Aus dem Max-Delbrück-Centrum für Molekulare Medizin (MDC)
&
der Abteilung für Nephrologie der Northwestern University

DISSERTATION

Functional and morphological intrarenal changes in mice lacking Aminopeptidase A and their susceptibility to glomerular injury

Funktionale und morphologische intrarenale Veränderungen in Aminopeptidase A knock-out Mäusen und ihre Anfälligkeit für glomeruläre Schädigung

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von
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Datum der Promotion: 03.12.2021

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1. Abstract (English)

Aminopeptidase A(APA) is an important Renin-Angiotensin-System(RAS) enzyme which is predominantly expressed in the glomerulus by podocytes and degrades both Angiotensin (Ang) I and AngII. Our objective was to determine the effect of APA deficiency on the kidney including functional as well as morphological alterations and examine whether APA-/- mice are more susceptible to glomerular injury.
We used a mouse model of APA deficiency on Balb/c genetic background. In kidneys harvested from APA-/- mice, AngII and AngI levels were measured by EIA. The activities and mRNA levels of ACE and several AngII degrading enzymes were determined as were ACE protein levels. APA-/- kidneys were examined by light microscopy(LM), electron microscopy(EM), immunohistochemistry and immunofluorescence. We measured blood pressure using a tail-cuff system. Albumin creatinine ratios were determined in APA-/- mice infused with AngII by osmotic mini-pumps and after 12 weeks of STZ-induced diabetes in APA-/- and WT.
APA-/- mice showed mild glomerular mesangial expansion and increased blood pressure without significant albuminuria. By EM, APA-/- exhibited a mild increase of the mesangial matrix and moderate thickening of the glomerular basement membrane(GBM) with a striking appearance of knob-like structures. The knob-like structures were observed in male and female mice and persisted after treatment with Telmisartan. APA-/- mice showed increased albuminuria after 1 week of AngII infusion. After 12 weeks of STZ induced diabetes albuminuria was significantly increased in diabetic APA-/- as compared to diabetic WT. Surprisingly, AngII levels in APA-/- kidneys, were not different compared to WT and the activities of other AngII degrading enzymes were downregulated rather than upregulated. However, kidney lysates from APA-/- mice showed a profound decrease in ACE activity, mRNA and protein levels.
Deficiency of APA results in glomerular morphological alterations in the mesangial stalk and the GBM. The downregulation of ACE likely counterbalances the impaired AngII degradation due to APA deficiency. Our findings support a role of APA in maintenance of glomerular structure and intrarenal Ang peptide homeostasis.

2. Abtsrakt (Deutsch)

Aminopeptidase A(APA) ist ein wichtiges Enzym des Renin-Angiotensin-Systems(RAS), welches vorwiegend im Glomerulus von Podozyten exprimiert wird und sowohl Angiotensin (Ang) I als auch AngII abbaut. Unser Ziel war es, die Auswirkungen eines APA-Mangels auf die Niere zu untersuchen, einschließlich funktioneller und morphologischer Veränderungen, und zu ermitteln ob APA-/- Mäuse anfälliger für glomeruläre Schädigungen sind.
Wir verwendeten ein Balb/c Mausmodell mit totalem APA-Mangel. In Nieren von APA-/- und WT, wurden die AngII- und AngI-Spiegel mittels EIA gemessen. Die Aktivitäten und mRNA-Spiegel von ACE und verschiedenen AngII-abbauenden Enzymen wurden ebenso bestimmt wie die ACE-Proteinspiegel. APA-/- Nieren wurden mittels Lichtmikroskopie(LM), Elektronenmikroskopie(EM), Immunhistochemie und Immunfluoreszenz untersucht. Der Blutdruck wurde mit einem Tail-Cuff-System gemessen. Der Albumin-Kreatinin-Quotient wurde in APA-/- Mäusen, die mit AngII über osmotische Minipumpen behandelt wurden, und nach 12 Wochen STZ-induziertem Diabetes in APA-/- und WT, bestimmt.
APA-/- Mäuse zeigten eine milde glomeruläre mesangiale Expansion und einen erhöhten Blutdruck ohne signifikante Albuminurie. In der EM zeigten APA-/- eine milde Vergrößerung der mesangialen Matrix und eine moderate Verdickung der glomerulären Basalmembran(GBM) mit einem auffälligen Erscheinungsbild knotiger Strukturen. Die knotigen Strukturen wurden bei männlichen und weiblichen Mäusen beobachtet und blieben nach der Behandlung mit Telmisartan bestehen. APA-/- Mäuse zeigten eine erhöhte Albuminurie nach 1 Woche AngII-Infusion. Nach 12 Wochen STZ-induziertem Diabetes war die Albuminurie in diabetischen APA-/- im Vergleich zu diabetischen WT Mäusen signifikant erhöht. Überraschenderweise unterschieden sich die AngII-Spiegel in APA-/- Nieren nicht von denen des WT und die Aktivitäten anderer AngII-abbauender Enzyme waren herunter- statt kompensatorisch hochreguliert. Allerdings zeigten Nierenlysate von APA-/- Mäusen eine starke Reduktion der ACE-Aktivität, der mRNA und der Proteinkonzentrationen.
Ein Mangel an APA führt zu glomerulären morphologischen Veränderungen im Mesangium und der GBM. Die Reduktion von ACE gleicht wahrscheinlich den beeinträchtigten AngII-Abbau aufgrund des APA-Mangels aus. Unsere Ergebnisse unterstreichen die Wichtigkeit von APA bei der Aufrechterhaltung der glomerulären Intaktheit und des intrarenalen Gleichgewichts der Angiotensine.

3. Synopsis

The following synopsis describes the current state of research, methodology, essential new results, and future scientific questions including the clinical implications of the publication (1):

Marahrens B, Schulze A, Wysocki J, Lin MH, Ye M, Kanwar YS, Bader M, Velez JCQ, Miner JH, Batlle D. Knockout of aminopeptidase A in mice causes functional alterations and morphological glomerular basement membrane changes in the kidneys. *Kidney Int. 2020*.
DOI: https://doi.org/10.1016/j.kint.2020.11.012

A. State of research

The Renin-Angiotensin-System

The Renin-Angiotensin-System (RAS) is one of the most sophisticated hormonal systems and plays an important role for blood pressure regulation. The RAS is used as a pharmacological target in a variety of disease such as hypertension, congestive heart failure and kidney disease. (2, 3)

A simple version of the RAS - the “classical” RAS, is studied by medical personal around the world. (4) However, over the last decades the complexity of the RAS increased intensively as novel enzymes and substrates were discovered and lead to a complex version of the RAS, called the “non-classical" RAS. (5)

The classical RAS cascade consists of five major components: Angiotensinogen, Angiotensin(Ang)-I, AngII, Renin and Angiotensin-Converting-Enzyme (ACE). Angiotensinogen, AngI and AngII are peptides and build a cascade linked by enzymatic cleavage of amino acids. Renin and ACE are the enzymes mediating the reactions. First, Renin cleaves 10 amino acids of Angiotensinogen to form AngI. AngI is further converted into the octapeptide AngII by ACE. (4) In the classical RAS AngII is the only major effector particle of the system and binds to receptors: AngII-type1 (AT1R) and AngII-type2 (AT2R) (Figure 1).

Renin is an Aspartyl-Protease, which is produced in the juxtaglomerular cells of the tubule in the kidney and mediates the first step of the RAS cascade. Renin release is thought to be the rate-limiting step and is directly regulated by the macula densa, baroreceptors and the sympathetic nervous system. (6) The second step of the

cascade is the cleavage of the octapeptide AngII from the decapeptide AngI. This reaction is catalyzed by ACE, which can be found primarily in the endothelium. (7) AT1R binding leads to an increase of blood pressure, the primary effect of the system. AT1R can be located in the walls of blood vessels and can initiate a vasoconstriction. Ligands binding to AT1 receptors also set off a cascade which releases aldosterone from the adrenal gland as well as Antidiuretic-Hormone (ADH) from the pituitary gland. (8) Both substances increase Na+ retention in the kidneys increasing the blood pressure (Figure 2). Due to the Aldosterone release the system is sometimes also referred to as Renin-Angiotensin-Aldosterone-System (RAAS). Thus, AngII contributes to blood pressure control mainly through vasoconstriction, vasopressin release, sodium retention. (4, 9-12) However, AT2R binding of AngII shows anti-proliferative effects and leads to remodeling due to differentiation. (13)

The non-classical RAS includes a kaleidoscope of AngII degrading pathways that has been studied extensively over the last decades. Aminopeptidase A(APA) degrades AngII and AngI(1-10) and forms AngIII(2-8) and Ang(2-10) respectively by N-terminal cleavage of Aspartate. The enzyme is abundantly expressed in the kidney. (14-18) ACE2 converts AngII to Ang(1-7) by cleaving Phenylalanine from its C-terminus. This enzyme can be found with high abundancy in the tubules. (12, 19) Two more enzymes which mediate this pathway of the non-classical RAS are Prolyl-Oligo-Endopeptidase (POP) and Prolylcarboxypeptidase (PRCP). (20-22) Neprilysin (NEP) forms Ang(1-7) by cleavage of AngI and also degrades AngII (Figure 2). (23, 24)

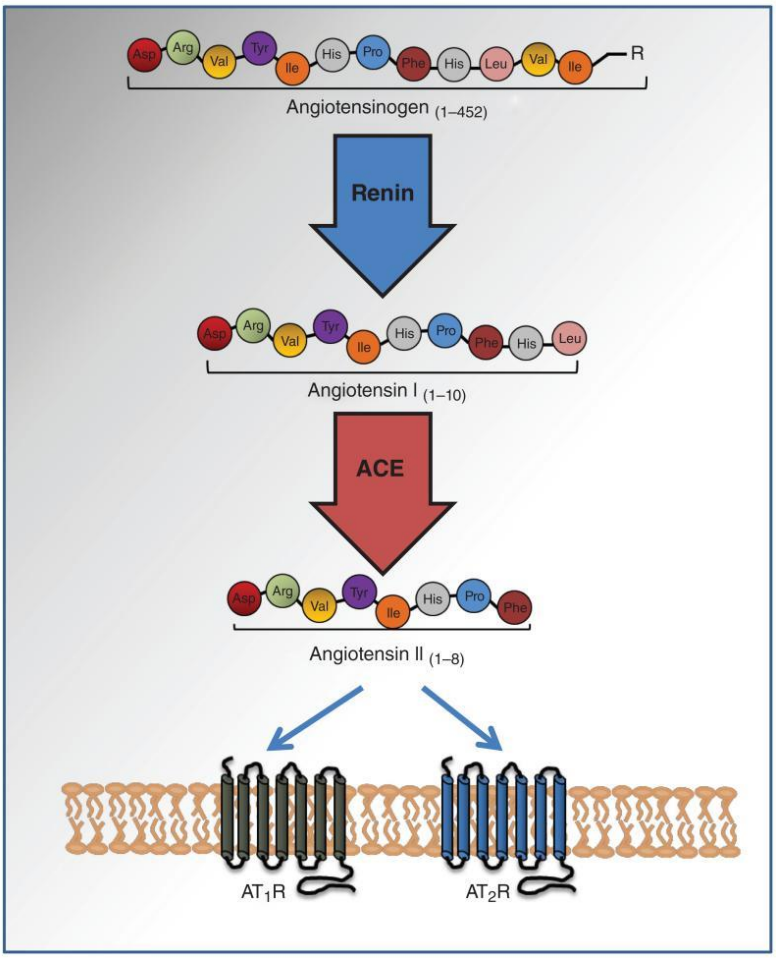

Figure 1: Classical Renin-Angiotensin-System by Sparks et al. (4)
In the classical Renin-Angiotensin-System(RAS) AngI is formed from Angiotensinogen by enzymatic activity of Renin. ACE forms AngII from AngI by cleaving off Histidine and Leucine. AngII has two major receptors: AT1-R and AT2-R.

AngII is not the only peptide of the RAS having an effect by binding to a receptor and in fact, many of the products of AngII degradation have been shown to have a variety of effects. Ang(1-7), the product of AngII degradation by ACE2 has been shown to have several beneficial effects. (25, 26) Thus, the non-classical RAS has at least two major axis: the ACE/AngII/AT1-R and the ACE2/Ang(1-7)/Mas-R axis (Figure 2). The actions of these two axis are thought to counteract each other with the ACE2/Ang(1-7)/Mas-R axis having positive effects such as vasodilation and antiproliferative effects and on the other side the ACE/AngII/AT1-R axis having negative effects such vasoconstriction and cell proliferation. (5) AngIII the product of AngII degradation by APA has been shown to have a high affinity to the AT2-R. (27)

Hypertension annually causes around 7.5 million deaths and is a great thread in modern society. It is the number-one risk factor for an increased cardiovascular mortality and has an increasing prevalence in western cultures. (28) Thus, the RAS has been in the spotlight of the pharmacological industry to develop drugs that reduce the mortality in hypertension and other disease. First-line treatment for essential hypertension are ACE-inhibitors. (29) AT1-inhibitors are drugs that are also targeting hypertension and its comorbidities by reducing the actions of AngII. ACE-inhibitors are also used to reduce the mortality in chronic heart failure. (30)

In other disease such as diabetes a disbalance of the RAS can aggravate the disease especially if AngII is accumulating as this peptide can mediate oxidative stress and inflammation. (19, 31, 32) The group around Dr. Batlle at Northwestern University, Chicago, USA, focus their research on RAS enzymes such as ACE2, APA, ACE, POP and PRCP including their importance for AngII homeostasis in different tissues. (22, 33) The group investigated the localization of ACE2 and other RAS enzymes within the kidney and how this might affect the pathology of disease such as diabetes. (19, 32, 34, 35) Lately, they investigate the potential use of ACE2 as a therapeutical target for different types of kidney damage. (36, 37)

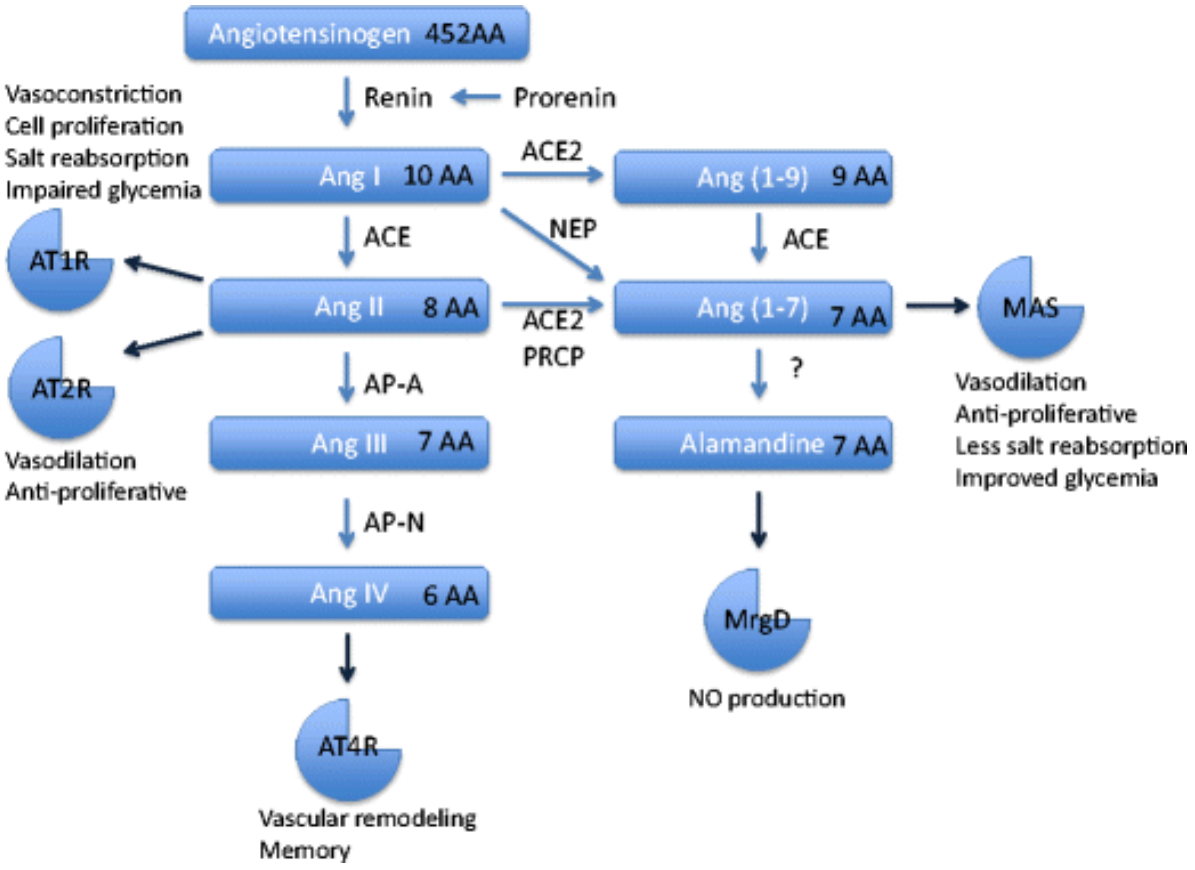

Figure 2: The RAS system by Friedrich C. Luft (5)
"Not all of the putative functions are documented in detail, and the schema is subject to change. AA amino acids, ACE angiotensin-converting enzyme, ATR angiotensin receptor, NEP neprilysin, AP aminopeptidase, PRCP prolylcarboxypeptidase, NO nitric oxide, MAS G protein-coupled receptor, MrgD mas-related G protein-coupled receptor D" (5)

Aminopeptidase A

Aminopeptidases are enzymes that catalyze the cleavage of amino acids from the N-terminus of peptides and proteins. (38) APA can be found abundantly in a broad range of tissues, but in the kidney this enzyme has its highest relative tissue activity where it is expressed mainly in podocytes. (14-17) APA has, besides AngI and AngII, other substrates within the RAS such as Ang(1-7). (39) The enzyme has substrates outside the RAS too including but not limited to Cholecystokinin-8 and Amyloid-ß. (40, 41)
The AngII degrading activity of APA is important for blood pressure regulation. APA deficient mice on C57/129sv background show higher baseline systolic blood pressure

(SBP) and hypersensitivity to chronic AngII infusion. (42) Recombinant APA decreased blood pressure in spontaneously hypertensive rats. (43)

Increased albuminuria has been observed not only after a week of chronic AngII infusion to APA deficient mice on Balb/c background but also after acute depletion of APA activity by an antibody targeted against the enzymes active center in WT mice. (15, 44, 45) This acute APA deficiency also causes elevated AngII levels in the kidney. (46) Compensatory APA upregulation can be observed in different models of kidney disease such as renal ablation, two-kidney-one-clip hypertension, and AngII-mediated hypertension. (47-49) Nephrotoxic serum (NTS), a model of immune mediated glomerular nephritis, which also causes albuminuria was linked to APA previously. (50, 51) During diabetes APA activity is also upregulated as shown in isolated glomeruli of STZ treated rats. (52)

In summary, these findings suggested that APA plays a role in kidney disease and AngII metabolism but before our study there was very limited evidence pointing towards the involvement of APA in glomerular integrity. However, three decades ago Scherberich et al. showed that APA activity protects against glomerular destruction during progressive renal disease in humans and already suggested the involvement of AngII. (53, 54)

Glomerular injury

The glomerulus is the place within the kidney where the first step of urine production takes place by making a selective ultrafiltrate of the blood. To fulfill this function the glomerulus has a unique architecture consisting of a capillary convolute which connects the efferent to the afferent arteriole. These capillaries lie within the Bowman's capsule which is connected the proximal tubule (Figure 3). (55) Specialized cells called podocytes lay on the outside of the capillaries within a Bowman's capsule. The foot processes of the podocytes cover the outside of the capillaries by interdigitating each other (Figure 3). Structural support to the convolute of capillaries in the glomerulus is provided by mesangial cells. These cells produce matrix proteins and also play a role in signaling pathways. Mesangial cells are also thought to be able to contract as part of an autoregulative mechanism of the glomerulus. (55, 56)

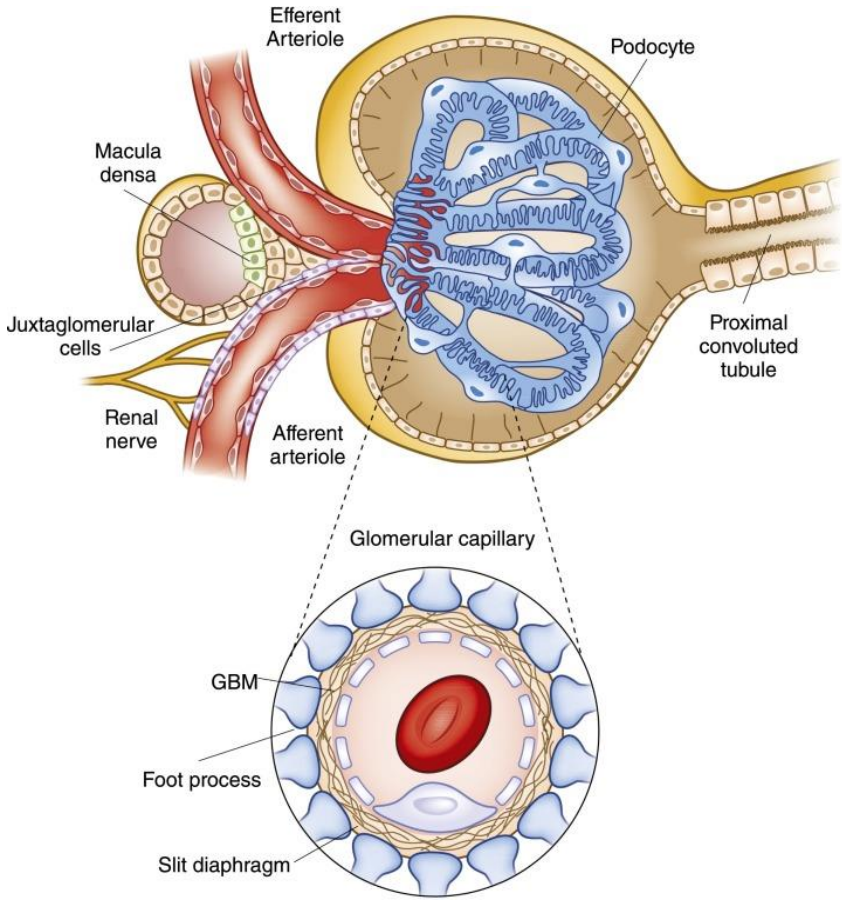

Figure 3: Structure of a glomerulus by Pollak et al. (55)
The schematic presentation of the glomerulus shows the glomerular capillary convolute connecting the efferent to the afferent arteriole in the Bowman's capsule. The glomerular filtration barrier is enlarged in the lower circle showing the endothelium of the capillary, the glomerular basement membrane (GBM) and the foot processes of the podocytes with the slit diaphragm in between.

The blood is filtered by passing through the fenestrations of the vascular endothelium within the lumen of the capillaries, the glomerular basement membrane(GBM) and the slit diaphragm between the foot processes of the podocytes (Figure 4). The GBM has a unique composition of substances synthesized by the endothelial cells and podocytes. The components include agrin, LAMB2 and Collagen α3, α4, α5. (57, 58) Damage to the delicate architecture of the glomerulus can occur in many ways. When the glomerular filtration barrier is damaged and loses its function this most likely results in albuminuria. (59) The functional changes can be often associated with histologic changes which might be only observable by electron microscopy (EM) but not light microscopy.

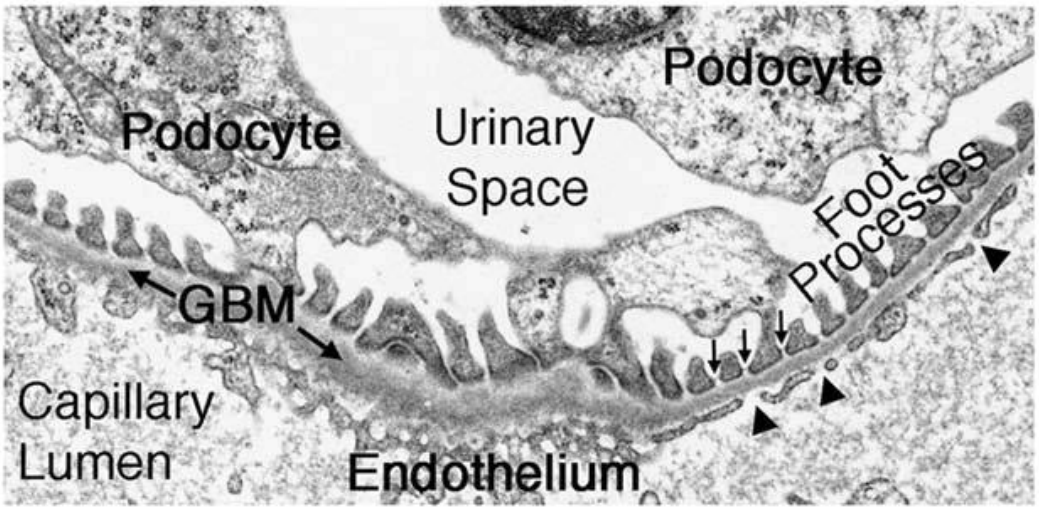

Figure 4: The ultrastructure of the glomerular filtration barrier by J. Miner (60)
The Electron microscopic image shows the glomerular filtration barrier with the endothelium lining out the capillary lumen. The endothelium of the glomerular capillaries shows fenestrations (arrow heads). On the opposite side of the glomerular basement membrane (GBM) the foot processes of the podocytes interdigitated with the slit diaphragm (arrows) in between.

The unique composition of the GBM can be disrupted if one of its components cannot be synthesized correctly. During Alport's syndrome, a genetic disease, effecting the structure of COL-IV the GBM develops structural irregularities with area of thinning as well as thickening of the GBM. To compensate for the defect COL-IV other components may be implemented into the GBM such as LAMA1 or COL4A1/A2. The altered composition of the GBM can lead to outpocketting of the GBM. (60) Patients with Alport's syndrome usually first develop hematuria followed by proteinuria and end stage renal disease in early adolescence. (61) The glomerular filtration barrier can also be damaged by acquired disease such as hypertension and diabetes. (62)

In diabetes, high blood glucose levels seem to damage the glycocalyx, a layer of membrane-bound matrix proteins covering the endothelial cells within the capillary lumen. This damage can already lead to proteinuria. Other mechanism that are thought to lead to glomerular injury in diabetes are a disrupted balance of cytokines, growth factors and the renal RAS which leads to an altered crosstalk between the different types of glomerular cells. These mechanisms result in podocyte injury and a thickening of the GBM by an overproduction of matrix components. (63)

During hypertension an auto-regulatory mechanism in the afferent arteriole usually protects the glomerular capillaries from increased blood pressure. However, if the

hypertension exceeds the capacity of this mechanism the blood pressure also increases in the glomerular capillaries. (64) This may result in glomerular capillary stretching and endothelial damage leading to damage to the glomerular filtration barrier and again to a disorder of intercell communication. The RAS not only plays a key role for blood pressure regulation, but also in non-hemodynamic effects on a much smaller level including but not limited to increased oxidative stress and proliferative signaling. In the glomerulus mesangial cells are thought to be highly affected by RAS disbalance. (64)

The distinct architecture and the interaction between the glomerular cells make the pathological mechanisms of glomerular damage complex, and thus, they remain in the focus of research. (65)

B. Scientific methods

Most of the experiments were conducted in Dr. Batlle's laboratory in the Division of Nephrology and Hypertension at Northwestern University, Feinberg School of Medicine during a research stay funded by the Biomedical Exchange Program (BMEP). All animals for our studies were housed, bred and maintained at the Center for Comparative Medicine at Northwestern University, according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Approval for animal care and experiments was granted by the Institutional Animal Care and Use Committee.

Localization of Aminopeptidase A in the kidney

From the literature and previous experiments from Dr. Batlles group we knew that APA is predominantly expressed in the glomerulus by podocytes. First, we confirmed the predominant glomerular localization of APA by Immunohistochemistry and used Immunofluorescence to determine the exact localization with co-localization studies of Podocin and PECAM as described previously. (19) For staining of APA we used goat anti-mouse-APA antibody (1:200, AF2809, R/D systems). For analysis of histologic Images, we used ImageJ (NIH) and Aim Image Browser (Zeiss).

As we also wanted to investigate the localization of APA in a functional way we isolated glomerular mass from WT mice and performed AngII degradation studies as described previously. (34) To compare the contribution of ACE2 and APA, two major known AngII

degrading enzymes in the glomerulus, we performed the degradation studies with an ACE2 inhibitor (MLN-4760) and an APA inhibitor (Amastatin) respectively and compared them to degradations without inhibitors.

Aminopeptidase A knock-out mouse model

For our studies we also used a global APA-/- mouse model on Balb/c genetic background. This mouse model was first described by Lin et al. (66) and was bred from a C57/129sv to a Balb/c genetic background by our collaborators Velez et al. which is why this model was first used in 2017 by this group. (15)

As the phenotype of this mouse model had not been described extensively before our study, we started with basic physiologic measurements which we were interested in such as BP. For the APA deficient mouse model on C57/129sv genetic background elevated SBP had been show by Mitsui et al. (42)

We measured SBP using CODA mouse tail-cuff system (Kent Scientific Corporation) during Ketamine anesthesia (133µg/gBW i.p) and obtained average SBP values for each mouse by taking the average of 25 consecutive measurements over a period of 12.5 minutes (1 measurement every 30 seconds).

We confirmed the results of Velez et al regarding AngI and AngII levels measured with Liquid Chromatography / Mass Spectrometry (LC/MS) in kidneys from APA-/- on Balb/c background by EIA. (15) We measured AngII concentrations in plasma and kidneys by EIA (SPI-BIO Chemical (Cayman)) following the manufacturers' protocols. We also tried measuring AngIII by EIA, but the cross-reactivity of the AngIII-EIA to AngII was far greater than described in the manufacturer's protocol. AngI concentrations were also measured by EIA (Peninsula Laboratories International), but this assay was not sensitive enough to measure this peptide in plasma samples. To measure AngII in glomerular isolates we had to pool samples from 5 WT and 5 APA-/- mice respectively due to prosperity.

Assessment of the renal RAS in APA-/- mice

In our next step we wanted to assess the effect of APA deficiency on other RAS enzymes in the kidney as this had not been studied before. Fluorometric measurements of the activities of common AngII-degrading enzymes are performed daily in Dr. Batlle's laboratory and the protocols have been perfected by Dr. Jan

Wysocki over the years. We measured the enzymatic activites of APA, ACE2, NEP and POP using the specific substrates and inhibitors (Table 1) for each enzyme as described previously. (14, 32, 34, 67)

Table 1: Substrate and inhibitors with concentrations for each enzyme (1)

Enzyme	Substrate	End conc.	Inhibitor	End conc.	Excitation/Emission wavelength
APA	H-Glu-AMC (Bachem)	10^{-2}M	Amastatin (Sigma)	10^{-5}M	380/460
ACE2	Mca-APK-Dnp (Anaspec)	10^{-2}M	MLN-4760 (Millennium Pharmaceuticals)	10^{-5}M	320/400
NEP	Dansyl-D-Ala-Gly-4-nitro-Phe-Gly-OH (Bachem)	10^{-1}M	Thiorphan (Bachem)	10^{-3}M	340/560
POP	Z-Gly-Pro-AMC (Bachem)	10^{-5}M	ZPP (Enzo)	10^{-5}M	380/460

We also investigated changes in ACE in the kidney. We measured ACE activity using the substrate hippuryl-L-histidyl-L-leucine(HHL) as described by Schwager et al. (68) For specificity of the reaction an additional measurement for each sample with initial administration of 1µl of 10^{-4}M captopril were taken and subtracted from wells without inhibitor. After finding such a profound decrease in ACE activity in kidneys from APA-/- we also measured ACE protein levels with densitometry after western blot using rat anti-ACE (5C4) as 1° antibody with goat anti-rat as 2° antibody. Afterwards we measured mRNA levels of ACE and AngII-degrading enzymes in kidney lysates from APA-/- and WT using Trizol reagent and reverse transcription. For every corresponding enzyme we used specific primers (Table 2) with Sybr-green reagent as described previously. (32)

Table 2: Forward and reverse primers used for each enzyme (1)

Enzyme	Forward sequence	Reverse sequence
APA	5'- CAT CAG GGA GAC CAA GAT CAC -3'	5'- CCA CGT ACT CCT GCT TCT TAT AC -3'
ACE	5'- GCC ATA CGT CAG GTA CTT TGT -3'	5'- CTG CTT CCT TGG ATT GGT AGA T -3'
ACE2	5'- CCC AAA GAG CAG TGG ATG AA -3'	5'- GAG ATG CAG GGT CAC AGT ATG -3'
NEP	5'- GCC AAA GCA AGC TAA AG -3'	5'- CTG ATT TCG GCC TGA GGA ATA A -3'
POP	5'- GAG CAC GAG AAG GAT GTC TTA G -3'	5'- GGT CGT GAA GCT GTA GAA TGT -3'

Microscopy of kidneys from APA-/- mice

Assessment of kidney sections stained by PAS from APA-/- mice and their WT littermates was performed by an experienced pathologist with a 2-point scoring system. After the pathologist observed mild glomerular mesangial expansion in kidneys sections from APA-/- mice we performed electron microscopy of APA-/- and WT kidneys after preparing them as previously described. (69)

In EM pictures we quantified GBM thickness with ImageJ (NIH) by measuring the distance between podocytes and endothelial cells in 20 points per picture (as 5 points per loop have been shown to be enough) and 5 pictures for each mouse. (70)

To examine the composition of the observed knob-like GBM structures we performed Immunofluorescence for markers normally found in an intact GBM such as agrin, nidogen, COL4A5 and Laminin-ß2. We also stained for non-GBM markers such as fibronectin, perlecan, COL1, Laminin-α1 and others. These markers can sometimes be observed as a compensatory mechanism if the GBM composition is disrupted in a state of disease. (71)

To further investigate the knob-like GBM structures and their origin we examine kidney sections from female mice by EM. We also treated female mice with Telmisartan for 3 weeks to rule out the association of the knobs to hypertension. To confirm the effect of Telmisartan on blood pressure we measured SBP in treated and untreated female mice as described above.

Susceptibility of APA-/- mice to glomerular injury

Considering the primarily glomerular localization of APA we wanted to challenge our APA deficient mouse model on Balb/c background with models of glomerular injury. First, we used a model of AngII mediated hypertension because of the well-known importance of APA for the degradation of AngII. We treated APA-/- mice with chronic AngII infusion for one week with a higher dose than used by Velez et al. (15)

In a second study we treated APA-/- and WT mice on Balb/c background with STZ to induce diabetes. As a marker for glomerular damage, we assessed urinary albumin creatinine ratio (UACR) 12 weeks after STZ application. Albumin and creatinine were both measured in spot urine by ELISA (Exocell / Albuwell M and Parameter Creatinine Assay kit (R&D systems), respectively) as per manufacturer’s instructions. After 12 weeks of diabetes kidneys from WT and APA-/- mice were harvested and prepared

for light microscopy with PAS staining and for EM to be examined by an experienced pathologist regarding markers of glomerular injury.

Statistical analysis

For the statistical analysis of our study we used SPSS Statistics 25 (IBM) and Windows Excel 2016 (Microsoft). To display our data, we used Prism 5 (GraphPad Software).
Normally distributed data were analyzed by t-test (2 groups) or ANOVA (3 groups) with Bonferroni post-hoc for pairwise comparison. Non-parametric data was analyzed by Mann-Whitney-U-Test (2 groups). A p-value <0.05 was considered significant. Normal distribution was determined using a two-step protocol. First, we conducted the Shapiro-Wilk-Test and ruled out false results by visual interpretation of the histogram in SPSS.
In figures significance levels are expressed as: not significant=ns, $p<0.05$=*, $p<0.01$=**, $p<0.001$=***.

C. Essential new results

In our study we demonstrated for the first time that there is a specific kidney phenotype associated with APA deficiency which includes functional and morphological alterations. In the GBM of APA-/- mice striking knob-like structures and moderate thickening of the GBM were observed (Figure 5). We show that these knob-like structures consist of regular GBM material as the knobs were not stained by immunofluorescent antibodies for non-regular GBM markers. (1)
Knowing the importance of APA for AngII degradation and the predominant localization of this enzyme in the glomerulus we expected increased AngII levels within the glomerulus to lead to the observed changes. Due to prosperity of glomerular mass we had to pool glomerular isolates from 5 WT and 5 APA-/- mice respectively but we found a 3-fold increase in AngII levels in glomerular isolates of APA-/- mice. (1)
The knob-like structures were found in male mice of different ages and as early as 4-weeks of age. However, in the EM pictures the knob-like structures appear to be less frequent in young mice. We also observed the knob-like GBM structures in female mice and after treatment with Telmisartan, an AT1R-Blocker, for 3 weeks. (1)

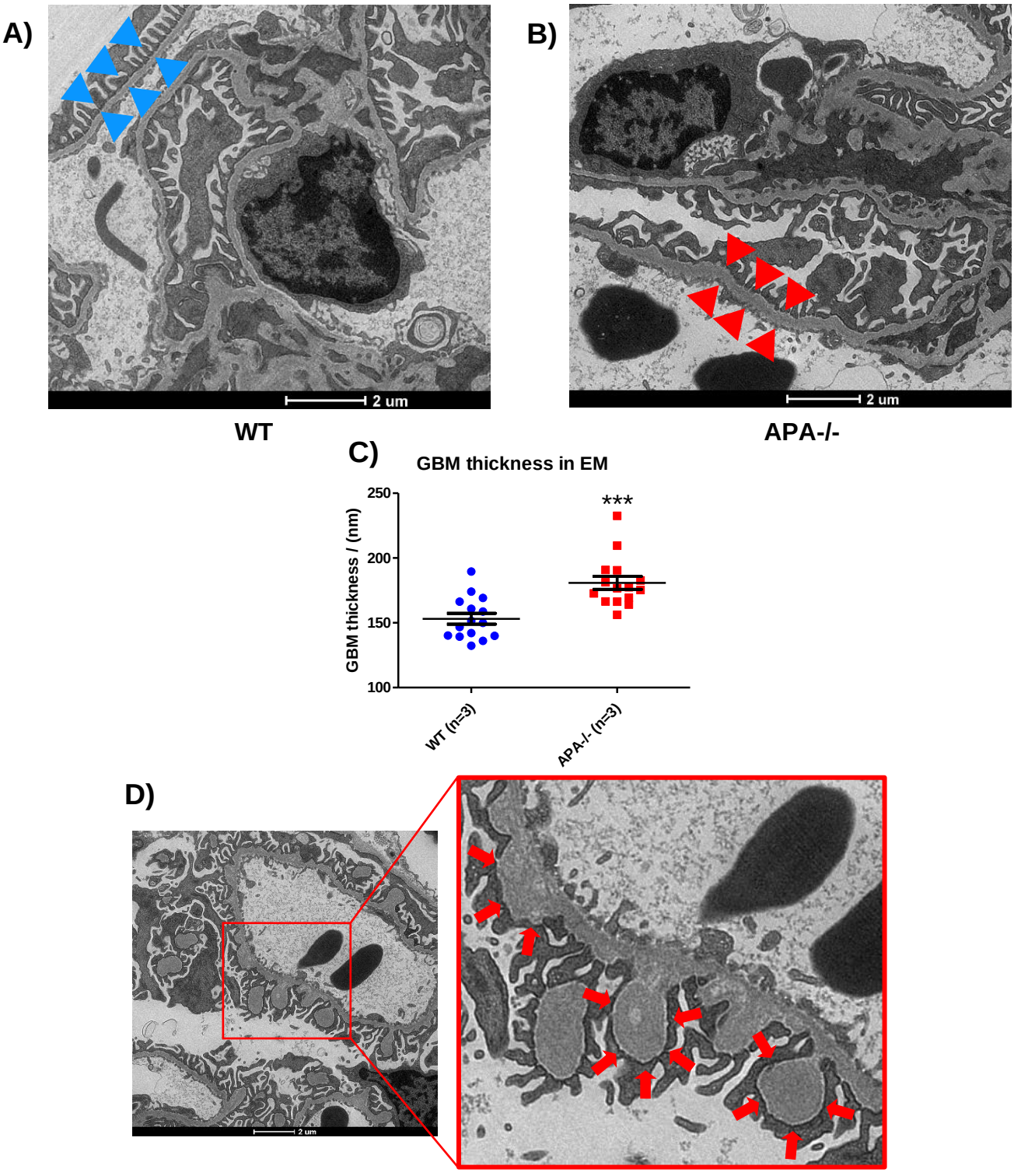

Figure 5: Electron microscopy of APA-/- kidneys by Marahrens et al. (1)
*"**A:** Electron microscopy (EM) of kidneys from WT mice showed no pathologies and a normal glomerular basement membrane thickness (GBMT) (distance between arrow heads). **B:** EM of kidneys from APA-/- mice showed an increase in GBMT (distance between arrow heads). **C:** GBMT measured in EM pictures confirmed the visual increase of the GBMT in APA-/- as compared to their WT littermates (p<0.001). **D:** In EM pictures of APA-/- kidneys the GBM showed knob-like structures (arrows). Scalebars=2µm" (1)*

We also investigated the functional effects of APA deficiency on the renal RAS. Surprisingly, the activity of other AngII degrading enzymes such as ACE2 and NEP were downregulated to about 50% in kidneys of APA-/- compared to WT rather than upregulated (Figure 6A). However, we found a marked downregulation in ACE on enzymatic activity, protein and mRNA levels in APA-/- kidneys (Figure 6B). (1) We interpreted these data as a compensatory mechanism to reduce AngII levels in the kidney as we did not find increased levels of AngII in kidneys of APA-/- mice. Our results on kidney AngII and AngI levels obtained by ELISA confirm the results by Velez et al. measured by LC/MS in the same model. (1, 15) Unlike Velez et al. we showed significantly increased blood pressure in Bab/c APA-/- mice. However, they did not study blood pressure extensively during their project. (15)

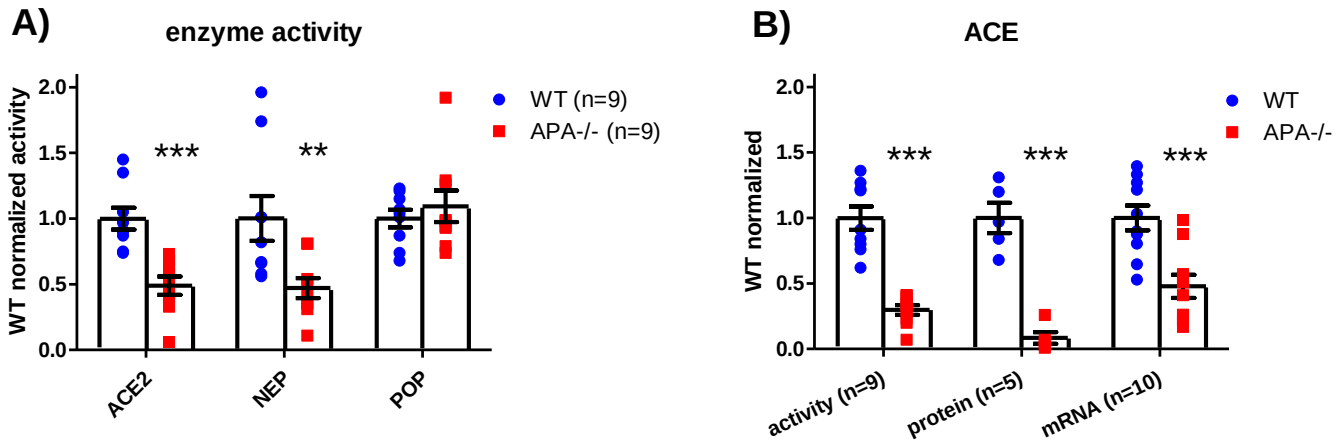

Figure 6: Non-APA AngII-degrading enzyme activities and ACE in the kidney modified after Marahrens et al. (1)
A) *Enzymatic activity of ACE2 and NEP were decreased markedly in kidneys from APA-/- (red) as compared to WT (blue). No difference in POP activity was observed.* ***B)*** *In kidneys from APA-/- (red) ACE activity, protein and mRNA levels were decreased markedly as compared to WT (blue).*

For systemic ACE the lung has proven to play an essential role as ACE is an endothelial enzyme. (72) So, we also measured ACE mRNA in lungs of APA-/- and WT mice as plasma AngII levels were slightly elevated in APA-/- mice. (1)
ACE mRNA levels in lungs of APA-/- were not different to WT suggesting that the lung does not compensate in the same way as the kidney does in this model (Figure 7). (unpublished data by Marahrens et al.)

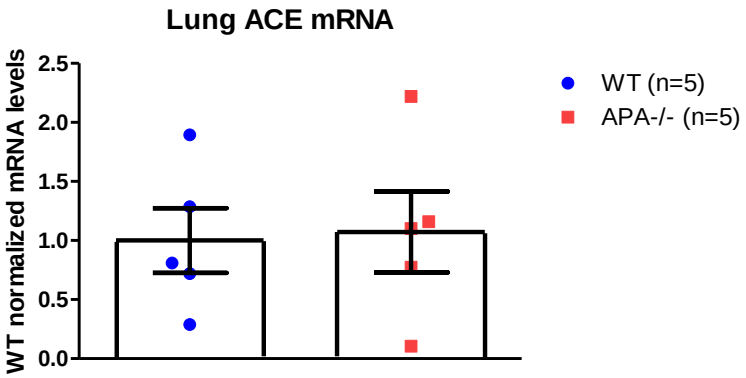

Figure 7: Lung ACE mRNA levels in APA-/- and WT mice by Marahrens et al. (unpublished)
APA-/- mice showed no difference in ACE mRNA levels in lung tissue as compared to their WT littermates.

PAS stained kidney slides were evaluated by an experienced pathologist and kidneys from APA-/- mice showed an increase in glomerular mesangial expansion. (1) However, at baseline we did not find a difference in urinary albumin creatinine ration (UACR) between APA-/- and WT mice (Figure 8A). To investigate the susceptibility of this model to glomerular injury we infused AngII and in a different experiment we induced diabetes with STZ to APA-/- mice.

After one week of chronic AngII infusions APA-/- mice showed an enormous increase in UACR (Figure 8B). Twelve weeks after diabetes induction UACR in APA-/- were significantly increased as compared to STZ treated WT (Figure 8C). Hypersensitivity of APA-/- mice to chronic AngII infusion has been shown before. (15, 42) However, a different concentration of AngII was used and to our knowledge there were no data on the susceptibility to glomerular damage caused by diabetes in APA deficiency before this study. An increased susceptibility to diabetes was also observed in the examination of PAS stained kidney slides by the experienced pathologist. (1) Of note, diabetes had no effect on the knob-like structures observed in the GBM of APA-/- mice.

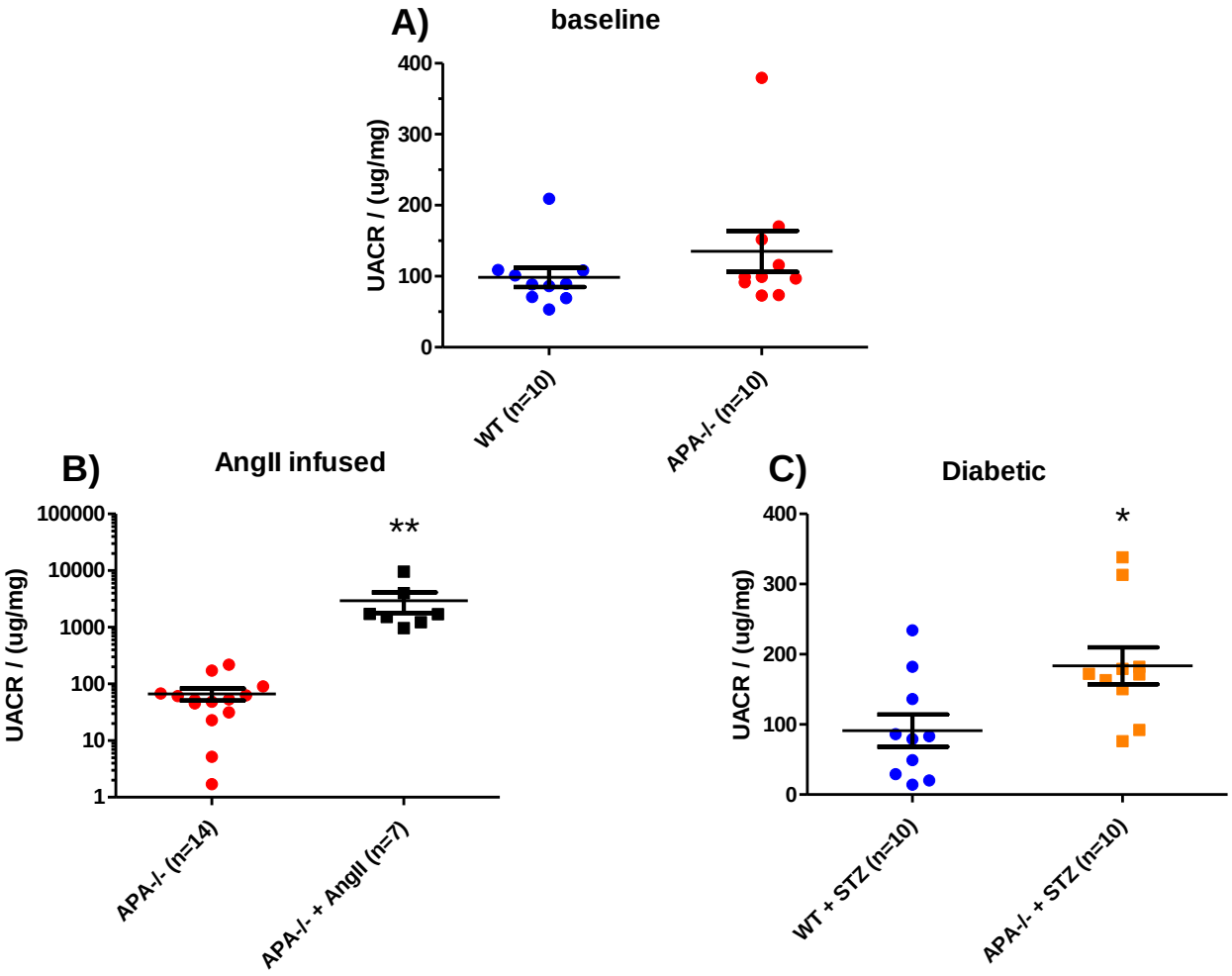

Figure 8: UACR at baseline, after AngII infusions and after 12 weeks of diabetes modified after Marahrens et al. (1)
A) *APA-/- (red) show no significant difference in urinary albumin creatinine ratio (UACR) compared to WT (blue) mice.* ***B)*** *After one week of chronic AngII infusions (black) APA-/- show a marked increase in UACR.* ***C)*** *After twelve weeks of diabetes UACR was significantly increased in diabetic APA-/- (red) as compared to their diabetic WT (blue) littermates.*

In summary, we demonstrate a link between APA and the glomerular integrity as shown by the glomerular morphological alterations in the mesangial stalk and striking changes in the GBM with extensive knob-like structures observed in APA-/- mice. The experiments on glomerular isolates provide a plausible cause for the observed changes and underline the importance of this enzyme for the metabolism of Ang peptides in the kidney. We further demonstrate an increased susceptibility of APA-/- mice to glomerular injury caused by STZ induced diabetes.

D. Further scientific questions

APA in other models of kidney disease

The role of APA for the protection of glomerular function has only been elucidated to some extent. Two different models of APA deficient mice were challenged by chronic AngII infusion and in our study diabetes, as another model of glomerular injury, was investigated. (1, 15, 42) Investigating the importance of APA in other models of glomerular disease is of interest. The involvement of this enzyme in models of acute kidney injury (AKI) should also be investigated in the future as the RAS seems to be involved in this type of injury and a potent therapy is yet to be found. (73)

The knob-like GBM structures in APA-/- mice have not been observed in other knock-out models of RAS enzymes such as ACE2. (74) In the literature the closest we could find resembling knob-like structures observed in the GBM of kidneys from APA-/- mice were in knock-out models of agrin and COL-IV, structural components of the GBM. (71, 75) These models resemble genetic diseases effecting the GBM such as Alport's syndrome. To our knowledge the role of APA in such disease effecting the GBM has not been elucidated yet and might help finding novel therapeutic targets for such disease.

The role of other APA substrates for the observed kidney phenotype

As the importance of APA for AngII degradation in the glomerulus was established in early experiments we measured AngII levels pooled glomeruli of WT and APA-/- to solidate the link of the observed glomerular basement membrane phenotype to the RAS. The AngII levels in APA-/- were 3-fold increased. So, it is reasonable to conclude that the knob-like structures are the result of increased AngII levels in the glomerulus when the degradation of this peptide is impaired during APA deficiency. (1) However, one cannot rule out the possibility of other substrates of APA playing a role for the development of the observed GBM alterations. Cholecystokinin-8 (CCK-8) is a substrate of APA outside the RAS and has been shown to have an effect on a variety of tissues including the kidney. (41, 76) This peptide is degraded by APA and thus, deficiency of APA might lead to increased levels of this peptide. (77) CCK-8 transgenic mice showed an irregular GBM and tubular pathologies, but no knob-like structures in the GBM were observed. (41) Although increased levels of this peptide seem to lead to a different kidney phenotype, future experiments should be aimed on measuring

some of these substrates that might also play a role for the development of the distinct GBM phenotype observed in APA-/-.

The origin of the knob-like GBM structures in kidneys from APA-/- mice

We observed the knobs in mice 4 weeks of age. (1) As younger APA-/- mice seem to have fewer GBM knobs than older mice we conclude that there might be a dynamic in the observed GBM structures. The kidney phenotype of different ages and in mice younger than 4 weeks of age should help to investigate a dynamic or correlation between age and different aspects of the kidney phenotype such as size and number of knob-like structures. The knobs might prove to develop before birth and be a developmental phenomenon possibly caused by increased AngII levels or an imbalance of other APA substrates in the developing kidney.

E. Clinical applications

Lately, recombinant enzymes of the RAS have been a research target as treatment for various disease. Dr. Batlle's lab has shown the beneficial effect of recombinant ACE2 in a variety of models such as diabetes and hypertension. (12, 36, 78, 79) Recently, recombinant ACE2 has also been proposed as a treatment for COVID-19 as the SARS-CoV-2 virus uses membrane-bound ACE2 as a receptor to enter the cell. (80-83)

Wysocki et al. also used advanced methods to shorten the enzyme to make it more filterable in the glomerulus and be most beneficial during disease with RAS over reactivity. (36, 83) Recombinant APA has been shown to decrease hypertension significantly in spontaneous hypertensive rats. (43) Bearing in mind the importance of APA as an AngII degrading enzyme and the localization of APA in the kidney, it also seems a promising novel pharmacological target to be used as a recombinant formula. In our studies we discovered a link between APA and the glomerular integrity and demonstrate the significance of this enzyme for AngII degradation in the glomerulus. In future, recombinant APA might not only be used as a novel treatment for hypertension, but also for the treatment of a variety of glomerular diseases. Due to the importance of APA for glomerular AngII degradation this novel target is most promising in a state of RAS overreactivity in the glomerular compartment.

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4. Eidesstattliche Versicherung

„Ich, Benedikt Marahrens, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Functional and morphological intrarenal changes in mice lacking Aminopeptidase A and their susceptibility to glomerular injury“ / „Funktionale und morphologische intrarenale Veränderungen in Aminopeptidase A knock-out Mäusen und ihre Anfälligkeit für glomeruläre Schädigung“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

5. Anteilserklärung an der erfolgten Publikation

Benedikt Marahrens hatte folgenden Anteil an der folgenden Publikation:
Marahrens B, Schulze A, Wysocki J, Lin MH, Ye M, Kanwar YS, Bader M, Velez JCQ, Miner JH, Batlle D. Knockout of aminopeptidase A in mice causes functional alterations and morphological glomerular basement membrane changes in the kidneys. *Kidney Int. 2020.*

Herr Marahrens war maßgeblich an allen Schritten, von der Planung bis zur Veröffentlichung, beteiligt und hat insgesamt den größten Beitrag zu dem Projekt geleistet, weshalb er als alleiniger Erstautor der zur Dissertation gehörigen Publikation gelistet ist.
Er war der Hauptverantwortliche für das Projekt, inklusive der Pflege des Bestands der Aminopeptidase A knock-out Mäuse, und plante in enger Zusammenarbeit mit seinen Betreuern seine Experimente.
Herr Marahrens gestaltete Abbildungen 1-7 selbst, indem er teils eigene und teils bereits existierende Primärdaten analysierte. Die Primärdaten der Abbildungen 2-4 sammelte und analysierte er vollkommen selbstständig. Die Experimente mit diabetischen Mäusen plante und führte Herr Marahrens auch durch. Dr. Wysocki sammelte die Primärdaten der Experimente, welche die AngiotensinII Infusionen und die glomerulären Isolate beinhalteten. Die elektronenmikroskopischen Aufnahmen wurden, nach der Vorbereitung der Nieren durch Dr. Ye, bei der EM-core-facility der Northwestern University in Auftrag gegeben. Die Immunhistochemischen Bilder mit Färbungen für Marker der glomerulären Basalmembran in den Abbildungen 8&9 wurden von unserem Partnerlabor von Dr. Miner erstellt.
Die Publikation schrieb und veröffentlichte Herr Marahrens primär in Zusammenarbeit mit Dr. Batlle.
Die anderen Koautoren der Publikation leisteten jeweils Beiträge zur Datengewinnung Überarbeitung und/oder der Kritischen Interpretation der Publikation.

Unterschrift, Datum und Stempel des/der erstbetreuenden Hochschullehrers/in

Unterschrift des Doktoranden/der Doktorandin

6. Auszug aus der Journal Summary List

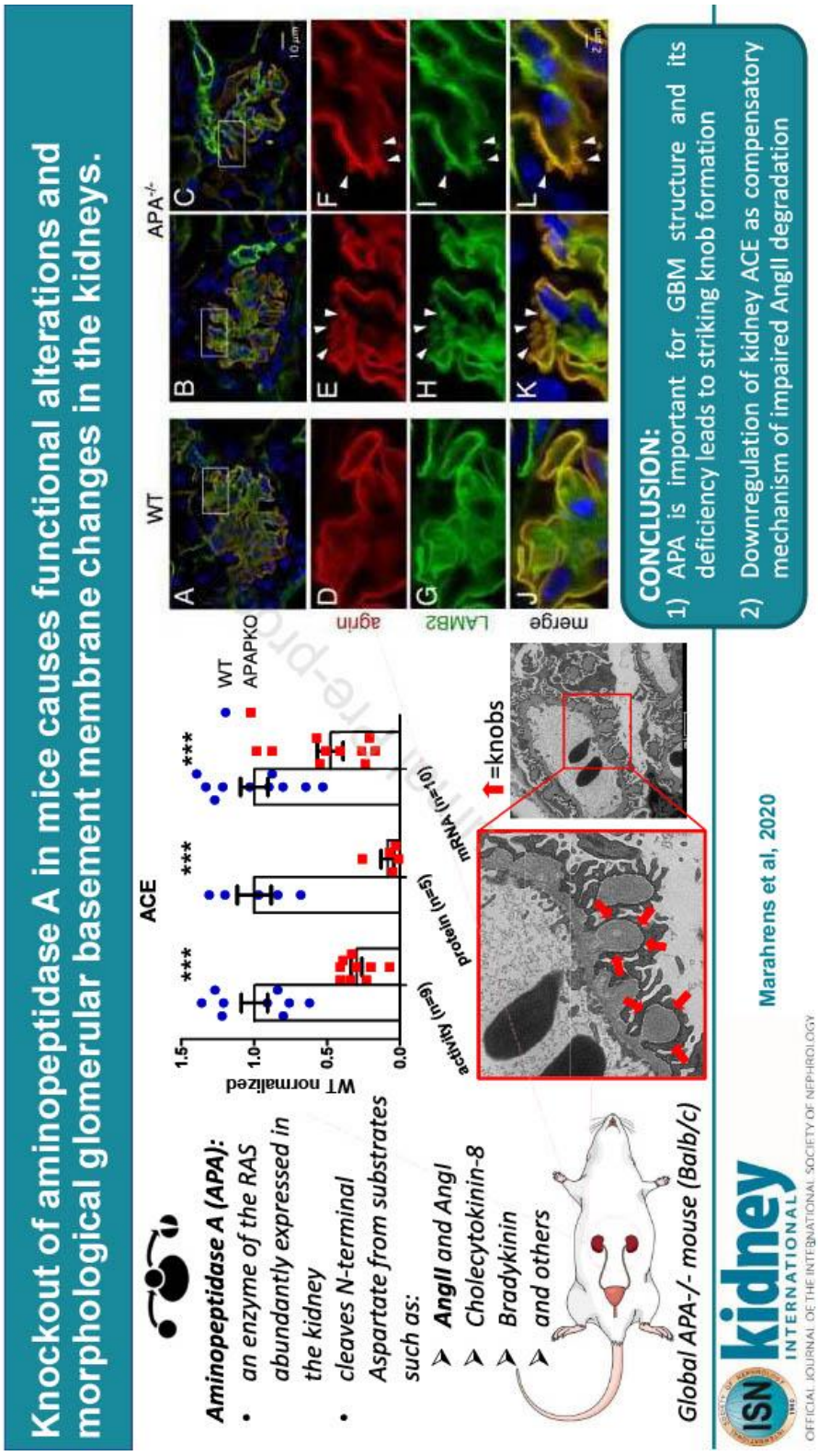
Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **"UROLOGY and NEPHROLOGY"** Selected Category Scheme: WoS

Gesamtanzahl: 80 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	Nature Reviews Nephrology	5,767	19.684	0.017080
2	EUROPEAN UROLOGY	30,782	17.298	0.070930
3	Nature Reviews Urology	3,262	9.333	0.009550
4	JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY	38,177	8.547	0.055540
5	KIDNEY INTERNATIONAL	42,627	8.306	0.043340
6	AMERICAN JOURNAL OF KIDNEY DISEASES	23,401	6.653	0.030950
7	Clinical Journal of the American Society of Nephrology	16,951	6.243	0.032570
8	Kidney International Supplements	2,062	5.842	0.004680
9	JOURNAL OF UROLOGY	48,298	5.647	0.045970
10	PROSTATE CANCER AND PROSTATIC DISEASES	2,144	4.600	0.005380
11	BJU INTERNATIONAL	19,938	4.524	0.025070
12	NEPHROLOGY DIALYSIS TRANSPLANTATION	25,423	4.198	0.029210
13	JOURNAL OF NEPHROLOGY	3,065	3.698	0.004370
14	Journal of Sexual Medicine	9,915	3.649	0.014910
15	SEMINARS IN NEPHROLOGY	2,881	3.629	0.004130
16	AMERICAN JOURNAL OF PHYSIOLOGY-RENAL PHYSIOLOGY	15,642	3.323	0.017660
17	EUROPEAN UROLOGY SUPPLEMENTS	666	3.121	0.000950
18	CURRENT OPINION IN NEPHROLOGY AND HYPERTENSION	3,228	3.013	0.005510

7. Publikation

Marahrens B, Schulze A, Wysocki J et al. Knockout of aminopeptidase A in mice causes functional alterations and morphological glomerular basement membrane changes in the kidneys. *Kidney International 2020* (Published: December 11, 2020)
DOI: https://doi.org/10.1016/j.kint.2020.11.012



8. Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

9. Publikationsliste

Paper:

Marahrens B, Schulze A, Wysocki J et al. Knockout of aminopeptidase A in mice causes functional alterations and morphological glomerular basement membrane changes in the kidneys. *Kidney International 2020* (Published: December 11, 2020)

Oral presentations:

GRS - Angiotensins 2020 *Lucca (Barga), Italy*	Functional Intrarenal Alterations and Morphological Glomerular Basement Changes in Mice Deficient of the Angiotensinase Aminopeptidase A
Kidney week - ASN 2019 *Washington D.C., USA*	Functional Intrarenal Alterations and Morphological Glomerular Basement Changes in Mice Deficient of the Angiotensinase Aminopeptidase A

Poster:

AHA - Hypertension Meeting 2018 *Chicago, USA*	Predominance of formation over degradation as mechanism of Angiotensin-II(1-8) regulation in Aminopeptidase A deficient kidneys

10. Acknowledgments

Firstly, I would like to give a special thanks to Prof. Dr. Batlle, my supervisor at Northwestern University, who supported me throughout the whole project with unbelievable devotion. He is a great mentor who inspires me constantly with his expertise and passion for the field of nephrology. Another special thanks goes out to Jan Wysocki, MD/PhD who tough me the art of benchwork and helped me whenever help was needed. I consider myself lucky to have such a kind character as my colleague and friend. I also would like to thank Prof. Dr. Bader, my supervisor at Max-Delbrück-Center(MDC) in Berlin, for the opportunity to pursue my doctoral degree and his support throughout this project. Both my supervisors I would like to thank again for their unbelievably quick responses to any matter. Representing all co-authors, I want to thank Minghao Ye and Arndt Schulze for their contributions to the project. I am also truly grateful for the support from the Biomedical Exchange Program(BMEP) stipend which I received during my research stay in Chicago. And finally, I would like to thank my family: my parents and my brothers for always believing in me, and my wife, the best woman in the world who waited for me in Berlin while I was in Chicago, for her patience and her support.