 contrast to “diabetes-resistant” C57BL/6 mice (20).

308 In human patients as well as in animal models, albuminuria is used as the hallmark
309 biomarker of DN. In mice, it has been shown that genetic factors modulate the levels of
310 albuminuria in models of type-1 diabetes including the STZ-induced and Akita models (14, 29, 34)
311 as well as in type-2 diabetes in the db/db model (31). In this study, we observed robust
312 albuminuria by 8 weeks of age in both female and male db/db DBA/2J mice. Furthermore, we saw
313 a development in albuminuria with ACR fold changes in db/db relative to age- and gender-

matched controls resulting in a 56-fold increase in females and a 44-fold increase in males by 12 weeks of age. As far as we are aware this is the first study that has examined the development of albuminuria in the db/db model on a DBA2J background. We decided to compare this genetic strain to the most widely studied strain that develops early DN, BLKS/J. Importantly we studied both strains of mice in the same environment, using the same feeding regimen, and collected all samples in a similar manner. Despite these measures, the different vendors of the mouse strains cannot be excluded as a confounding factor. Our head-to-head strain comparison showed significantly higher levels of urinary albumin excretion in male db/db DBA/2J mice compared with db/db BLKS/J mice as assessed by urinary ACR. This feature was mainly driven by an increase in crude urinary albumin excretion rather than a reduction in urinary creatinine levels. Furthermore, although we didn't measure formal glomerular filtration rates in our mice we did measure serum creatinine levels in a subset of mice which were not significantly different. This suggests that there wasn't a major difference in the level of hyperfiltration between DBA2J and BLKS mice. GFRs have not been previously assessed in db/db DBA2J mice however they have been performed in BLKS db/db and wild-type mice. These studies have revealed either no difference in the level of GFR between these groups at 24 weeks (35) or a small increase in the db/db mice at 18 weeks (4). In the future it will be interesting to rigorously assess the progression of hyperfiltration in the db/db DBA2J model using formal GFR assessment but this wasn't the focus of this study.

Alongside albuminuria, features of DN in the db/db mouse include histopathological findings such as glomerulosclerosis and glomerular enlargement as well as ultrastructural changes including foot process effacement and GBM thickening. Our data showed that glomerulosclerosis was increased in db/db compared to wild-type DBA/2J mice by 8-weeks of age and glomerular enlargement was detectable by 12 weeks of age. Compared with the db/db BLKS/J cohort, we detected no differences in these features in the db/db DBA2J males. It has previously been described in mouse models of DN that increased levels of urinary albumin excretion do not

translate into exacerbation of histopathological changes (14, 28). This may simply be due to the superior sensitivity of the urinary albumin excretion relative to semi-quantitative histological analysis in detecting renal damage. Furthermore, the ultrastructural changes of the glomeruli described here do not explain the observed increase in ACR in db/db DBA/2J mice compared to the BLKS/J strain. Together, our data suggest that the increased susceptibility to albuminuria of the DBA/2J mouse may be caused by underlying genetic makeup leading to differences in the molecular susceptibility to albuminuric kidney disease.

We characterized the development of metabolic parameters during the transition from systemic insulin sensitivity to resistance, and through to overt diabetes. We observed positive correlations between ACR and systemic insulin resistance as well as glomerulosclerosis scores in the db/db DBA/2J mouse model. Clinically, these findings are potentially important as they mimic the association between microalbuminuria and insulin resistance observed in non-diabetic metabolic syndrome patients (24, 27) and in diabetic subjects (9, 15). Thus, our findings support the applicability of the db/db DBA/2J mouse to study these early stages of insulin resistant glomerulopathies and DN in order to decipher the pathophysiological events that may precede and accelerate the progression to late stage DN.

Together with a growing amount of experimental data from mouse models of diabetes (13, 14) and models of diet-induced obesity (32), the present study confirms the notion that DBA/2J display enhanced sensitivity to metabolically-induced albuminuria kidney disease. When compared to BLKS/J mice, at its onset, systemic insulin resistance was less in the db/db DBA/2J mice, but they exhibited significantly more albuminuria. This suggests that the inherent cellular insulin resistance in DBA/2J mice must be greater than that in the BLKS/J strain, which has previously been described by others (3). Studies have been conducted to identify genetic contributors to diabetes susceptibility in the db/db mouse model (8), but fail to distinguish diabetes and DN-susceptibility loci. Further genetic analyses are therefore required to identify

genes that are differentially expressed between the diabetes-prone DBA/2J and BLKS/J strains and that may explain the molecular difference underlying the present observations as previously achieved in a comparison between the DBA/2J and C57BL/6 strains (29). Finally, as the DBA/2J is a pure inbred strain, it is attractive as it will be possible to generate db/db mice carrying specific transgenes that can be back-crossed onto this albuminuric susceptible genetic background to decipher the molecular mechanisms of this underlying insulin resistance and the influence on the establishment and development of DN in the kidney, glomerulus and even in individual cell populations such as the podocytes in this kidney disease-prone strain.

In summary, we have demonstrated that the db/db DBA/2J mouse develops robust albuminuria by 8 weeks of age alongside histopathological features of early DN including glomerulosclerosis and glomerular enlargement by 8 and 12 weeks of age, respectively. This is closely linked to systemic insulin resistance. The observed correlations between albuminuria and metabolic parameters support the applicability of this model as a clinically relevant model for biomedical research and drug development in insulin resistant states, diabetes and early DN. Further studies are required to explore whether the DBA/2J background also display an enhanced acceleration of the progression to late stage DN in the db/db mouse model of diabetes.

**Disclosure**

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**Figure Legends**

**Figure 1** | **Metabolic phenotype of the db/db DBA/2J mouse. A |** Body weight and **B |** non-fasted blood glucose in female and male db/db DBA/2J mice and wt controls from 6-12 weeks of age. Data are mean ± SEM, n=7-12. *P<0.05, **P<0.01, ***P<0.001: Within same gender and age, groups are significantly different. **C |** Plasma insulin in female and male db/db DBA/2J mice and wt controls at 12 weeks of age. Data are mean ± SEM, n=3-5. ***P<0.001: Within same gender, groups are significantly different.

**Figure 2 | Systemic insulin resistance in the db/db DBA/2J mouse.** Systemic insulin resistance as assessed by Insulin Tolerance Tests (ITT) in female and male db/db and wt DBA/2J mice at 6, 8 and 12 weeks of age. **A, C |** Mice were fasted for 6 h and blood glucose measured before and after intraperitoneal administration of insulin (0.20 IU/kg in females, 0.50 IU/kg in males). **B, D |** Systemic insulin resistance quantified as Area under the curve (AUC) from ITT curves in females and males, respectively. Data are mean ± SEM, n = 3-11. **P<0.01, ***P<0.001: At individual time-points, groups are significantly different.

**Figure 3** | **Urinary albumin excretion in the db/db DBA/2J mouse.** Development of urinary albumin excretion quantified as the albumin-to-creatinine ratio (ACR) in spot urine samples from **A |** female and **B |** male db/db DBA/2J mice and wt controls through 6-12 weeks of age. Dots represent individual animals and lines indicate medians, n = 4-12. **P<0.01, ***P<0.001: At individual time-points, groups are significantly different. **C |** ACR fold change in db/db relative to gender and age-matched wt controls. Bars are mean ± SEM, n = 4-12. ***P<0.001: Independent of gender, significant difference between time-points. **D-F |** Correlations between urinary ACR and **D |** body weight, **E |** non-fasted blood glucose and **F |** systemic insulin resistance in the db/db DBA/2J mouse. Dots represent individual animals and lines are fitted by linear regression within

515 each gender (⊖ females, ⊖ males). All correlations are statistical significant (P<0.001, $R^2$ are
516 given in individual graphs), n = 34-71.
517
518 **Figure 4 | Renal histopathology in the db/db DBA/2J mouse. A |** Representative micrographs of
519 renal cortex and glomeruli from db/db and wt DBA/2J mice by 12 weeks of age stained with
520 Periodic acid-Schiff and Masson's trichrome as well as nephrin and collagen IV antibodies. All scale
521 bars are 50 µm. **B |** Distribution of glomerulosclerosis scores (GS) in db/db DBA/2J mice by 8 (n =
522 2) and 12 weeks of age (n = 4) compared with wt DBA/2J controls (n = 6, compiled across 8-12
523 weeks of age). **C |** Mean GS in db/db DBA/2J and wt mice at 8 and 12 weeks of age. Bars are mean
524 ± SEM, n = 2-4. ****P*<0.001: At individual time-points, groups are significantly different. **D |**
525 Correlation between urinary ACR and mean GS. Dots represent individual animals (n = 16), the
526 correlation was fitted by linear regression (P<0.001, $R^2$ = 0.7686) and the dotted line indicate the
527 95% confidence interval.
528
529 **Figure 5 | Glomerular ultrastructure in the db/db mouse model.** The ultrastructural changes of
530 glomeruli were evaluated by transmission electron microscopy (TEM) at 12 weeks of age. **A |**
531 Representative TEM micrographs of podocyte foot processes and the glomerular basement
532 membrane (GBM) in 12 week old wt and db/db DBA/2J and BLKS/J mice. Glomeruli were
533 systematically evaluated to quantify **B |** the podocyte foot process width and **C |** the GBM
534 thickness in wt and db/db both within and between mouse strains. Bars are means ± SEM.
535 *P<0.05, ***P<0.001: Groups are significantly different.
536
537 **Figure 6 | Modulation of urinary albumin excretion by genetic background in the db/db mouse**
538 **model.** The urinary albumin excretion was evaluated in spot urine samples from male db/db and
539 wt DBA/2J and BLKS/J mice through 8-12 weeks of age. **A |** Evaluation by SDS-PAGE (5 µl urine per

lane) followed by comassie stain using BSA (~66kDa) as positive control. Urinary **B |** ACR and **C |** albumin concentrations in spot urine samples. Dots represent individual animals (n = 4-12) and lines indicate medians. **D |** Urinary creatinine concentrations in spot urine samples. Bars are mean ± SEM, n = 4-12. *P<0.05, **P<0.01: Within same group, strains are significantly different. **E |** Dot blot of serum creatinine measured in 12 week old mice. Analyzed with a one-way ANOVA revealed no significant difference between any groups. N=3-6 for each group.

## Tables

**Table 1 |** DBA/2J breeding outcome

| | | Female | Male | Total |
|---|---|---|---|---|
| **Litters** | | - | - | 12 |
| **Mice per litter (mean)** | | 2.7 | 2.8 | 5.5 |
| **Gender (fraction)** | | 0.49 | 0.51 | 1.00 |
| **Genotype (fraction)** | **db/db** | 0.32 | 0.41 | 0.36 |
| | **db/wt** | 0.52 | 0.44 | 0.48 |
| | **wt/wt** | 0.16 | 0.15 | 0.16 |

**Table 2 |** Metabolic parameters in the DBA/2J and BLKS/J db/db mouse models

| | Body weight (g) | | Non-fasted blood glucose (mM) | | Area under Curve (mM*min) | |
|---|---|---|---|---|---|---|
| | wt | db/db | wt | db/db | wt | db/db |
| **8 weeks** | | | | | | |
| **DBA/2J** | 24.0±1.5 | 33.1±0.7*‡ | 7.88±0.39 | 16.94±2.09*‡ | 450.9±64.2 | 1007.3±87.4*‡ |
| **BLKS/J** | 25.7±0.5 | 39.7±0.8* | 6.95±0.29 | 26.05±1.52* | 372.4±20.2 | 2264.8±147.5* |
| **10 weeks** | | | | | | |
| **DBA/2J** | 25.5±1.6 | 34.9±0.7*‡ | 7.75±0.45 | 27.31±1.19* | ND | ND |
| **BLKS/J** | 27.8±0.8 | 42.2±0.9* | 7.30±0.32 | 29.9±1.21* | ND | ND |
| **12 weeks** | | | | | | |
| **DBA/2J** | 27.3±1.5 | 33.0±1.1*‡ | 7.77±0.32 | 31.01±0.86* | 402.3±42.8 | 1836.8±326.1*† |
| **BLKS/J** | 28.7±0.8 | 42.1±1.2* | 7.10±0.21 | 28.63±1.21* | 405.9±15.3 | 2530.5±121.0* |

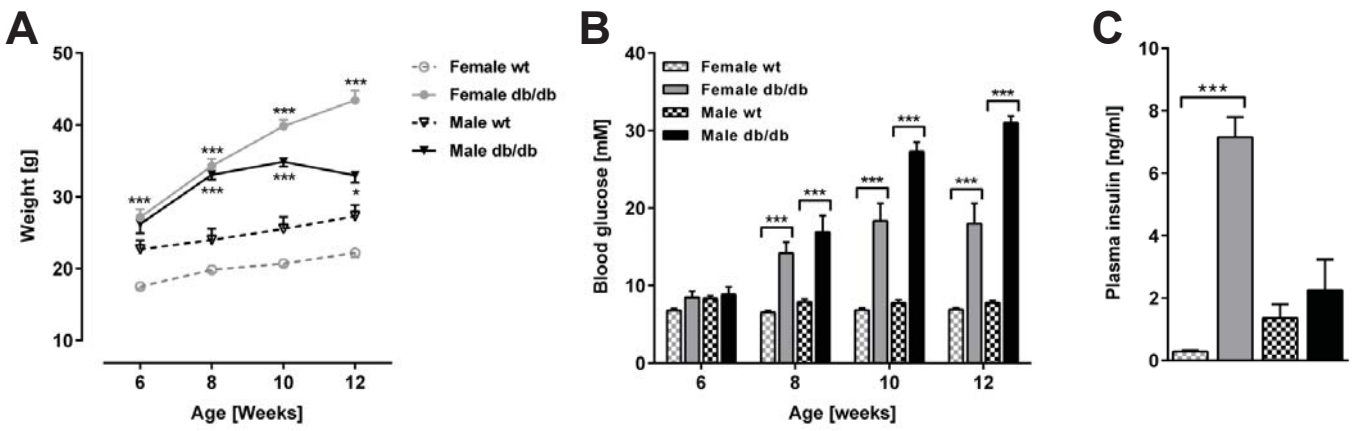
Data are mean ± SEM, n = 3-12 for DBA/2J mice, n = 6 for BLKS/J mice. *P<0.001: Within same strain and age, groups are significantly different. †P<0.05, ‡P<0.001: Within same group and age, strains are significantly different. ND: Not determined.

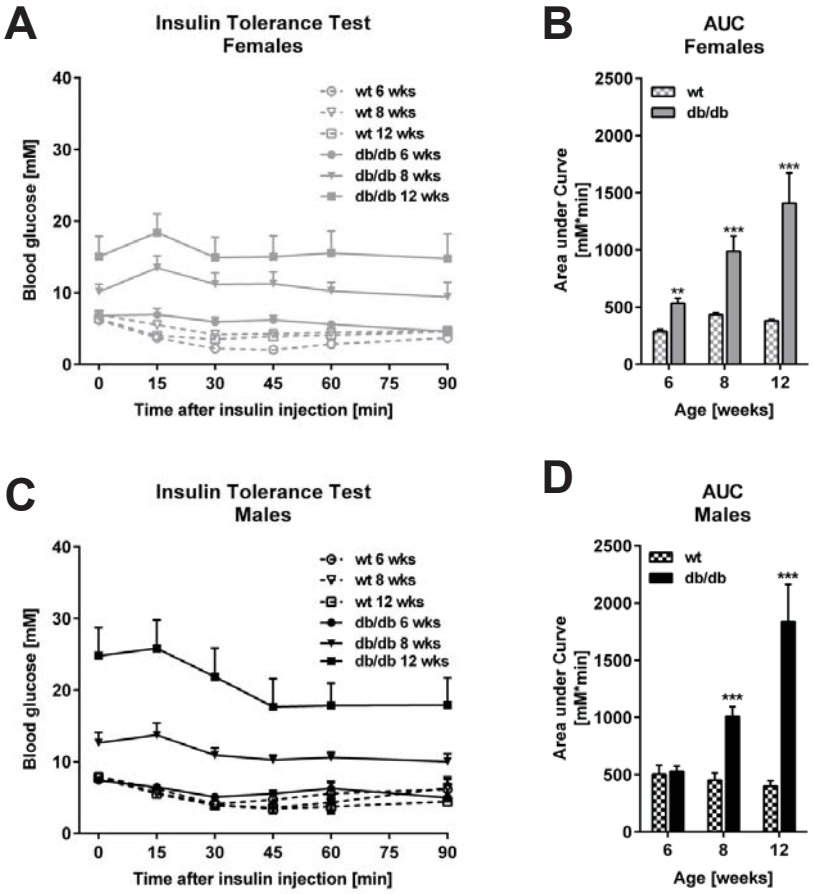
552 **Table 3 |** Kidney weights and glomerular structure in the DBA/2J and BLKS/J db/db mouse models

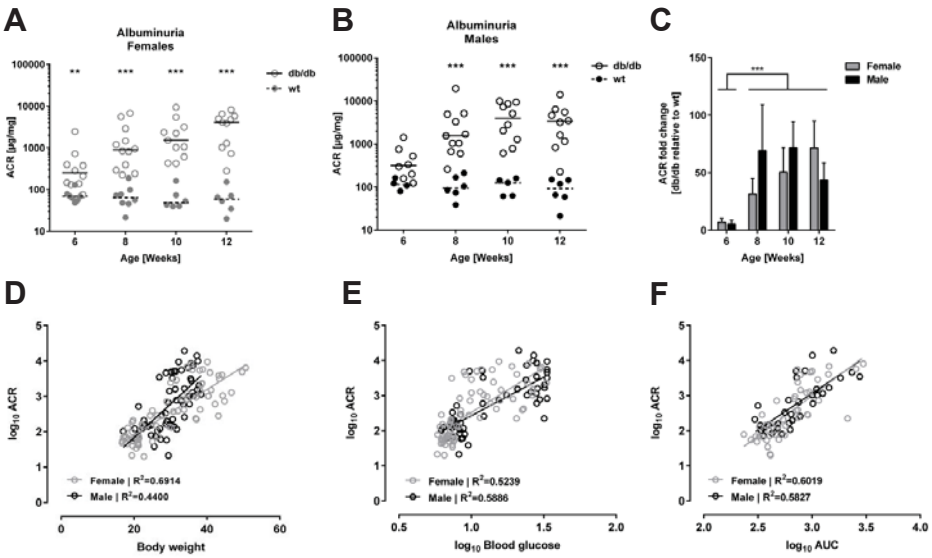
| | Absolute weight (g) | | Relative weight (mg kidney/g body weight) | | Mean glomerulosclerosis score | | Glomerular area ($\mu m^2$) | |
|---|---|---|---|---|---|---|---|---|
| | wt | db/db | wt | db/db | wt | db/db | wt | db/db |
| **DBA/2J** | 0.489±0.034† | 0.524±0.068 | 18.42±0.71‡ | 16.79±3.17‡ | 0.21±0.10 | 1.35±0.13* | 3878±86 | 4653±238* |
| **BLKS/J** | 0.379±0.016 | 0.410±0.024 | 13.77±0.51 | 9.78±0.63* | 0.45±0.13 | 1.47±0.10* | 4274±197 | 4765±220 |

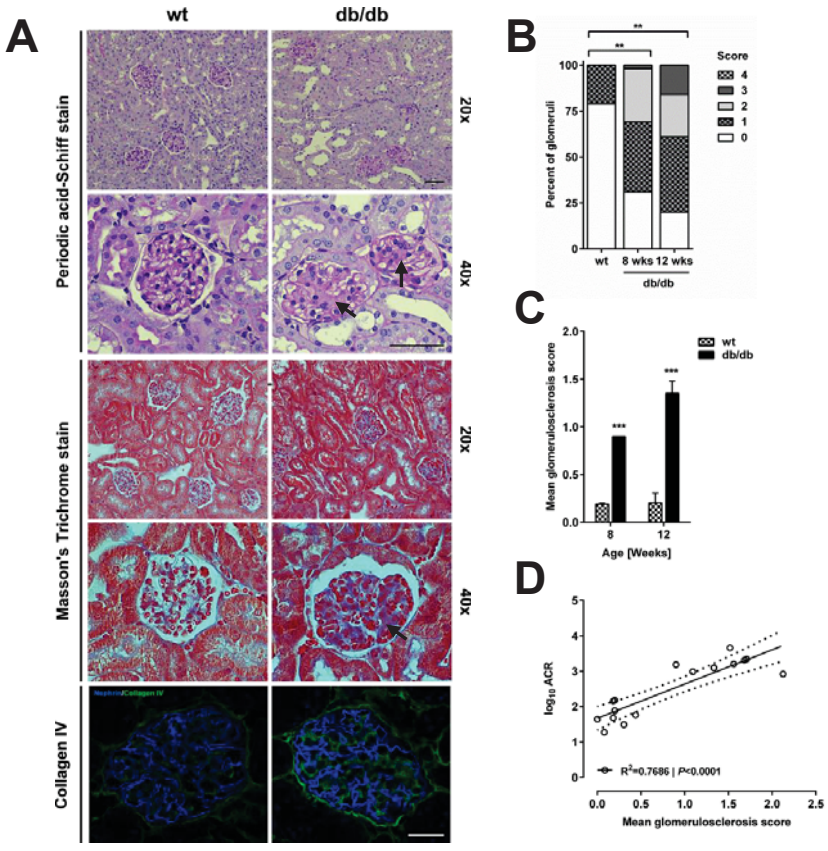
553 Data are mean ± SEM, n = 4-6. *$P$<0.001: Within same strain, significant difference between groups. †$P$<0.05, ‡$P$<0.01:

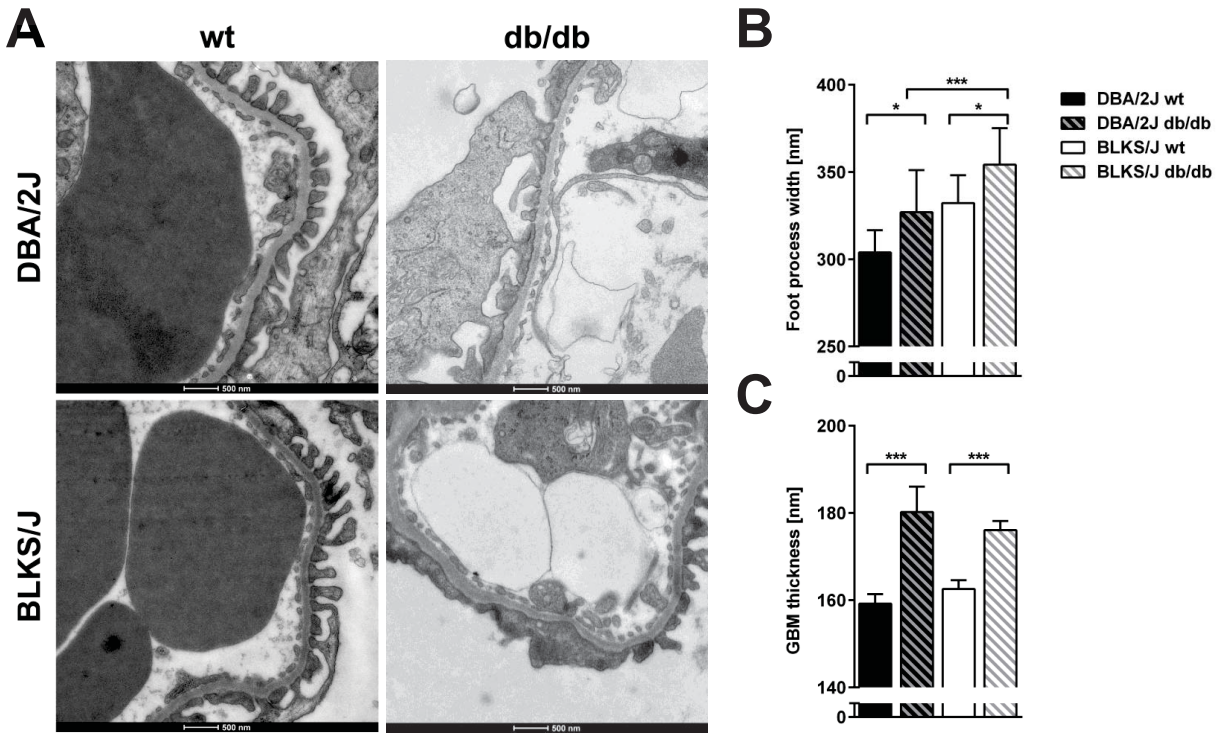
554 Within same group, strains are significantly different.

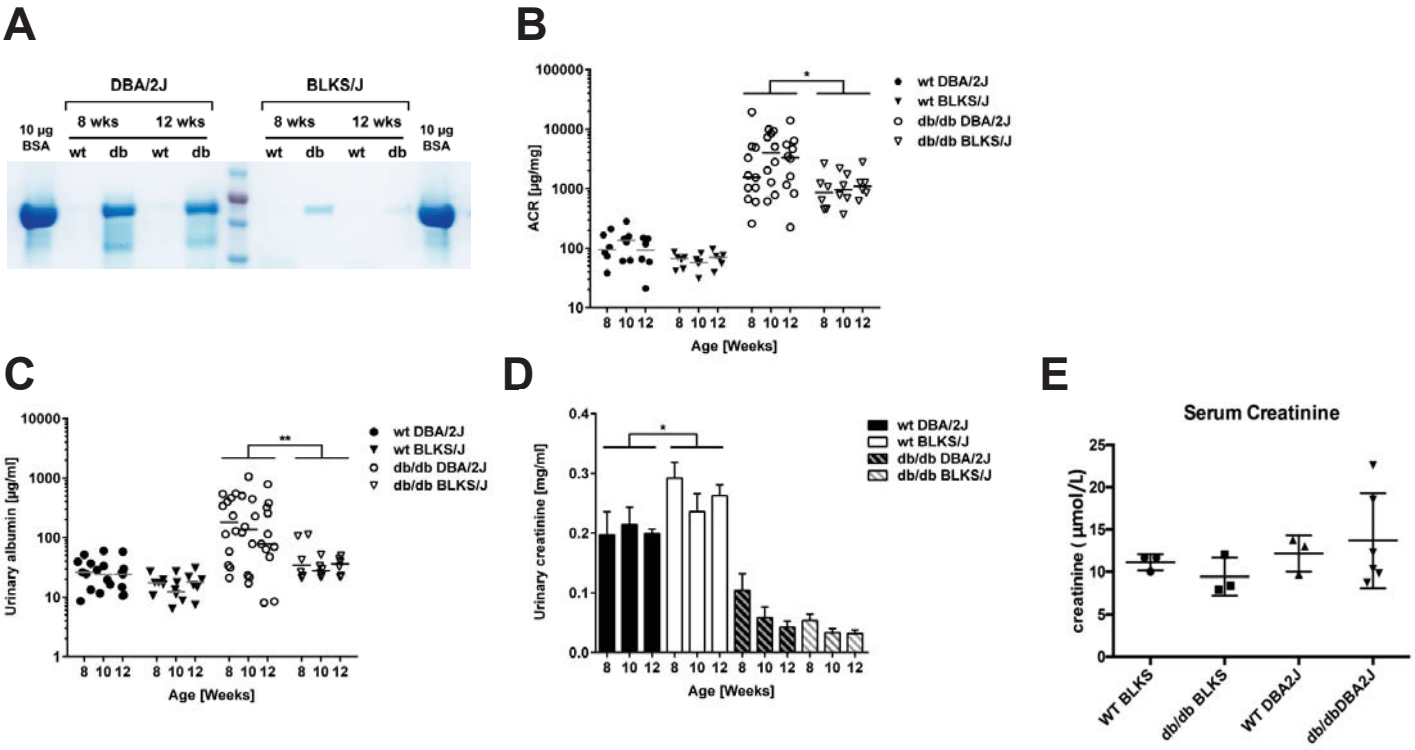

A
Weight [g]
Age [Weeks]
Female wt
Female db/db
Male wt
Male db/db
B
Blood glucose [mM]
Age [weeks]
C
Plasma insulin [ng/ml]


A
Insulin Tolerance Test
Females
wt 6 wks
wt 8 wks
wt 12 wks
db/db 6 wks
db/db 8 wks
db/db 12 wks
Blood glucose [mM]
Time after insulin injection [min]
B
AUC
Females
wt
db/db
Area under Curve [mM*min]
Age [weeks]
C
Insulin Tolerance Test
Males
D
AUC
Males


A
Albuminuria
Females
ACR [µg/mg]
Age [Weeks]
db/db
wt
B
Albuminuria
Males
ACR [µg/mg]
Age [Weeks]
db/db
wt
C
ACR fold change
[db/db relative to wt]
Age [Weeks]
Female
Male
D
log10 ACR
Female | R2=0.6914
Male | R2=0.4400
Body weight
E
log10 ACR
Female | R2=0.5239
Male | R2=0.5886
log10 Blood glucose
F
log10 ACR
Female | R2=0.6019
Male | R2=0.5827
log10 AUC


A
wt
db/db
Periodic acid-Schiff stain
Masson's Trichrome stain
Collagen IV
20x
40x
20x
40x
B
Score
4
3
2
1
0
Percent of glomeruli
100
75
50
25
0
wt
8 wks
12 wks
db/db
**
**
C
wt
db/db
Mean glomerulosclerosis score
2.0
1.5
1.0
0.5
0.0
***
***
8
12
Age [Weeks]
D
log10 ACR
5
4
3
2
1
0
R2=0.7686 | P<0.0001
0.0
0.5
1.0
1.5
2.0
2.5
Mean glomerulosclerosis score


A
wt
db/db
DBA/2J
BLKS/J
500 nm
B
Foot process width [nm]
400
350
300
250
0
*
***
*
C
GBM thickness [nm]
200
180
160
140
0
***
***
DBA/2J wt
DBA/2J db/db
BLKS/J wt
BLKS/J db/db


A
DBA/2J
BLKS/J
8 wks
12 wks
8 wks
12 wks
10 µg BSA
wt db wt db
wt db wt db
10 µg BSA
B
ACR [µg/mg]
Age [Weeks]
wt DBA/2J
wt BLKS/J
db/db DBA/2J
db/db BLKS/J
C
Urinary albumin [µg/ml]
Age [Weeks]
D
Urinary creatinine [mg/ml]
Age [Weeks]
E
Serum Creatinine
creatinine ( µmol/L)
WT BLKS
db/db BLKS
WT DBA2J
db/dbDBA2J