

Podocyte Density and Albuminuria in Aging Diabetic Ins2± Mice with or Without Adenosine A1 Receptor Signaling

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Robert Faulhaber-Walter ¹⁻³
Lanping Jiang⁴
Diane Mizel²
Patricia M Zerfas²
Jeffrey B Kopp ²
Jurgen B Schnermann²
Limeng Chen ^{2,4}
Mario Schiffer^{3,5}

¹Facharztzentrum Aarberg, Waldshut-Tiengen, Germany; ²NIDDK, National Institutes of Health, Bethesda, MD, USA; ³Department of Nephrology, Medical School Hannover, Hannover, Germany; ⁴Peking Union Medical College Hospital, Beijing, People's Republic of China; ⁵Department of Nephrology, University of Erlangen, Erlangen, Germany

Aim of Study: To investigate podocyte density in aging diabetic Ins2± and Ins2±, A1AR-/- mouse models in C57Bl/6 background.

Methods: Ins2± mice and especially Ins2±, adenosine A1 receptor knockout mice (Ins2±, A1AR-/-) are mouse models with a phenotype of diabetic nephropathy. Aged mice (at ~40 weeks) were assessed for glomerular filtration barrier function by measuring albuminuria, glomerular filtration, glomerular damage by electron microscopy, and podocyte numbers by Wilms Tumor protein (WT-1) staining.

Results: Compared to healthy wild-type mice, both diabetic mouse models developed diabetic nephropathy, including hyperfiltration (p<0.01) and albuminuria (p<0.05). Typical diabetic structural glomerular and podocyte damage was visualized by electron microscopy. Podocyte count per glomerular area (podocyte density) was significantly decreased in both diabetic mouse models (p<0.01). In contrast, no significant correlation was detected between albuminuria and absolute podocyte count per glomerulus.

Conclusion: The amount of albuminuria as marker of diabetic nephropathy does not correlate with the podocytes density; however, a relative podocyte deficiency became evident with an increase in glomerular area in the diabetic animals, suggesting a relative podocytopenia.

Keywords: diabetes nephropathy podocyte mouse Akita WT-1

Introduction

Diabetic nephropathy is a serious complication of type 1 and type 2 diabetes mellitus and the most frequent prevalent cause for progression to end-stage renal disease in the developed world with rising incidence.¹ Diabetic nephropathy typically progresses through distinct stages with increasing amounts of protein being excreted, advancing from microalbuminuria to overt gross proteinuria and possibly nephrotic syndrome.² In advanced stages, kidney damage includes glomerulosclerosis, demise of glomerular capillaries, tubulointerstitial atrophy and fibrosis associated with progressive loss of glomerular filtration rate.³ Very early after the onset of diabetes, podocytes frequently show distinguished pathologic and functional features like broadening of the width of foot processes, reduced nephrin expression, increased urinary excretion of nephrin, increased VEGF or TGF-β/Smad 3.⁴ Podocyte proteins like nephrin or podocin among others are crucial for maintaining the intact glomerular filtration barrier⁵ and their dysregulation in the hyperglycaemic milieu leads to diabetic podocytopathy with foot process fusion, effacement and detachment from the glomerular basement membrane.⁶

Correspondence: Robert Faulhaber-Walter
Facharztzentrum Aarberg, Tannenstrasse 4,
Waldshut-Tiengen 79761, Germany
Tel +49-151-27164882
Fax +49-321-21036268
Email rofaulhaber@web.de

In addition, a decline in podocyte numbers has been demonstrated in mouse models of both type 1 and type 2 diabetes, and apoptosis and epithelial-to-mesenchymal transition likely contribute to podocyte loss.^{7,8} The reduction of podocyte numbers has been associated with the progression of diabetic glomerulosclerosis.^{9,10} The loss of the intact glomerular filtration barrier seems to be implicated with the increasing amounts of proteinuria which in turn results in tubulointerstitial inflammation and fibrosis.¹¹ The ongoing decline of podocyte numbers has a stronger correlation with the progressive decline of GFR in diabetic nephropathy, than the amount of protein excreted in the urine. This indicates a major role of the podocytes not only in early stages but also in advanced diabetic nephropathy.^{12,13}

To our knowledge, only one study reports data for podocyte numbers of older diabetic mice (OVE26 at ~64 weeks).¹⁴ Our own experience with severely diabetic mice suggested that a reduced life expectancy (as for instance in *Ins2[±]* models¹⁵) causes difficulties maintaining sufficient high sample numbers in the time course of a longitudinal study to gain valid data.^{16,17} Therefore, in view of the major role of podocyte dysfunction and loss in initiation, development and progression of diabetic nephropathy, our study aimed to further clarify the role of podocytes in aged mice with long-term diabetic nephropathy.

Materials and Methods

Animals

Experiments were performed in mice on a C57Bl/6 background that were bred in our laboratory as described previously.¹⁸ In brief: Male *Ins2[±]* mice (*Ins2[±]*; C57Bl/6 background) from Jackson Laboratories (Bar Harbor, ME, USA) were crossed with female C57Bl/6 wild-type mice. To generate *Ins2[±]*, *A1AR^{-/-}* double mutants, female *A1AR^{-/-}* mice (C57Bl/6 background) were crossed with male F2 mice heterozygous for both the *Ins2[±]* and *A1AR* mutations. Mice had unrestricted access to tap water and received standard rodent chow (NIH-07 rodent diet), containing 24 cal% protein 12 cal % fat, and 64 cal% carbohydrate. Experiments were performed in male mice of 35–42 weeks of age to assure an advanced diabetic renal phenotype. For electron microscopy, mice from 16 to 46 weeks of age were examined for signs of developing and/or evolved diabetic nephropathy. All animal studies were examined and approved by the Animal Use and Care Committee of the National Institute of Diabetes and Digestive and

Kidney Diseases, and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Glomerular Filtration Rate (GFR)

GFR was measured by single injection FITC inulin clearance as described by Qi et al¹⁹ modified to minimize plasma withdrawal. FITC-inulin (5%), dialyzed overnight against 0.9% NaCl (final concentration ~3%), was injected at 3.7 μ L/g BW into the retroorbital plexus during brief isoflurane anesthesia (recovery within about 20 s). At 3, 7, 10, 15, 35, 55 and 75 min, the mice were placed in a restrainer, the tail vein was punctured with a 30 g atraumatic needle, and ~2 μ L blood was collected into heparinised 5- μ L microcaps (Drummond Scientific, Broomall, PA). Five hundred nanolitres of plasma was diluted 1:10 in 500 mmol HEPES (pH 7.4) and measured against a standard curve (1 μ L of ~3%-FITC-inulin diluted 1:50, 1:100 and 1:500 in 500 mmol HEPES). Fluorescence was determined in 1.7 μ L in a Nanodrop-ND-3300 spectrometer (Nanodrop Technologies Inc, Wilmington, DE). GFR was calculated using a two-compartment model of two-phase exponential decay.

Blood Sampling and Determination of Blood Glucose

Blood samples were taken at midmorning after ~16 h fasting from conscious mice by tail vein puncture using 30 g needles. Blood glucose concentration was measured with Glucostix AccuCheck active (Roche Diagnostics, Indianapolis, IN, USA).

Urine Albumin and Glucose Excretion

Urine collections for 24 hr were obtained in metabolic cages (Hatteras Instruments, Cary, NC, USA) at ambient room temperature with unrestricted access to tap water and standard rodent diet. Urine albumin was measured by ELISA (Albuwell M, Exocell, Philadelphia, PA, USA) according to the manufacturer's guidelines. Twenty-fourhour albuminuria was calculated from concentration and volume. Urine glucose excretion was calculated from concentration and volume after measuring glucose in urine (for Wt) or 1:10 aqua bidest-diluted urine (for diabetic mice) with Glucostix AccuCheck active (Roche Diagnostics, Indianapolis, IN, USA).

Tissue Sampling and Histochemistry

Selected mice were euthanized by rapid cervical dislocation. Kidneys were excised, gently squeezed to release urine and

then weighted to measure the wet kidney weight. Thereafter, the kidneys were cut longitudinally and frozen in liquid nitrogen. Frozen sections were cut under a layer of Tissue-Tek O.C.T. Tissue preparation for histochemistry was provided by Histoserv (Germantown, MD, USA). Three μm sections were cut from paraffin-embedded tissue and stained with haematoxylin–eosin (HE), Masson's trichrome and periodic acid Schiff (PAS)-staining.

Immunolabeling Podocytes and Measurement of Glomerular Area

Tissue sections were dewaxed with xylene and rehydrated with graded ethanol. After boiling in Tris-EDTA buffer (10 mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0) for 25 min, sections were blocked in 10% normal goat serum with 1% BSA in TBS for 2 hrs at room temperature. Sections were stained with primary WT-1 (Wilms Tumor protein) antibody (Ab89001, Abcam, Cambridge, MA, USA) using ABC Kit (PV-9001, Zhongshan Goldenbridge, Beijing, China) that functions as podocyte marker.²⁰ Figure 1 shows representative WT-1 stained glomeruli. Analysis software (Image-Pro Plus 6.0), Media Cybernetics, Rockville, MD) generated a binary image in which the stained number, density and area could be automatically calculated as percentage of every glomerular area. Fifty fields per specimen were randomly selected that covered nearly the whole piece of cortex. Staining and scoring was performed by a single observer who was masked to slide identity.

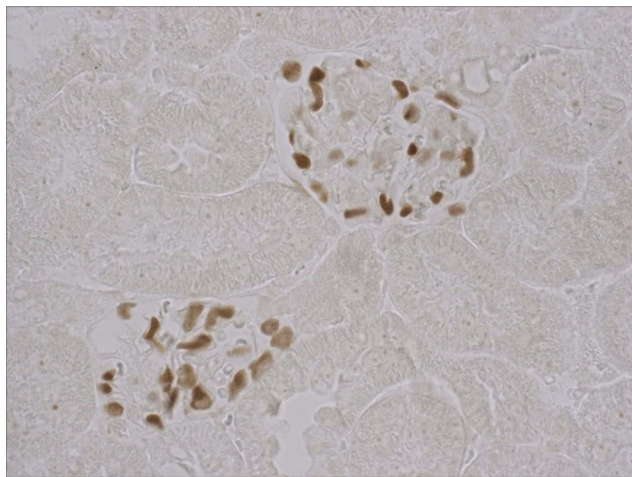


Figure 1 WT-1 stained glomeruli. Sections from kidney tissue s were stained with primary WT-1 (Wilms Tumor protein) antibody (Ab89001, Abcam, Cambridge, MA, USA) using ABC Kit (PV-9001, Zhongshan Goldenbridge, Beijing, China) that functions as podocyte marker 400 \times .

Electron Microscopy

Kidney tissue specimens were prepared, processed and visualized as described previously.¹⁸ Visual analysis was descriptive.

Statistical Analyses

Data are presented as mean \pm standard deviation (SD). Multivariable data were compared by one-sided ANOVA with Tukey or Bonferroni post-hoc testing. Two-sided Student's test was used for univariable comparison, adding Welch's correction in case of unequal variances. Correlations were tested by linear regression analysis.

Results

Diabetic Phenotype

The diabetic phenotype of the aged diabetic mice was shown in both $\text{Ins}2^{\pm}$ strains. Mice lacking also the A1-adenosine receptor ($\text{Ins}2^{+/-}$, $\text{A1AR}^{-/-}$) develop a more severe diabetic phenotype and more severe diabetic nephropathy, as described below. Several mice from our study groups died early and did not reach an advanced age of about 40 weeks, as intended in our study. However, those surviving displayed a continuously increasing severity of the diabetic phenotype with age. The same applies for the development of the diabetic nephropathy. Despite our demonstration of severe scarring and sclerosis of the kidney at 27 weeks of age, as reported previously,¹⁸ especially in the $\text{Ins}2^{\pm}/\text{A1AR}^{-/-}$ model the hallmarks of diabetic nephropathy, like hyperfiltration and overt proteinuria, did not climax but were continuing to worsen further within the observed age span. Thus, both diabetic mouse models developed diabetes and diabetic nephropathy with a progressing phenotype with increasing age. At about 40 weeks of age, the kidneys were significantly hypertrophic compared to wild-type mice (WT) and at least doubled in wet weight. The high fasting blood glucose (WT: 208 ± 29 mg/dl vs. $\text{Ins}2^{\pm}$: 786 ± 99 mg/dl vs. $\text{Ins}2^{\pm}$, $\text{A1AR}^{-/-}$: 914 ± 100 mg/dl, $n=4-6$; $p < 0.001$ [Figure 2A]) led to a significant rise in glucosuria that was accompanied by a very large increase of daily urine volume in both diabetic mouse models. Moreover, as expected, the double knockout model displayed a more pronounced diabetic phenotype compared to the $\text{Ins}2^{\pm}$, excreting up to ~ 12 g of glucose daily in a urine volume that reached about half its body weight. The metabolic disturbance caused significant albuminuria, the hallmark of diabetic nephropathy. However, the glomerular filtration rate per g of bodyweight was still hypercompensated, with a significant hyperfiltration in both diabetic mice models, and particularly pronounced in

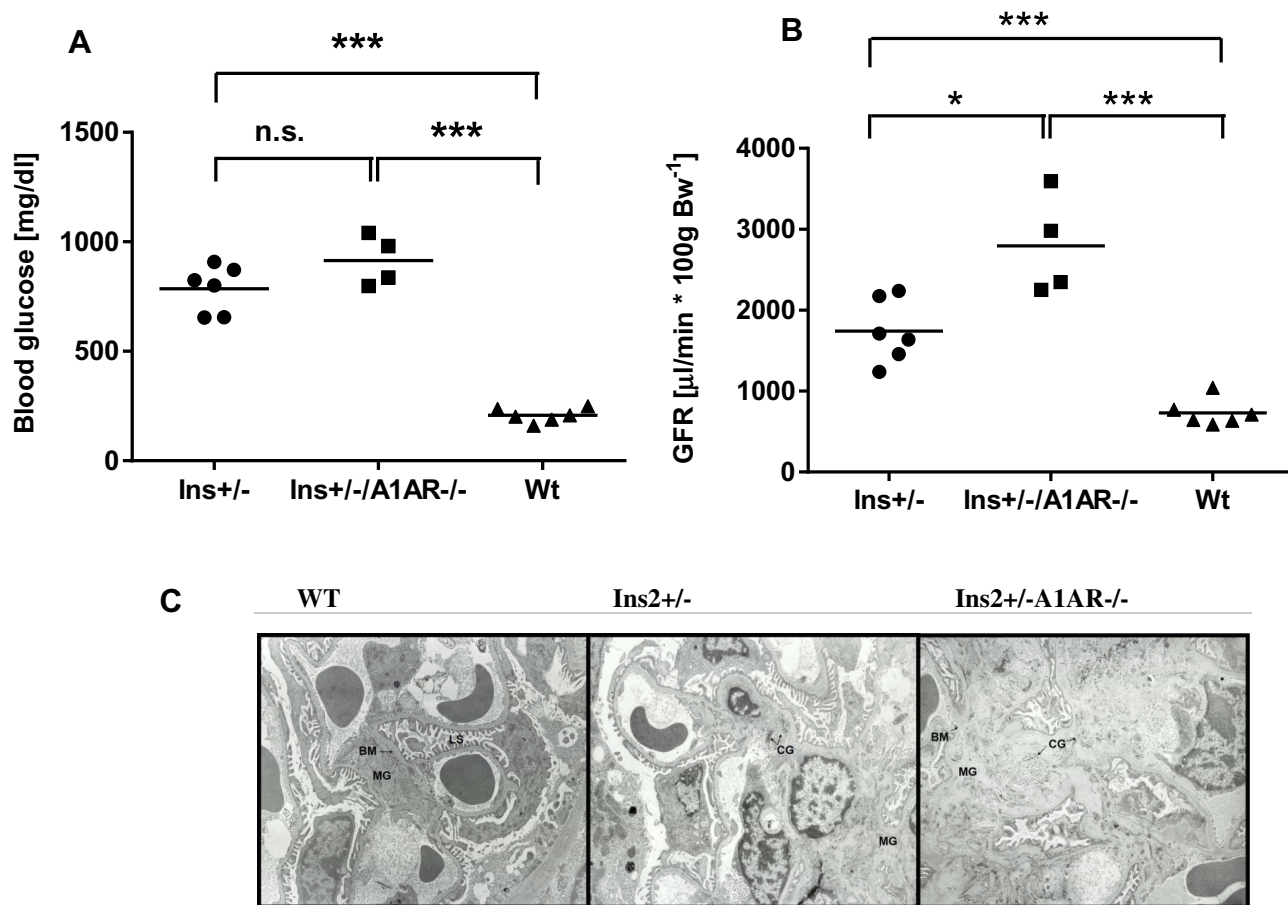


Figure 2 (A) Fasting blood glucose. Both diabetic phenotypes increased fasting blood glucose compared to the Wt mice, with the highest levels in the double mutant mice lacking A1AR. Whole blood glucose in mg/dl after 12–16 h fasting period. Wt vs. Ins[±] and Ins[±]/A1AR^{-/-} mice. n=4-6; age 39 weeks; *** p<0.0001. **(B)** FITCI-GFR. Significant hyperfiltration in the mutant diabetic mice, with the highest values in the double mutant mice lacking A1AR, shown by Fluorescence-inulin-thiocyanate (FITCI) single bolus excretion kinetics glomerular filtration rate calculated as $\mu\text{L}/\text{min}$ by 100 g of body weight. Wt vs. Ins[±] and Ins[±], A1AR^{-/-} mice. n=4-6; age 39 weeks; *p<0.05, ***p<0.0001. **(C)** Fibrotic lesions in diabetic nephropathy of adult mice. Electron-microscopic images of mice kidney samples at ~25 weeks of age demonstrate pronounced mesangial expansion with thickening of basal membrane, compression of luminal space and diffuse deposition of collagen fibres in Ins^{2±}A1AR^{-/-} kidney, consistent with severe diabetic nephropathy. Milder diabetic lesions in Ins^{2±} kidney. Enlargement $\times 11880$.

Abbreviations: n.s., non-significant; MG, mesangium; BM, basal membrane; LS, Luminal Space; CG, Collagen fibres.

the double knockout model (GFR/g/Bw: WT: $732 \pm 149 \mu\text{L}/100\text{g BW}$ vs. Ins^{2±}: $1743 \pm 360 \mu\text{L}/100\text{g BW}$ vs. Ins^{2±}/A1AR^{-/-}: $2795 \pm 541 \mu\text{L}/100\text{g BW}$, n=4-6; p<0.001 [Figure 2B]). Table 1 summarizes the main findings.

Histology

In our previous study light microscopy analysis revealed a significant increase of PAS-positive material in the mesangial areas of the adult diabetic Ins^{2±}, A1AR^{-/-} mouse, present at 26 weeks, compared to the diabetic Ins^{2±} mouse and normal glomeruli in the WT mouse.¹⁸ Interestingly, in that study we could not find significant mesangial expansion in the Ins^{2±} mice despite the diabetic phenotype, suggesting a protecting effect of the tubular glomerular feedback mechanism in diabetic nephropathy. However, progressive diabetic nephropathy features interstitial fibrosis and glomerulosclerosis. Therefore,

in this study, electron microscopic sampling was employed to visualize the extent of structural diabetic lesions: The differences between WT, Ins^{2±} and Ins^{2±}/A1AR^{-/-} mice were clearly apparent at 25 weeks of age. Both diabetic mouse models displayed an increased amount of collagen fibers, thickening of the glomerular basement membrane and compression of the glomerular capillary lumens. Effacement of podocyte foot processes as one sign of diabetic podocytopathy was clearly visible in both Ins^{2±} models, but again much more pronounced in the Ins^{2±}/A1AR^{-/-}, consistent with a more advanced phenotype (Figure 2C).

Podocyte Count and Glomerular Size

We found no difference in absolute podocyte numbers, as determined by WT-1 staining. The means of 50 measurements per specimen in each group were $n=17.6 \pm 5.2$ in

Table 1 Diabetic Phenotype: Characteristics of Mice, Age >37 Weeks

Genotype	WT	Ins2± "Ins2±"	Ins2+/-A1AR-/-	p
Age (weeks±SD)	42±2.5	42±3.0	38±1.8	n.s.
Body weight (g±SD)	36±6.3	26±1.9	28±3.5	**
Wet kw/bw (mg/g±SD)	10±1.3	20±1.7	25±1.8	***
Urine volume (mL/24h±SD)	820±349	5001±2579	15,453±9599	***
Glucose excretion (mg/24h±SD)	5±2.6	5148±2878	12,791±5319	***
Albumin excretion (µg/24h±SD)	12±6.5	79±91	103±39	*

Notes: Determination of the diabetic renal phenotype in mutant mice. Increase of body weight, kidney weight (shown as wet kidney weight to body weight ratio), urine volume, glucosuria and albuminuria are shown according to the genotype. Most severe diabetic renal phenotype developed in the double mutant mice lacking A1AR. Statistics: One-way ANOVA. ***p<0.001; **p<0.01; *p<0.05.

Abbreviations: SD, standard deviation; kw, kidney weight; bw, body weight; n.s., non-significant.

Ins2± vs. n= 18.9±5.4 in Ins2±, A1AR-/- vs. 18.3±5.6 in WT mice (ANOVA: n.s.) (Figure 3A). The WT-1 positive area as well did not differ significantly, but an enlarged WT-1 positive area was obvious in the Ins2±, A1AR-/- mice compared to the Ins2± and the wild-type mice (308.8 ±122 µm² vs. 256.8 ±100 µm² vs. 259.7±103.5 µm²; ANOVA: n.s.; *t*-test diabetic groups: p=0.159, data not shown). The effect could possibly be explained by a trend towards enlargement of the podocyte nuclei in A1AR-/- mice, but further inquiries to enlighten this finding were not part of the current project.

As expected, the diabetic phenotype led to a highly significant glomerular hypertrophy of both aged diabetic phenotypes when compared to the WT mice (Ins2±, A1AR-/-: 4.65±1.2 mm² vs. Ins2±: 4.05±1.0 mm² vs. wild-type 3.32±1.0 mm²; ANOVA p<0.01) (Figure 3B). Loss of the adenosine A1 receptor function resulted in a more pronounced increase of the glomerular area when compared to the pure Ins2± diabetic phenotype; however, due to the small sample size the trend was not significant (p=0.062). Therefore, the relative podocyte densities were significantly reduced in both diabetic phenotypes compared to the WT mice (Figure 3C).

Albuminuria versus Podocyte Numbers

Although glomerular hypertrophy was not clearly different between the two diabetic models, the Ins2±, A1AR-/- mice had a significantly higher urine albumin excretion compared to the WT and the Ins2± mice. Of note, there was no proportional correlation of albuminuria of the Ins2±,

A1AR-/- model in comparison to the Ins2± mouse model. In addition, there was no correlation of albuminuria and absolute podocyte numbers (data not shown). Therefore, the amount of albuminuria as an indicator of disease severity was not determined by the absolute number of podocytes in this model of diabetic nephropathy. When we correlated albuminuria in the 24 hr urine with the number of podocytes per unit glomerular area (podocyte density), we found, that with aggravating diabetic phenotype, the correlation was increasingly lost, with R²=0.0613 in the Ins2± model and R²=0.0089 in the Ins2±, A1AR-/- model (Figure 4).

Discussion

In our study, we focused on the correlation of a diabetic nephropathy phenotype with the podocyte numbers in two different Ins2± mice strains. Next to the well-characterized and commercially available (Jax laboratories) Ins2± (Akita) strain, we used a self-bred double mutant strain (Ins2±, A1AR-/-), that lacks the adenosine A1 receptor and develops a more pronounced diabetic phenotype and nephropathy.^{17,18} Our main findings were to demonstrate the progressive worsening with age of the dysregulated hyperglycaemic phenotype with increasing blood glucose levels, rising urinary glucose excretion, rising glomerular hyperfiltration and increasing albuminuria in both diabetic mouse models, but especially in the Ins2+, -A1AR-/. In contrast, this progression of severity does not seem to concur with a progressive loss of absolute podocyte numbers. We conclude that in the late phases of diabetic nephropathy in aging mice, podocytopathy does not play the same pathophysiological role in the Ins2± mouse model of type 1 diabetes as it does in early and moderate diabetic nephropathy.

Podocytes are responsive to insulin, glucose, AGE and TGF-β, which are important factors of the diabetic milieu that promote podocyte survival, cellular hypertrophy and apoptosis.²¹⁻²³ We previously described that in the early phase of diabetes there is an initial loss of podocytes that is detectable coinciding with the beginning of hyperglycaemia. After that, the number of podocytes appears stable, while proteinuria further increases.⁷ This indicates that the remaining podocytes are able to adapt to the diabetic conditions, presumably via a change in their metabolic profile.²²

Our results confirm now, that in our chosen mouse model of type 1 diabetes with diabetic nephropathy, the podocyte numbers do not play a major role for the

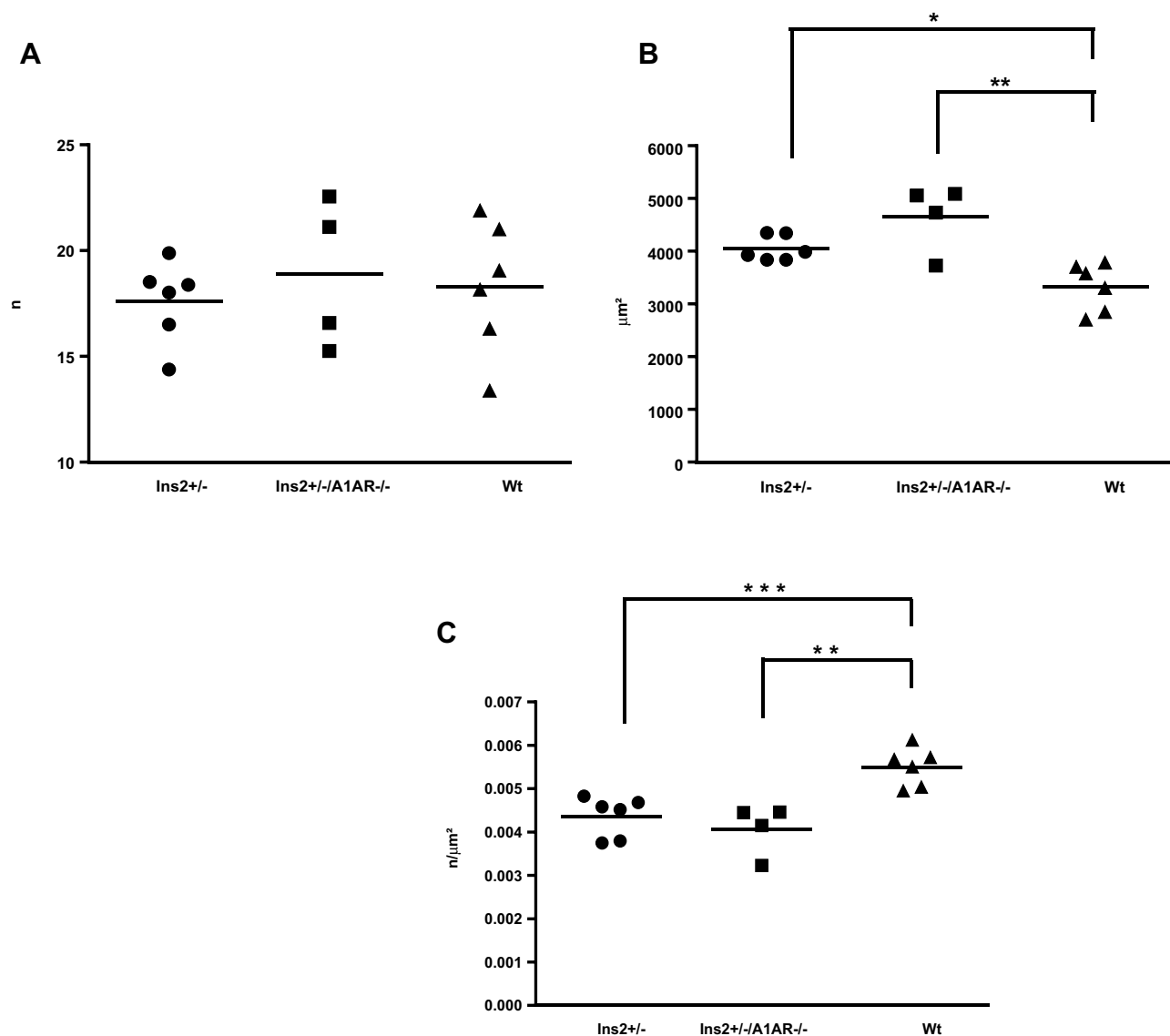


Figure 3 (A) Absolute podocyte numbers. No significant differences of absolute numbers of podocytes after immunostaining of glomeruli for the specific podocyte marker Wilms-tumor-1 antigen (WT-1). n =mean of 50 measurements per specimen. Mean per group. (B) Glomerular area. Glomeruli were enlarged in both *Ins2±* and *Ins2±/Al1AR-/-* mice, with a non-significantly greater effect in the latter. Glomerular area calculated by computerized automated scoring of 50 glomeruli. Mean per group. * p <0.05, ** p <0.01. (C) Podocytes per glomerular area (podocyte density). Podocyte density was reduced both in *Ins2±* and *Ins2±/Al1AR-/-* mice compared to WT mice. Mean per group. ** p <0.01, *** p <0.001.

peculiarity of the phenotype in terms of proteinuria. Although we could clearly define a much more pronounced diabetic phenotype in the *Ins2±/±/Al1AR-/-* model compared to the *Ins2±/±* mouse, the differences in absolute podocyte numbers and podocyte density were not significant. Therefore, these data do not support a leading role for the injured podocyte function in progression of the diabetic nephropathy in the chosen models of aging diabetic mice. It is well known that the amounts of proteinuria correlate with the dynamics of loss of glomerular function since it is a measure of glomerular and tubular injury in diabetic nephropathy.

After an initial loss of podocyte integrity, detectable as microalbuminuria,²⁴ with progress through the stages of diabetic nephropathy, the amount of proteinuria gradually increases, e.g. to overt proteinuria or even a nephrotic range proteinuria, and may change in pattern to a, for instance, selective-glomerular type or even non-selective-glomerular and tubular type of proteinuria. The occurrence of protein in the urine is also a function of an increased glomerular filtration and reduced tubular reabsorption urinary protein. Thus, the changing pattern that occurs during the progression of diabetic nephropathy probably reflects a differentiation of the causal

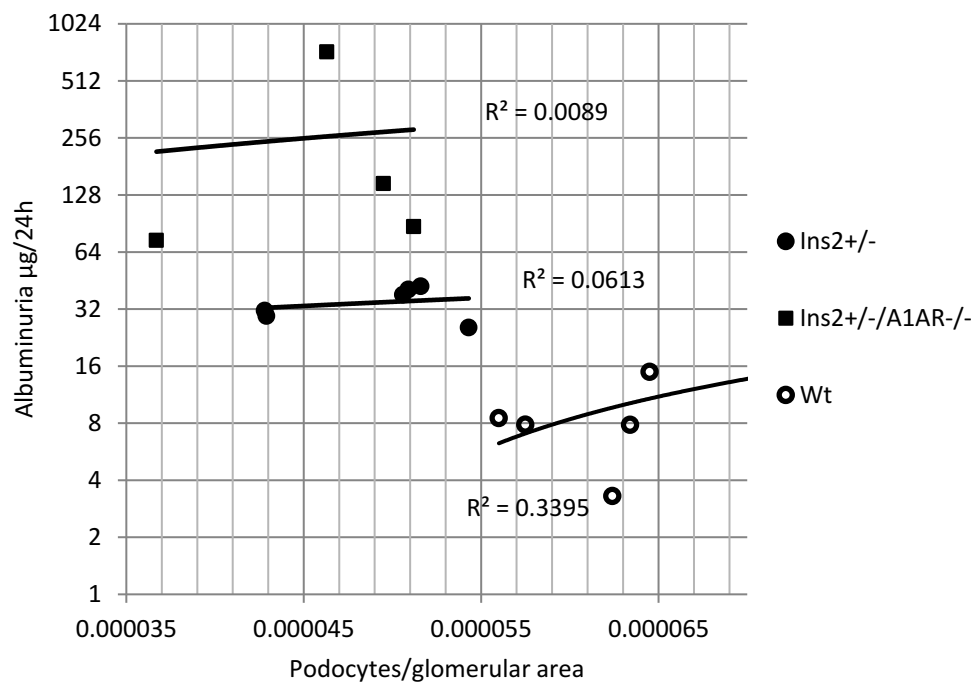


Figure 4 24h-albuminuria vs. podocyte density. Log₂- display of total 24 h albuminuria versus podocytes per glomerular area (podocyte density). Linear regression analysis. R² values (=coefficients of determination) show a decreasing trend of correlation of albuminuria and podocyte density with the severity of diabetic phenotype.

mechanisms. Due to cytokines like TGF- β and others, the progressive diabetic nephropathy in later stages is eventually dominated by interstitial fibrosis and nephron loss, which finally terminates the kidney function. Thereby, in view of our results, the role of the podocyte after initiating the disease process seems not to be a leading trigger for further progress through the later stages. Our data show that absolute podocyte numbers remain relatively stable in the advanced stages of diabetic nephropathy in the Ins2 \pm mouse model and do not correlate to the amount of albuminuria. However, we speculate, that the decreased podocytes density of the increased glomerular size of the diabetic kidney indicates reduced total functional efficacy of the remaining podocyte population, thus nevertheless contributing to the diabetic renal phenotype.

The pursuit of the optimal mouse model for advanced diabetic nephropathy continues.

Two well-established contenders are the Ins2 \pm mouse²⁵ and STZ induced eNOS $^{-/-}$ ^{26,27} model of type 1 diabetes. Alpers and Hudkins, in a recent review,²⁸ have discussed two newer models: BTBR ob/ob, a genetically engineered type 2 mouse model in C57bl/6 background which lacks leptin,²⁹ the other, a diabetes type 1 model, the OEV26 mouse.³⁰ Investigations into the role of podocytes in these diabetic nephropathy models mostly were obtained by

studying the mouse models in early or moderately progressed diabetic nephropathy the Ins2 \pm model with 28 weeks being an example.⁷ Our study is limited since we studied podocyte number but not podocyte function or differentiation. Thus, more studies in advanced aged study populations of diabetic mice should be undertaken since this age group might mirror the long-term diabetic nephropathy in many of the human patients we treat.

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Disclosure

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