Diplomarbeit

Evaluation of nephroprotective effects of glucagon-like peptide 1 receptor in renal ischemia reperfusion injury

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Graz, 17. Dezember 2018 *Moritz Uhlig (eh)*

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Abstract

Liraglutide affects various mechanisms that are decisive in IRI like the RAAS, reactive oxygen species and inflammation (155). Therefore we aimed to test the hypothesis that liraglutide exhibits a nephroprotektive effect in a murine model of renal IRI. Here, we followed two approaches: first, we compared the outcome of GLP-1R -/- mice with wild-type mice, and secondly wild-type mice with liraglutide treatment (200µg/kgBW/d starting 7 days prior to surgery) versus vehicle. We hypothesized an improved outcome of wild-type compared to the knock-out mice and a further improvement with the administration of liraglutide as a protective agonist.

The results of this study suggest a protective effect of GLP-1R activation in renal IRI. There was a significant increase in the retention parameters urea ($p = 0.043$) and creatinine ($p = 0.0473$) in GLP-1R deficient mice, on the other hand there was an alleviation of renal IRI when the GLP-1 receptor was activated. In animals pretreated with liraglutide we observed significantly decreased serum urea ($p = 0.0149$) and BUN levels ($p = 0.03$). Unfortunately, this improvement in outcomes was not directly reflected in the histology, although a significantly higher number of macrophages ($p = 0.0286$) was detected in mice treated with liraglutide. The following L-Arg stain showed a non-significant trend ($p = 0.1111$) towards the benefit of the M2 phenotype, which at least suggests that the majority of the additional macrophages might have been of the M2/repair type

In summary, the absence of the GLP-1R aggravated the impairment of kidney functions in renal IRI, as well as the activation of the GLP-1R by liraglutide ameliorated the AKI and preserved kidney functions. Therefore, we conclude that liraglutide has a reno-protective effect in mice subjected to renal IRI. Not only does this further elucidate the underlying mechanism of the improved renal outcome that was seen in large-scale clinical trials, but also reveals the potential that Liraglutide might have for the treatment of AKI.

Zusammenfassung

Liraglutid wirkt auf verschiedene Mechanismen, die im IRI von entscheidender Bedeutung sind, wie das RAAS, reaktive Sauerstoffspezies und Entzündungsprozesse (155). Daher wollten wir die Hypothese testen, dass Liraglutid eine nephroprotektive Wirkung in einem murinen Modell des renalen IRI zeigt. Hier verfolgten wir zwei Ansätzen: Erstens verglichen wir das Ergebnis von GLP-1R-knockout Mäusen mit Wildtyp Mäusen und zweitens Wildtyp Mäuse mit Liraglutidvorbehandlung (200µg/kgKG/d ab 7 Tage vor der Operation) gegenüber den Vehicle Mäusen. Wir gingen von einer geringeren Schädigung des Wildtyps im Vergleich zu den Knock-out Mäusen aus und von einer weiteren Verbesserung nach der Vorbehandlung mit dem GLP-1R Agonisten Liraglutid.

Die Ergebnisse dieser Studie suggerieren eine protektive Wirkung der GLP-1R-Aktivierung bei einem Ischämie-Reperfusionsschaden. Es zeigte sich ein signifikanter Anstieg der Retentionsparameter Serumharnstoff ($p = 0,043$) und Serumkreatinin ($p = 0,0473$) bei GLP-1R-knockout Mäusen. Außerdem konnte eine Milderung der Nierenschädigug bei Aktivierung des GLP-1-Rezeptors beobachtet werden. Bei Tieren, die mit Liraglutid vorbehandelt wurden, beobachteten wir signifikant niedrigere Serumharnstoff- ($p = 0,0149$) und Blood Urea Nitrogen (BUN) Werte ($p = 0,03$). Leider spiegelte sich diese Verbesserung nicht in der Histologie wider, obwohl eine signifikant höhere Anzahl von Makrophagen ($p = 0,0286$) bei Mäusen nachgewiesen wurde, die mit Liraglutid vorbehandelt wurden. Die folgende L-Arginase Färbung zeigte einen nicht signifikanten Trend ($p = 0,1111$) zu Gunsten des M2 Phänotyps, was zumindest darauf hindeutet, dass die Mehrheit der zusätzlichen Makrophagen möglicherweise vom Typ M2/repair war.

Zusammenfassend führt die fehlende Expression des GLP-1R zu einer verstärkten Beeinträchtigung der Nierenfunktionen nach Ischämie-Reperfusionsschaden. Weiters schwächt die Aktivierung des GLP-1R durch Liraglutid die akute Nierenschädigung ab und zeigte damit eine nephroprotektiveprotektive Wirkung. Dies verdeutlicht nicht nur den zugrunde liegenden Mechanismus des verbesserten renalen Outcomes, das in klinischen Studien beobachtet wurde, sondern zeigt auch das Potenzial, das Liraglutid für die Behandlung des AKI haben könnte.

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Abbreviations

a-ANCA	atypical ANCA
AAV	ANCA associated vaskulitides
AB	Antibody
ABC	Avidin-Biotin Complex
ADH	antidiuretic hormone
ADQI	Acute Dialysis Qualitive Initiative
AEC	3-Amino-9-ethylcarbazole
AGPC	Acid guanidinium thiocyanate-phenol-chloroform
AIN	Acute Intrstitial Nephritis
AKI	acute kidney injury
AKIN	Acute Kidney Injury Network
ANCA	anti-neutrophil cytoplasm antibodies
ARF	Acute Renal Failure
ATN	Acute tubular Necrosis
ATP	adenosine triphosphate
BIR	brain insulin resistance
BSA	Bovine Serum Albumin
c-ANCA	cytoplasmic ANCA
cAMP	Cyclic adenosine monophosphate
CHCC	Chapel Hill Consensus Conference
CKD	Chronic Kidney Diesease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CNS	central nervous system
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cells
DMF	Dimethylformamide
DNA	deoxyribonucleic acid

dNTP	deoxyribose nucleoside triphosphate
DPP-4	Dipeptidyl peptidase-4
DTT	Dithiothreitol
eGFR	estimated Glomrular Filtration Rate
EGPA	eosinophilic granulomatosis with polyangiitis
ELISA	Enzyme-linked immunosorbent assay
FcRs	Fc receptors
FCS	Fetal Calf Serum
FDR	False Discovery Rate
GBM	Glomerular Basement Membrane
GFB	Glomerular Filtration Barrier
GFR	Glomerular Filtration Rate
GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
GN	Glomerulonephritis
GPA	Granulomatosis with polyangiitis
GPI	glycophosphatidylinositol
GPLR	G-protoein linked receptor
HLA	human leucocyte antigen
HPF	High Power Field
HRP	Horseradish peroxidase
ICAM-1	Intercellular Adhesion Molecule 1
IF	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
iNOS	inducible nitric oxide synthase
IRI	ischemia reperfusion injury

KDIGO	Kidney Disease Improving Global Outcomes
KO	knockout
LPF	Low power field
LPS	lipopolysaccharide
MDRD	Modification of Diet in Renal Disease
MHC	Major Histocompatibilty Complex
mPA	microscopic polyangiitis
MPO	Myeloperoxidase
mPR3	membrane-PR3
MPS	mononuclear phagocytic system
NC1	noncollagenous-1
NET	neutrophil extracellular trap
NGAL	Neutrophil Gelatinase-Associated Lipocalin
nitric oxide	NO
p-ANCA	perinuclear ANCA
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCI	percutanous coronary intervention
PCR	polymerase chain reaction
PCSK1	proprotein convertase 1
PR3	proteinase 3
qPCR	quantitative polymerase chain reaction
RAAS	Renin Angiotensin Aldosterone System
RBF	renal blood flow
RIFLE	Risk, injury, Failure, Loss of kidney function, End-stage renal disease
RNA	ribonucelic acid
ROS	reactive oxygen species
ROUT	Robus Regression and Outlier Removal
RPGN	rapid progressive glomerular nephritis

RT	reverse transcription
SLE	Systemic Lupus Erythematodes
STAT 3	signal transducer and activator of transcription 3
STEMI	ST elevation myocardial infarct
T-regs	regulatoy T-cells
TGF	Tubule Glomerular Feedback
TLR	Toll Like Receptor
TMB	tetramethybenzidine
TNF	tumor necrosis factor
tPA	anti-tissue plasminogen activator
WT	wild-type

1 Introduction

In 1953 the Danish anesthetist Björn Ibsen founded the first intensive care unit in Europe after a polio epidemic in Copenhagen made artificial respiration necessary for more than 300 people. Since then, intensive care medicine has developed steadily and has made great progress, especially in the management of partial or complete organ failure (2). While the invention of continuous arteriovenous hemofiltration by Dr. Scribner in the 1960s can certainly be considered a milestone, both in nephrology and intensive care medicine, the mortality of acute kidney Injury (AKI) is still very high and has not changed much over the last decades: the reported overall hospital mortality is at 60% (3, 4). More recent studies suggest that AKI may be the cause of death rather than a symptom of the underlying disease. It is therefore discussed that the treatment of AKI as such, and not just the replacement of renal function, may affect mortality (5). Recent studies provide data on the antiinflammatory effects of the incretin hormone glucagon-like peptide 1 (GLP-1) in addition to its well-studied *incretin effect* (cf. Chapter GLP-1) and since the GLP-1 receptor was also found to be expressed in the human kidney, it is tempting to hypothesize that GLP-1 could exert beneficial effects on the course and the treatment of AKI. In the present study, we aimed to evaluate whether the GLP-1R analogous liraglutide has a nephroprotective effect in a murine model of renal ischemia reperfusion injury.

1.1 Acute kidney injury (AKI)

1.1.1 Definition

AKI or more formally acute renal failure (ARF) is defined as a sudden loss of renal function over the course of hours to weeks, that is basically reversible under appropriate therapy. The loss or impairment of renal functions leads to oliguria or anuria with accumulation of nitrogen waste products, that shows as elevated levels of blood urea nitrogen (BUN) and serum creatinine. The renowned non-profit organization KDIGO (Kidney Disease Improving Global Outcomes) defines AKI in their *Practice Guidelines for Acute Kidney Injury* (7) as an increase in serum creatinine of at least 0.3 mg/dL or more within 48h or an 1.5 fold increase within 7 days or a volume of urine less than 0.5mL/kg/h within 6h. Apart from the retention of nitrogen waste products, the disorder of electrolytes, fluid and acid-base balances is also of major concern in AKI (8). Acute kidney injury affects approximately 5% of all intensive care patients and causes an increase in in-hospital mortality. Furthermore, a multicenter study of Clec'h *et al.* has shown that critically ill patients with AKI had longer stays in intensive care units and in hospitals in general compared to critically ill patients without AKI (8, 9).

1.1.2 Classification

As the spectrum of acute renal failure ranges from minimum increases in retention parameters to complete loss of kidney function, a classification system is mandatory to meet the communicative requirements of daily clinical as well as scientific life. In the history of AKI/ARF there have been more than 35 definitions, which makes comparability almost impossible (10). In order to meet these demands, the *Acute Dialysis Quality Initiative* (ADQI) published the *Risk, Injury, Failure, Loss of kidney function, End-stage Kidney Disease* (RIFLE) classification in May 2004 (cf. table 1). RIFLE assesses serum creatinine and the amount of excreted urine for classification into three categories (*risk, injury and failure*). It also includes the outcome categories *Loss of Kidney Function* and *End-Stage renal Disease* (11). However, RIFLE definition for acute renal failure requires the knowledge of serum creatinine changes relative to baseline, which raises some difficulties in clinical routine, as baseline serum creatine has to be defined first.

Depending on the time the patient is presenting himself in the course of disease, and since the baseline value cannot be known for a new patient, already increased serum creatinine values must be assumed as baseline, which leads to a delay in diagnosis and staging and thus therapy (7). To face this problem in 2010 the *Modification of Diet in Renal Disease (MDRD) Study equation* for estimating baseline serum creatinine can be used, with the drawback that the calculation is performed on the basis of an assumed normal GFR of 75ml/min/1.73m2 (12). This is especially difficult in patients with chronic kidney disease (CKD), where these problems reduce the sensitivity of the diagnosis as previously unidentified CKD patients may falsely be diagnosed with AKI (13). In March 2007 the *Acute Kidney Injury Network* (AKIN) published the AKIN classification (cf. table 1), a modified version of RIFLE, with the aim of improving the sensitivity and the specificity of the RIFLE criteria. According to the AKIN criteria, the diagnosis of AKI may now be considered if i) a proper hydration status was ascertained and ii) serum creatinine values increased at least 0.3 mg/dL within 48h without the necessity of baseline serum creatinine; it is therefore no longer dependent on an estimated GFR or estimated

RIFLE	AKIN	KDIGO	Increase in creatinine	urinary excretion
R = Risk	**1**	**1**	1,5-2 fold ↑ (KDIGO/AKIN) 1.5 fold ↑ of baseline or GFR >25% (RIFLE) ↓ 0.3 mg/dL ↑ (KDIGO/AKIN)	< 0,5ml/kgBW/h for 6-12h
I = Injury	**2**	**2**	2-3 fold ↑ (KDIGO/AKIN) 2 fold ↑ of baseline or GFR >50% ↓ (RIFLE)	< 0,5ml/kgBW/h ≧ 12h
F = Failure	**3**	**3**	> 3-fold ↑ (KDIGO/AKIN) 3 fold ↑ of baseline or GFR>75% (RIFLE) ↓ Serum creatinine > 4mg/dL with acute increase by >0.5mg/dL (RIFLE/AKIN) absolute serum creatinine > 4mg/dL (KDIGO) initiation of renal replacement Therapy (KDIGO/AKIN) in patients < 18a decrease in eGFR to < 35ml/min per 1.73m^2 (KDIGO)	< 0,3ml/kgBW/h ≧ 24h anuria ≧ 12h
L= Loss	-	-	loss of kidneyfuncton > 4 weeks	
E = ESRD	-	-	loss of kidneyfuncton > 3 months	

Table 1 comparision of acute kidney injury classification systems
ESRD = endstage renal disease

baseline serum creatine (14). The categories, into which AKI is divided, are very similar to the RIFLE classification. AKIN stage 1 is equivalent to the risk category of the RIFLE classification with the exception that the AKIN classification takes an absolute increase in serum creatinine of more than 0.3 mg/dL into account; 2 and 3 are equivalent to injury and failure categories, but stage three also includes patients on dialysis treatment regardless of the stage when dialysis treatment started. In contrast to the RIFLE classification the AKIN classification does not feature any outcome classes. The fact that the AKIN classification relies on two serum creatinine values within a 48h timeframe excludes the identification of AKI, when serum creatinine levels rise more slowly. Another drawback in terms of comparability could be the different standards concerning dialysis treatment among hospitals.

To offset their respective disadvantages and to finally have a common international and inter-hospital basis for assessing AKI it seems appealing to use a combination of both definitions. This approach was implemented by the KDIGO initiative in their *Practice Guidelines for Acute Kidney Injury* (7) (cf. table 1). The KDIGO guidelines adopted the AKIN classification system with the minor change of patients reaching stage three at absolute serum creatinine values greater than 4.0 mg/dL without the need of an acute increase.

1.1.3 Etiology

Potential causes of AKI are divided into three main groups: AKI can either be caused pre-renal, intra-renal or post-renal, with pre-renal causes being most common (60%). In the beginning of pre-renal AKI, the reno-tubular and glomerular structure itself is undamaged. Here, the loss of kidney function is due to reduced renal blood flow (RBF), either as a result of reduced cardiac output, volume loss, loss of peripheral resistance or renal vasoconstriction. As a reaction to reduced renal perfusion the renin angiotensin aldosterone system (RAAS) is activated as well as the secretion of antidiuretic hormone (ADH) and catecholamines. This neurohumoral activation is vital to maintain arterial pressure but may worsen AKI (15).

The temporary renal failure is reversible, as long as the primary issue is fixed in time (i.e. recovery of normal blood pressure and RBF). However, lengthy ischemia can damage the nephrons and the tubular endothelium, resulting in secondary renal AKI. The ischemia causes an 0_2 shortage

that in turn induces necrosis of the tubular endothelium. Tubular necrosis either leads to the detachment of the basement membrane with ensuing obstruction or to an inadequate resorption of sodium. Therefore, the sodium concentration at the *macula densa* is increased, which in turn activates the tubular-glomerular feedback (TGF) mechanism. As a result, the activation of TGF constricts the afferent arteriole, resulting in a more enhanced reduction of renal perfusion and an exacerbation of AKI (8, 15).

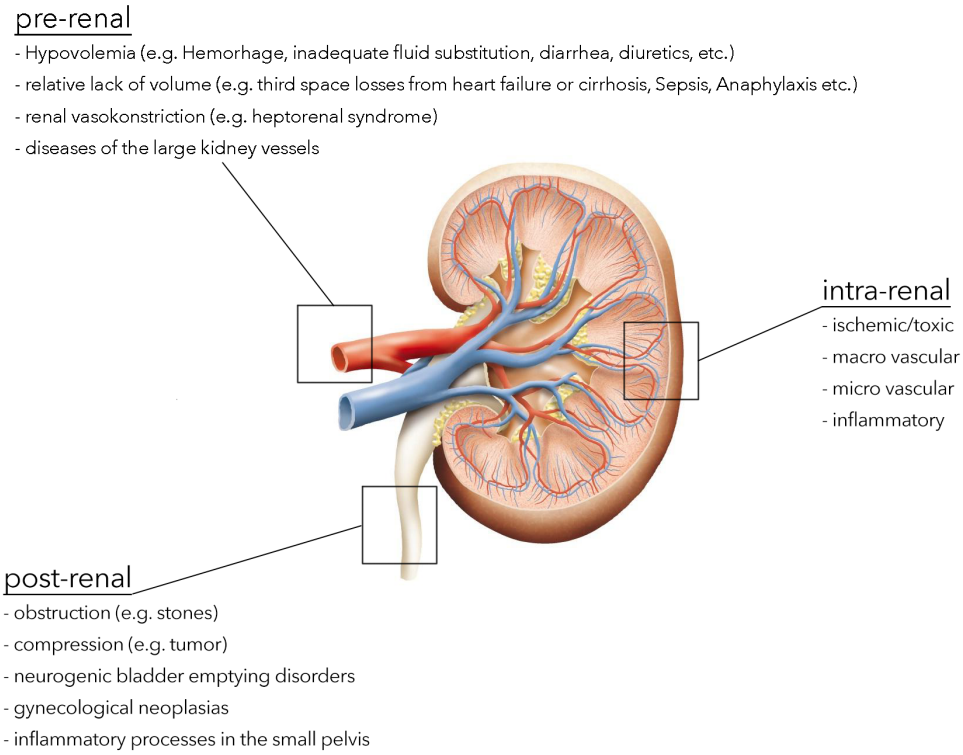

Fig 1. pathogenesis of acute renal injury
modified after(1)

Intra-renal AKI does not occur as often as pre-renal AKI, but is with 35% still the second leading cause (8). In case of intra-renal failure, the disease process focuses directly on the kidney tissue itself, where either the entire kidney or individual structures such as tubules, glomeruli or the interstitial parenchyma can be impaired. The specific trigger may be either ischemic/ toxic, macrovascular, microvascular or inflammatory (cf. Fig 1). According to their respective focus, three common phenotypes are described: acute interstitial nephritis (AIN), acute tubular necrosis (ATN) and glomerulonephritis (GN) (8). In the context of the present work it seems reasonable to pay more attention to the ischemic (cf. secondary renal AKI) and the microvascular causes, and among those to the rapid progressive glomerular nephritis (RPGN) that is one of

the most aggressive forms of renal diseases that – if untreated – leads to ARF and death within months (cf. RPGN). Fully elaborating all causes in detail would go beyond the scope of this work, but has been reviewed in depth in the past (16).

Lastly, in about 5% of the cases of AKI an obstructive urinary flow disorder is causative, which is referred to as postrenal AKI (8). The exact pathophysiology is provided in the editorial review *Pathophysiology of obstructive nephropathy* by D.R. Wilson (17).

1.1.4 Therapy and prognosis

The foremost concern in the therapy of patients with renal failure is to eliminate the underlying cause and to treat the shifts in water- and electrolyte balance, that can be life-threatening. For an appropriate management of fluid balance, an exact monitoring of all fluids supplied and disposed is mandatory. Moreover, potentially nephrotoxic medication should be stopped or switched to less harmful alternatives and all renally excreted drugs should be adjusted in dosage. Diuretics are not generally recommended except for therapy of volume overload. Depending on severity, hemodialysis is an option in order to treat conditions such as azotemia, severe shifts in water- and electrolyte balance as well as metabolic acidosis (7, 8).

Despite the huge progress intensive care medicine has made since its early beginnings in the 1950's, the mortality rate of ICU patients suffering from AKI remained almost the same over the last decades. The overall hospital mortality is still at 60.3% (52% in the ICU, 8% after discharge from ICU) and of those patients who survive AKI 13.8% are still dependent on hemodialysis after discharge from the hospital (3). While the severity of the underlying disease certainly contributes to the high mortality rate, Hoste *et al.* were able to show that despite dialysis patients with AKI RIFLE class F have a fivefold higher risk of death than patients without (18). It was concluded, that patients are actually dying from and not just with AKI. In line with these findings, the work of Thakar et al. suggests that the prevention or at least alleviation of AKI could reduce the mortality rate (5).

1.2 Rapidly Progressive Glomerulonephritis

1.2.1 Glomerulonephritis

Various immune-mediated diseases are summarized under the term glomerulonephritis (GN). Glomerulonephritis is a non-bacterial inflammation, that affects both kidneys usually in a symmetrical manner; in particular it affects the cortex and leads to inflammatory processes in the glomeruli. As a consequence, there is loss of kidney function, that – when untreated – progresses to tubulointerstitial fibrosis. The GN can either be primarily located in the glomeruli without a sign of a systemic illness, which is referred to as a primary GN, or manifest as a renal disease in the course of various systemic diseases such as collagenoses, vasculitides or diabetes mellitus, that are referred to as a secondary GN (8, 19).

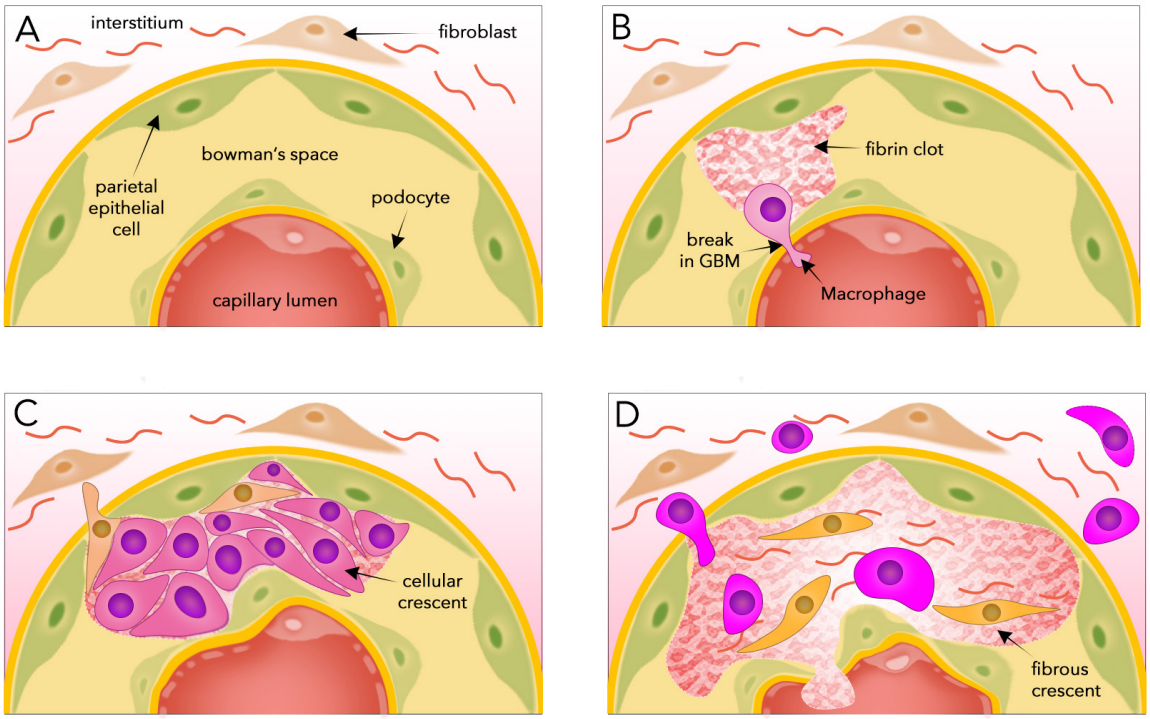

Fig. 2 pathogenesis of glomerular crescents
In a healthy glomerulum (A) the formation of glomerular crescents can be induced by inflammatory or immunologocal processes resulting in increased permeability of capillary walls with leakage of inflammatoy cells and immune material into the bowman space (B) forming a cellular crescent (C) further evolving into a fibrous crescent with compression of the capillary lumen (D). Redrawn from (21).

1.2.1.1 Definition and Epidemiology

The RPGN is, with one in 100.000 cases per year, a relatively rare subform of the glomerulonephritis with a highly aggressive course. If left untreated, it leads to a decrease in GFR of at least 50% over a short period, from a few days to 3 months or terminal renal failure within 6 months from clinical onset (8). As a result of immunological and inflammatory processes, the capillary walls are ruptured, resulting in increased permeability. As a consequence, there is a leakage of inflammatory cells and immune material into the Bowman space that in turn leads to an activation and proliferation of parietal cells with the compression of capillary loops (20) (cf. Fig. 2(21)). The deposits formed in this way show the characteristic crescent shape of the RPGN. These histological findings are an expression of severe glomerular damage (22). As RPGN is a rather clinical term and based on the histological image, RPGN is pathologically spoken referred to as crescentic GN (cf. Fig. 3(230)).

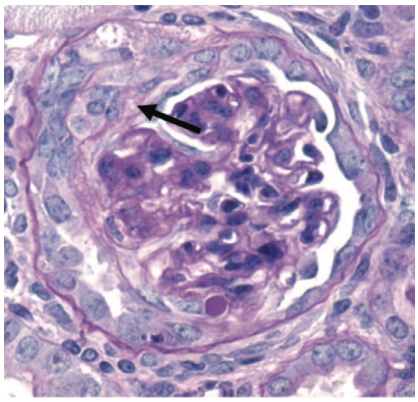

Fig. 3 glomerular crescent
glomerular crescent (arrow) in kidney tissue of mice at day 14 after the administration of nephrotoxic serum.
Obtained from Smeets et al. „Tracing the Origin of Glomerular Extracapillary Lesions from Parietal Epithelial Cells" (230)

1.2.1.2 Classification and Etiology

Based on immunohistological findings, such as the occurrence and the distribution of immunological residues (linear vs. granular), the crescentic GN can be split into three groups according to *Couser (1988)*(23): (1) Anti-glomerular basement membrane (GBM) antibody, (2) immune complex and (3) Pauci-immune GN with no antibody deposition in the glomeruli (cf. Table 2). These findings directly reflect the respective mechanism of glomerular injury.

1.2.2 Anti-glomerular basement membrane antibody disease (Anti-GBM-AB disease)

Anti GBM-AB disease is characterized by circulating ABs against specific parts of the glomerular or alveolar basement membrane. Opsonization of the GBM by this ABs causes severe glomerular injury usually resulting in crescent formations and the clinical picture of RPGN. If the alveolar basement membrane is affected, which is found frequently due to structural similarities, a combined disorder consisting of RPGN and lung hemorrhage is seen clinically. This entity is

known as *Goodpasture syndrome* (8, 24).

1.2.2.1 Pathogenesis

Anti-GBM ABs are responsible for about 10-15% of crescentic GN cases (25). The GBM is a substantial part of the glomerular filtration barrier (GFB), that is separating the vascular system from the urinary space, formed by podocytes, endothelial cells and the glomerular basement membrane between the two of them. The GBM itself is a highly specialized extracellular matrix consisting mainly of the four macromolecules laminin, type IV collagen, nidogen and the heparan sulfate proteoglycan agrin (26). In the case of anti-GBM AB crenscentic GN the type IV collagen is the decisive building block (cf. Fig. 4).

Immunopathogenic Classification of RPGN

Anti-GBM Antibody (20%)
- **with lung hemorrhage (Goodpasture's Syndrome)**
- **without lung hemorrhage**
- **complicating membranous nephropathy**

Immune Complex (40%)
- **postinfectious**
  - poststreptococcal
  - viseral abscess
  - Other
- **Collagen vascular disease**
  - Lupus nephritis
  - Henoch-Schonlein purpura
  - mixed cryoglobulinemia
- **Primary Renal disease**
  - IgA nephropathy
  - Membranoproliferative glomerulonephritis
  - idiopathic

Pauci immune GN (40%)
- **Vasculitis**
  - Polyarteritis
  - GPA
  - MPA
  - Hypersensitive vasculitides
- **idiopathic**

Table 2 immunogenic classification of RPGN (23)

In contrast to other collagens, collagen IV can only be found as a component of basal membranes. It is a collagen family comprising six different α-chains (α1-6) forming a variety of trimers also referred to as protomers. Out of the various combinations possible only three are found in mammals: α1α1α2, α3α4α5 and α5α5α6. Two of such protomers fuse at noncollagenous-1 (NC1) domains forming hexamers. In GBM only the α345NC1 hexamer is relevant as only this subtype occurs in the basal membranes of kidney, lung, testis and eye. Enzymatically cross-linked they form a network that is vital for the resilience of the GBM. The α3NC1 and α5NC1 subunits of those hexamers could be identified as the target of anti-GBM-ABs. The fact that α345NC1 can be found not only in the kidney, but also in the lung tissue explains the clinical picture of RPGN in combination with lung hemorrhage (26-29).

Even though it has not yet been conclusively clarified, there are reasonable approaches to explain the formation of anti-GBM-ABs. The work of Pedchenko, Bondar *et al.* (29) suggests that *Goodpasture's disease* is in fact a *conformeropathy*, that only develops due to a change in

the quaternary structure of the α345NC1 hexamer. It is assumed that various inhalative noxae, among others, are responsible for this structural degradation (30-32). By changing its quaternary structure as a hexamer, the α3NC1 and α5NC1 subunits also change their configuration and get exposed, which in turn triggers an immune response. It was shown that the anti-GBM ABs do not bind to the intact cross-linked α345NC1 hexamer, but only to the altered α3NC1 and α5NC1 monomers (33).

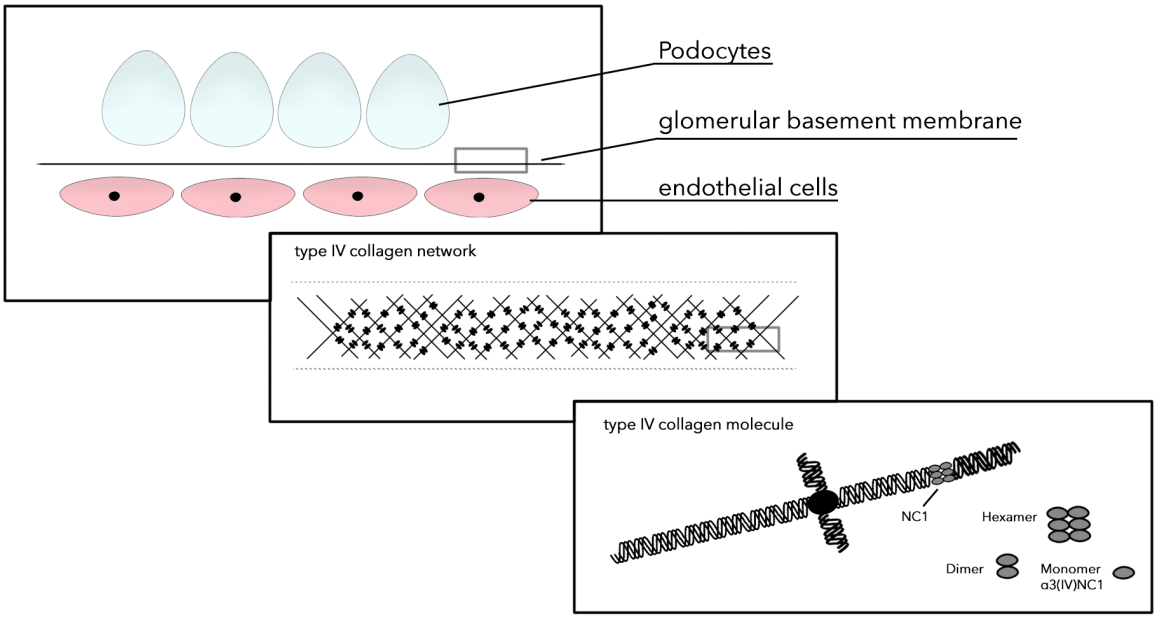

Fig. 4 glomerular filtration barrier
the glomerular filtration barrier consists of podocytes, the glomerular basement membrane und endothelial cells. The GBM itself is a network formed from type IV collagen molecules consisting of triple helices formed from α3(IV)-chains. Redrawn from p.1540 (24).

1.2.2.2 Mechanism of renal injury

Anti-GBM-ABs, that usually belong to the subclasses immunoglobulin G (IgG) 1 and 3 and occasionally to IgA and IgM, adhere to the thus altered GBM in a linear pattern and thereby activate the complement system and cysteine-proteinases such as cathepsin L (33-35). The activated complement cascade caused lesions in the GBM, through which cells, fibrin and other macromolecules enter *Bowman's space* where they initiate crescent formation promoted by interleukin (IL)-12 and interferon (IF)-γ (20, 36-39). It also leads to the chemotaxis of neutrophils and macrophages mediated via CD4+ and CD8+ T-Cells and the renal epithelium. These inflammatory reactions lead to interstitial nephritis with proteinuria and subsequently fibrosis (40-43).

The work of Merkel, Kalluri *et al.* suggests that in addition to anti-GBM-Abs, autoreactive T-cells may also make a contribution to the pathomechanism, as they showed that T-cells obtained from patients with *Goodpasture's disease* also respond to α3NC1-epitopes (44). In additional studies more evidence on cell-mediated glomerular injury playing a role in anti-GBM crecentic GN was provided. Dean, Wilson *et al.* were able to demonstrate that in B-cell-deficient mice – and thus in absence of anti-GBM-ABs – the histopathological and clinical picture of an anti-GBM crecentic GN develops nonetheless (45). Usually, auto-reactive T-cells are depleted in the thymus and consequently the α3NC1 antigen could not only be isolated from the thymus tissue of ill patients but also of healthy controls. In order to explain the T-cell reaction that still occurs in patients with *Goodpasture's disease* it is discussed that here the auto-reactive T-cells escape depletion because of a low-avidity binding to the auto-antigens expressed by the thymus (46). However, once *Goodpasture's disease* has been overcome, relapses are rare and furthermore, anti-GBM-ABs in untreated individuals disappear spontaneously within three years. It is assumed that this is due to CD25+ regulatory T-cells (T-regs), which are able to attenuate the autoimmune response and thus reconstitute tolerance against the α3NC1 auto-antigen (47). Wolf *et al.* demonstrated this effect in a mouse model of experimental glomerulonephritis (48). In that study the transfer of CD4+CD25+ T-cells into nephritic mice significantly reduced macrophage migration, CD4+ and CD8+ T-cell infiltration, glomerular damage and resulting proteinuria, but not the deposition of anti-GBM-ABs. One can therefore assume that T-regs suppress the inflammatory response but not the humoral immune response.

1.2.2.3 Genetics

Genetic studies have identified certain subtypes of the human leucocyte antigen (HLA) genes as potential risk genes for the development of an anti-GBM crescentic GN. Based on these studies, the detection of HLA-DRB15 and DR4 is associated with a higher risk of disease, whereas the gene variants DR1 and DR7 appear to have a rather protective effect (49).

1.2.2.4 Diagnosis

Rapidly increasing retention parameters without anamnestic explanation for AKI make the diagnosis of RPGN very likely. In any patient suffering from RPGN and lung hemorrhage or even lung hemorrhage alone, an anti-GBM-AB disease should be ruled out as a differential diagnosis.

To diagnose anti-GBM-AB disease it is necessary to provide evidence of anti-GBM ABs either from serum or biopsy; a biopsy is more precise and may give additional information regarding the severity of renal injury and the time course of disease. This is why any RPGN represents an absolute indication for renal biopsy. In the histological examination, linear deposition of anti-GBM-ABs along the GBM is seen as well as the above-mentioned crescent formations (8, 50).

An important differential diagnosis of anti-GBM-AB disease is vasculitis, as pulmonary edema and lung involvement occur also during anti-neutrophil cytoplasm antibody (ANCA) associated vasculitis, Lupus erythematodes or other systemic vasculitides may also cause pulmonary hemorrhage. Boyce and Holdsworth showed that in 45 patients presenting with the symptomatic complex of acute glomerulonephritis and pulmonary hemorrhage only eight cases of anti-GBM-AB disease could be identified as underlying cause, but systemic vasculitis in 25 cases (51). In addition, 10 to 40 % of patients with anti-GBM-AB disease may be ANCA positive. In line with that, these patients' serum should always be tested regarding ANCA as well since newer retrospective studies provide evidence that the co-presentation of ANCA and anti-GBM-ABs is associated with a higher risk for relapse and therefore requires, besides an aggressive anti-GBM AB therapy in the first place, a prolonged follow-up interval with focus on ANCA-associated vasculitis in the second place (52, 53).

1.2.3 Immune-complex GN

The Immune-complex GN accounts for about 40 percent of all cases of RPGN and is histologically characterized by discontinuous granular deposits distributed in the renal capillaries or the mesangium in contrast to the linear deposits seen within Anti-GBM-AB disease. It usually develops secondary to a primary disease such as *systemic lupus erythematosus (SLE),* mixed cryoglobulinemia, IgA nephropathy or *post infectious GN* following systemic infection with

streptococci/staphylococci. Crescent formations are barely seen in immune-complex GN, but if they do occur, they indicate a severe course of events and the number of crescent formations is disproportionate to prognosis (8, 23, 54).

1.2.3.1 Pathogenesis

The immunological deposits can form due to different mechanisms, that could be distinguished depending on where the target structure of the respective antibody is located. If the circulating ABs are targeting proteins, that are regularly expressed by podocytes in the GBM, it is referred to as an *in situ* complex formation. This pathomechanism is discussed in the context of SLE (55, 56). A second mechanism of complex formation is the implantation of antigens into the GBM. In this case, proteins in the circulation are bound by GBM components. The proteins bound in this manner then serve as antigens that trigger the formation of antigen-antibody complexes. In addition to that, complex formation in the prerenal circulation can occur. The complexes formed in this way are then consecutively flushed into the GBM where they get stuck. Independent of the respective mechanism of complex formation, the complex itself triggers a classical complement activation, that leads to a damage of the filtration barrier mediated by the C5b-9 membrane attack complex resulting in proteinuria and hematuria (57).

1.2.3.2 Diagnosis

Similar to the anti-GBM-AB disease it is a clinical diagnosis with rapid development of AKI without anamnestic explanation in the first place. However, only biopsies can provide a reliable diagnosis. Here the aforementioned discontinuous granular deposits with or without crescent formation can be seen.

1.2.4 Pauci-immune GN

Pauci-immune GN describes the picture of a necrotizing crescentic glomerulonephritis in which either very few or no immune deposits can be seen in immuno-histological examination (58). The word "*pauci*" is classical Latin and means "*few*". The Pauci-immune GN accounts for about 50% of the reported RPGN cases. These cases are due to mostly ANCA-associated

vasculitides. *Granulomatosis with polyangiitis (GPA)* or *microscopic polyangiitis (mPA)* could be identified as the predominant underlying diseases (59, 60). In only 10% of Pauci-immune GN cases ANCAs could not be detected. However, Eisenberger *et al.* were able to demonstrate that prognosis and histology as well as the underlying cause is very similar to ANCA-positive forms. In a retrospective multicenter cohort study with 20 patients with ANCA-negative Pauci-immune GN, 17 were diagnosed with mPA, two with GPA and only one with renal limited vasculitis (61).

1.2.4.1 Pathogenesis/ANCA-associated vasculitis

Vasculitis in general is defined as an immune-mediated inflammation of vessels and consecutive impairment of the respective organ or organ system. The variety of clinical signs and symptoms can be ascribed to the great differences in distribution and localization (8). In order to establish a systematic nomenclature, the *Chapel Hill Consensus Conference* has developed the *International Chapel Hill Consensus Conference Nomenclature of Vasculitides,* that has been continuously refined since. The most recent version is the *2012 CHCC* (62). The *CHCC* differentiates between large, medium, small and variable vessel vasculitides as well as organ specific vasculitis and vasculitis in the course of a systemic disease and secondary vasculitis. In the context of this study the small vessel vasculitides are of particular importance, as they include ANCA-associated vasculitides (AAV), immune-complex vasculitides and Anti-GBM-AB disease.

The AAV include mPA, GPA (formerly known as *Wegner's granulomatosis*) and eosinophilic granulomatosis with polyangiitis (*Churg-Strauss*; EGPA) as well as renal limited vasculitis. Additionally, drug-induced vasculitides have been subject to recent studies (59, 63, 64). Anti-neutrophil cytoplasmic autoantibodies were first described in Pauci-immune GN and later on also linked to GPA, mPA and also EGPA. Nowadays they are a crucial part of the diagnostics of vasculitides (65-68).There are three different subclasses of ANCA: perinuclear ANCA (p-ANCA), cytoplasmic ANCA (c-ANCA) and atypical ANCA (a-ANCA) are distinguished. These subclasses can be assigned to two main target antigens, c-ANCA to proteinase 3 (PR3) and p-ANCA to myeloperoxidase (MPO). For convenience, they are also referred to as PR3-

ANCA and MPO-ANCA. All antigens with an atypical staining behavior are attributed to the a-ANCA group. The predominant type of ANCA gives a good indication for the respective entity of disease. 70-80% of patients with GPA are positive for PR3-ANCA and 60% of Patients with mPA are positive for MPO-ANCA. In Patients with EGPA only in 40% MPO-ANCA could be identified and PR3-ANCA in below 5% (69-72).

1.2.4.2 Etiology

The exact etiology of ANCA and thereby AAV's themselves remains still unclear, but there are many promising approaches. Since early symptoms patients suffering from AAV may present, e.g. fever, malaise and weight loss, overlap with those of infection, it is assumed that AAV is at least partially triggered by infectious processes. The work of Stegeman *et. al* suggests a connection with S. aureus infection and here especially with nasal carriage. There are other studies that support the hypothesis that S. aureus contributes to the pathophysiology of AAV and the frequency of relapses (73, 74).

There is evidence that there is also a genetic component contributing to AAV. Data provided by Cao *et al.* indicate that MHC Class II allele HLA-DRB1-15 leads to an increased risk of African Americans and Caucasians to develop PR3-ANCA associated vasculitis (75). Furthermore, a nucleotide polymorphism in the PTPN22-gene (PTPN22 620W), which is encoding for tyrosine phosphatase, was linked to AAV. Jagiello *et al.* were able to demonstrate that the PTPN22 620W allele appears with a significantly higher frequency in ANCA-positive GPA than it does in healthy controls. Interestingly, this single nucleotide polymorphism is also indicating for a couple of other autoimmune-disorders, such as diabetes mellitus type I and SLE (76).

Alpha-1-antitypsin, since it is the main inhibitor of PR3, is also assumed to play a role in the pathophysiology of AAV, especially GPA. In line with alpha-1-antitrypsin being the main inhibitor of PR3, patients suffering from alpha-1-antitrypsin deficiency show increased levels of PR3, more precisely an insufficient depletion of PR3 in the course of an inflammatory reaction. The imbalance between proteases and their respective antiproteases may contribute to the

development of immunogenic forms of PR3 and thus PR3-ANCA (77, 78).
PR3 is mainly expressed by neutrophils and can be cell-surface dependent here, in which case it is termed membrane-PR3 (mPR3). The amount mPR3 varies in neutrophils of a distinct individual , so that there are neutrophils with high levels and such with low levels of mPR3, but the ratio of high to low mPR3 is genetically determined, even though there are variations in the total amount of mPR3 depending on cytokines (79-81). Moreover, patients who show a high level of high-mPR3 seem to be more prone to develop GPA and those who already suffer from GPA for relapse (82, 83).

As already mentioned above, some drugs such as hydralazine, minocycline, propylthiouracil and levamisole potentially effect an ANCA-seroconversion. Especially hydralazine-associated AAV can show a severe course with significant damage to the kidneys and therefore hydralazine should be used critically. Levamisole, actually an anthelmintic, is particularly interesting, as it has been used more and more often as a thinner for cocaine since 2003 and is thus consumed accidently by drug users and may also lead to AAV (63, 64, 84, 85).
It is also assumed, that inhalative noxae play a role in the development of AAV, since at the onsets patients often present themselves clinically with upper respiratory tract symptoms like sinusitis, cough, and hemoptysis. The work of Hogan *et al.* suggests that silica dust is contributing to the pathogenesis of AAV, maybe as a trigger substance, although the precise mechanism is still unknown (86). Other possible triggers, as for instance farming, solvent exposure or earthquake are a subject of discussion but have yet to be verified (87).

ANCA production itself is considered to be initiated by the revelation of cryptic sites as new epitopes. Such cryptic sites are epitopes that only develop the respective immunogenic form or become accessible within the framework of an inflammatory immune reaction. The extension of the immune response from the initial antigen by the newly exposed cryptic sites is also referred to as epitope spreading (88) (cf. Fig. 5 (88)). Epitope spreading in the context of AAV may develop from an initial tissue injury or irritation by noxae as already mentioned above. Whether ANCA's are actually directly pathogenic is still subject to current research. However,

there is increasing evidence that ANCA's certainly fulfill a function in the causal chain of AAV. Xiao *et al.* for instance have shown in a murine model that transfer of antibodies specific for MPO into RAG 2- deficient mice leads to a crescentic GN (89). Nevertheless, the fact that at least 10% of patients with mPA or GPA show no evidence of ANCA suggests that although ANCA's can play a pathogenic role, they are not absolutely necessary for the pathogenesis of the disease (72).

1.2.4.3 Mechanism of Injury

Role of T-Cells

T-cells subdivide into a variety of different subsets. For AAV one subset of particular interest are CD4+ T-cells, also known as T helper cells. CD4+ T-cells detect specific antigens presented by antigen presenting cells (APC) via MHC II molecules. If they detect their specific antigen, they get activated and differentiate depending on different cytokines either to Th1- or Th2-cells. In the course of AAV, Th1 cells are thought to be the relevant subset. Th1 cells are induced by IL-12 and are involved in the defense against microorganisms such as S. aureus. Activated Th1 cells release IFNg and TNFa, that in turn prime neutrophils. It is more than likely that AAVs are T-cell dependent, since higher levels of CD4+ T-cells and their respective cytokines can be found in patients with GPA than in healthy controls. The assumed mechanism is a dysregulated IL-12 production of monocytes and thus an overproduction of IFNg and TNFa (90, 91).

Role of Neutrophils

The purpose of neutrophils is to identify and destroy microorganisms. There are a variety of methods available for this task. They can either eliminate them by phagocytosis or degranulation and thus secretion of anti-microbial enzymes and reactive oxygen species (ROS) or by neutrophil extracellular traps (NETs). The latter is a strategy of neutrophils where they release webs of decondensed chromatin and serine proteases into the extracellular space. NETs render microorganisms harmless and it is assumed that the physical barrier prevents the further spread of pathogenic organisms. NET formation may even occur intravascularly, suggesting

that NETs may also play an important role in sepsis (92-94). More importantly, especially in the context of AAVs, intravascular NETs appear to have thrombogenic effects and may cause endothelial damage (95, 96). Neutrophils stimulated by ANCA seem to release NETs containing PR3 and MPO autoantigens even in absence of infectious agents and can thus maintain an ongoing production of ANCA itself (97).

In addition to the aforementioned thrombogenic effect of intravascular NETs, Berden *et al.* could demonstrate that in patients with AAV anti-plasminogen- and anti-tissue plasminogen activator (tPA) antibodies could also be identified (98). Bautz *et al.* described in a former study that the anti-plasminogen ABs are recombinant proteins, translated from the antisense strand of PR3-cDNA (99). As plasminogen is the inactive precursor of plasmin, which is of central importance for fibrinolysis, antibodies against plasminogen result in a delayed degradation of

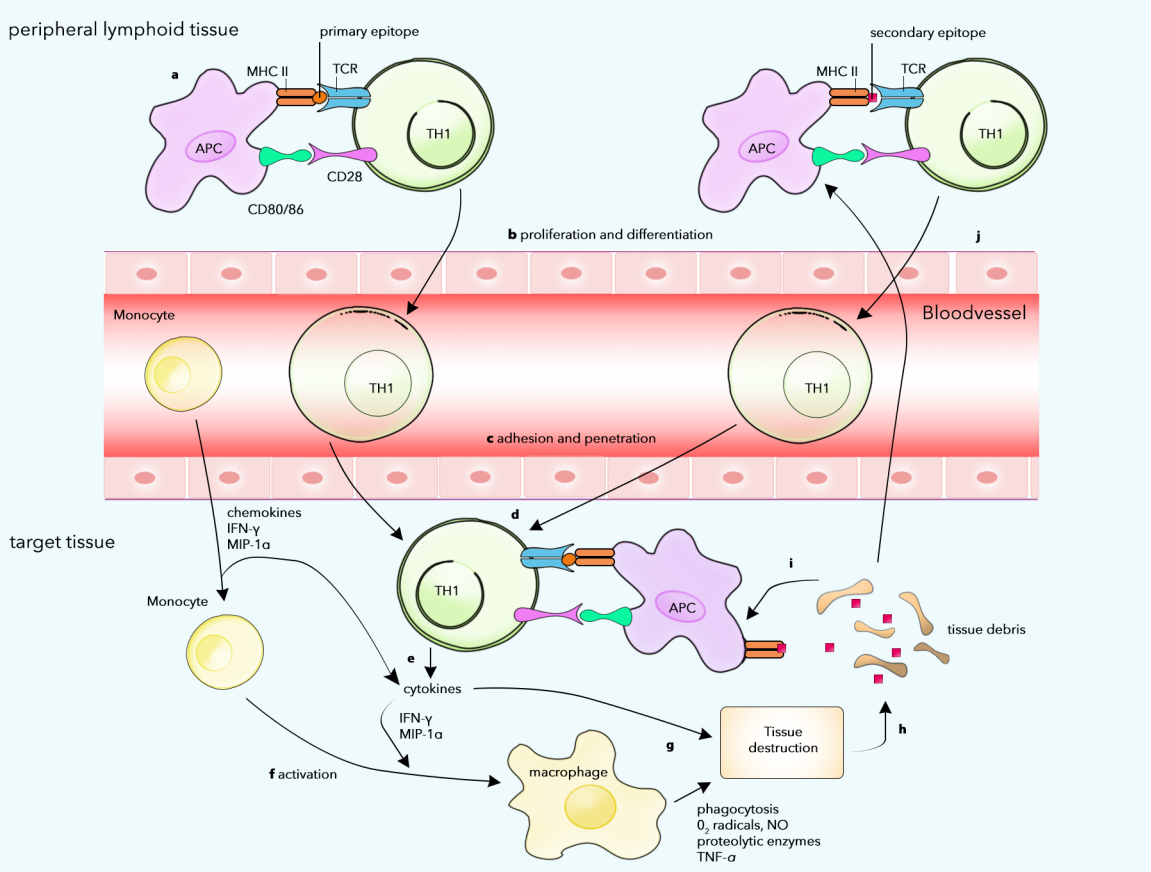

Fig. 5 epitope spreading
APC's present the primary epitope (either virus or endogenous) in peripheral lymphoid tissue (a) to Th_1 Cells, which effects proliferation and differentiation of autoreactive t-cells (b). In the target tissue (c) resident APC's restimulate Th_1 Cells by antigen-presentation (d). Restimulation effects cytokine and chemokine release (e). As a result monocytes are recruited and differentiate to macrophages (f). Macrophages cause tissue destruction by release of TNF-α, proteolytic enzymes, NO and O2 radicals as well as phagocytosis (g). The resulting tissue debris (h) is presented to APC's in the target tissue as well as peripheral lymphpod tissue (i), effecting a second wave of Th_1 cells causing additonal tissue destruction (j). Redrawn from Fig.1 (88).

thrombi. In line with these findings, Berden *et al.* showed that anti-plasminogen ABs correlate with crescentic kidney lesions (98).

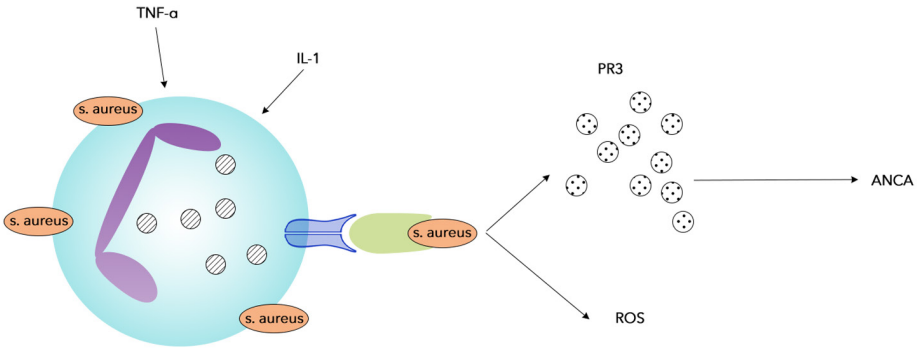

Fig. 6 effect of s. aureus on primed neutrophils
neutrophils primed by proinflammatory cytokines like TNF-α and IL-1 can be fully activated by binding of opsonized s. aureus. As a result PR3 and ROS are released, which in turn could damage the glomerular filtration barrier and stimulate ANCA production. Redrawn from Fig. 3 (74).

As the target antigens of ANCA are mainly located in the cytosol of neutrophils, except for the membrane dependent part, they are fairly inaccessible. If neutrophils are activated by cytokines released by CD4+ T-cells, such as TNFa the expression of mPR3 increases and thus ANCAs are able to bind (100). Via binding of ANCA the now membranous target antigens get crosslinked and thus primed neutrophils become activated. Another mechanism of primed-neutrophil activation is the binding to Fc receptors (FcRs). FcRs are surface antigens of immune cells that bind to the Fc fragment of immunoglobulins and thus opsonized pathogens or cells according to the key-lock principle and thereby trigger an immune response (221) Especially binding to the Fcγ RIIa is known to trigger tissue injury and inflammation induced by neutrophils. In particular, tissue injury is due to the release of lytic oxygen radicals, which is also referred to as oxidative or respiratory burst, and antimicrobial enzymes such as PR3 (101, 102). Furthermore, primed neutrophils can adhere to the endothelium and attack the vascular walls. Additionally, they release chemokines that attract other neutrophils and, in this manner, initiate a vicious circle. These membranous target antigen-dependent mechanisms may explain why patients with higher rate of high mPR3 are more prone to develop AAV (82, 103). The same pathway that is activated by ANCA binding on the FcRs, can also be triggered by S. aureus and therefore activate primed neutrophils and effects not only the secretion of reactive oxygen species (ROS) but also of PR3, which in turn may stimulate ANCA production (74) (cf. Fig. 6).

Role of B-cells

It is evident, that the level of activated B-cells in active AAV is higher than the level of activated T-cells and besides that, B-cell activity levels correlated with disease activity scores. Moreover, selective B-cells depletion with cyclophosphamide shows a significant effect in AAV therapy. In contrast to that, T-cell levels are higher during disease remission (104, 105).

Renal impairment is not necessarily present in the beginning of AAV, but up to 85% of patients show signs of a renal involvement in the further course of disease (106, 107). The neutrophil-triggered inflammatory processes also impair the glomerular microcirculation and thus leads to a damage of the filtration barrier. Hematuria, proteinuria and a rise in serum creatinine may be the consequence. Development of a RPGN in the course of AAV is commonly seen.

1.2.4.4 Diagnosis

In diagnosing AAV and thus pauci-immune GN a biopsy of the most likely affected organ (usually skin, kidney or lung) is still considered the gold standard and therefore mandatory. However, in certain cases, it may not be possible to perform a biopsy, depending on the individual condition of the patient. In this case a tentative diagnosis based on the clinic, potential pulmonary nodules, alveolar hemorrhage or positive ANCA testing can be made. Especially ANCA serology has a very high positive predictive value, in some cases up to 98% with corresponding symptoms (108). If another cause for a clinical presentation suspicious for AAV or pauci-immune GN respectively is very unlikely, a tentative diagnosis can be made with a high probability, which allows an early start of adequate treatment even without biopsy (72). For assessment of kidney involvement and function, creatinine, proteinuria, estimated glomerular filtration rate (eGFR) by means of the *Chronic Kidney Disease Epidemiology Collaboration formula* (CKD-EPI) and urinalysis can be used. A closer evaluation of the lungs can be done by chest x-ray, computer tomography or bronchoalveolar lavage.

As already mentioned above, a biopsy is the most reliable way to detect and assess AAV, particularly with renal involvement. Depending on the histological findings the progression of disease can be estimated. The picture varies from patients presenting with asymptomatic hematuria and either normal or slightly impaired renal function, showing mild focal and

segmental glomerulonephritis, to patients suffering from acute renal injury with the full picture of a crescentic GN, frequently along with mononuclear tubulointerstitial infiltrates. In contrast to other sub forms of RPGN, patients with pauci-immune GN show only few or no immune deposits (61).

1.2.5 Therapy and prognosis

Referring to RPGN, early diagnosis and immediate therapy is vital to prevent progression to end stage renal disease. Thus, it is recommended to start an empiric therapy with pulse intravenous methylprednisolone as soon as RPGN is suspected. Depending on the severity of the symptoms present, a plasmapheresis should be considered. The further therapeutic procedures depend on a prompt identification of the respective RPGN subform.

Anti-GBM-antibody disease

In 2011 Cui *et al.* compared (1) combination therapy of plasmapheresis and immunosuppression with (2) steroids and cytotoxic agents and (3) steroids alone in a retrospective study involving 221 patient and found that the combination of plasmapheresis and immunosuppression is the most promising approach (109).

It is therefore recommended to ensure low serum levels of anti-GBM-ABs by means of plasmapheresis for two to three weeks at least in combination with methylprednisolone over a period of three days followed by daily oral prednisone. Immunosuppression may be supplemented with cyclophosphamide. After three months, or after remission is established, a de-escalation of therapy from cyclophosphamide to azathioprine as maintenance therapy is to be considered, since the latter is less toxic. Maintenance therapy can either consist of prednisolone only or a combination of prednisolone and azathioprine and should be continued for six to nine months. An important limitation to therapy is end-stage renal disease, when neither plasmapheresis nor immunosuppression is of significant benefit if the patient is already depending on dialysis at the time of diagnosis (23, 110, 111).

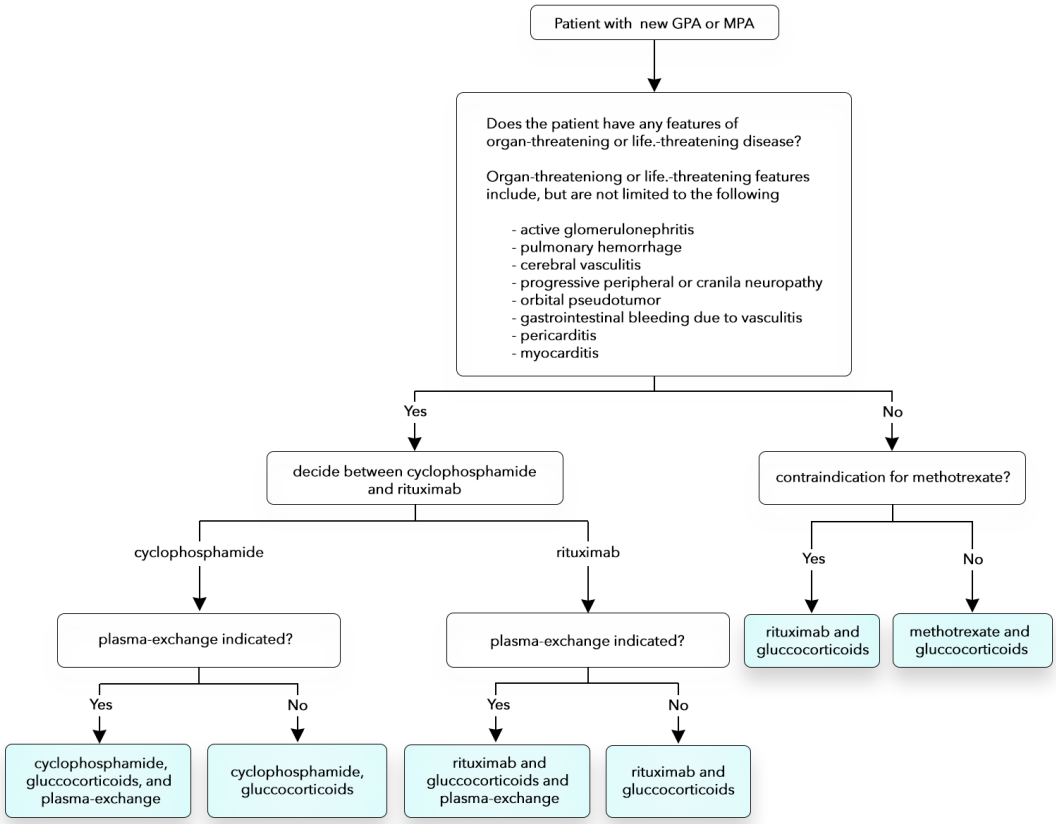

Fig 7 Therapyalgorithm for non-pregnant patients with MPA or GPA.
Modified after (114)

Immune-complex GN

Since an immune-complex GN describes the renal component of various underlying diseases such as lupus, IgA nephropathy, membranous nephropathy, cryoglobulinemia, and others, the respective therapeutic approaches differ. Implementing all therapy concepts here would go beyond the scope of this work. Nevertheless, immunosuppression is also one of the key components here. Methylprednisolone and optionally cyclophosphamides are commonly used pharmaceuticals for this purpose (8).

Pauci-immune GN

AAV therapy is divided into two stages: first to induce remission, followed by the maintenance of remission and prevention of relapse. Both goals are achieved by aggressive immunosuppression, which is essential, as the mortality rate in untreated patients is up to 90% within two years. Glucocorticoids, which can be supplemented with cyclophosphamide, rituximab or methotrexate as desired, serve as the basic immunosuppressive agent (112, 113). As a further

option, plasmapheresis is also possible. (cf. Fig. 7(114)) A key factor in the therapy of AAV is the severity of the disease. If the patient is not showing any life- or organ-threatening conditions yet, methotrexate is still an option. However, as soon as there is evidence of a life- or organ-threatening course, either a combination of glucocorticoids and rituximab or glucocorticoids and cyclophosphamide is indicated. Both, rituximab and cyclophosphamide, represent an effective treatment for AAV, the choice depending on individual contraindications as well as preferences of the respective physician and patient (115, 116). Additional plasmapheresis should be considered, if kidney function is rapidly decreasing, the patient already requires dialysis, or is also positive for Anti-GBM-AB's or shows symptoms of lung-hemorrhage (117, 118). At this point, however, it should be noted that it is very likely that the existing algorithms will have to be revised due to the results of the PEXIVAS trial, which has already been completed but not yet published (229).

In summary the RPGN could be considered as a medical emergency and requires immediate diagnosis and treatment to prevent a serious course of events.

1.3 Renal Ischemia Reperfusion murine model

AKI is a very common condition in hospitalized patients, especially in intensive care, and is associated with a high mortality rate (120). As mentioned before, the etiology is manifold (cf. AKI) and therefore different models based on the principles of sepsis, ischemia and toxicity have been developed over time, in order to provoke and investigate mechanisms and consequences of AKI. A well-established murine model in investigation of AKI is the renal ischemia-reperfusion-injury (IRI) model, that forms the basis of this thesis. Despite the fact that there are some well documented and significant differences in the pathophysiology of humans and rodents and hence the results are not fully transferrable, renal IRI has made a major contribution to understanding mechanisms of acute renal failure as well as warm ischemia in the setting of kidney transplantation (121). Nevertheless, I will focus mainly on aspects of AKI throughout this thesis.

By the time renal IRI models were established in 1960's, predominantly large animals were

used for testing. The first experiments were conducted in dogs and rabbits, while subsequently investigators tended to use rats in the 1990's before mice became the model of choice (122, 123). A likely reason is the increasing availability of genetically modified mouse strains. As a consequence, today there are over 5000 publication on the keyword *renal ischemia reperfusion injury*, with exception of the reviews, more than half of which were conducted in rodents (mice and rats).

Renal IRI (cf. Material and Methods) has various effects on the kidney, especially on the S3 segment of the straight proximal tubule (cf. Fig. 8 (124)). Both, necrotic and apoptotic cell death is observed in this segment, which leads to the formation of tubular casts due to epithelial desquamation. As a consequence, the passage of filtrate through the nephron is blocked. Furthermore, the effects differ depending on the time from reperfusion to examination of the respective tissues. Total loss of the brush border in S1, S2 and S3 can be observed 15 minutes after ischemia, which results in reversibly impaired sodium exchange. After 30 minutes post-ischemia the brush border starts to reappear, 4h post-ischemia a *restitutio ad integrum* in S1 and S2 can be seen as well as a partial repair in S3, but for some signs of irreversible cellular damage (125).

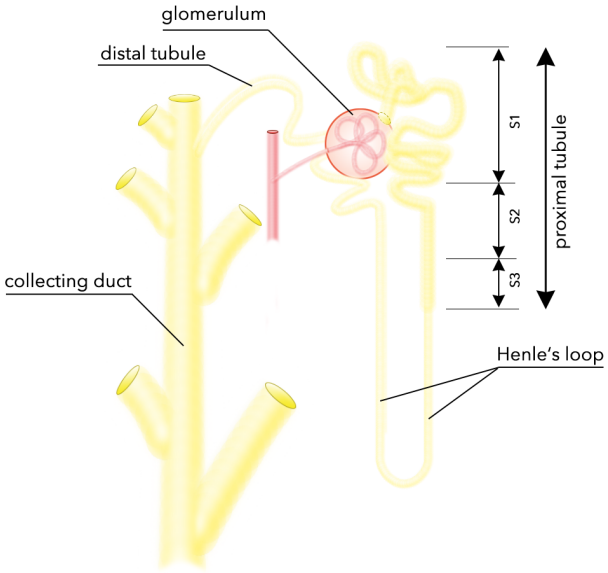

Fig. 8 nephron anatomy (124)

In addition to that, there are studies illustrating a proliferative effect on the tubular epithelium, starting from 24h until 120h after ischemia. Moreover Witzgall *et al.* demonstrated that surviving epithelial cells of the most severely damaged S3 segment could serve as progenitor cells for post-ischemic repair processes (126). However, because in the present study mice were euthanized at 24h after reperfusion (cf. Material and Methods), the proliferative effects can be neglected.

It has become clear that the exact selection of animals has great impact on the outcome. It is

recommended to use rodents of the same age, gender and weight. Age and weight are important, because surgery duration and quality affect the outcome. Older or heavier animals tend to have a higher intraabdominal fat content, which worsens the surgical conditions. Gender is of relevance, as Park *et al.* could show that testosterone enhanced the susceptibility to ischemia. They demonstrated that castration of male rodents reduced and administration of testosterone in females increased susceptibility to renal injury (127).

A very recent paper by Owji *et al.* criticized the previously established renal IRI models to the effect that they always block arterial inflow and venous outflow simultaneously. One of the main criticisms was that complete obstruction of the pedicle occurs rarely in human AKI. They showed that clamping the venous vessels results in a more severe damage and dysfunction than clamping either the artery or the pedicle (128). Thus, this work could contribute to a further development of the model itself.

The most commonly used technique to monitor renal IRI is blood urea nitrogen (BUN), serum-creatinine as well as the histologic processing of the kidneys (cf. Material and Methods) (129). However, it must be said that serum creatinine is not an ideal marker in the murine model, since it can be falsified by the significantly higher tubular secretion and the greater fluctuations in body weight and muscle mass of the rodents compared to humans (222).

1.3.1 Mechanism of injury

Obstruction of the pedicle and thus inhibition of blood flow results in a lack of oxygen and therefore a depletion of adenosine triphosphate (ATP) and an insufficient glycogen supply. As a consequence, metabolic changes lead to tissue damage resulting in leukocyte and complement activation (130, 131).

Neutrophil granulocytes infiltrate the injured tissue and degranulate, releasing ROS and proteases. In addition to that, they start secreting the pro-inflammatory cytokines IL-4, IL-6, TNFα and IFNγ (132). Macrophages release cytokines in response to tissue injury. Here IL-1, IL-6, IL-12 and TNFa are released. Experimental depletion of macrophages or suppression of cytokine receptors involved in the macrophage response significantly improved the outcome, up to a complete prevention of renal IRI. Hence it can be assumed, that macrophages are a vital

element in the pathogenesis of renal IRI (133-136).

1.4 Macrophages

Macrophages are very likely some of the oldest players of the immune system (137). In the current literature, macrophages are attributed to the mononuclear phagocytic system (MPS), which also includes osteoclasts, microglia or dendritic cells and many more. They are the primary mediator of innate immunity and therefore constantly scan their environment for pathogenic microorganisms, cell debris or foreign material. This scanning process is primarily mediated by *Toll Like Receptors* (TLR), by *pathogen associated molecular patterns* (PAMP) and by *damage associated molecular patterns* (DAMP). Thereby, pathogenic microorganisms and exogenous structures can be identified and distinguished from damaged but endogenous structures, which is crucial to macrophage function (138, 139). Depending on the recognized structure, a macrophage can call up different programs: It can either kill pathogenic microorganisms, or repair, if damaged tissue is identified, or as a third option phagocytize identified foreign material and present it via MHC class II molecules to T-cells in order to direct adaptive immunity. Hence the name *macrophage*, derived from the ancient Greek, which means *"big eater"* and refers to the cells' ability to perform phagocytosis. Until recently, macrophages were reduced to their function as *trash collectors* and considered merely as effector cells of the adaptive immune response, thus their actual importance for the immune system was completely underestimated. Thanks to the work of Mills *et al.* who demonstrated that macrophages also act in the absence of T-cells and thus independent of T-cell activation we now know that not only do T-cells direct macrophages, but that it can also be the other way around (140, 141). Macrophages are capable of eliciting either a Th1 (IFNg dominant) or a Th2 (IL-4 and TGFb dominant) response. IFNg in turn leads to a further activation of macrophages and IL-4 triggers an antibody-based reaction, thus T-cell activation can be considered as amplification and specification loop in macrophage response (140, 142).

1.4.1 M1/M2-type Macrophages

Mills *et. al.* also made another fundamental discovery for the understanding of the functioning

of macrophages. Initially, they studied macrophage responses in sterile wounds and later on in growing and regressing tumors. As a result of these studies, they stated that macrophages were able to metabolize the substrate arginine either to the cytotoxic nitric oxide (NO) or ornithine, the precursor of many proliferative mediators, depending on the respective stimulus. In sterile wounds and in proliferating tumors, they produce ornithine and in regressing tumors and in the initial phase of wounding NO (143, 144). This per se contradictory result could be attributed to the induction of either inducible nitric oxide synthase (iNOS) or arginase, respectively. Based on these findings M1/kill and M2/repair-type macrophages can be distinguished. M1 and M2-type macrophages do not only differ in the direct cell-cell interaction as mentioned above, but in their cytokine pattern as well. M1/kill type macrophages show increased IL-12, IL-8 and CCL2 production, whereas M2/repair type macrophages mainly release EGF, VEGF and TGFβ. Furthermore M1/kill-type macrophages show increased expression of MHC class II molecules and M2/repair-type mainly mannose-receptors. Other cytokines which can be observed within macrophage responses, such as IL-6, TNF or IL-1 and IL-10, are not specific for either type but rather part of the general macrophage response (145).

1.4.2 Origin of macrophages

For a long time it was thought that macrophages derive from monocytes, originating from bone marrow and are then distributed via bloodstream to their target tissues and differentiate accordingly. This is certainly still a fact, but more recent studies provide evidence that macrophage progenitor cells are ubiquitously implanted prior to birth and are merely supplemented by immigration. Moreover, due to these progenitor cells, tissue resident macrophages have the ability to regenerate themselves (146-148).

1.5 Glucagon-like peptide-1 (GLP1) Pathway

1.5.1 GLP-1

In order to react adequately to ingested food in terms of metabolization and digestion, a complex regulatory system is needed. Pancreatic and intestinal hormones are vital to coordinate this

interplay. The pancreas' b-cells secrete insulin and amylin and its antagonist glucagon from a-cells, whereas the gastrointestinal tract produces various gastrointestinal peptides such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), a peptide of 30 amino acids that is synthesized from proglucagon by posttranslational processing (15, 149).

GLP-1 is an incretin hormone that is expressed in intestinal L-cells as a response to oral food intake. L-cells belong to the group of enteroendocrine cells and are spread throughout the ileum and colon. The peptide is released in two equally effective isoforms, GLP-1(7-36)-amide und GLP-1(7-37), with GLP-1(7-37) accounting for the significantly larger share (150, 151).

1.5.2 Glucagon-like peptide-1 receptor (GLP-1R)

GLP-1 unfolds its effects through specific binding to the GLP-1 receptor (GLP-1R), which is expressed in many different tissues such as pancreatic b-cells, pancreatic ducts, gastric mucosa, kidneys, lung, heart, skin, immune cells and hypothalamus (152). It is a member of the same subgroup as the GIP-receptor or the glucagon receptor, for they are all G-protein linked receptors (GPLR). These are seven-transmembrane proteins which, once activated, in this case by GLP-1, start an intracellular signaling pathway. The stimulatory Gs-protein couples to adenylatcyclase and thereby induces the formation of cyclic adenosine monophosphate (cAMP), which in turn leads to a change in cell function by modulating the state of ion channels. The specific effect depends on the respective target cell (149, 153).

1.5.3 Effects of GLP-1 activation

1.5.3.1 Incretin effect

A commonly known and extensively studied effect of GLP-1R activation is the *incretin effect*. Interestingly, the *incretin effect* was discovered prior to the identification of GLP-1 and GIP. It describes a significantly increased insulin secretion in response to oral glucose intake, that is two to three times higher than following intravenous glucose administration (149). The respective incretins were eventually identified, and it is now known that GLP-1 is the main agonist for the

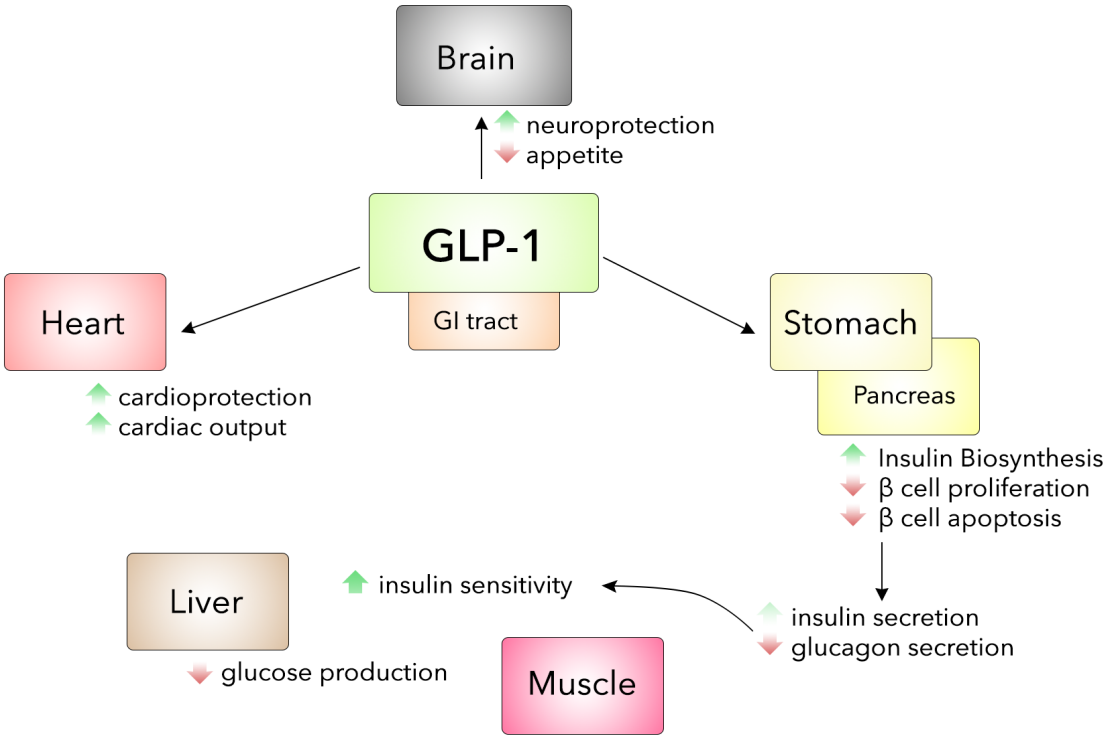

Fig. 9 effects of GLP-1
Redrawn after (154)

increased insulin secretion. It is believed that neural as well as endocrine factors are involved in GLP-1 secretion from distal L-cells, hence the release can be seen before food pulp reaches the corresponding intestinal section. However, the induced insulin peak also drops off extremely quickly, as GLP-1(7-36)-amide und GLP-1(7-37) are excreted quickly by the kidneys and are almost immediately cleaved to GLP-1(9-36)amide and GLP-1(9-37) by dipeptidyl peptidase-4 (DPP4), which are both unable to bind to the GLP-1R (154). In addition to that, it is known that GLP-1 has stimulatory and proliferative effects on pancreatic b-cells as well as an enhancing effect on proinsulin biosynthesis, gene expression and also sufficiently inhibits glucagon release from pancreatic a-cells (154, 155). It is also known that GLP-1 signaling delays gastric emptying and reduces small bowl motility resulting in deferred glucose uptake (156). Complementing direct effects on metabolism, GLP-1 also has an effect in the central nervous system (CNS). As noted earlier, the GLP-1R is likewise expressed in the hypothalamus, especially in regions that are relevant in terms of food intake. It was shown that administration of GLP-1 increases the feeling of satiety and thus reduces food intake (157, 158). These effects taken together (cf. Fig. 9)

made the GLP-1R signaling pathway particularly interesting for research into new antidiabetics and obesity treatments.

1.5.3.2 Protective effects of GLP-1R activation

Brain insulin resistance (BIR) describes an insufficient reaction of CNS-cells to insulin. It has been identified as a major risk factor for the development of neurodegenerative disease. Epidemiological studies have shown a correlation between the beginning of peripheral and central insulin resistance, which is suggesting a connection between metabolic diseases such as type 2 diabetes mellitus and neurodegeneration. These findings made GLP-1R agonism particularly interesting as a new therapeutic approach in neurodegenerative diseases such as *Alzheimer's disease* (159-161).

During *et al.* showed that central GLP-1R activation in a rodent model leads to improved learning and retentiveness. Their work also suggests that GLP-1 has neuroprotective effects, as kainate-induced apoptosis of hippocampal neurons could be prevented by GLP-1R activation (162). Other studies show additional evidence, in particular that GLP-1R agonism could reduce chronic inflammatory processes and amyloid plaques in the course of neurodegenerative disease. In addition to that it is assumed, that synaptogenesis as well as neurogenesis and cell repair are positively influenced by GLP-1R activation (163).

Furthermore, several studies have examined a potential cardioprotective effect of GLP-1. In preclinical as well as clinical trials reduction of infarction size and improved survival of myocardium following the administration of GLP-1R agonists was shown (164-166). Woo *et al.* could also demonstrate in a clinical trial with ST elevation myocardial infarction (STEMI) patients who underwent primary percutaneous coronary intervention (PCI) that supplemental administration of exenatide improves the left ventricular function (167). Additionally, a blood pressure lowering effect of GLP-1R was reported in several studies, mainly encompassing diabetic, hypertensive participants (168, 169). The underlying mechanism is not entirely understood yet.

In terms of nephroprotective effects, Matthias Thelen from our group of the Medical University of Graz demonstrated that treatment with GLP-1R agonist liraglutide improved the outcome and severity of glomerular injury in a model of nephrotoxic serum nephritis, suggesting that

GLP-1 might have a reno-protective effect (6). In addition to his findings, a very recent study of Moellmann *et al.* has proven a reno-protective effect, not of GLP-1(7-37) or GLP-1(7-36)-amide, but of its cleavage products in murine diabetic nephropathy (170).

1.5.3.3 Role of GLP-1 in inflammation

Besides the *incretin effect*, there is increasing evidence that GLP-1 is involved in the regulation of inflammatory processes. Rodents showed elevated GLP-1 levels in a model of experimental inflammation as well as human patients in a study of 155 ICU patients compared to 134 healthy controls. Kahles *et al.* were able to demonstrate an increasing effect of IL-1, IL-6 and lipopolysaccharide (LPS) on GLP-1 plasma levels (171, 172). IL-6, IL-1 and TNF are important cytokines in the orchestration of early immune responses and inflammation and are involved in the chemotaxis of leukocytes to the site of inflammation (173). In terms of IL-6 mediated GLP-1 release Elinsgaard *et al.* demonstrated that IL-6 alters the gene expression in pancreatic islet a-cells such that proprotein convertase 1 (PCSK1) is expressed and proglucagon is processed to GLP-1. Elinsgaard *et al.* concluded that IL-6 is a key element of an endocrine loop facilitating adaption to different insulin demands in the course of inflammation. GLP-1 is also believed to attenuate macrophage accumulation and thereby modulate the inflammatory response (171, 174).

In line with these findings, Yusata *et al.* found a significant dysregulation of anti-inflammatory and cytoprotective genes in the intestinal mucosa of GLP-1R deficient mice effecting a higher susceptibility to inflammation related injury, which was reversible by transplantation of bone marrow obtained from wild-type mice (175).

1.6 Liraglutide

Since the spectrum of effects mediated by GLP-1R activation, as discussed above, is very promising concerning anti-inflammatory effects, a further investigation and pharmacological utilization of these effects is of obvious interest. One of the main shortcomings in using GLP-1 as a pharmacological agent is the extremely short half-life of 2 minutes. At this point, great progress was made towards the development of DPP-4 inhibitors and the discovery of extendin-4.

DPP-4 inhibitors prolong the half-life of the metabolically active isoforms of endogenous GLP-1 (11-15h vs. 1-2 min) and therefore serve as antidiabetic drugs themselves (176). Exenatide in turn is a short acting synthetic GLP-1R agonist, which is derived from extendin-4 that was first isolated in the 1990's from the venom of a lizard called *gila monster*. Extendin-4 had the major advantage of being resistant to degradation by DPP-4 (177). It was subsequently approved as an antidiabetic drug by the FDA in 2005. Later on, other GLP-1R agonists were launched on the market, including liraglutide in 2009 by *Novo Nordisk*. Liraglutide was initially only registered as an antidiabetic agent but is meanwhile also approved as a treatment for obesity because of its positive effects on satiety and food intake as mentioned above.

Liraglutide utilizes the *incretin effect* (cf. Effects of GLP-1 activation) to reduce postprandial hyperglycemia. A tremendous advantage over conventional antidiabetic drugs like sulfonylurea is the fact that GLP-1-mediated insulin secretion is glucose- dependent, and therefore does not entail the risk of iatrogenic hypoglycemia (178, 179).

1.6.1 Mechanism of action

Liraglutide is a long acting GLP-1 receptor agonist that has been modified to bind to serum albumin, so it is degraded slower by DPP-4 and its renal elimination is delayed; thus the half-life of liraglutide is prolonged (11-15h vs. 1-2 min). Receptor binding of liraglutide induces the same effects as endogenous GLP-1 (cf. Effects of GLP-1 activation).

1.6.2 Pharmaceutical potential

Investigations into the pharmaceutical potential of GLP-1R agonism have been carried out by the LEADER, SUSTAIN 6 and ELIXA trials among others.

The primary objective of the LEADER trial (randomized double-blind trial with 9340 participants) was to investigate cardioprotective effects of liraglutide in patients with type 2 diabetes mellitus. This revealed a protective effect with regard to cardiovascular events. A further analysis of the LEADER data showed a positive effect on the development and exertion of diabetic nephropathy, as well as a lower occurrence of pancreatitis compared to the placebo group (223-225).

The SUSTAIN 6 trial (double-blind trial with 3297 participants) investigated the protective effects on the cardiovascular system of a weekly dose of semaglutide in diabetes mellitus type 2 patients. The Investigators observed a significant reduction of major cardiovascular events in patients at high cardiovascular risk in the semaglutide group compared to placebo. The SUSTAIN 6 trial also revealed that the risk of progression of an existing nephropathy or a newly emerging nephropathy is significantly lower with semaglutide therapy (226).

Another large trial examining the protective effects of GLP-1R agonism is the ELIXA trial (randomized, triple-blinded trial with 6068 participants). ELIXA studied potential cardioprotective effects of Lixisenatide in patients with type 2 diabetes mellitus and acute coronary syndrome. For cardioprotective effects, the hypothesis was not confirmed, but a further analysis of the data regarding the renal outcome showed that lixisenatide reduces the urinary albumin-to-creatinine ratio in patients with microalbuminuria and also reduces the risk of new microalbuminuria compared to placebo (227, 228).

In another study examining the effects of liraglutide monotherapy in 165 patients with type 2 diabetes mellitus in comparison to a placebo group, the authors demonstrated a reproducible lowering effect on blood glucose and HbA1c. They also reported positive effects on bodyweight and blood pressure. A further study by Garber *et al.* confirmed these results (180, 181). After the effectiveness of liraglutide monotherapy was proven, the value of a combination therapy with either metformin, the current first line oral antidiabetic, or basal insulin or both was investigated. As a result, a significantly greater reduction of HbA1c could be achieved by addition of liraglutide to conventional antidiabetics. As unwanted side effects mainly gastro-intestinal problems or headaches were observed (182-185).

Multiple publications provided data concerning protective and anti-apoptotic effects of GLP-1 activation in various tissues (cf. Protective effects of GLP-1R activation), including the kidney. Deducing from these findings, we first hypothesized that GLP-1R activation might have protective effects on acute kidney injury in the experimental model of nephrotoxic serum nephritis, which finally showed promising outcome (cf. Thelen 2013(6)). Since liraglutide also affects various mechanisms that are decisive in IRI like the RAAS, reactive oxygen species and

inflammation, we now aimed to test this hypothesis in a murine model of renal IRI (155). Here, we followed two approaches: first, we compared the outcome of GLP-1R -/- mice with wild-type mice, and secondly wild-type mice treated with liraglutide or vehicle were subjected to the IRI.

2 Materials and Methods

2.1 Animals and renal ischemia reperfusion injury model

In all experiments 8-12-week-old littermates on C57Bl/6J (Charles River Laboratories International, Inc., Sulzfeld, Germany) background and GLP-1R-KO mice (Drucker Laboratories, Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada) were used. Necessary genotyping was done by isolating genomic DNA with the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). The subsequent PCR was performed with the following primers: primer A Glp1r 5' (TAC ACA ATG GGG AGC CCC TA), primer B Glp1r 3' (AAG TCA TGG GAT GTG TCT GGA), primer C Neo1 (CTT GGG TGG AGA GGC TAT TC), primer D Neo2 (AGG TGA GAT GAC AGG AGA TC). During growth and time of trial mice were kept on a standard diet and water ad libitum. Liraglutide was administered for 7 days prior to surgery in a dose of 200µg/kg BW. The experiments were performed as already described by Hochegger *et al.* (186). Mice were anesthetized using 6–8 mg/kg bodyweight xylazine and 90–120 mg/kg bodyweight ketamine. Afterwards an incision was made on the central abdomen avoiding intestines and bowels. Utilizing this incision, the bilateral renal vessels were carefully dissected and mobilized to clamp both renal pedicles using microvascular clamps. After 30 min of ischemia the clamps were removed, and the incision sutured. While performing the entire procedure, the opened intestinal cavity was kept humidified by administration of 0.9% NaCl. Furthermore, body temperature was controlled using a rectal probe and adjusted accordingly with an adjustable heating pad to maintain 37°C body temperature. Mice were killed 24 h after removing the clamps. Intraperitoneal injection of 0.5mL Avertin (Sigma-Aldrich, Inc., St. Louis, MO) was used for deep sedation in combination with dislocation of the cervical spine. The investigator performing the intervention was blinded for genotype and pre-treatment. All experiments were done within the Austrian "Law on animals used for experimental purposes" (187) and have been approved by the Committee on the Ethics of Animal Experiments of the Austrian Ministry (BMBWF-66.010/0010-V/3b/2018).

2.2 Evaluation of renal impairment

For assessment of renal function serum urea, serum creatinine and blood urea nitrogen were evaluated. Blood samples were taken by cardiac puncture after euthanizing the mice.

2.2.1 Serum urea and creatinine detection

Serum urea and creatinine were measured by biochemical analysis (COBAS, Roche Diagnostics, Mannheim, Germany)

2.2.2 Blood Urea Nitrogen

Urea accumulates as a by-product of protein metabolism and is eliminated by renal filtration. The amount of circulating urea nitrogen allows conclusions regarding kidney function (188). Blood Urea Nitrogen (BUN) was measured using a Urea Nitrogen (BUN) Colorimetric Detection Kit (Invitrogen by Thermo Fisher Scientific; Life Technologies Corporation, Frederick, MD) according to the manufacturer´s indications.

Prior to the actual assay the samples were diluted 1:100 in distilled water. The standard was prepared according to the protocol provided by Thermo Fisher Scientific resulting in the following standard concentrations: Std1 = 10mg/dL, Std2 = 5mg/dL, Std 3 = 2,5mg/dL, Std 4 = 1.25 mg/dL, Std 5 = 0.625mg/dL, Std. 7 = 0.156mg/dL. 50µL standard or diluted sample were added to the appropriate wells of a 96-well plate followed by the addition of 75µL color reagent A to each well. Afterwards 75µL of color reagent B were added into the wells and the entire plate incubated for 30 min at room temperature. The reaction of urea with color reagent A and B results in a colored product, which can be quantified using a colorimeter. For colorimetric detection a FLUOstar Omega multi-mode microplate reader (BMG LABTECH GmbH, Ortenberg, Germany) was used at 450 nm. A standard-curve was generated from the data obtained with a four-parameter fit algorithm using MARS Data Analysis Software (BMG LABTECH GmbH, Ortenberg, Germany) as curve fitting software. From this standard curve the unknown sample concentrations were estimated.

2.3 Histological evaluation of tubular injury

To evaluate general histopathological changes, tissue sections were stained with periodic acid Schiff's reaction (PAS). Half a kidney of each mouse was fixed in 4% buffered formalin overnight (Donau Chemie GmbH, Vienna, Austria) and embedded in paraffin the next day. Prior to staining, the kidneys were cut in 4µm slices. The evaluation was done using an Olympus BX43 optical microscope. The degree of tubular injury was assessed as described before (186, 189) with a semiquantitative scale that evaluates the amount of cast formation, loss of brush border membranes, sloughing of tubular epithelial cells, and dilation of tubules throughout the cortex and outer medulla in 6 high power fields (HPF; 1 HPF = 40x) per kidney respectively. In doing so, the kidneysections were scored as follows: 0 = no tubular necrosis; 1 = 10%; 2 = 10–25%; 3 = 26–75%; and 4 = 75% tubular necrosis. (186). The scoring took place in a blinded manner.

2.3.2.1 PAS

Periodic acid-Schiff's reaction is used to stain glycol-containing cellular elements such as polysaccharides, glycoproteins, mucin and glycolipids and due to the attached glycoproteins, cellular basement membranes. The two main reagents here are *periodic acid* and *Schiff's reagent*. *Periodic acid* oxidizes free hydroxyl groups in macromolecules containing carbohydrate residues to aldehyde groups.

Schiff's reagent is the reaction product of fuchsine with sulfur dioxide. The reaction with sulfur dioxide causes a decolorization of fuchsine. If *Schiff's reagent* gets into contact with aldehydes, the sequential reaction will restore the initial purple color. In combination, these two are used to visualize the structures mentioned above in tissues of interest, in our case the kidney (cf. Page 632 Lüllmann-Rauch (190)).

PAS staining technique

To stain the prepared samples, it was first necessary to dewax and rehydrate the samples using a standard xylol- and a descending alcohol series and a final rinse in distilled water. After having the slides prepared like this, staining began with 5 minutes of incubation in *periodic acid* (Merck KGaA, Darmstadt, Germany), rinsed with distilled water for 3 minutes and then incubated with

Schiff's reagent (Merck KGaA, Darmstadt, Germany) for another 15 min. Following another rinsing in distilled water, the slides were counterstained with hematoxylin no. 3 (Sigma-Aldrich, Inc., St. Louis, MO). Eventually the samples were dehydrated using an ascending alcohol series and covered with Roti-Histokitt II (Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

2.3.1 Immunohistochemistry

In order to identify different subsets of immune cells, such as macrophages, cytotoxic T- cells, T-helper cells and neutrophils, immunohistochemistry was conducted. For immunohistochemical staining the kidney tissue was embedded directly in Scigen Tissue-Plus O.C.T Compound (Scigen Scientific, Gardena, CA) and flash frozen in liquid nitrogen. The kidneys were also cut in 4 µm slices using a standard cryomicrotome.

Basic principle of immunohistochemistry

By using immunohistochemistry (IHC), mediators and cells of interest can be identified and located in tissues by means of antigen-antibody reactions. The respective primary antibody for antigen-detection of a substance, is harvested from the serum of an animal of a species other than the tissue under investigation, which is immunized against the substance of interest (cf. pages 633-634 Lüllmann-Rauch (190)). Methods to make the specifically bound primary antibody visible to conventional optical microscopy are differentiated in direct and indirect methods. Either the primary antibody itself is labeled, which is referred to as direct or a labeled secondary antibody that specifically binds to the primary antibody is used, which is referred to as indirect staining. Indirect detection methods have a higher sensitivity than direct methods because the number of labels per molecule of primary AB is higher, which in turn increases the reactions intensity (191). With indirect methods there are various labels and methods available from which we chose the avidin-biotin complex (ABC) method, which will be discussed in more detail below.

Avidin-biotin complex method

Avidin is a rather large glycoprotein which was first described in egg white. It consists of four

identical subunits, each of which contains one binding site for biotin respectively (192, 193). Biotin, a water-soluble vitamin of the B family (vitamin B7), has a high affinity to avidin and can be either bound to the Fc region of an AB or enzymes or marked directly by fluorochromes. Enzymes or ABs conjugated with biotin are referred to as biotinylated agents (194). In the present study, a sandwich technique was used: a primary AB targeting the protein of interest, a biotinylated secondary AB targeting the primary AB and as third layer the avidin-biotin-conjugated peroxidase (ABC). The ABC consists of avidin and biotin with a conjugated peroxidase. Since avidin has four binding sites for biotin, other than the peroxidase and the secondary AB, two additional biotinylated proteins could bind, either more peroxidase or another ABC. This increases the amount of peroxidase per target threefold at least, resulting in signal amplification. (cf. Fig 10)

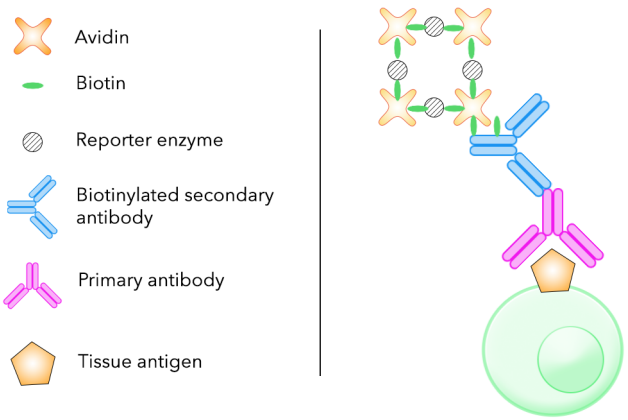

Fig. 10 Avidin-Biotin Complex (ABC) Method
redrawn from (195)

CD68+-staining and quantification

CD stands for "cluster of differentiation", which is an international nomenclature of surface molecules identifying different cell types and their respective stage of differentiation (190).

CD68 is a surface molecule specific for the monocyte lineage, most importantly macrophages, microglia, osteoclasts and myeloid dendritic cells (DCs) (195, 196). As CD68 is mainly expressed in the monocytic lineage, it is considered an appropriate marker to show the infiltration of kidney tissue by macrophages and DCs. In the present study, a rat anti-mouse AB (Bio-Rad Laboratories, Inc., Hercules, CA) was used. To quantify the results 6 low power fields (LPF; 20x) were counted in a blinded manner and transferred into a semiquantitative score to facilitate evaluation. The score was designed as follows: 0 = 0-4 positive cells, 1+ = 5-15 positive cells, 2+

= 11-50 positive cells, 3+ = 50-200 positive cells, 4+ = >200 positive cells.

2.3.1.1 CD8+-staining and quantification

CD8 is a surface molecule specific for cytotoxic T-cells. Cytotoxic T-cells identify and eliminate cells either infected by a virus or otherwise compromised, thus they are also referred to as T-killer cells. The identification process is mediated by major histocompatibility complex (MHC) class I molecules (190, 197). Due to its specific nature, CD8 is often used as a marker in immunohistochemistry. Here, a rat anti-mouse AB (Bio-Rad Laboratories, Inc., Hercules, CA) served as primary AB. In order to quantify the results, 6 HPF (40x) were counted regarding CD8 positive cells in a blinded manner.

2.3.1.2 CD4+-staining and quantification

CD4 is a surface molecule commonly known to be expressed on T-helper cells, but is also found on B cells, macrophages, and granulocytes. CD4 positive T-lymphocytes play a major role in early T-cell activation. Since CD4 is predominantly expressed in CD4+ T-cells, it can be used as T-cell marker in IHC. (91, 198, 199) As primary AB rat anti-mouse anti CD4 ABs were used (Bio-Rad Laboratories, Inc., Hercules, CA). Evaluation and quantification were done as described above (cf. Chapter CD8+).

2.3.1.3 Ly6G-staining

Ly6G, a small protein of 25 kD, is anchored to the outer cell membrane by a glycophosphatidylinositol (GPI) linker (200). As Daley *et al.* could demonstrate, Ly6G is specific for blood neutrophils and can therefore be used as a target in IHC to identify neutrophils in kidney sections (201). Here, a rat anti-mouse AB (Abcam, Cambridge, UK) was used. To quantify the results, 6 HPF (40x) with at large 25 glomeruli per mice were counted regarding positive, intraglomerular cells.

Immunohistochemical staining technique

For immunohistochemical staining the kidney tissue was embedded directly in Scigen Tissue-Plus O.C.T Compound (Scigen Scientific, Gardena, CA) and flash frozen in liquid nitrogen.

4 µm-kidney slices were dried at room temperature for 30 min and then fixed in 4°C acetone (Merck KGaA, Darmstadt, Germany) at room temperature for 8 min. Afterwards the samples were circled with a dako pen (Dako Denmark A/S) to provide a barrier to restrict the reagents to the tissue. To prevent non-specific binding, those sites were blocked using a solution consisting of 20% fetal calf serum (FCS; Merck, KGaA, Darmstadt, Germany), 10% heat-inactivated goat serum (Sigma-Aldrich, Inc., St. Louis, MO), 70% phosphate buffered saline (PBS; Dulbeccos Phosphate Buffered Saline/D8537-500mL, Sigma-Aldrich, Inc., St. Louis, MO) and avidin from a standard Avidin/Biotin Blocking Kit (Vector Lab. Inc. Burlingame, CA). By adding the avidin, it is ensured that the endogenous biotin activity is inhibited to prevent unspecific binding in following steps. 200µL of this solution was added to each sample respectively and incubated at room temperature in a moist chamber for 20min. The slides were then washed twice in PBS for 1min. During incubation time, the primary AB solution was prepared in a dilution of 1:500 and biotin from a standard Avidin/Biotin Blocking Kit (Vector Lab. Inc. Burlingham, CA). 200µL of the primary AB solution were applied on each sample and then incubated in a moist chamber at room temperature for 60min. In the next step the slides were washed in PBS for 4min for three times. Hereafter 200µL of the secondary AB (biotinylatetd goat anti-Rat AB; Jackson ImmunoResearch Europe Ltd., Newmarket, Suffolk, UK) was applied per sample in a concentration of 5µL per mL total volume and incubated in a moist chamber for 45min. In the meantime, the ABC (Vectastain ABC Kit, Elite PK6100 Standard, Vector Lab. Inc. Burlingame, CA) was prepared. Following the incubation, the slides were washed again in PBS as already described before. Subsequently the samples were incubated with 200µL ABC per slide in a moist chamber for 45min followed by another washing procedure. Afterwards the slides were submerged into 0,1M acidic acetate buffer for at least 3min. In order to visualize the targets, a chromogenic solution is added and was prepared for 10 slides as follows: 3mL 3-Amino-9-ethylcarbazole (AEC) stock solution (6 AEC tablets (Sigma-Aldrich, Inc., St. Louis, MO), 30mL dimethylformamide (DMF)), 60mL 0.1 M acidic acetate buffer (300mL 0.2 M sodium acetate, 100mL acetic acid, 400mL distilled water) and 30µl H2O2. Progress on colour development was checked by microscopy. On average, it took 8min to fully develop the slides. Counterstaining was conducted by submerging the slides in hematoxylin no. 3 (Sigma-Aldrich, Inc., St. Louis, MO)

for 10sec followed by rinsing with tap water. Eventually the slides were washed in distilled water twice and finally covered using Aquatex (Merck KGaA, Darmstadt, Germany) for embedding and storage.

2.3.1.4 L-Arg-staining

Two isoforms of arginase are currently known, arginase-I and arginase-II. Arginase-I is an enzyme that catalyzes the last step of the resin cycle, it converts arginine and H2O to ornithine and urea. As aforementioned in the chapter 'Macrophages' (cf. Macrophages), expression of arginase instead of iNOS is the main differentiation criterion between M1 and M2. Hochstedler *et al.* validated immunohistochemical staining of arginase-I as a method to distinguish M1 from M2 macrophages (202). As primary AB Anti-liver Arginase antibody 100µg (Cat.No: ab91279; Abcam, Cambridge, UK) was used.

L-Arg staining Technique

For immunohistochemical staining half a kidney of each mouse was fixed in 4% buffered formalin overnight (Donau Chemie GmbH, Vienna, Austria), embedded in paraffin wax the next day. Prior to staining the kidneys were cut in 4µm slices and mounted on Vectabond slides (Vector Laboratories, Inc. Burlingame, CA, USA) and fixed at 56°C for at least 1h. To stain the prepared samples, it was first necessary to dewax and rehydrate the samples using a standard xylol- and a descending alcohol series and a final rinse in distilled water. Additionally, the antigen unmasking solution (Cat.No:H-3300; Vector Laboratories, Inc. Burlingame, CA, USA) needs to be prepared, therefor 4680µl antigen unmasking solution were added to 500ml distilled water and mixed well. Now the slides were incubated in the unmasking solution 20 minutes at 120°C and subsequently 20 seconds at 85°C. After the slides have cooled down, they were rinsed with tap water and stored in phosphate buffered saline (PBS; Dulbeccos Phosphate Buffered Saline/D8537-500mL, Sigma-Aldrich, Inc., St. Louis, MO). From here on, the procedure was the same as described above in the 'Immunehistochemistry – Immunohistochemical staining technique' section with the only exception that an goat-anti-Rabbit AK (Cat.No: 111-065-144; Jackson ImmunoResearch Europe Ltd., Newmarket, Suffolk, UK) was used as secondary AB.

2.3.2 Real-time Reverse Transcription (RT) polymerase chain reaction (PCR)

Real-time PCR is a quantitative method that monitors the production of target DNA (deoxyribonucleic acid) in a given sample. As the prefix *real-time* indicates, it does so parallel to testing using fluorescent labels and not, as in usual assays, in the end. There are two different labeling methods, dyes of the SYBR family with lower and the use of TaqMan probes with high specificity. SYBR dyes may also stain single stranded DNA or even RNA to a lesser extent, which causes lack of specificity. In contrast to that the TaqMan probes will only emit light in case of actual amplification of double stranded DNA, which implies a substantially higher specificity. Due to its quantitative character it is often referred to as qPCR. RT-qPCR differs from qPCR in terms of target. In contrast to qPCR, which, as mentioned above, amplifies DNA, RT-qPCR amplifies expressed genes via cDNA reversely transcribed from its RNA (ribonucleic acid) using the enzyme reverse transcriptase (RNA-dependent DNA polymerase). The cDNA transcribed in this way can now be cloned by means of traditional qPCR. This method allows the qualitative evaluation of gene expression as well as the quantitative measurement of the respective genes (203).

Thus, in preparation for real-time RT-PCR the isolation of RNA (ribonucleic acid) is required. RNA was isolated from frozen kidney slices by acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction. AGPC disrupts cells and their various organelles to liberate RNA (204).

Technique

Shortly before working with tissue or RNA, the workspace as well as all working materials were thoroughly cleaned from omnipresent RNase using RNase Away spray (Molecular BioProducts, Inc., San Diego, CA). The tissue was homogenized crudely first by using a scalpel and then, after being mixed with 800µL TRI Reagent (AGPC; Sigma-Aldrich, Inc., St. Louis, MO), by resuspending the solution with a syringe. Afterwards additional 200µL TRI Reagent and 200µL Chloroform (Sigma-Aldrich, Inc., St. Louis, MO) were added. Now the suspension was mixed for 15sec and then incubated for 15min at room temperature. The suspension was then centrifuged at 4°C and 12000g. The supernatant containing the isolated RNA was transferred into a 1,5mL tube. By adding 500µL of isopropyl, mixing and subsequent incubation for 8min,

the RNA precipitated and centrifuged again for 15min at 4°C and 12000g for pelleting. After discarding the supernatant by aspiration, this pellet was washed by using 500µL 70% ethanol, then centrifuged 5min at 7000g and had the supernatant discarded. This washing procedure was repeated twice. Following the second washing procedure the pellet was air-dried. The then whiteish pellet was dissolved in 20µL of distilled water and heated up to 56°C for 3min. The RNA solution was put on ice immediately afterwards. RNA-concentration was measured using NanoDrop Onec (Thermo Fisher Scientific; Life Technologies Corporation, Frederick, MD).

As intermediate step it is necessary to transcribe the RNA in cDNA by means of RT using SuperScript™ III Reverse Transcriptase kit (Invitrogen by Thermo Fisher Scientific; Life Technologies Corporation, Frederick, MD). Therefore, first of all a mastermix of 0,5µL random primer (Roche Molecular Systems, Inc., Pleasanton, CA) and 1µL of 10mM deoxyribose nucleoside triphosphate (dNTP, Invitrogen by Thermo Fisher Scientific; Life Technologies Corporation, Frederick, MD) per sample was prepared and mixed with 2µg of respective RNA (total volume: 11,5µL RNA + 1,5µL mastermix = 13µL). For incubation purposes the suspension was heated up to 65°C for 5min using a thermocycler (Primus 96 plus, MWG Biotech AG, Ebersberg, Germany), and subsequently put on ice for at least 1min. A second mastermix was prepared consisting of 4µL 5x first-strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2), 1µL Dithiothreitol (DTT), 1µL RNase-inhibitor diluted in a 1:4 ratio (Invitrogen by Thermo Fisher Scientific; Life Technologies Corporation, Frederick, MD) and 1µL RT III (Invitrogen by Thermo Fisher Scientific; Life Technologies Corporation, Frederick, MD) at 200U/µL. 7µL of second mastermix were added to each sample and incubated in the thermocycler in the following (5min at 25°C, 45min at 50°C, 15min at 70°C). Right after that, 150µL distilled water was added to each sample, which were then stowed away at -25°C.

The actual real-time PCR was performed on 96-well PCR plates (Bio-Rad Laboratories GmbH, Vienna, Austria) using a Cf.X96 Real-Time PCR-System (Bio-Rad Laboratories GmbH, Vienna, Austria). iQ SYBR Green Supermix 1,25mL (Bio-Rad Laboratories GmbH, Munic, Germany) was used with the housekeeping gene HPRT (HPRT forward Primer: 5´-GCT TCC TCC TCA GAC CGC TTT TTG C´-3; HPRT reverse Primer: 5´ ATC GCT AAT CAC GAC GCT GGG ACT G-´3). Additionally the following Taqman probes (Applied Biosystems part of Thermo

Fisher Scientific; Life Technologies Corporation, Frederick, MD) :

- **IL17a**: Mm00439619_m1; Lot: 1444051
- **MPO**: Mm00484683_m1; Lot: 602965
- **INFj**: Mm00801778_m1; Lot: P160920_001 C04
- **IL6**: Mm00446190_m1; Lot: P160920_001 C05
- **TNFa**: Mm00443258_m1; Lot: P160920_001 C06
- **Gata3**: Mm00484683_m1; Lot: P160920_001 C03
- **RORjt**: Mm01261022_m1; Lot: P160920_001 C02

were used with the TaqMan Universal PCR Master Mix (Applied Biosystems part of Thermo Fisher Scientific; Life Technologies Corporation, Frederick, MD).

2.3.3 Statistics

Data were analyzed and presented using GraphPad Prism 7 (version 7.0c, GraphPad Software, Inc., La Jolla, CA), except for data from experiments that were performed on different days that were analyzed by mixed model analysis with genotype as the fixed and day of experimentation as the random variable (JMP 13.2, SAS Institute GmbH, Heidelberg, Germany). Firstly, outliers were identified by means of robust regression and outlier removal (ROUT) (205). The statistical analysis was based on the null hypothesis, that the outcome of GLP-1R KO mice or WT mice with liraglutide treatment are not different from their respective controls. Therefore the direction of the hypothesis was known, justifying unilateral testing (i.e. one tailed t-test)(206). The residuals of the t-test were tested for normality using the D'Agostino & Pearson normality test at $p<0.01$ (207). If normality was accepted, a t-test was performed, otherwise the non-parametric Mann-Whitney test was used. Additionally, an F-test was conducted to check for heteroscedasticity; in case of unequal variances ($p<0.01$) the t-test was performed with Welch's correction. $P<0.05$ was considered as significant. Power and sample size were calculated using *www.powerandsamplesize.com* (208), a web application based on *R*, an established software for statistical analysis. For data from histological evaluations, as for all data obtained by counting, a Poisson distribution must be assumed. In this case, a parametric t-test may not be performed, which is why a non-parametric Mann-Whitney U-test was used (206, 209) .

3 Results

Of the four test-series performed, three experiments were performed in with GLP-1R -/- mice (n=11) versus wild-type mice (n=10) and one experiment was performed in wild-type mice treated with liraglutide (n=5) versus vehicle (n=4). The difference in the group size of the respective setups was due to breeding circumstances. The setup and protocol used has been the same in all four runs, which allowed us to combine the data from the three sessions conducted in GLP-1R deficient mice, i.e. the same conditions, taking the day factor into account as a random variable in a mixed model analysis and to evaluate them as a whole.

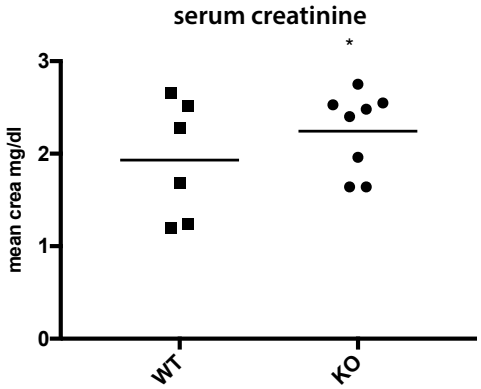

Fig. 11A GLP-1R knockout does aggravate serum creatinine levels in renal IRI
*Serum creatinie levels in wild-type mice (black squares n=6) and GLP1- R -/- mice (black dots, n=8) were analysed after mice were subjected to 30min of ischemia followed by 24h of reperfusion. Shown are the individual data points and the mean (black line). *p<0,05 vs wild-type*

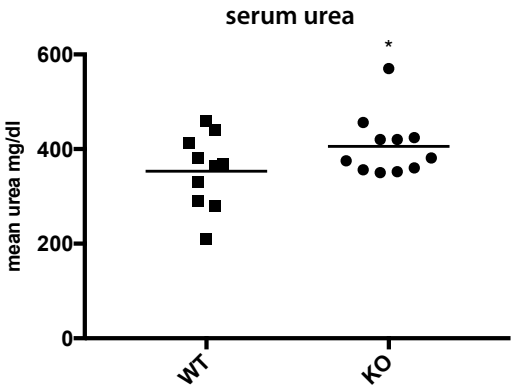

Fig. 11B GLP-1R knockout does aggravate serum urea levels in renal IRI
*Serum urea levels in wild-type mice (black squares, n=10) and GLP1- R -/- mice (black dots, n=11) were analysed after mice were subjected to 30min of ischemia followed by 24h of reperfusion. Shown are the individual data points and the mean (black line). *p<0,05 vs wild-type*

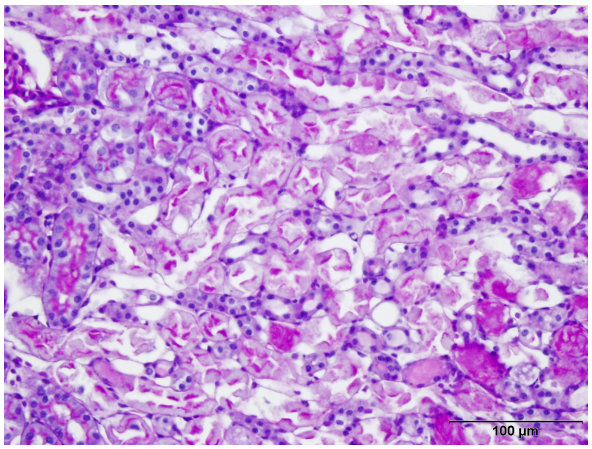

Fig. 12A (wild-type mice)

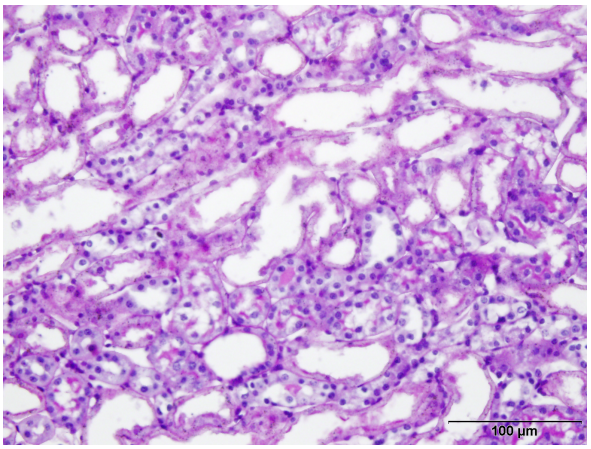

Fig. 12B (GLP-1R -/-)

Fig. 12 HE stain after wild-type mice (1A) and GLP-1R -/- (1B) were subjected to 30 min of ischmemia followed by 24h of reperfusion

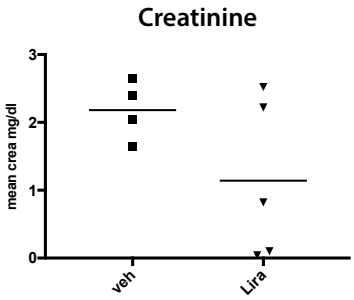

Fig. 13A an effect of treatment with liraglutide on serum creatinine levels in renal IRI could not be shown
Serum creatinine levels in wild-type mice with liraglutide treatment (black triangels, n=5) and vehicle (black dots, n=4) were analysed after mice were subjected to 30min of ischemia followed by 24h of reperfusion. Shown are the individual data points and the mean (black line).

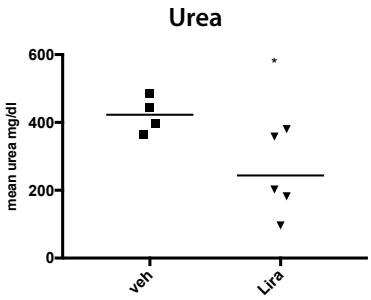

Fig. 13B Treatment with liraglutide ameliorates serum urea levels in renal IRI
*Serum urea levels in wild-type mice with liraglutide treatment (black triangels, n=5) and vehicle (black dots, n=4) were analysed after mice were subjected to 30min of ischemia followed by 24h of reperfusion. Shown are the individual data points and the mean (black line). *p<0,05 vs vehicle*

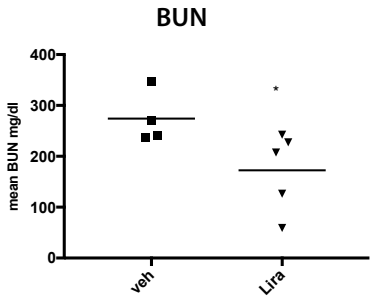

Fig. 13C Treatment with liraglutide ameliorates blood urea nitrogen levels in renal IRI
*Blood urea nitrogen levels in wild-type mice with liraglutide treatment (black triangels, n=5) and vehicle (black dots, n=4) were analysed after mice were subjected to 30min of ischemia followed by 24h of reperfusion. Shown are the individual data points and the mean (black line). *p<0,05 vs. vehicle*

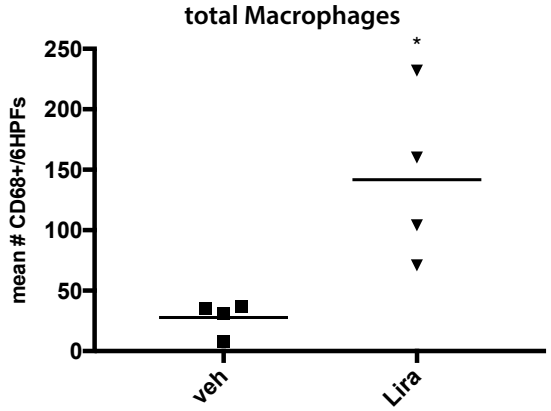

Fig. 14A Treatment with liraglutide leads to higher amount of total Macrophages in renal IRI
*Macrophages in wild-type mice with liraglutide treatment (black triangels, n=4) and vehicle (black dots, n=4) were analysed after mice were subjected to 30min of ischemia followed by 24h of reperfusion. Shown are the individual data points and the mean (black line). *p<0,05 vs. vehicle*

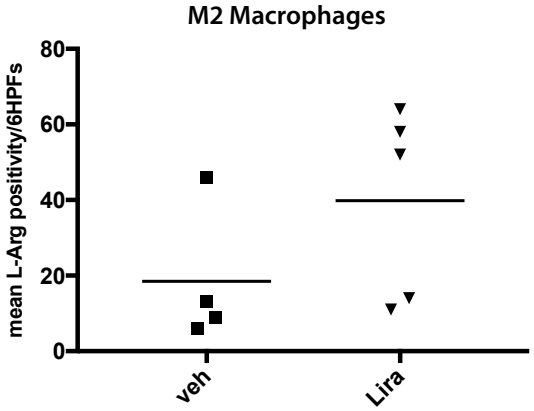

Fig. 14B Treatment with liraglutide did not show significantly higher amount of M2-Macrophages in renal IRI
M2-Macrophages in wild-type mice with liraglutide treatment (black triangels, n=4) and vehicle (black dots, n=4) were analysed after mice were subjected to 30min of ischemia followed by 24h of reperfusion. Shown are the individual data points and the mean (black line).

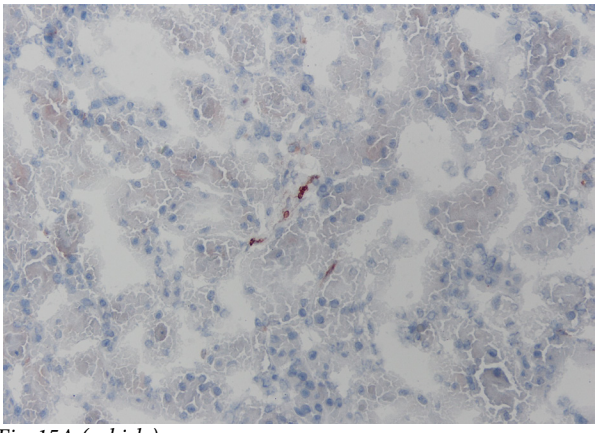

Fig. 15A (vehicle)

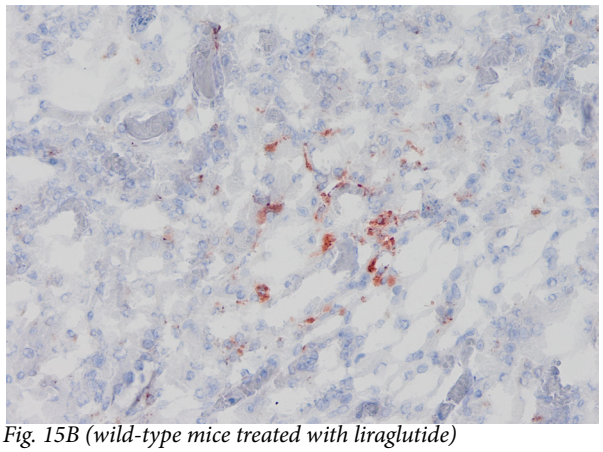

Fig. 15B (wild-type mice treated with liraglutide)

Fig. 15 Macrophages (CD68+) in kidney tissue after subjecting vehicle and wild-type mice treated with liraglutide to 30 min of ischemia followed by 24h of reperfusion.

3.1 GLP-1R KO present with an aggravated Phenotype when subjectd to renal IRI

Kidney injury after subjection to 30 minutes of ischemia followed by 24h of repersfusion was assessed by three parameters: urea, creatinine and PAS staining evaluated using a semiquantitative scale (cf. Material and Methods).

GLP-1R KO exhibit significantly higher retention of urinary excreted substances

The assessment of serum creatinie levels of the GLP-1R -/- and the wild-type mice, showed a significantly higher value in GLP-1R -/-mice ($p = 0.0473$, Fig 11A) after 24h of reperfusion. The same applies to serum urea values, where GLP-1R -/- mice presented significantly higher values ($p = 0.043$, Fig 11B) as well. From this, we conclude that renal IRI results in a more severe impairment of renal function in GLP-1R -/- mice than in wild-types.

Histological changes in GLP-1R KO and wild-type mice after 24h of reperfusion

In histological evaluation both the wild-types and the GLP-1R -/- mice exhibited clear structural damage (cast formation, loss of brush border membranes, sloughing of tubular epithelial cells, and dilation of tubules) as can be seen in figure 12. Even if at first glance there seems to be a clear difference, the semi-quantitative analysis showed no significant difference (Fig. 12).

3.2 Liraglutide treatment improves renal IRI

Here the kidney injury was also assessed by measuring serum creatinine as well as urea levels, but the evaluation was supplemented by a BUN-assay. Similar to the examination of the GLP-1R results a histological assessment by means of PAS staining and IHC was conducted (cf. Materials and Methods).

Liraglutide ameliorates serum urea and BUN levels

The wild-type mice that were treated with liraglutide prior to subjection to renal IRI, showed significantly decreased serum urea ($p = 0.0149$; Fig 13B) and BUN levels ($p = 0.03$; Fig 13C), in comparison to vehicle. Serum creatinine values showed only a non-significant trend towards benefits for the liraglutide group ($p = 0.1481$, Fig. 13A).

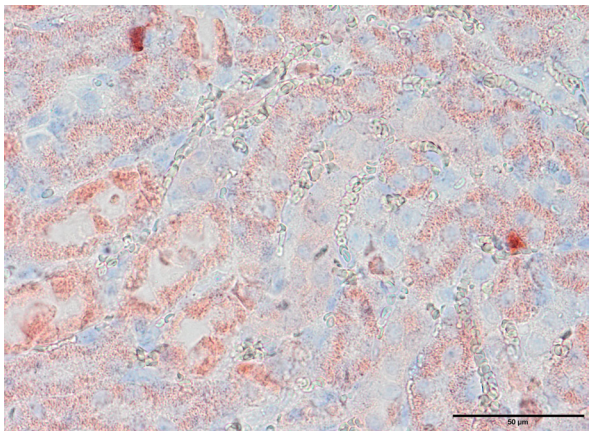

Fig. 16A (vehicle)

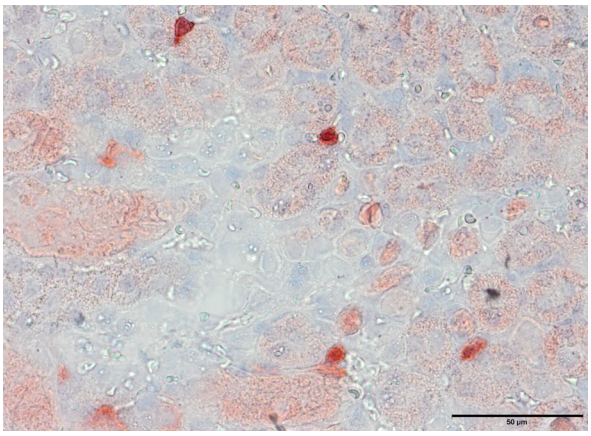

Fig. 16B (wild-type mice treated with liraglutide)

Fig. 16 M2/repair-Type Macrophages (L-Arg+) in kidney tissue after subjecting vehicle and wild-type mice treated-with liraglutide to 30 min of ischemia followed by 24h of reperfusion

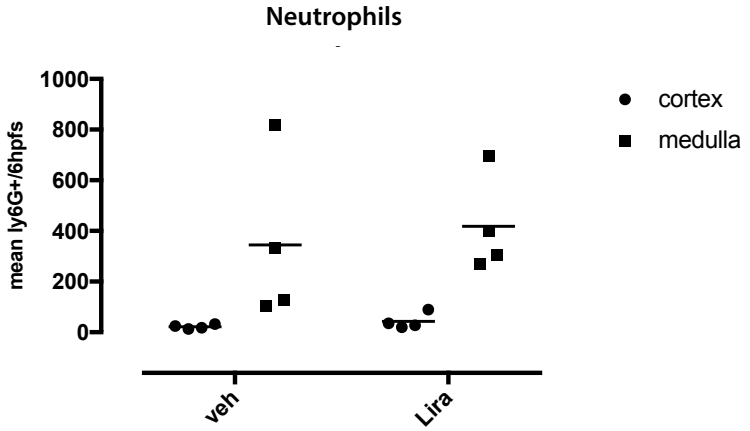

Fig. 17 Treatment with liraglutide does not effect a difference in neutrophils in renal IRI
Neutrophils in wild-type mice with liraglutide treatment (right, n=4) and vehicle (left, n=4) were analysed after mice were subjected to 30min of ischemia followed by 24h of reperfusion. Shown are the individual data points and the mean (black line).

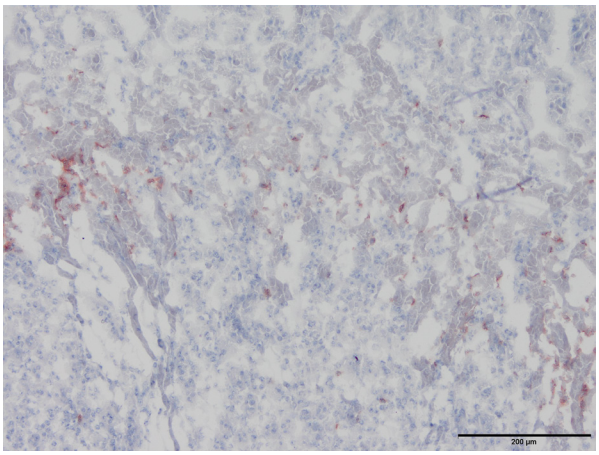

Fig. 18A (vehicle)

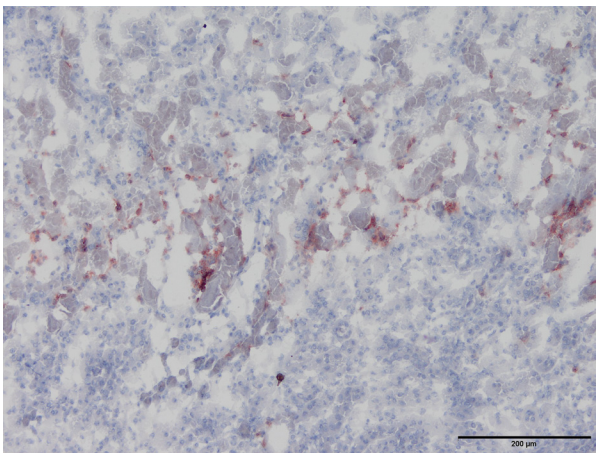

Fig. 18B (wild-type mice treated with liraglutide)

Fig. 18 Neutrtrophils (Ly6G+) stained in kidney tissue after subjecting vehicle (3A) and wild-type mice treated with liraglutide (3B) to 30min of ischemia followed by 24h of reperfusion

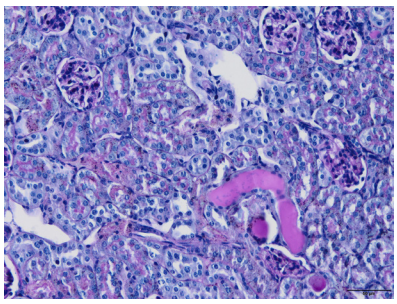

Fig. 19A (wild-type)

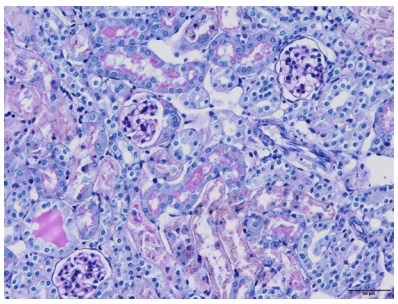

Fig. 19B (GLP-1R -/-)

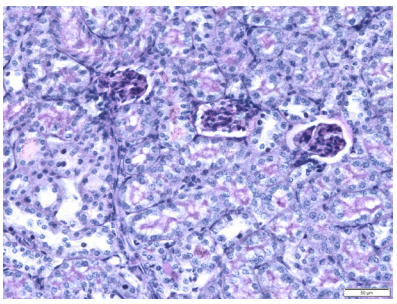

Fig. 19C (wild-type mice treated with liraglutide

Fig 19 PAS stain of cortical kidney tissue after subjecting wild-type mice (A) and GLP-1R -/- (B) and wild-type mice treated with liraglutide (C) to 30 min of ischemia followed by 24h of reperfusion

Immunohistological changes in wild type mice treated with liraglutide and vehicle after 24h of reperfusion

In addition, the number of macrophages in the kidney tissue was evaluated by means of an immune histological staining for CD68+ cells (Fig. 15; cf. Material and Methods – IHC). This analysis showed a significantly higher number of overall macrophages in the group treated with liraglutide ($p = 0.0286$, Fig. 14). Subsequently, an L-Arg stain was performed to identify the proportion of M2/repair type macrophages. As a result of this stain, only a trend could be seen, to the effect that the animals previously treated with liraglutide tended to develop an M2 phenotype ($p = 0.1111$, Fig 16). The harvested kidneys were also tested regarding their number of neutrophils using a Ly6G stain (Fig. 17 and 18; cf. Materials and Methods – IHC). The medullary and cortical neutrophils were evaluated separately, but did not show any difference between the animals treated with liraglutide and vehicle (cortical neutrophils $p =0.2$; medullary neutrophils $p = 0.6857$, Fig 16).

Histological changes in mice treated with liraglutide and vehicle after 24h of reperfusion

The histological findings in the PAS stained samples confirm that there was a structural damage in the kidney for both groups (cast formation, loss of brush border membranes, sloughing of tubular epithelial cells, and dilation of tubules), but no difference could be determined by semi-quantitative analysis (Fig. 19).

Chemokine gene expression in kidney tissue

A PCR was performed testing for gene expression of the macrophage relevant chemokines TNF-α , IL-6, RORC, Gata-3, IL-17, IFN-γ, MPO, CCL2, CCR2, CCR5, CCL4, CCL5, CCL17, CCL22 and IL4. Here we did not see any significant difference in the expression patterns between the two groups (Fig. 20).

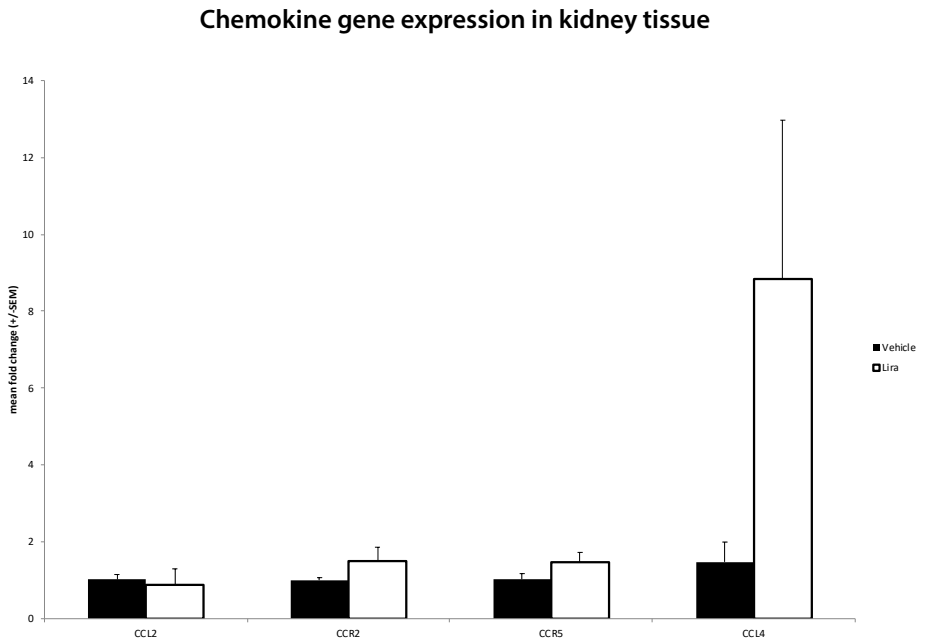

Fig. 20 Treatment with liraglutide does not effect a groupdifference in chemokine expression pattern in renal IRI
Chemokine gene expression levels of wild-type mice with liraglutide-treatment (white) and vehicle (black) after 30 min of ischmemia followed by 24h of reperfusion.

4 Discussion

The results of this study suggest a protective effect of GLP-1R activation in renal IRI. There was a significant increase in the retention parameters (Fig. 11) urea and creatinine in GLP-1R deficient mice, on the other hand there was an alleviation of renal IRI when the GLP-1 receptor was activated (Fig. 13A). Unfortunately, this improvement in outcomes was not directly reflected in the histology, although a significantly higher number of macrophages was detected in mice treated with liraglutide (Fig. 14, Fig. 19). The following L-Arg stain showed a non-significant trend towards the benefit of the M2/repair type, which suggests that GLP-1 receptor agonism might promote an anti-inflammatory M2/repair type (Fig. 14, Fig. 15).

4.1 Is the outcome of GLP-1R deficient mice worse than their respective control?

GLP-1R deficient mice showed a significant increase in retention parameters compared to their respective control (Fig. 11). Although histology did not directly reflect these results, we definitely saw a deterioration in kidney function, if GLP-1R is not expressed. This observation further supports the initial hypothesis, that the GLP-1R has a protective effect in renal IRI and moreover in AKI.

4.2 Does administration of liraglutide improve the outcome of mice subjected to renal IRI?

The mice treated with liraglutide prior to subjection to renal IRI showed a significantly improved outcome than their respective controls. A preserved kidney function marked by lower increase in the retention parameters, i.e. serum urea and BUN, was observed (Fig. 13). Thus, we conclude, that GLP-1R activation exhibits a nephroprotective effect in renal IRI. As already mentioned, we did not see a significant difference in terms of tissue damage, but we saw a significantly higher proportion of macrophages in the kidneys of mice pretreated with liraglutide.

Macrophage infiltration versus activation/induction

To this point it is not entirely clear, whether the macrophages are infiltrating the kidney tissue or if it is a matter of activation or enhanced proliferation of tissue-resident macrophages. As

mentioned above, recent studies have provided evidence, that the majority of tissue macrophages is independent of immigration from the bloodstream (146-148). Despite these new insights, it is nevertheless not unlikely that the additional macrophages in the mice treated with liraglutide are due to infiltration. As Persy *et al.* were able to demonstrate, a suppression of macrophage recruitment leads to a decrease of reparative processes (210). Furthermore, several studies provide evidence that a blockade of the monocyte adhesion cascade, especially E- and P-selectin as well as a knockout of the Intercellular Adhesion Molecule 1 (ICAM-1) results in reduced macrophage infiltration and therefore attenuated renal IRI (211-214). These findings support the thesis that the immigrated macrophages play a more important role in the renal IRI than the tissue resident macrophages. In contrast, the fact that we found more macrophages in the liraglutide group in total, while CCL2 expression, that is central for the recruitment of macrophages, did not differ between the groups (Fig. 20) supports a crtitical role of resident macrophages in our study (215).

M1 versus M2

Although we could not show a significantly higher proportion of M2/repair type Macrophages after IRI in mice treated with liraglutide, a trend was discernible (Fig 14). It is likely that the lack of significance here is due to the small size of the group. However, as macrophages express the GLP-1R and because Shiraishi *et al.* already showed that GLP-1 induces signal transducer and activator of transcription 3 (STAT 3), which in turn is known to play a vital role in induction of M2/repair type macrophages and because they further showed that macrophages after administration of GLP-1 exhibited increased levels of the M2/repair type marker IL-10, we consider it very likely that GLP-1R activation by liraglutide could actually lead to an M2 phenotype in renal IRI (216). Since several studies have already proven a protective and potentially therapeutic effect of M2 activation, it seems very likely that the M2/repair type macrophage pathway is part of the mechanism underlying the published reno-protective effect after GLP-1R activation (217, 218).

Role of neutrophils

The examination of cortical and medullary neutrophil granulocytes after subjection to renal

IRI showed no difference between mice treated with liraglutide and vehicle. However, since neutrophils definitely contribute to the genesis of IRI, it can be concluded that the mechanism by which liraglutide acts nephroprotectively in renal IRI is probably independent of neutrophil granulocytes (131).

Role of GLP-1R cleavage products

Besides the above-mentioned possible induction of M2/repair type macrophages, a recent study of Moellmann *et al.* has proven a reno-protective effect of GLP-1 cleavage products in diabetic mice (170). In their study, treatment with GLP-1(7-36)amide , GLP-1(9-36)amide and GLP-1(28-36)amide in a model of unilateral renal ischemia led to the reduced infiltration of CD4+ and CD8+ T-cells. Their study design did also incorporate liraglutide administered as a single shot 12h prior to surgery, which however did not fully reproduce the effect that was observed after administration of GLP-1. To explain this merely partial immunomodulatory effect, the authors argued that the metabolites are responsible for the immunomodulatory effects and thus that the slower metabolism of liraglutide delayed their availability; it was assumed that 12h were not sufficient to achieve the necessary concentration of active metabolites (219). Since in the present work the animals received daily doses of 200µg/kgBW liraglutide already 7 days before surgery, it was expected, that the concentration of active metabolites was significantly higher if not approximately equal to that of GLP-1 degradation, and over a much longer period of time. However, as already mentioned, we observed a very high variance in our data, which could possibly also be attributed to inter-individual differences in liraglutide metabolism and thus the available cleavage products. One limitation of our study is that we did not consider measuring the cleavage products in our study design.

On the other hand, we observed a significant deterioration in kidney function in GLP-1R deficient mice (Fig 11), which suggests that the reno-protective effect is at least partially mediated via GLP-1R directly. Since it might also be possible, that these cleavage products mediate their effect via receptors other than GLP-1R, it might be useful, as a complement to the present data, to administer liraglutide to GLP-1R -/- mice in comparison to wild-type mice before subjecting them to renal IRI. This might reveal whether the beneficial effect in our study is related exclusively

to the GLP-1R (cf. Material and Methods – ischemia reperfusion).

The creatinine assay was not significant in the liraglutide-group

In contrast to serum urea and BUN analysis the evaluation of serum creatinine levels did not show a significant difference between mice with liraglutide treatment and vehicle. A possible explanation might be the relatively low number of mice used in the liraglutide experiments. To test this hypothesis, a calculation of power and sample size was performed. It turned out, that with the given variance a group size of 33 animals per group would have been needed to achieve a significant result, in line with that it was also found that the statistical analysis of the serum creatine values only had a power of .34. For a meaningful test design, it is recommended to work with a power of at least 0.8, therefore our analysis was underpowered. As a conclusion, another run using the same setup, but a higher n-number would be useful to achieve higher power and to be able to prove the assumed difference statistically, however a total of 66 animals may not be justified. Furthermore, the data of the liraglutide group generally showed a very high scattering (Fig. 13), which could have led to a non-significant result of the creatinine assay, despite the significantly increased values in the urea and BUN analysis (Fig. 13). The fact, that the results of all assays performed with data obtained from the liraglutide-experiments are highly scattered, makes it very unlikely that the procedure of creatine analysis itself had a negative effect on the results. A study which examined cardioprotective effects of liraglutide demonstrated that, given at 200µg/kgBW twice daily, it also effects weight loss in mice. Liraglutide was also tested by Noyan-Ashraf *et al.* in a dose of 75µg/kg, with the result that the observed protective effects were maintained but no weight loss occurred (220). Therefore, a possible explanation for the observed scattering in liraglutide data may be, that the dose may have been chosen too high, and that the side effects occurring at higher doses may have affected the outcome.

4.3 Is the administration of liraglutide in renal IRI clinically relevant?

As already mentioned, AKI is suspected to be a relevant cause of death and not just a symptom of an underlying disease, especially when it comes to intensive care (5). Since different large-scale clinical trials such as LEADER, ELIXA and SUSTAIN 6 have all demonstrated a nephroprotective

effect for the administration of GLP-1R agonists, it is safe to say that the results are highly relevant for further clinical research (224, 226, 228). So far, however, the underlying mechanisms for the shown positive effects on the renal outcome are insufficiently understood. Nevertheless, with this work we were able to contribute to a further understanding of the nephroprotective effect of GLP-1R agonism. The anti-inflammatory effect by the conversion of M1/kill-type to M2/repair-type macrophages after liraglutide administration may explain the improved renal outcome in studies like LEADER, ELIXA or SUSTAIN 6 at least partially. Since we know that liraglutide influences ROS, a factor also decisive in renal IRI, it is necessary to investigate the development of ROS after subjection to renal IRI in mice treated with liraglutide in a further study (155). This may help to fully elucidate the nephroprotective potential of GLP-1R agonism. Altogether, liraglutide therapy could be a promising approach to improve the outcome of patients suffering from AKI. Whether it can actually contribute to an improvement in mortality and morbidity has yet to be confirmed in further clinical trials.

In summary, the absence of the GLP-1R aggravated the impairment of kidney functions in renal IRI, as well as the activation of the GLP-1R by liraglutide ameliorated the AKI and preserved kidney functions. Therefore, we conclude that liraglutide has a reno-protective effect in mice subjected to renal IRI. Not only does this further elucidate the underlying mechanism of the improved renal outcome that was seen in large-scale clinical trials, but also reveals the potential that liraglutide might have for the treatment of AKI.

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