Aus der Klinik für Nieren- und Hochdruckerkrankungen

der Medizinischen Hochschule Hannover

Molecular mechanisms of surgery associated acute kidney injury (AKI) by blood degradation products in experimental mouse models

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1 Introduction

1.1 Acute kidney injury (AKI)

Acute kidney injury (AKI) formerly known as “acute renal failure” has been traditionally described as a rapid decline in kidney function ranging from several hours to several weeks. Kidney function normally is measured by an increase of serum creatinine and blood urea nitrogen (BUN). The Acute Kidney Injury Network (AKIN) defined it more accurately as “an abrupt decline in kidney function”[1] (within 48 hours) and provided laboratory and clinical values to guide clinical diagnosis. AKI is classified into three categories according to AKIN, depending on increase of serum creatinine, decrease of the glomerular filtration rate (GFR)[1] or reduction of urine output. From a clinical perspective, AKI is categorized into prerenal, intrinsic and post-renal depending on its primary cause. Prerenal AKI can be caused by the decline of the renal blood flow due to low blood pressure, hypovolemia or peripheral vasodilatation with systemic hypotension. In these cases, kidney tissue is not permanently affected if treated timely and the elimination of the cause can lead to restoration of normal kidney function[2]. Intrinsic AKI is caused by diseases of kidney itself such as interstitial nephritis, glomerulonephritis or by nephrotoxic medication (i.e. analgetics, aminoglycoside antibiotics or contrast media). Five percent of AKI have post-renal causes such as urinary tract obstruction because of prostatic hyperplasia, posterior urethral valves or urolithiasis. By early detection and early treatment, the irreversible damage of the kidney can be avoided[2]. The long-term effects of AKI on patients is a high risk to develop chronic kidney disease (CKD), and even end-stage renal disease (ESRD)[3]. The incidence of AKI is associated with increased morbidity and mortality, especially with patients on Intensive Care Units (ICU)[4]. In hospitalized patients AKI is associated with high mortality which can reach 30 to 70%[5].

Furthermore, there is a high incidence of AKI after major surgeries, especially after solid organ transplantation, ranging from 5%-50% for kidney transplantation (depending on cold ischemia time and causing delayed graft function[6]), 50-60% for lung transplantation and 40-70% for liver transplantation[7, 8]. Mortality due to AKI occurs in high-income countries (HIC) as well as in low- and middle-income countries (LMIC). However, the majority of deaths due to AKI in LMIC nations (i.e. Kuwait, North India and Brazil) would be avoidable if better medical care would be available[9,10]. AKI has a severe economic burden on healthcare expenditure, especially in poor countries. The International Society of Nephrology (INS) has launched the '0 by 25' project. This program advocates zero people should die from untreated AKI by 2025 in the poorest countries of Asia, Africa and Latin America[10].

1.2 Ischemia reperfusion injury

Ischemia causes reduced oxygen supply of tissues and the following reperfusion initiates the inflammatory response[11]. IRI affects aerobic cells in tissues and organs e.g. brain, heart, kidney, liver and intestine. The energy of these cells can only be supplied by mitochondrial oxidative phosphorylation[12-20]. In solid organ transplantation IRI is a relevant cause of organ dysfunction and also restricts the long-term survival of the transplanted organs[21]. As mentioned, IRI is characterized by hypoxia which leads to free radical generation and inflammation. This causes damage of the tubular epithelial cells and decrease of renal blood flow, which induces AKI. Especially, the outer medulla is very sensitive to hypoxia due to the renal architecture with low oxygen saturation in this area (Fig.1).

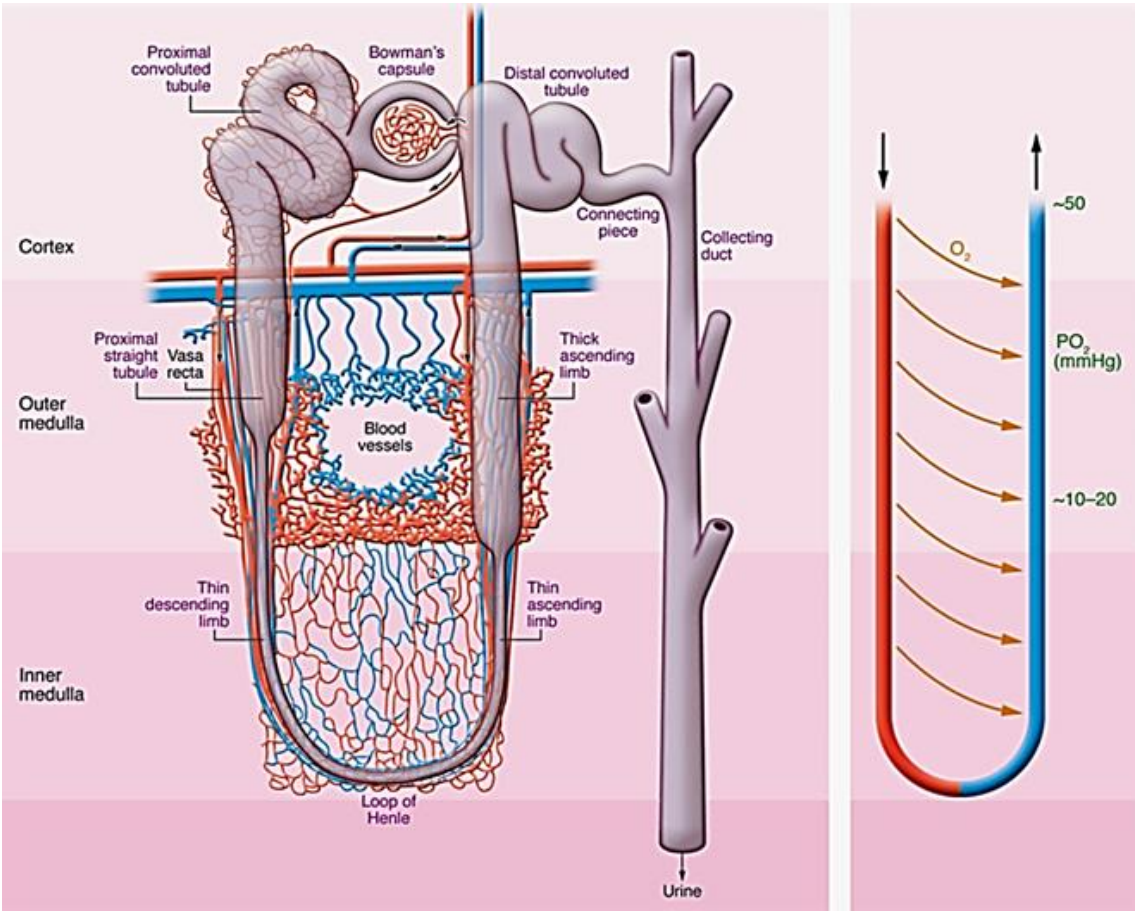

Figure 1: Renal architecture
The normal kidney is divided into the cortex, the outer medulla and the inner medulla. The oxygen supply varies in the different areas with high pO2 in the cortex (50-60 mmHg) and a decrease towards the outer medulla (10-20 mmHg; Bonventre, Yang; JCI; 2011).

IRI is a complex phenomenon including intracellular injury processes and an inflammatory response. Both of them are interconnected. In the ischemic phase, anoxic cell injury is predominant which results in activation of hydrolases and loss of selective permeability of cell membranes. After reperfusion the inflammatory response with leukocyte infiltration follows[21]. In the ischemic phase, anoxic injury starts with the decrease of ATP content followed by a decrease of the cytosolic pH and acidification. This disturbs cellular ion homeostasis, activates hydrolases, and increases the permeability of cellular membranes. All of these alterations interact in a network structure. The cold storage (0° to 4°C) used for allografts during transportation, decreases oxygen consumption of the cells and therefore the rate of anoxic injury can be reduced[21]. Following ischemia the phase of an inflammatory

response starts after reperfusion. Due to the sequence of anoxia and reoxygenation, cells especially endothelial cells and macrophages are intrinsically activated contributing to the inflammatory response. The endothelial cells have many ways to contribute to the pathology of ischemic AKI[25, 26]. Increased expression of intercellular adhesion molecule 1 (ICAM-1) which originates from damaged endothelial cells binds to integrin complexes on the surface of circulating leukocytes[27]. This causes a series of inflammatory responses, including the activation of leukocytes, vasoconstriction and obstruction of capillaries and production of pro-inflammatory cytokines (Fig.2B).

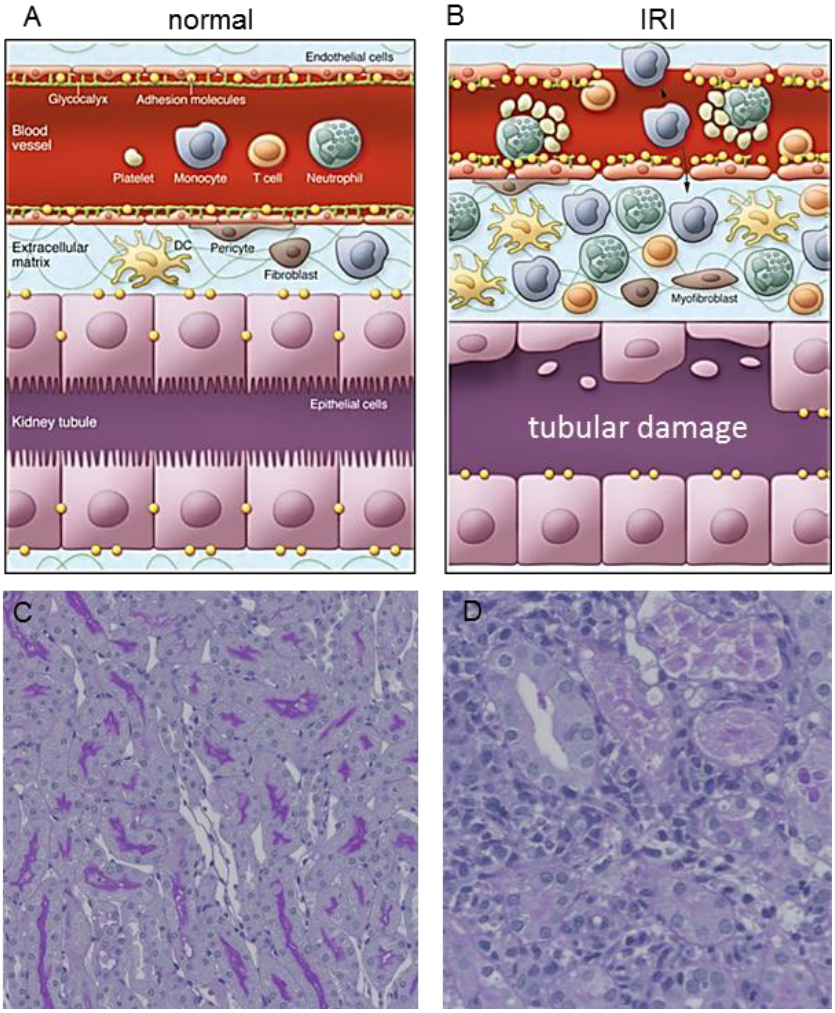

Figure 2: Endothelial injury in IR-induced AKI
In the healthy kidney (A) proximal tubular epithelial cells are stained with the prominent pink brush border membrane in PAS (C). IRI causes an inflammatory response (B, D) with loss of the brush border flattening of the tubular epithelial cells and leukocyte infiltration (A and B from Bonventre, Yang; JCI; 2011)

The injury of endothelium causes the loss of the glycocalyx, disruption of the actin cytoskeleton and the endothelial cell-cell contacts, with the result of increased microvascular permeability and fluid loss into the interstitial space[25, 26]. The loss of the endothelial barrier function has been documented within two hours of reperfusion in the cortex by two-photon microscopy in a rodent model of AKI[28]. In this phase, macrophages, neutrophils, lymphocytes, natural killer (NK) cells, dendritic cells (DCs), as well as non-cellular elements, including the blood coagulation cascade, the complement system, nitric oxide, reactive oxygen species (ROS), and many inflammatory cytokines may be involved[29] (Fig.3 A).

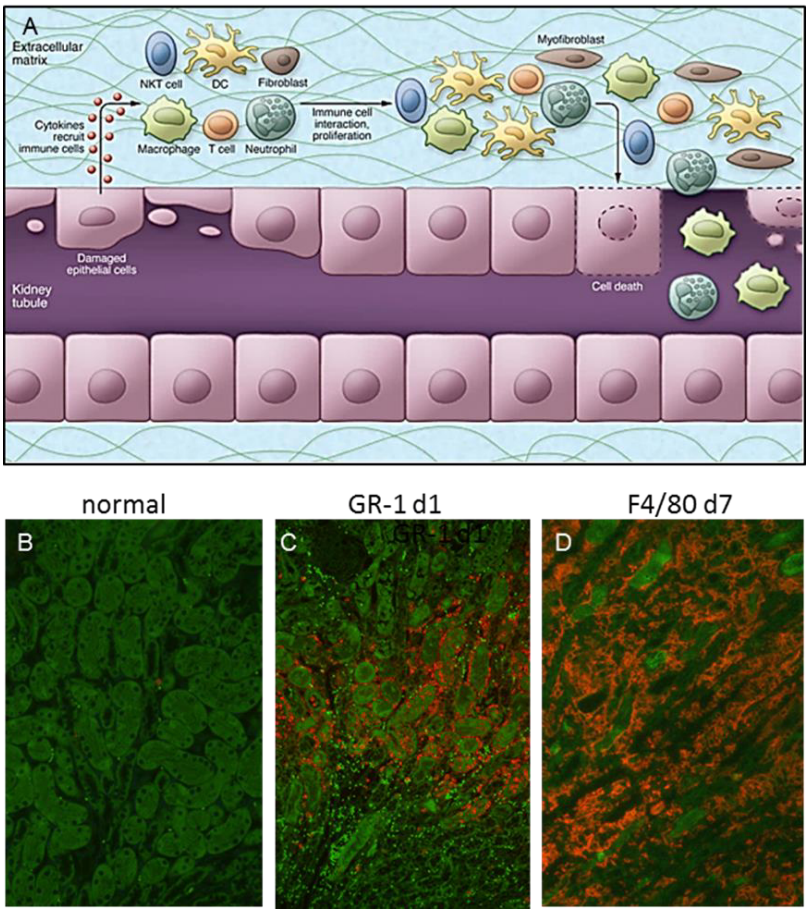

Figure 3: The inflammatory response in IRI
After IRI, tubular epithelial cells are injured and leukocytes are activated and transmigrate (A, (Bonventre, Yang; JCI; 2011). Inflammation after IRI is characterized by invasion of Gr-1+ neutrophils (C) within 3-4 hours till day 1. Later they are replaced by F4/80+ macrophages (D). (Magnification 200x, green: auto-fluorescence of intact tubuli, red: Gr-1+ neutrophils or F4/80+ macrophages).

Both innate and adaptive immune responses also play an important role in the pathology of ischemic injury. NK cells, DCs, macrophages and neutrophils are the innate component, which mediates the early immune response in a non-antigen-specific manner (Fig. 3 C, D). The adaptive phase is activated by certain antigens and leads to DC maturation and antigen presentation, followed by T lymphocyte activation and proliferation, and also T to B lymphocyte interaction. It lasts several hours and even over the course of several days after injury[28].

The complement system is another critical contributor to inflammation after IRI. The complement activation occurs very early after renal IRI[29] and upregulates the expression of endothelial cell adhesion molecules[30]. Furthermore, the activation of various signaling pathways, including c-Jun N-terminal kinase (JNK) or nuclear factor-κB (NF-κB), and both the death receptor signaling pathway and the mitochondrial apoptotic pathway are all involved in ischemia reperfusion injury[21].

Histopathological signs of tubular damage in the kidney are various and are characterized by a loss of brush border of the proximal tubuli, flattening, vacuolization and detachment of the epithelial cells, tubular dilatation and the loss of the tubular nuclei and tubular obstruction by protein casts. Furthermore, tubular necrosis and the apoptosis along the nephron and leukocyte infiltration are representative signs for AKI.

1.3 Transfusion of packed Red Blood Cell (pRBC)

The British obstetrician James Blundell was the first who carried out a successful blood transfusion in 1818 and he used only small volumes of fresh blood at the beginning[31]. Afterwards transfusion of packed red blood cells (pRBCs) gradually started to play an important role in clinical medicine. Transfusion can rescue many patients from life threatening situations or prevent the deterioration of a disease.

Every year millions of units of various separated blood components (red blood cells, plasma or platelets) and sometimes whole blood[32] are transfused to overcome life threatening situations.

According to the World Health Organization (WHO) statistics show that approximately 112.5 million units of whole blood are donated in the about 180 countries every year[33]. In 2012 European Union (EU) Member States reported that more than 20 million blood components and whole blood donations were collected by about 1,350 blood donation services. In addition to this, millions of donations of plasma by apheresis are used for transfusion or for producing plasma derivatives including albumin, coagulating factors and immunoglobulins as medication[34]. In the United States, approximately 4.5 million patients get about 21 million blood products every year.

Nowadays, blood transfusion is an important part of daily clinical practice[35]. Stored pRBCs are very often used in medical procedures, especially in the context of critical care[36]. The purpose of transfusion is to increase the hemoglobin levels or in case of fresh frozen plasma to substitute protein loss or promote coagulation. Administration of pRBC is also increasing the intravascular oxygen-carrier capacity and improves tissue oxygenation. Data showed that about 40% of ICU patients are transfused with a mean of five pRBC units per patient[37]. Especially, during surgery on thoracic organs or liver transplantation bleeding occurs and pRBC are frequently transfused.

Although, blood transfusion has many beneficial effects, more and more studies report that the blood products, especially pRBCs transfusions have various potential adverse effects. In a large meta-analysis on ICU patient's mortality was linked to the number of pRBC transfusion[38]. There are several controversial reports about the benefits and risks of pRBC transfusion. RBCs are currently stored for up to 50 days in Germany. To increase blood reserves and to avoid wasting of pRBC units, the

worldwide standard practice is transfusing the oldest available and compatible pRBC first. Despite, improved methods for preservation storage lesions develop over time. They are characterized by pRBC shape change, metabolic dysfunction, the loss of membrane carbohydrates, increased adhesion of RBCs to endothelial cells, proteins and lipids, changes in oxygen affinity and delivery and they cause shorting of RBCs lifespan[39]. There are classifications to distinguish between young (<14-21 days) and old (>21 days) pRBCs[40]. Previous research has shown that the toxicity of cell free hemoglobin (Hb) and heme are contributing to the storage lesions[41]. It has been published that heme toxicity originating from RBCs storage lesions seriously worsens the outcome in trauma-induced hemorrhage mouse models[42, 43].

1.4 Pathophysiology of heme

Heme is a complex of iron and the tetrapyrrole protoporphyrin IX. Under physiological heme is bound by hemoproteins and plays important roles for many biological processes, ranging from reversibly binding oxygen to transport electrons of the respiratory chain. In some pathological states such as sickle cell disease and malaria serious hemolysis and myolysis cause heme release from hemoproteins (i.e. hemoglobin (Hb) and myoglobin (Mb)). Hb and Mb contain the largest stock of bioavailable heme, and are easily oxidized and then release their prosthetic heme. Released heme is unstable can be exchangeable between low molecular weight ligands, macromolecules and receptor proteins. The unstable heme is called *labile* heme (previously *free* heme).

Excessive labile heme is cytotoxic, because it can promote oxidative stress leading to membrane injury and apoptosis. Therefore, labile heme metabolism needs to be tightly controlled in order to avoid pathological conditions. For this reason the amount of intracellular labile heme is regulated by multiple different ways: heme synthesis,

utilization by hemo-proteins, heme degradation and both intracellular and intercellular heme trafficking.

1.4.1 Physiological functions of heme

Heme is the very important functional constituent of several hemoproteins, such as hemoglobin, myoglobin and cytochromes. Heme has a multitude of relevant biological functions. In hemoglobin and myoglobin, it is relevant for oxygen transport and storage, whereas in cytochromes it participates in energy generation electron transport and chemical transformation. Heme functions in hydrogen peroxide inactivation or activation, in catalases, peroxidases and in tryptophan pyrolases respectively. Furthermore, it catalyzes the oxidation of tryptophan[44] and is essential for a wide-range of other important enzyme systems, like cyclooxygenase and nitric-oxide synthase[45].

In addition to serve as a prosthetic group in hemoproteins, heme may also effect the expression of many genes. Heme can up-regulate its metabolism and down-regulate heme biosynthesis to regulate its own production in non-erythroid cells[46]. In turn, heme plays a role as a positive feedback regulator for its synthesis and controls its degradation in erythroid cells[47,48]. Heme can regulate gene transcription, mRNA stability, synthesis of proteins and post-translational modification to modulate almost all levels of gene expression[49,50]. In addition, heme participates in regulation of the differentiation and proliferation of many cell types. It stimulates erythroid differentiation of erythro-leukemia cells[51] and neuronal differentiation of mouse neuroblastoma cells[52] and formation erythroid colonies in human and mice bone marrow cultures[53, 54]. Heme has also been linked to differentiation of 3T3 fibroblasts into adipocytes[55], and cell growth of cultured fibroblasts[56].

1.4.2 Toxicity of labile heme

There are several pathological conditions, such as malaria, sickle cell anemia, thalassemia, paroxysmal nocturnal hemoglobinuria (PNH) and rhabdomyolysis, associated with hemolysis and myolysis. This results in tissue exposure to large amounts of labile heme[57] which is also linked with inflammation, vascular dysfunction and local tissue injury (Fig.4)[58].

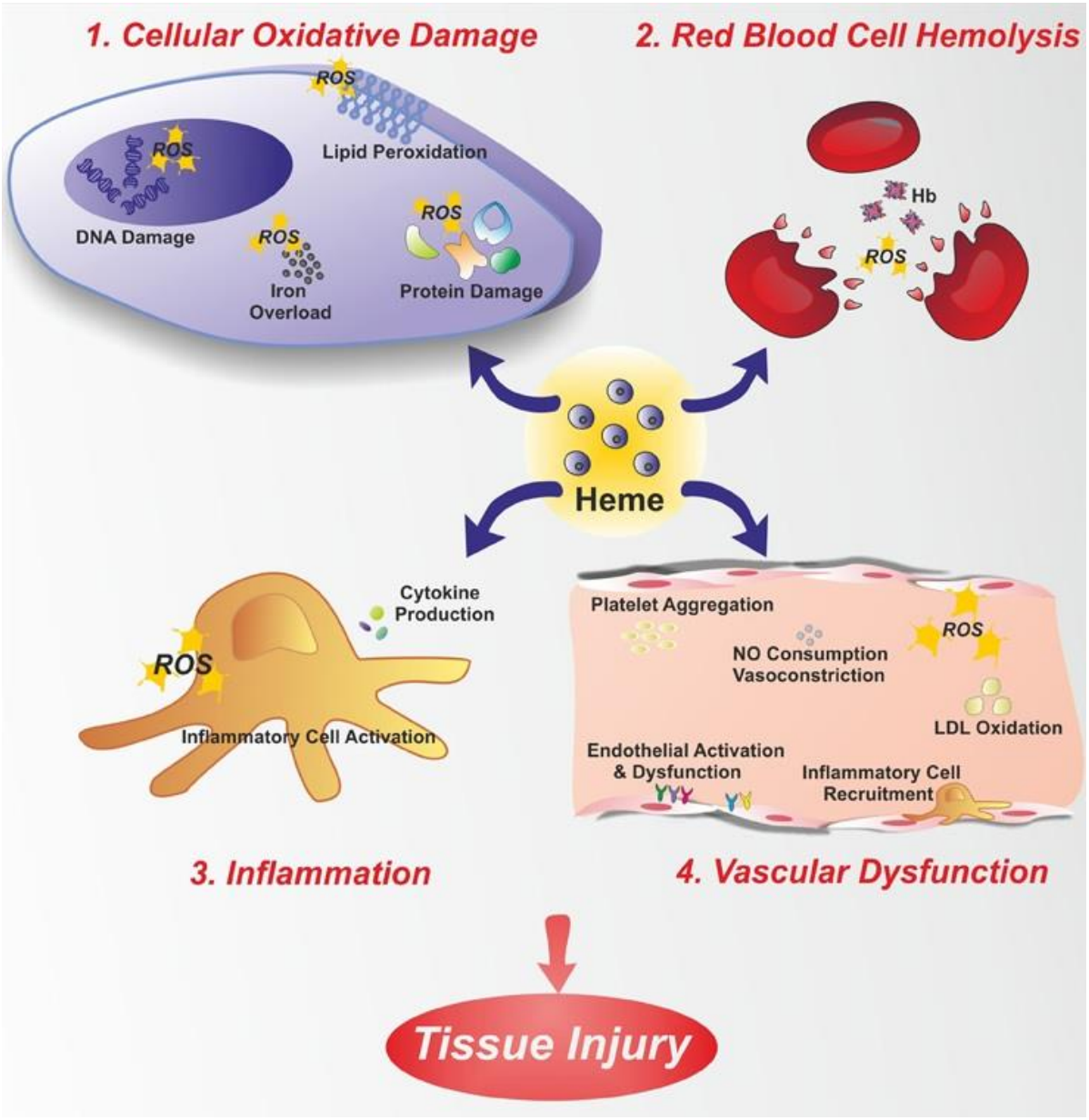

Figure 4: The effects of heme toxicity
Heme is released by necrotic cells after tissue injury and also after hemolysis and hemoglobin degradation from red blood cells. Heme promotes inflammation and vascular dysfunction causing tissue injury (Chiabrando, Vinchi et al. 2014).

Labile heme catalyzes the oxidation and participates in the Fenton reaction, which leads to the production of ROS[59] which in turn cause oxidative stress. Heme is a lipophilic molecule that intercalates in the cell membrane. ROS stimulate the formation of cytotoxic lipid peroxide and causing lipid bilayer dysfunction and destabilization of the cytoskeleton, thus promoting cell lysis and death[60]. On the genomic level labile heme further causes damage of DNA through oxidative stress[44]. Labile heme is also a potent inducer of further hemolysis. ROS enhance membrane permeability and change the conformation of cytoskeletal proteins in erythrocytes, which affects membrane stability. Storage lesions of red blood cells are directly linked with cell free Hb and labile heme generation. For a variety of inflammatory disorders such as sepsis, arteriosclerosis and peritoneal endometriosis heme induced pathomechanisms have been described[60, 61]. Labile heme promotes inflammation by activation of NF-κB (nuclear factor-κB) and MAP kinase (mitogen-activated protein kinase) transcription factors to stimulate secretion of pro-inflammatory cytokines. The pro-inflammatory property of labile heme causes endothelial cell injury, especially in the vascular endothelium and induces the recruitment of leukocytes and platelets. This results in vascular inflammation, enhanced expression of intracellular adhesion molecules and finally impaired vascular function[62].

1.4.3 Heme recycling

There are two major ways for heme recovery. One is erythro-phagocytosis which plays the principal role. Spleen macrophages, as well as liver Kupffer cells and bone marrow macrophages finally eliminate 90% of senescent erythrocytes. During aging, macrophages recognize senescent red blood cell because of changes in the erythrocyte shape and a series of biochemical modifications including membrane

fission. After recognition, the red blood cells are phagocytosed degradation[63]. In the macrophage, heme is catabolizes by heme oxygenase (HO)[64].

Besides these physiological processes, many pathologic conditions cause the release of labile heme. In these situations, erythrocytes release plenty of hemoglobin (Hb). It stimulates the formation of stable complexes with haptoglobin (Hp)[65]. Hb-Hp complexes are delivered to hepatocytes and also macrophages, and then recognised by CD163, the main scavenging receptor which then mediates endocytosis[66, 67]. After overwhelming the buffering capacity of Hp, Hb rapidly converses to met-Hb releasing labile heme which binds to albumin and hemopexin (Hx). The heme-albumin complex exists only as a short-lived deposit before the heme-Hx complex transports the heme to the liver[68]. Once the heme-Hx complexes are absorbed by hepatocytes, heme is released and used for new hemoprotein synthesis or is catabolized by HO[62]. Several studies have shown that heme alone or in the complex with albumin or Hx is a strong inducer for cytoprotective hemeoxygenase (HO) production[69-72].

2 Aims of the thesis

In this study we developed a translational mouse model to investigate the effects of free Hb or labile heme on renal ischemia reperfusion injury (IRI). This translational study mimics the clinical situation during major surgeries where pRBCs are transfused in situations with low blood pressure and decreased renal perfusion. First, we established a mouse model for subclinical AKI and studied the effect of free hemoglobin of human origin on renal IRI. Next, the degradation product labile heme was used in the renal IRI model and molecular mechanisms of labile heme induced renal damage were analyzed by qPCR, histology, immunohistochemistry, Western blotting and flow cytometry. Finally, we demonstrated that albumin was able to attenuate AKI due to labile heme release.

3 Material and Methods

3.1 Material for animal surgeries

Name	Company	City	Country
Stellar S-30 aquarium air pump	Oscar Enterprises	California	USA
Surgery microscope M690	Leica Service	Bensheim	Germany
Heating circulator bath table C10-B3	HAAKE GmbH	Karlsruhe	Germany
Univentor 400 anaesthesia unit	TSE systems	Bad Homburg	Germany
Syringe pump	TSE systems	Bad Homburg	Germany

3.2 Surgical instruments and sutures

Name	Company	City	Country
Acutenaculum BM54	Aesculap	Tuttlingen	Germany
Micro forceps BD329	Aesculap	Tuttlingen	Germany
Nontraumatic vascular clamp FE690K	Aesculap	Tuttlingen	Germany
Scissor BC545	Aesculap	Tuttlingen	Germany
Surgical forceps BD537	Aesculap	Tuttlingen	Germany
Ethilon 4-0 PS-3 EH7761H	Ethicon	/	USA
Prolene 7-0 BV-1 8701H-S	Ethicon	/	USA

3.3 Anesthetics and analgetics

Name	Company	City	Country
Butorphanol	Chemos GmbH	Regenstauf	Germany
Isoflurane	Baxter	Unterschleiβheim	Germany

3.4 Antibodies for immunohistochemistry and Flow Cytometry

Immunohistochemistry (IHC) primary antibodies against mouse:

Antibody	Marker for	Company	Species	Dilution
Gr-1	Neutrophils	AbD serotec	Rat	1:1000
NGAL	Tubular damage, neutrophils	Dianova	Rat	1:1000
A1M	Tubular dysfunction	Lund, Sweden	Mouse	1:500
HO-1	HO-1	Enzo life sciences	mouse	1:75
haptoglobin	haptoglobin	Biozol	rabbit	1:100

IHC secondary antibodies:

Antibody	Company	Number	Dilution
Alexa Fluor-555 goat anti-rat IgG	Invitrogen	A21434	1:500
Alexa Fluor-555 goat anti-rabbit IgG	Invitrogen	A21428	1:500

Antibodies for Flow Cytometry:

Antibody	Company	Clone	Species	Dilution
CD11b eF780	ebioscience	M1/70	anti-mouse	1:600
CD11c PerCp-Cy5.5	ebioscience	N418	anti-mouse	1:400
CD45 eF450	biolegend	30-F11	anti-mouse	1:600
CD49b FITC	ebioscience	DX5	anti-mouse	1:100
F4/80 APC	biolegend	BM8	anti-mouse	1:100
Ly6G PE-Cy7	biolegend	1A8	anti-mouse	1:600
Ly6C PE-Cy7	biolegend	HK1.4	anti-mouse	1:600
Viability Dye- **eF506**	ebioscience		anti-mouse	1:1000

3.5 Primers for Real-Time PCR

Name	function	Company	Sequence
Col1a1	fibrosis	Qiagen	#QT02589482
CTGF	downstream target of TGF-beta, marker for fibrosis	Qiagen	#QT00096131
CXCL2	chemoattractant for neutrophils	Qiagen	#QT00113253
IL-6	inflammation marker	Qiagen	#QT00098875
MCP-1	macrophage chemoattractant	Qiagen	#QT00167832
PAI-1	downstream target of TGF-beta, marker for fibrosis	BioTez Berlin-Buch GmbH	Fwd:5'-ATGTTTAGTGCAACCCTGGC-3'
			Rev: 5'-CTGCTCTTGGTCGGAAAGAC-3'
TNFα	inflammation	Qiagen	#QT00104006
HPRT	house keeper	Qiagen	#QT00166768

3.6 Animals

Wild type (WT) C57BL/6J$^{Han\text{-}ztm}$, C57BL/6N and CD1 mouse strains were used. All experiments were performed with adult male C57BL/6 mice 11-13 weeks of age, bodyweight 23-28 g or CD1 mice 7 weeks of age, bodyweight 30-35 g). At least five mice were used (n≥5) in each group. Mice were housed and bred in the Institute of Laboratory Animal Sciences of Hannover Medical School or purchased from Charles River (Sulzfeld, Germany) and housed in the animal facility of Phenos GmbH. Mice had free access to drinking water and food. Day and night cycle was 14/10 h. Physical condition after surgery was monitored daily. Animals were treated according to the institution's guidelines for experimental animals which are in line with the international guidelines of animal welfare (GV Solas). All experiments were approved by the local animal protection committee of the Lower Saxony State department for animal welfare and food protection (33.9-42502-04-09/1637; 16/2295 and 33.19-42502-04-14/1657). We only used material and data from the tests that were carried out under applications 09/1637 and 16/2295 at Phenos GmbH. Studies were terminated if mice showed visible behavioral changes such as scrubby appearance, reduced motility, reduced food uptake and reduced activity. Body weight reduction of >20% or high s-creatinine elevation (more than 6 fold elevation compared to baseline) were reasons for study termination.

The study was terminated and the mice were sacrificed with a score of 3 or less. When a score of 4 was reached one hour follow up was done to reassess the mice.

Score	Quality	Characteristics
6	Very active	Strong, wide awake, quick movements
5	Active	Curious, quick, sporadic activity breaks
4	Limited active	Responds to affection, frequent activity breaks
3	Quiet, reduced food intake	Uninterested in the environment, rare activity, sleepy
2	Lethargic	No activities, decreased food intake
1	Moribund	No activity, expected death

3.7 Induction of ischemia reperfusion injury and heme infusion

Renal IRI causes transient hypoxia and results in AKI as described previously[73]. Briefly, mice were anesthetized with isoflurane (3-5% induction and 1,5-2% maintenance) and 1 mg/kg s.c. butorphanol was given prior to surgery for analgesia. Surgeries were carried out on a heating table at around 32-37°C. Median laparotomy was performed and the renal pedicles including renal vein and artery were clamped with a microaneurysm clip for 15min. Then clamps were released to allow reperfusion which was controlled visually (Fig.5). A sham group was also anesthetized and the abdominal cavity was opened but no clamps were placed. Free Hb (kind gift of Dr. Magnus Gram, Lund, Sweden) and labile heme (Logan, UT, USA) or vehicle was injected i.v. directly after IRI. When fully awake mice were returned to cages and monitored until they wake.

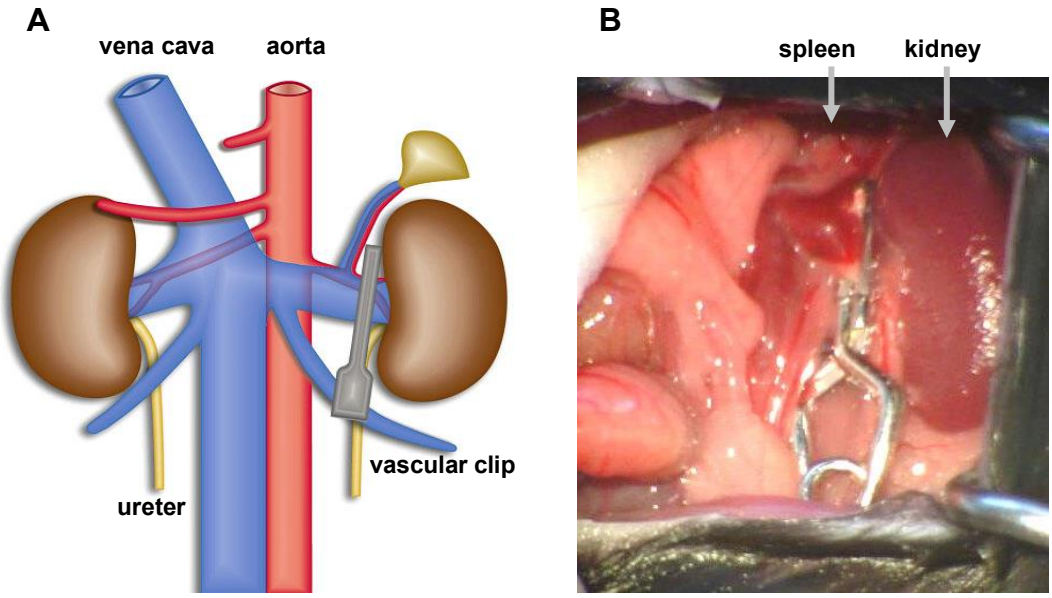

Figure 5: **IRI surgery to induce AKI**
IRI was induced by renal pedicle clamping (A). Either both renal pedicles were clamped or in some experiments unilateral clamping was done. (B: situs during surgery).

3.8 Albumin treatment to attenuate AKI

Human albumin (Kendrion Biopharma, purity 98%) was diluted by sterile Phosphate Buffered Saline (PBS). A final dose of 4mg/mouse was given i.v. 10 min before IRI

surgery was performed. The vehicle group received PBS injection.

3.9 Organ preservation

The mice were sacrificed at 2 or 24 hours and 7 days after IRI. Organ retrieval was done in deep isoflurane anesthesia (5-8%). Midline laparotomy was done followed by whole body perfusion via the left ventricle with ice-cold 0.9% PBS causing circulatory arrest during anesthesia. Organs were retrieved and dissected into 3-4 parts to undergo different fixation protocols:

- one part was shock frozen in liquid nitrogen and then stored at -80 °C and was used for Western blotting,
- one part was fixed in 4% formalin and embedded in paraffin for histological work-up,
- one part was snap frozen in isopentan at about -40°C and used for cryosections for immunohistochemical staining,
- one part was stored in RNA later (Thermo Fisher Scientific) for qPCR analysis.
- Alternatively, 75% of the renal tissue was placed in ice-cold PBS for flow cytometry.

3.10 Clinical chemistry

Blood was drawn by puncture of the venous plexus with an EDTA coated glass capillaries at baseline, before IRI and at day one or two days after IRI. The blood was centrifuged for 1 min at 3000 rpm and the plasma was transferred to a new tube for analysis. Clinical chemistry was measured by an automatic analyzer (Olympus AU400) according to the manufactures instructions. Creatinine, blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured.

3.11 Histology and immunohistochemistry

Renal tissue was fixed in 4% paraformaldehyde (PFA) with subsequent paraffin embedding. Two-µm paraffin sections were cut and staining was done according the different antibody protocols. For paraffin sections, deparaffinization and rehydration were performed as follows:

Slides were deparaffinated 3 times with Roti-Histol (Carl Roth) for 5 min and then rehydrate by 3 times incubation in 100% ethanol (Carl Roth) for 3 min each, 2 times in 96% ethanol for 2 min each and 1 time in 70% ethanol for 1 min short time in 50% ethanol and rinsed in distilled water.

3.11.1 Periodic acid Schiff staining (PAS):

PAS staining is used to investigate overall morphology and to score for acute kidney injury and inflammation.

Solution preparation:

- 0.5% Periodic acid solution (Merck)
- Sodium acid sulfite working solution:
    - 10% Sodium sulfite solution (Sigma-Aldrich) 12 ml
    - 1 mol/L Hydrochloric acid 10 ml
    - Distilled water 200 ml

Procedure:

- Slides were oxidized in 0.5% periodic acid for 10 min and rehydration.
- Washing was done 3 times for 5 min in distilled water
- Slides were incubated in Schiff´s reagent for 20 min.
- Incubation 3 times for 2 min in sodium acid sulfite working solution
- Rinsing with flowing tap water for 10 min.
- Nuclear staining with hematoxylin for 1 min
- Rinsing with flowing tap water for 10 min.
- Dehydration and mounting: 2 times incubation in 96% ethanol and 3 times in 100% ethanol for 3 min, and 3 times with Roti-Histol for 5 min.

- Covered with histoclear.

3.11.2 Immunohistochemistry:

Solution preparation:

- 0,01M Citric acid monohydrate (Sigma Aldrich): weights 1g citric acid in 300ml distilled water and adjusts pH to 6.0, then fills with distilled water to 500ml.
- Trypsin solution:
    - 1 mg trypsin pellet (Sigma Aldrich) was dissolved in 1000ml distilled water.
- Phosphate buffered saline (PBS):
    - 10 times PBS in distilled water for working solution.

Antigen retrieval:

Antigen retrieval was done by trypsin digestion or microwave incubation for 15min in citric acid according to antibody supplier recommendation and optimization studies.

Procedure:

- Heat-induced:
    - Slides were incubated citric acid in a microwave for 8 min on full power after deparaffinization and rehydration.
    - Kept warm for 2 min and then heated again with full power for 8 min.
    - Slides were cooled on ice for at least 30 min.
- Enzymatic

  Slides were incubated in trypsin solution for 15 min at 37 °C in a humid chamber after deparaffinization and rehydration.

Procedure for antibody staining:

- Slides were washed in PBS after antigen retrieval.
- Slides were incubated with the primary antibody (diluted with PBS) for at least 60 min at RT in a humid chamber in the dark.
- After washing in PBS incubation with the secondary antibody (labeled by fluorescence and diluted with PBS) was performed for 50-60 min at RT and then washed with PBS.
- Slides were embedded in mounting medium containing DAPI (Dianova) for nuclei staining.

Quantification:

Analysis of all stainings was conducted using a Leica imaging microscope in a blinded manner. Semi-quantitative scoring was used for leukocyte infiltration at 200-fold magnification (neutrophils: Gr-1$^+$ and macrophages: F4/80$^+$: 0 = no leukocyte infiltration per view field, 1 = 5-10 cells per view field (VF), 2 = 11-15 cell/VF, 3 = 16-25 cells/VF, 4 >26 cells/VF.

3.12 Quantitative PCR for mRNA expression

Solution preparation:

- 70% ethanol in distilled water
- RLT buffer working solution: 10 µl β-Mercaptoethanol (Sigma-Aldrich) was added to 1 ml buffer RLT
- DNase I stock solution:
    - Lyophilized DNase I (1500 units) (Promega) in RNase-free water

mRNA isolation from renal tissue:

- According to RNeasy Mini Kit (Qiagen) total RNA was isolated from animal tissues.
- Let the tissue in RNA later thaw at room temperature (RT). At the same time forceps and homogenizer were cleaned with RNase AWAY (Thermo Fisher Scientific) 2 times.
- About 30 mg kidney pieces were added 350 µl RLT buffer and tissue was homogenized by a conventional rotor-stator homogenizer.
- The lysate was transferred into a QIAShredder tube (Qiagen)and was centrifuged for 3 min at full speed.
- 1 volume of 70% ethanol was added to the supernatant and immediately mixed by pipetting.
- 700 µl sample including any precipitate that may have formed was transferred to an RNeasy spin column and centrifuged for 15 s at 10000rpm. The flow through was discarded. (Reuse the collection tube if the sample volume exceeds 700ml and centrifuge the left aliquots in the same tube.)
- 350 µl RW1 buffer were added to the RNeasy spin column and centrifuged for 15 s at 10000rpm. The flow through was discarded.

- 70 µl RDD buffer was added to 10 µl DNase I stock solution and then gently mixed. 80 µl mixtures was directly added to the RNeasy spin column and placed on the benchtop for incubation 15 min at RT.
- 350 µl RW1 was added buffer to the RNeasy spin column and centrifuged for 15 s at 10000 rpm. The flow through was discarded.
- 500 µl RPE buffer was added to the RNeasy spin column and centrifuged for 15 s at 10000 rpm. The flow through was discarded.
- Another 500 µl RPE buffer was added to the RNeasy spin column and centrifuged for 2 min at 10000 rpm. The flow through was discarded.
- RNeasy spin column were placed in a new 2 ml collection tube and centrifuged for 1 min at full speed.
- RNeasy spin column was placed in a new 1.5 ml collection tube and 30-50 µl of RNase-free water was added into the spin column. Then incubation for about 1 min was done. Spin column was centrifuged for 1 min at 10000rpm to elute the RNA.
- Eppendorf Bio Photometer was used for measuring RNA concentration at 260 nm.

Conversion RNA to cDNA

- cDNA was synthetized with Takara Prime Script RT reagent KIT.
- The mixture was prepared with 2 µl 5x Prime Script buffer, 0.5 µl Prime Script RT Enzyme Mix I, 0.5 µl Oligo dot Primer (50 µM) and 0.5 µl Random Hexamers (100µM).
- 3.5 µl mixtures were added to 10 µl of 0.1g/µl RNA.
- Then incubation was done at 37°C for 15 min for reverse transcription followed by 5 s at 85°C to denature the enzyme in a thermal cycler (MJ Research, BIO RAD).

Quantitative PCR

<u>Reaction mixture per well of 96-well plate:</u>

- Takara SYBR qPCR: SYBR Premix Ex Taq: 10 µl
- Forward Primer (10 pmol/µl): 1 µl

  Reverse Primer (10 pmol/µl): 1 µl
- or Quantitect Primer assay (Qiagen) Primer (100pmol/µl): 2 µl
- DEPC-treated water (Sigma Aldrich): 3 µl
- cDNA (0.01 µg/µl): 5 µl

Total volume 20 µL

q-PCR was conducted on a LightCycler 96 (Roche).

Procedure:

- 96°C for 10 min for pre-incubation to activate the polymerase
- 10 s at 95°C for denaturation

  10 s at 60°C for annealing

  10 s at 72°C for extension

  Repeated for 45 cycles for amplification

HPRT was used as house keeper for normalization. Quantification was performed by LightCycle 96 SW 1.1 software. Each sample was measured in triplicates. For qPCR analysis more than 5 mice each group were used.

3.13 Non-invasive blood pressure measurement

Materials

- CODA Non-invasive Blood Pressure System (CODA, Kent Scientific; Torrington, CT) from Kent Scientific can measure in up to eight mice simultaneously. The CODA system applies volume-pressure recording (VPR) technology to detect changes in tail volume, which correspond to systolic and diastolic BP (Fig.6 A).
- Mouse Restrainer (Fig.6 B): The restrainer is to minimize movement during the measurement session. The dark nose cone limits mice view and decreases the stress. The nose cone faces the CODA box and the rear with the mouse tail facing the user.
- Mouse Cuff Sets: the BP measurement relies on two tail-cuffs, occlusion cuffs (O-cuff) and volume–pressure recording cuffs (VPR-cuff). The O-cuffs and VPR-cuffs connected to the CODA box before the experiment. The O-cuff is placed near the base of the tail proximal to the VPR-cuff (Fig.6 C, D).
- Paper towels: paper towels were placed underneath each restrainer to collect urine and feces during measurement. It is also important to control the body

temperature. The restrainers are on a warming table. Sufficient blood flow to tail is critical for tail-cuff BP measurement, warming or cooling with paper towels is a key part of this method.

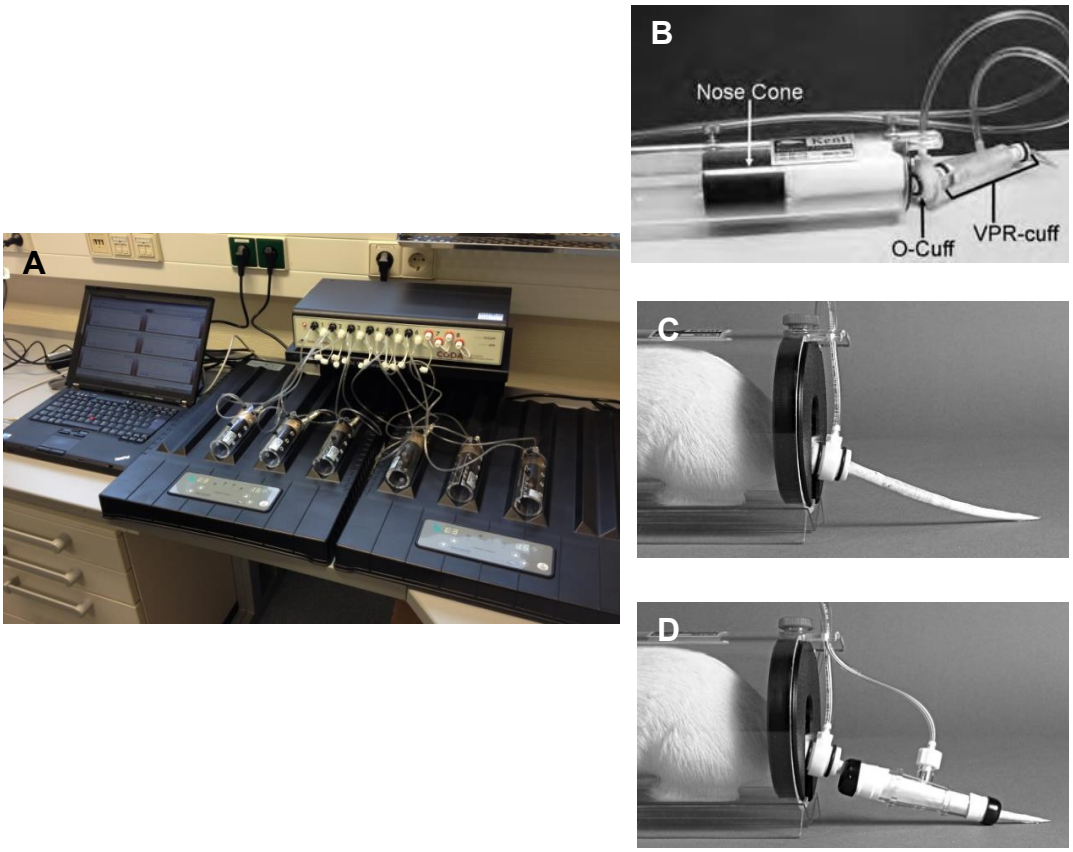

Figure 6: Non-invasive blood pressure Measurement in mice
Non-invasive blood pressure monitoring with a CODA system with 8 channels was done (A). Volume pressure recording (VPR) technology was used to detect changes in tail volume, correlating with systolic and diastolic blood pressure. Restrainer, occlusion-cuff (O-cuff), and volume–pressure recording cuff (VPR-cuff) are needed for monitoring (B). C and D show the positions of the cuffs.

BP measurement

- A warm quite room is needed for BP measurement. The mice should be transferred to house in a separate, nearby room one day before BP measurement. Room temperature (RT) should be controlled between 22°C and 27°C.
- At least 1h before BP measurement, the heating table should be turned on and the restrainers and paper towels should also be placed on the platform. The computer always should be tuned on prior to start the CODA box.
- The mice were gently guided into the restrainer without any forcing. Then the nose cone was adjusted so that the mice were comfortable but without moving

excessively. When the mice had entered, mice were gently held in the restrainer and the rear hatch was adjusted for fixation of the mice and the whole tail of the mice was outside. The O-cuff was gently positioned near the base of the tail proximal to the VRP-cuff.

- Once the mice were placed into the restrainers, they were left alone to acclimate for five minutes before starting the BP measurement protocol. During the acclimatization and measurement periods, the mice were kept warm by heating pads under the restrainer platform and the tail temperature was monitored.
- After taking all the measurements for one experiment, the data were exported and the average and standard deviation (SD) for each BP parameter of each mouse was calculated. Each measurement cycle included systolic, diastolic and mean arterial pressure. Any reading more than two SD from the mean for an individual mouse was excluded and a new average and SD was calculated as the final data for the single mouse.

Animals were trained to measure BP at least three times at different days before starting the study. Each measurement included five training inflations and 15 experimental measurements. Five mice or more were analyzed for each group.

3.14 Flow Cytometry

Fluorescence-activated cell sorting (FACS) was used to quantify the amount of infiltrating leukocytes in the tissue. The mice were sacrificed at d1 and d7 after IRI. 70% kidney were dissected and put into digestion medium on ice for flow cytometric analysis.

Solution preparation:

- 500 U/ml Collagenase II solution:
  - Collagenase II (Worthington) diluted with DMEM (liquid medium with stable glutamine, Biochrom)
- 1x RBC lysis buffer:
  - 10x RBC lysis buffer (biolegend) diluted with distilled water

- FACS buffer:
    - 0.5% BSA with PBS
- LD antibody for live / dead stain:
    - Fixable viability dye eflour 506 (eBioscience) 1:1000 in PBS

Procedure:

- Kidney tissue was cut into very small pieces and put in a C-tube (Miltenyi) with collagenase II solution. Incubation was performed in 37°C water bath for 22 min.
- Then tissue was homogenized on a gentleMACS dissociator (Miltenyi Biotec) on program B.01 in C-Tubes. Digestion was followed in 37°C water bath for 22 min.
- Lysate was filtered three times with 70 µm cell mesh (Thermo Fisher Scientific) and washed with 6 ml PBS to obtain single cell suspensions. After each filtering solution was centrifuged at 1250 rpm for 7 min.
- The spleen was sliced using a syringe, and then centrifuge all sample at 1250rpm for 3 min. The supernatant was thrown away.
- 1 ml in RBC lysis buffer was added to each sample and incubated for 1 min. Then 20 ml PBS was added to stop the reaction and centrifuged at 1250 rpm for 3 min at 4°C
- 5 ml PBS was added to the tube and counted. At least $4x10^5$ cell for surface staining and 10^6 for intracellular (IC) staining was used. At the same time about 300 µl spleen cells was heated at 95°C for 40 min to make cell death.
- All samples were centrifuged at 1250 rpm for 3 min at 4°C and were incubated with 250 µl life dead (LD) at 4°C for 30 min in the dark.
- Cells were washed with 1 ml FACS buffer and centrifuged at 1250 rpm for 3 min at 4°C.
- 300 µl antibody master mix in FACS buffer was added and incubated for 30 min at 4°C in the dark.
- Cells were washed with 1 ml FACS buffer and centrifuged at 1250 rpm for 3 min at 4°C.
- 300 µl FACS buffer was added and then analyzed.

BD Biosciences FACS Canto II was used for flow cytometry and Kaluza software 1.5a (Beckmann Coulter, Krefeld) was used for data analysis. More than five mice were used for each group.

3.15 Complement activity measurement in plasma samples

EDTA-plasma was collected at 4h after IRI 15min from B6 mice. Complement activation was induced by incubation of serial dilutions of plasma in ELISA plates (Nunc Maxisorp plates, Thermo Fisher Scientific) coated with human IgM, mannan and LPS to induce the classical, lectin and alternative pathway, respectively. Activation of complement was quantified at the level of C9 deposition, using a rabbit anti-mouse C9 polyclonal Ab. Complement activity in experimental samples was calculated using B6 serum as a standard which was set to 100 AU/ml. Activation of the complement system upon IRI was quantified in EDTA-plasma samples using a specific sandwich ELISA for the C3b/C3c/iC3b activation fragment. The measurements were performed by our collaborator Prof. Cees van Kooten (Nephrology, University Medical Center in Leiden, The Netherlands.

3.16 Labile heme measurement in tissue

The apo-HRP based assay was used to measure labile heme in the tissue and the peripheral blood.

Solution preparation:

- N-Formylmethionine-leucyl-phenylalanine (fMLP; St. Louis, MO)
- Apo horse radish peroxidase (apo-HRP; Gwent, UK) stock concentration was measured with Synergy™ 2 multi-mode plate reader (BIOTEK) using a molar extinction coefficient of 20,000 at 280 nm.
- Hemin (Frontier Scientific, Logan, UT, USA) stock was diluted in DMSO and detected the concentration using a molar extinction coefficient of 180 at 400nm.
- Peroxidase Substrate Kit TMB-ONE™ (Copenhagen, Denmark)
- RPMI 1640 medium (Carlsbad, CA)

Establishing the standard curve of Apo-HRP reconstitution with hemin

First, the hemin stock solution was diluted to 25 nM with DMSO. The following concentrations of heme standards (0.25, 0.5, 1, 1.5, 2 and 2.5 nM) were prepared in Hank's Balanced Salt Solution (HBSS) with 0.5% BSA. The final volume was 100µl with 0.75 µM of apo-HRP. The reaction mixture was incubated at 4°C for ten minutes. After incubation, 5µl reaction mixture was pipetted into a 96-well plate and then 200µl of TMB substrate was added in each well. The holo-HRP activity was detected at 652 nm absorbance for the enzymatic oxidation of tetramethyl benzidine (TMB). The absorbance of the highest standard was between 0.9 - 1.4. The absorbance data at 652 nm against concentration of hemin was plotted for the standard curve.

Kidney tissue sample preparation for labile heme assay

The kidney tissue samples were collected in ice cold PBS and freshly processed. The tissue was homogenized with 1ml HBSS and centrifuged at 1500 rpm for 10 min. The supernatant was transferred to a new Eppendorf tube and then centrifuged again. The new supernatant was aliquoted and immediately stored at -80 °C. Before assay, the supernatant was diluted in HBSS in a range between 1:50 to 1:16.000 (depending on the concentration of heme). The final concentration of labile heme was calculated depending on this standard curve.

To analyze specificity, an aliquot of the supernatants from 45min IRI tissue was incubated with hemopexin (Hx) for 15 min and the apo-HRP assay was repeated. Hx is labile heme scavenger with high binding affinity. This Hx-inhibitor experiment showed a reduction of labile heme compared with untreated supernatant. It indicated that the apo-HRP based assay is specific for detection of labile heme.

3.17 Functional magnetic resonance imaging (fMRI)

Using a 7 Tesla small animal scanner (Bruker, Pharmascan, PHS701, Ettlingen, Germany), MRI was conducted for kidney volume and investigation of renal perfusion at d1 after IRI or sham surgery in mice. For MRI, mice were anesthetized with 3% isoflurane and with 1-2% isoflurane to maintain anesthesia during the 60min protocol. Respiration of mice was monitored and kept between 50-60 breaths per minute in the whole procedure. Morphological images were acquired by using routine respiratory-triggered T2-weighted turbo spin echo sequences in axial and coronal planes. Kidney volume was quantified by analyzing manual segmentation of axial images through OsiriX software (v.6.0.2; Pixmeo, Switzerland).

According to the description previously, a respiratory-triggered, fat-saturated flow-alternating inversion-recovery (FAIR) ASL-sequence with an echo-planar readout was used for assessing the quantification of renal perfusion[73]. The long axis of the kidney was regulated by measuring of images in a central coronal plane. 13 inversion times = 30, 100, 200, 300, 500, 700, 1000, 1200, 1500, 2000, 3000, 5000 and 8000 msec, TR/TE = 18.000/16.4 msec, slice thickness = 2 mm, matrix = 128x128, field of view = 35x35 mm^2. Using OsiriX software (v.6.0.2, Pixmeo, Switzerland) the mean values of perfusion per minute and 100g kidney tissue of cortex were evaluated by manually placing regions of interest. Statistical analysis was performed at the single time points between each group. The loss of kidney volume was assessed by comparing with sham vehicle group. Three mice in the sham group and seven mice in the IRI group were used for analysis. Kidney volume of the sham group did not show differences between baseline and d1.

3.18 Statistical analysis

For statistical analysis GraphPad prism software, version 5.0 (GraphPad Software

Inc., San Diego, CA) was used. One way ANOVA was used for comparison between several groups and t-test was used to compare two groups. Data is shown as mean ± standard error (SEM). Significant differences were defined as *p < 0.05, **p < 0.01, *** p < 0.001.

4 Results

First, we aimed to establish a new animal model for subclinical IRI and to combine that with exposure to free Hb during the reperfusion phase. The free Hb was generated from human packed red blood cells (pRBC) and a gift from Dr. Grams (Lund University). Different ischemia times were tested first in CD-1 mice since they are more sensitive to renal injury[74]. Renal and liver function, kidney morphology, systemic blood pressure, inflammatory cytokine release and cell infiltration were investigated. Next, a comparison of the effect of labile heme in two different mouse strains (CD1 and B6) was done to choose the optimal model for further investigations. Finally, also the Hb degradation product labile heme was tested in the IRI mouse model and used for further studies.

4.1 The effect of free Hb on ischemia induced AKI in CD1 mice

CD1 mice had 15min bilateral IRI and immediately after releasing the clamps free Hb (2.5 mg/mouse) was injected intravenously. An IRI vehicle group received the same surgery with iv injection of the free Hb buffer (5 µl/g). A sham group was anesthetized and the abdominal cavity was opened. Follow-up was two days after the induction of IRI.

4.1.1 Free Hb injection aggravated renal and liver injury

Renal and liver function was investigated at baseline, day one and two after IRI. The IRI vehicle group had a slight increase of creatinine and BUN at day one which was

not significant.

In contrast, free Hb infusion induced a significant elevation of creatinine and BUN at day one and day two after IRI (Fig.7A and B). Similar results were seen when measuring liver function parameters. The liver enzymes ALT and AST both were elevated in the IRI free Hb group and were significantly different compared IRI vehicle group (Fig.7C and D). AKI can affect the liver by distant organ injury which explains the elevation of liver enzymes in this model.

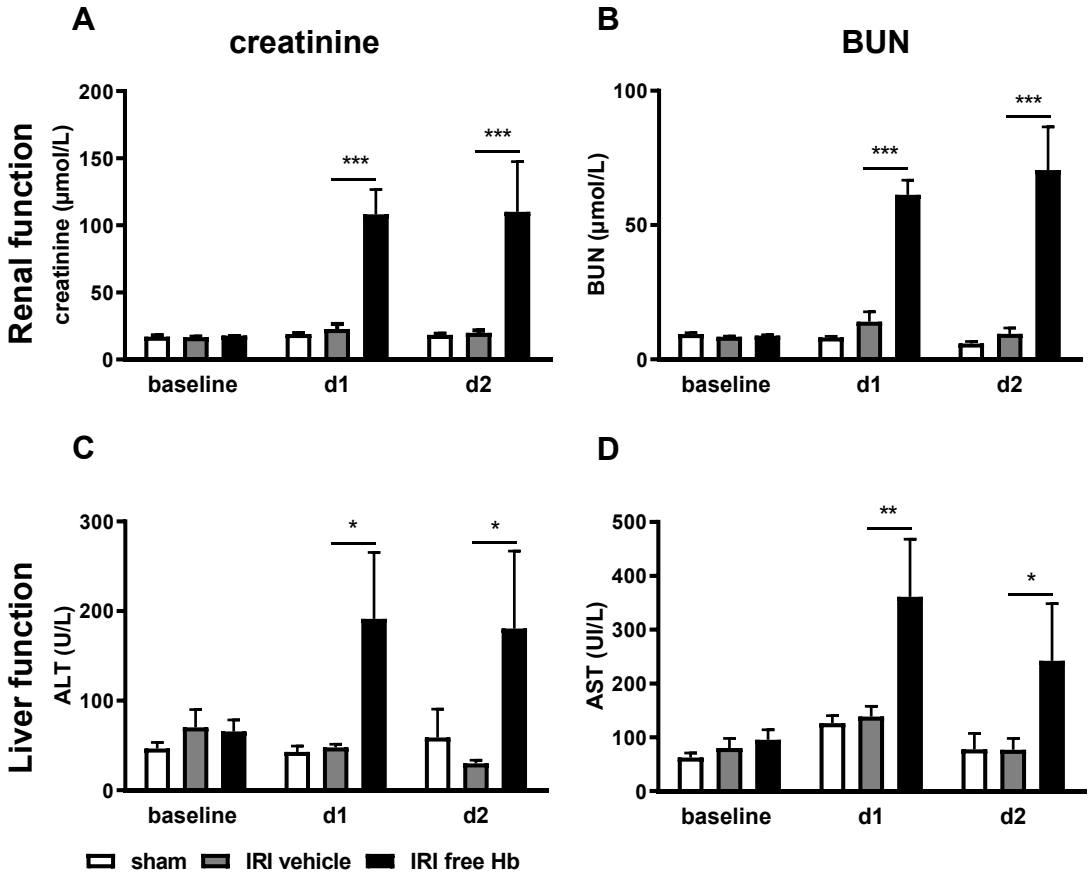

Figure 7: Free Hb injection after IRI caused renal and liver function deterioration Plasma samples from sham (white bars), IRI vehicle (grey) and the IRI free Hb (black) CD1 mice were analyzed at baseline, day 1 and 2 after 15min IRI. Renal function (upper row) was significantly impaired by free Hb injection (A, B). Liver enzymes were also elevated significantly at d1 after IRI (C, D). Shown are mean ± SEM, n = 2 sham group, n= 5 in all other groups, *p < 0.05, **p < 0.01, *** p < 0.001.

4.1.2 Free Hb injection after IRI enhanced neutrophil infiltration

PAS staining was done to analyze renal morphology. The IRI vehicle group showed mild tubular injury and very little leukocyte infiltration in the outer medulla (Fig.8A and

B). More severe tubular injury and more leukocyte infiltration were detected in the IRI free Hb group (Fig.8 D and E).

Neutrophils are the first leukocytes infiltrating the kidney after IRI. After two days the IRI-free Hb group had significantly more $Gr1^+$ neutrophils infiltrating in the outer medulla than the IRI-vehicle group (Fig.8 C,I and F). Furthermore, flow cytometry was performed to differentiate the infiltrating leukocyte subsets. Myeloid cells were analyzed and the data showed that $CD11b^+$ cells were enhanced significantly in kidneys of the IRI free Hb group. CD11b is expressed on many leukocytes surfaces, including monocytes, granulocytes, neutrophils, macrophages and natural killer cells. It indicated free Hb stimulated leukocytes infiltration during AKI (Fig. 8 J).

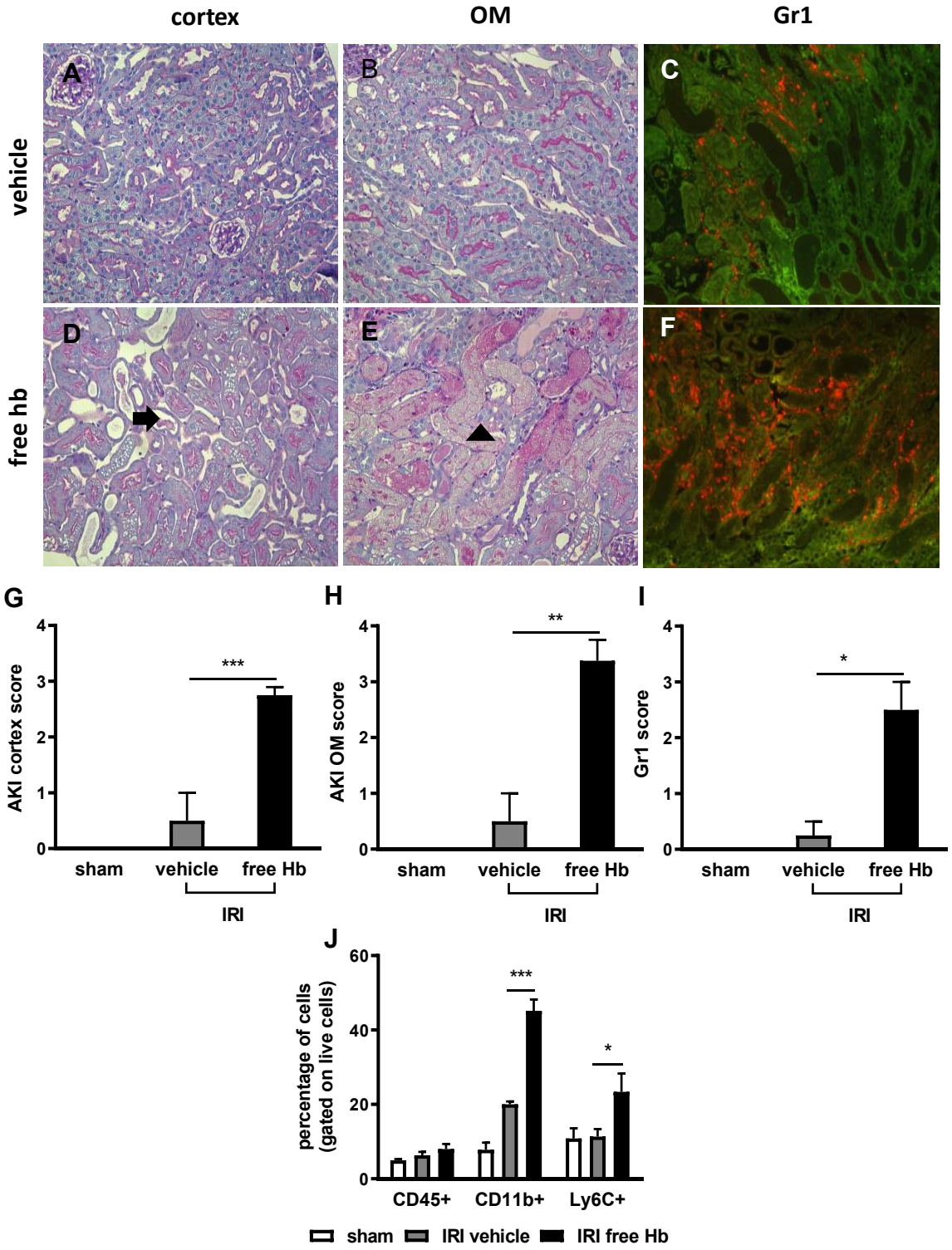

Figure 8: AKI and neutrophil infiltration were enhanced due to free Hb injection
Renal morphology was studied by PAS stain and showed that free Hb treatment (lower images, black bar) caused enhanced tubular injury in the cortex and the outer medulla compared to the vehicle IRI (upper images, grey bar) or sham group. The arrow marks tubuli with necrotic debris and the arrowhead indicates protein cast formation causing tubular obstruction (D, E). $Gr1^+$ neutrophil infiltration was aggravated in the free Hb IRI group and was most prominent in the outer medulla (C, F, I). FACS analysis confirmed enhanced CD11b+ leukocyte infiltration in the IRI free Hb group (J). Shown are mean ± SEM, n = 2 sham, n= 5 in IRI free Hb and IRI vehicle group,*p < 0.05, **p < 0.01, *** p < 0.001. (Magnification 200x)

NGAL is a tubular damage marker and is used as readout for kidney injury of renal tubular epithelia cells. Circulating NGAL is filtered by the glomerulus and then reabsorbed in the proximal tubulus[75, 76]. Free Hb enhanced the expression of NGAL in the cortical tubular epithelial cells (Fig.9C and E). A1M is a tubular function marker which is reabsorbed by the proximal tubular epithelial cells[77]. Our data showed that about 60% of the tubuli lost their transport function and many A1M positive casts appeared in the IRI-free Hb group as a sign of more severe tubular damage (Fig. 9D).

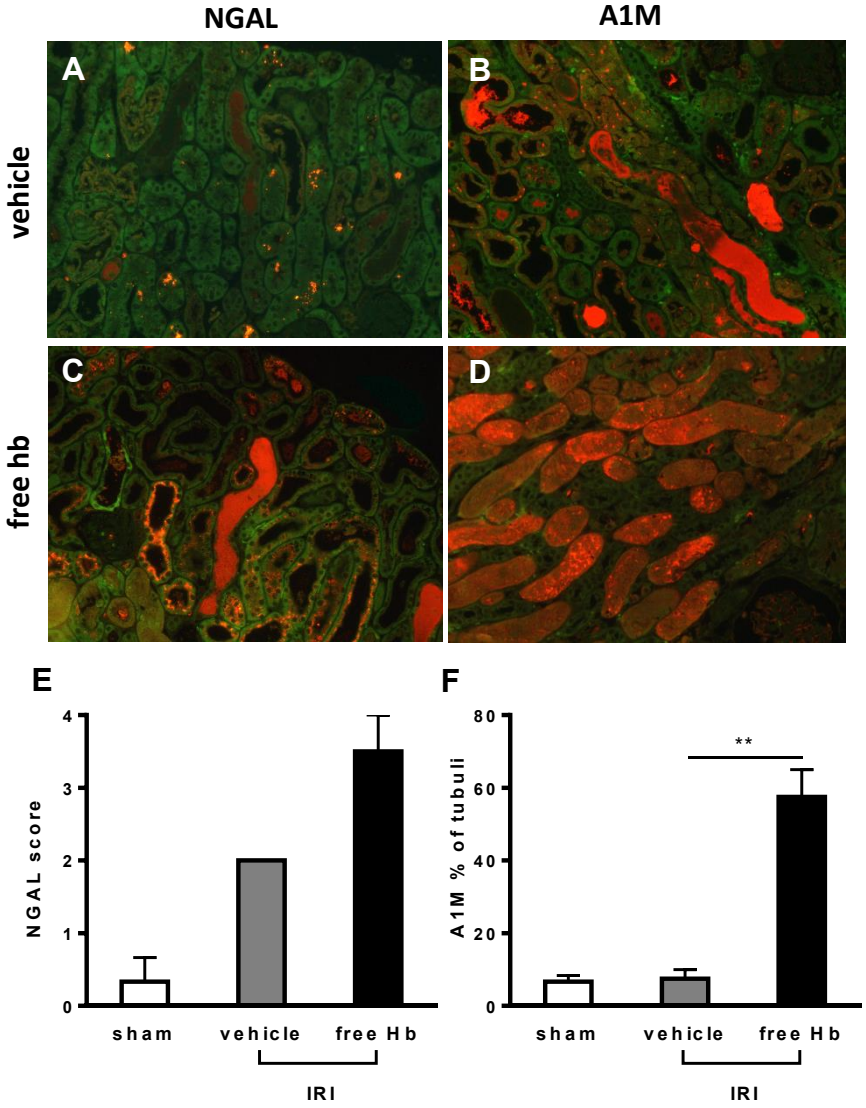

Figure 9: NGAL and A1M expression after IRI
NGAL is a biomarker for tubular damage. In the IRI-free Hb group (B, black bar) NGAL expression in the cortical tubuli was enhanced compared to the IRI vehicle group (A, grey). A1M is a tubular transport marker and is present in tubular casts after IRI (B, D). Mean ± SEM, n = 2 sham group, n= 5 in all other groups,*p < 0.05, **p < 0.01. (Magnification 200x)

4.1.3 Free Hb increased pro-inflammatory cytokines release

Pro-inflammatory cytokines mediate the inflammatory response[78-81]. Therefore, pro-inflammatory cytokines and chemokines were analyzed at d2 after IRI by qPCR. IL-6 is a general inflammation marker and was significantly increased in the kidneys of the IRI free Hb group compared to the IRI-vehicle group. MCP-1 is an important chemoattractant for monocytes/macrophages[82] and was significantly up-regulated in the IRI free Hb group (Fig.10A and B). PAI-1 is a downstream target of TGF-β. The expression of PAI-1 was enhanced in the IRI free Hb group (Fig.10C). TNF-α, another pro-inflammatory mediator was slightly increased in the kidney of both the IRI-vehicle and the IRI-free Hb group compared to the sham group (Fig.10D). There was no significant difference between the IRI groups.

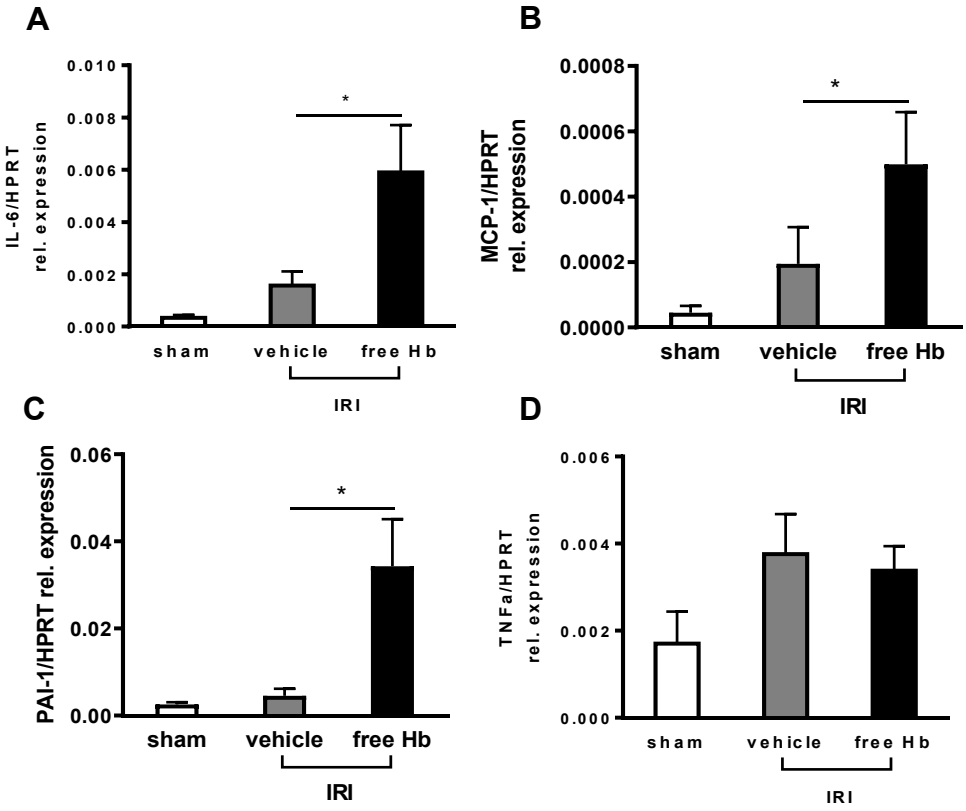

Figure 10: Cytokine expression at day 1 after IRI
Pro-inflammatory cytokine mRNA expression in the renal tissue was measured by qPCR. Free Hb induced significant upregulation of the pro-inflammatory cytokines IL-6, MCP-1, PAI-1 compared to the vehicle group (A-C). TNFα mRNA level was increased after IRI, but there were no significant difference between the IRI vehicle and the free Hb group (D). Mean ± SEM, n = 2 sham group, n= 5 in all other groups,*$p < 0.05$.

4.1.4 Blood pressure decreased after IRI

BP was measured with a tail cuff method using the CODA non-invasive blood pressure system, which is a computerized system that automatically performs three physiological parameters: systolic blood pressure, diastolic blood pressure and mean blood pressure. The mice had three different training days of BP measurement before starting the IRI study. Our data showed that BP was stable at baseline and remained stable after sham surgery. Systolic BP, diastolic BP and mean BP had a certain degree of decrease in the IRI vehicle and IRI free Hb group. However, no significant differences between IRI-vehicle group and IRI-heme group were detected (Fig.11).

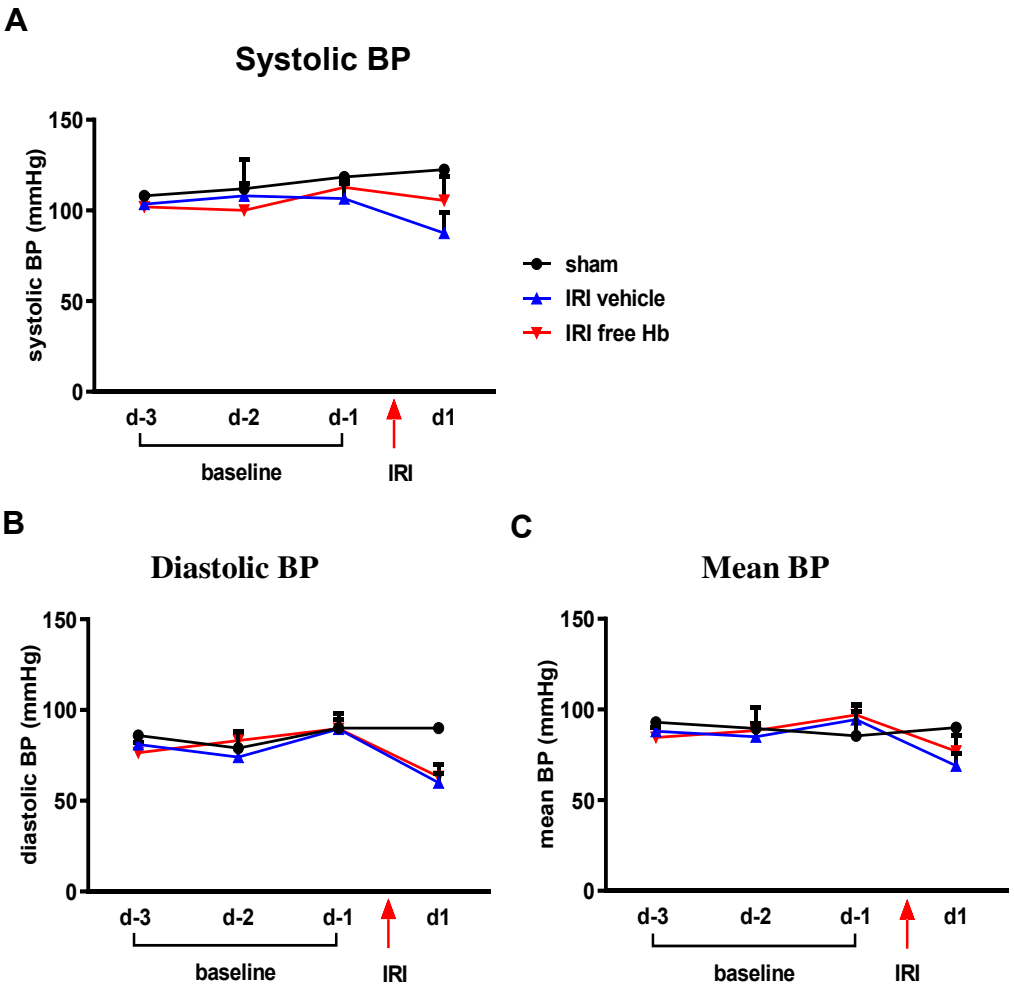

Figure 11: Blood pressure of CD1 mice decreased after IRI 15min
BP was measured in the sham group (black line), the IRI vehicle group (blue line) and the IRI free Hb group (red line) at three days before and one and two days after IRI 15 min. Systolic BP (A), diastolic BP (B) and mean BP (C) decreased following IRI and free Hb injection but without significant differences between groups. Mean ± SEM, n= 6-8 per group.

4.2 Labile heme in the context of ischemia induced AKI

Free Hb degrades to labile heme which also exerts cytotoxic effects. Since free Hb was of human origin the next experimental step was to test the effect of labile heme on renal IRI. The two commonly used mouse strains: CD1 and B6 were exposed to different labile heme concentrations after 15min of bilateral renal IRI. Escalating doses of labile heme: 15, 20, 30, 40 and 50 mg/kg bodyweight were injected iv after release of the clamps in the renal IRI model. Renal and liver function and pro-inflammatory cytokines were measured at the two day follow up. Normal mice without any surgery served as controls. In addition, a sham vehicle group underwent anesthesia with the abdominal cavity opening and was injected with the buffer of labile heme (5µl/g bodyweight). Another sham group received labile heme injection. The IRI vehicle group was injected the buffer of labile heme after IRI. Compared to healthy mice the sham vehicle group and the sham heme group showed very similar results.

4.2.1 Comparison of labile heme effects in CD1 and B6 mice after renal IRI

Renal and liver function impairment was compared between CD1 and B6 mice in the IRI model after 15 and 50 mg/kg heme infusion. In CD1 mice, creatinine and BUN increased 2-5 fold after one day after IRI compared to the baseline, but decreased to almost baseline levels at day two (Fig.12A and C). ALT and AST where also elevated at day one and returned to baseline at day two in the IRI groups. There was a trend towards higher elevation in the heme treated IRI groups. However, differences to the IRI vehicle group were not significant (Fig.12E and G). In B6 mice renal function impairment and elevation of creatinine and BUN were more pronounced than in CD1 at d1. In the 50mg/kg heme treated IRI group the BUN elevation persisted towards d2 in B6 mice. The liver enzyme elevation (ALT and AST) was higher in B6 mice and remained elevated at d2 (Fig.12B, D, F and H).

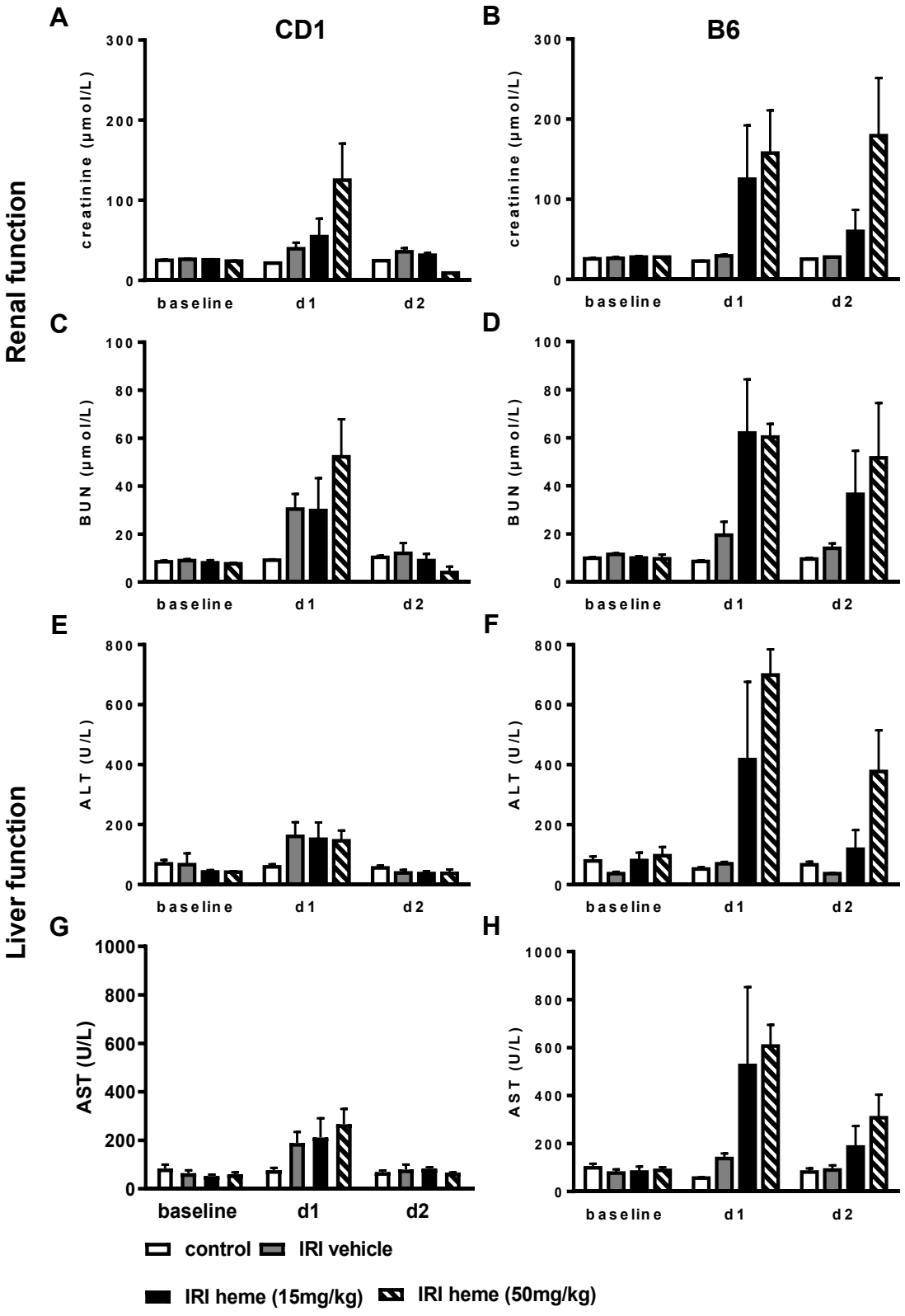

Figure 12: Comparison of CD1 and B6 mice in the IRI heme model – clinical chemistry
Renal function impairment was worse after heme injection in CD1 (A) and also in B6 (B) mice when compared to IRI vehicle. BUN elevation was comparable to creatinine elevation (C, D). Liver enzyme elevation was much higher in B6 mice (F, H) compared to CD1 mice (E, G). Injection of 15 and 50mg/kg heme/mouse had similar effects in B6 mice. Mean ± SEM, CD1 n=5, B6 n=4.

The expression of the pro-inflammatory cytokine IL-6 was measured by qPCR at d1 and revealed more pronounced elevation in B6 compared to CD1 mice in the IRI 15mg heme group. However, in the 50mg/kg heme treated IRI group the IL-6 expression was lower than in the 15mg/kg heme group. (Fig.13A and B).

In addition, the mortality was studied. The data showed that the survival was about 75% in CD1 mice and 100% in B6 mice in both the vehicle and the 15mg/kg heme treated IRI group. With 50mg heme/kg, the survival decreased to 35% in CD1 mice but was still 75% in B6 mice (Fig.13C and D).

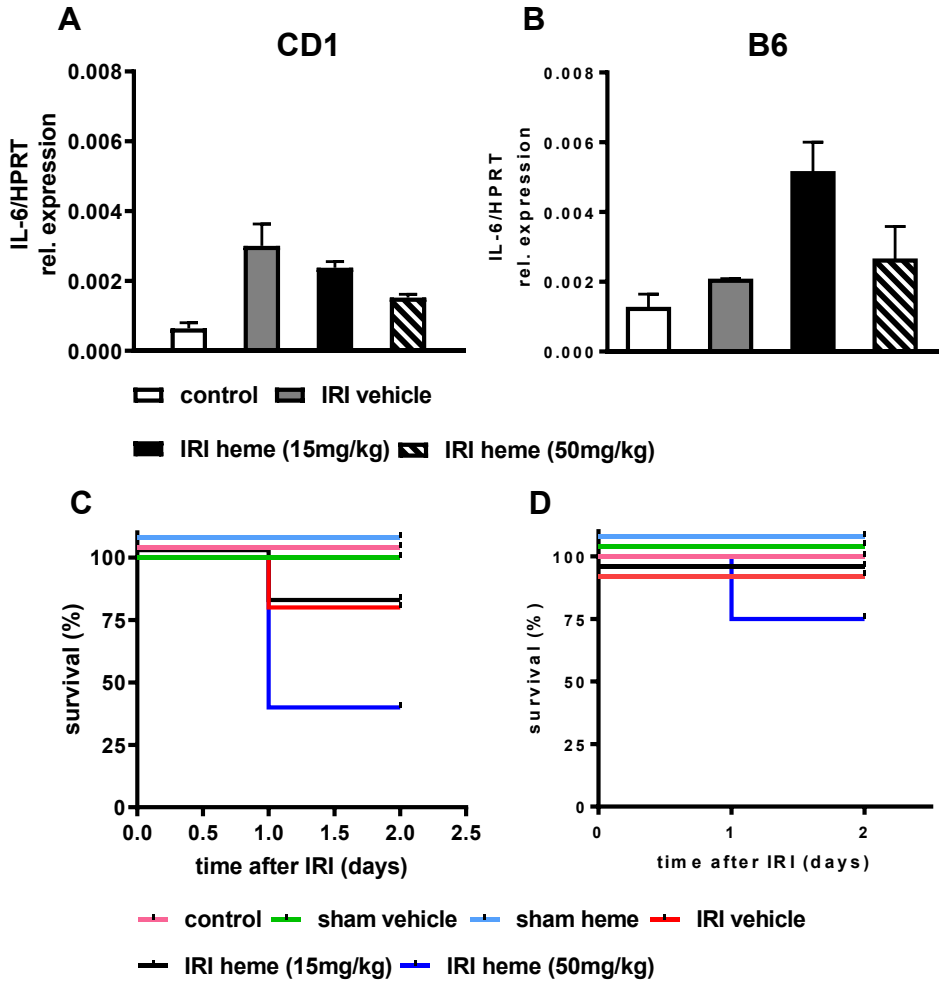

Figure 13: The effect of labile heme on IL-6 mRNA expression and survival in CD1 and B6 mice after IRI

The increase in IL-6 was more pronounced in B6 compared to CD1 mice at d1 after IRI (A, B). (mean ± SEM, control n=7, IRI-vehicle CD1 n=5, B6 n=4). IRI labile heme 15mg/kg black bars, CD1 n=5, B6 n=4 and the IRI-labile heme (50mg/kg hatched bar, CD1 n=5, B6 n=4).The control group (pink line, n=2), the sham vehicle group (green line, n=3) and the sham heme group (light blue line, n=3) had 100% survival in CD1 and B6 mice (C, D). In CD1 mice the IRI vehicle group (red line, n=5) and the IRI heme 15mg/kg group (black line,

n=5) had 75% survival and in B6 mice 100%. The higher labile heme application caused higher mortality in both groups (50mg/kg, navy blue line, n=5).

Overall, CD1 mice had quite high mortality despite rather mild increase of creatinine and BUN. B6 mice were less sensitive and showed better survival than CD1 mice. Therefore, B6 mice were used for the subsequent experiments. The high concentration of labile heme (50mg/kg) was linked to higher mortality compared to the moderate dose of 15mg/kg heme. Since there were high inter-individual variations further dose response studies were done in B6 mice (i.e. 20, 30 and 40 mg/kg bodyweight).

4.2.2 Dose response to labile heme in the renal IRI model in B6 mice

The results showed that creatinine and BUN of the 20 mg/kg IRI heme group was 1.5-2 fold higher compared with the 30 mg/kg group. In the 40 mg/kg group creatinine elevation was highest but was linked to higher mortality (Fig.14A, B, G). Similarly, ALT and AST were higher in the IRI heme groups compared to vehicle but did not differ between the different dose regimens of heme (Fig.14C and D). The pro-inflammatory cytokines MCP-1 and IL-6 showed the highest expression in the kidneys of the 20mg/kg IRI heme group compared to the other groups. Surprisingly, the higher heme doses had a trend towards less MCP-1 and IL-6 mRNA levels in the renal tissue (Fig.14E and F). The survival percentages with 20, 30 and 40mg/kg labile heme infusion after IRI were 100%, 80% and 50% respectively, the higher the concentration with the lower the survival proportion (Fig.14G).

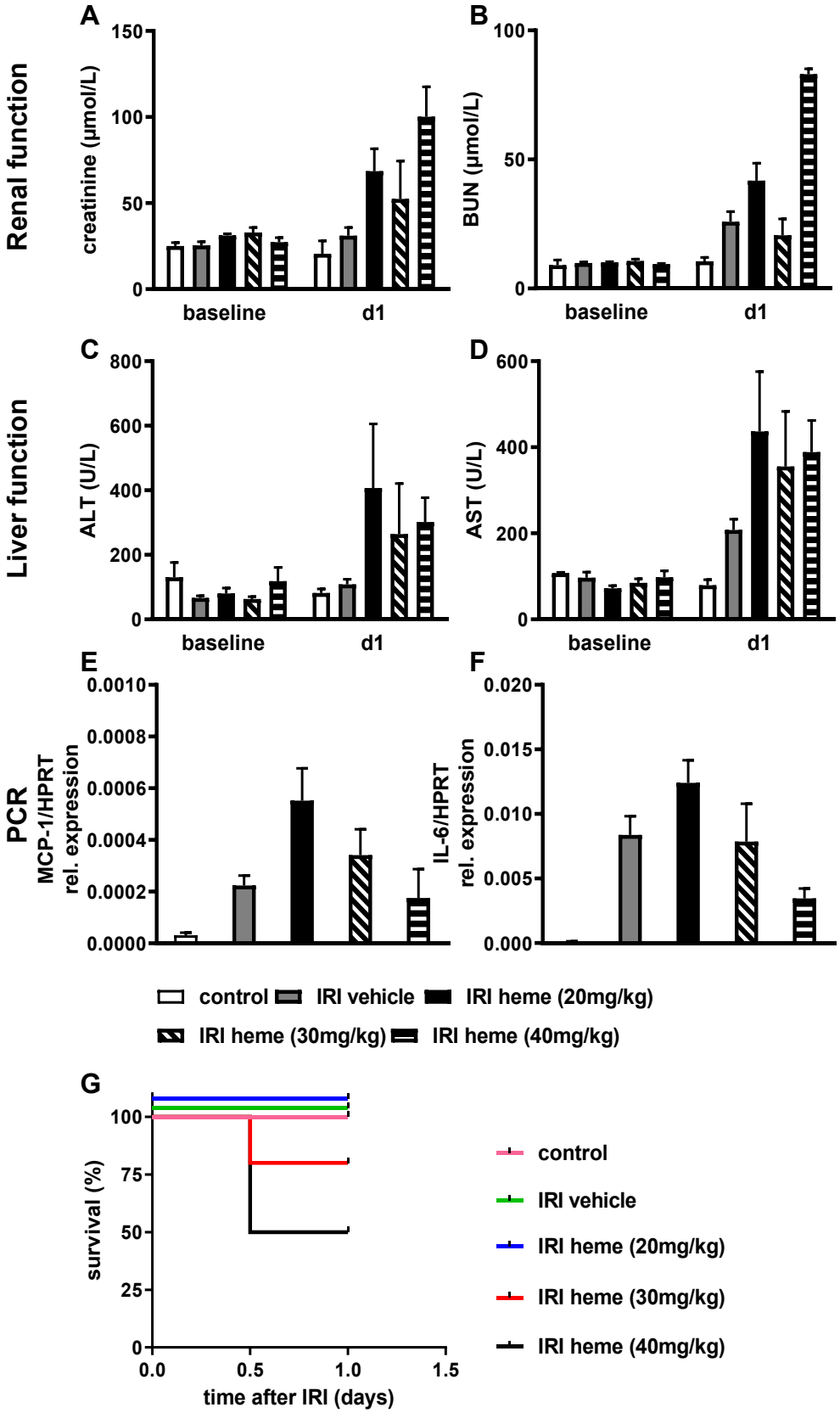

Figure 14: Escalating doses of labile heme after IRI in B6 mice – effects on clinical chemistry and pro-inflammatory cytokines

Renal function measured by creatinine and BUN (A and B), liver function by ALT and AST (C and D). The IRI labile heme (20mg/kg, black bars) group had relevant renal and liver function impairment and showed 100% survival (G). The higher heme doses caused 25 and 50%

mortality and had almost similar renal and liver function deterioration. MCP-1 and IL-6 mRNA levels (E, F) were highest in the 20mg/kg IRI heme group (black bars; mean ± SEM).

Based on these results the B6 mouse model with 15min bilateral IRI and i.v. injection of 20 mg/kg labile heme seemed to be stable and reproducible and was used for the further investigation of the molecular mechanisms of heme induced tissue injury.

4.3 Local labile heme release in the renal tissue after IRI

At d1 after IRI labile heme concentration of the kidney was measured by the apo-HRP assay at day 1 and revealed that IRI alone did not result in labile heme elevation but in the combination of IRI with iv. injection of labile heme after reperfusion there was significantly higher elevation (3-fold) of labile heme in the tissue compared to IRI vehicle or sham surgery (Fig.15A).

In the sham groups renal and liver function showed no difference when injected with vehicle or with labile heme. However, after induction of IRI additional labile heme injection caused deterioration of renal function with creatinine and BUN elevation and also liver enzyme elevation with ALT and AST increase (Fig.15B-E).

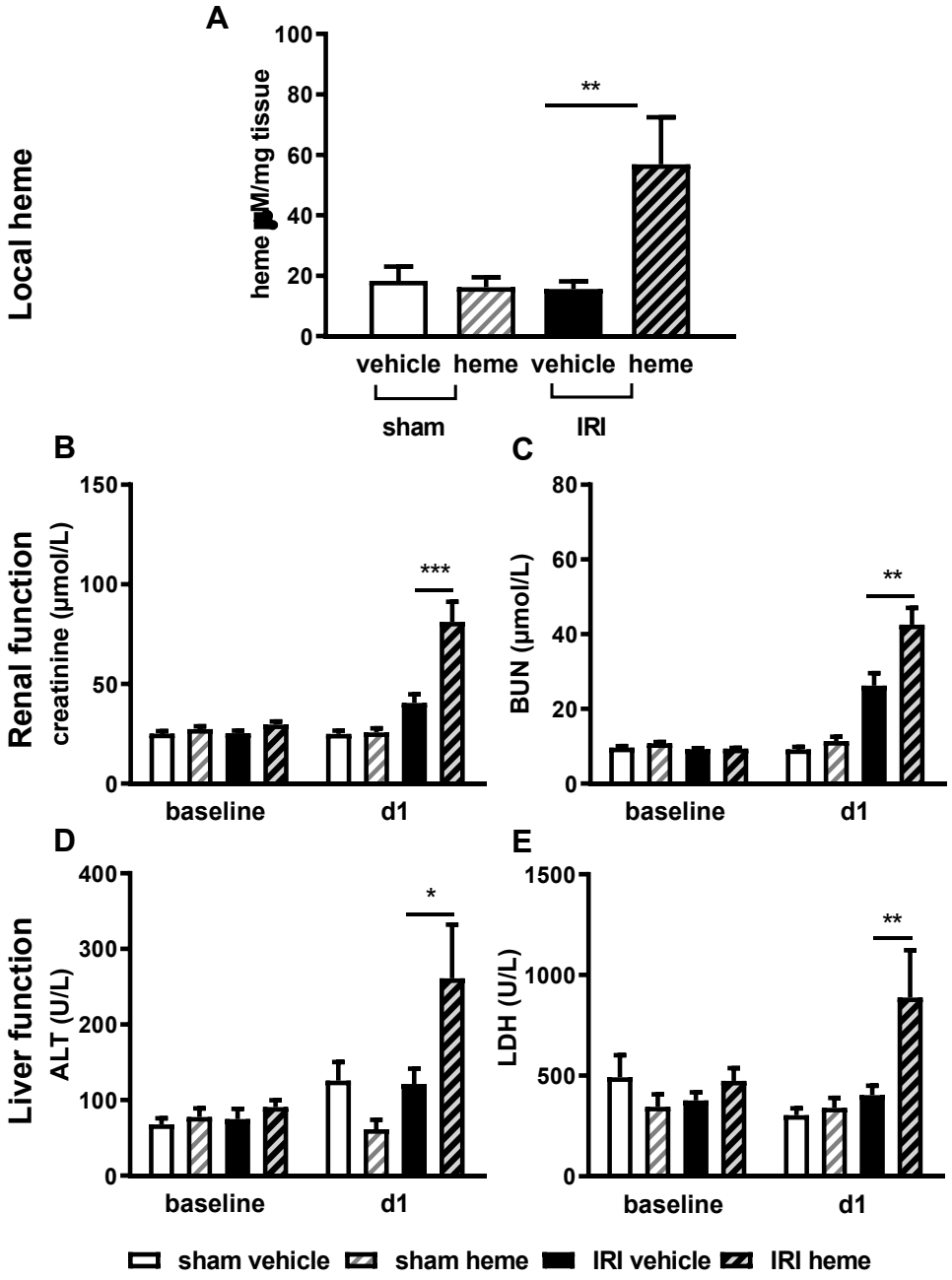

Figure 15: Effect of labile heme on renal and liver function
Labile heme levels were measured by apo-peroxidase assay. Additional heme injection (20mg/kg) after 15min IRI in B6 mice caused significant elevation of labile heme in the renal tissue at day 1 (A). Renal function impairment was aggravated after labile heme injection as measured by serum-creatinine and BUN elevation (B, C). Liver enzyme ALT (D) elevation was significantly higher after heme treatment. Similarly, LDH elevation was significantly higher (E, mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001)

4.3.1 Labile heme after IRI caused increase of pro-inflammatory cytokines

Pro-inflammatory cytokines and chemokines were analyzed by qPCR at d1 after IRI. IL-6, MCP-1, TNF-α and PAI-1 increased after labile heme infusion in both sham group and IRI group compared to vehicle treatment (Fig.16 - D).

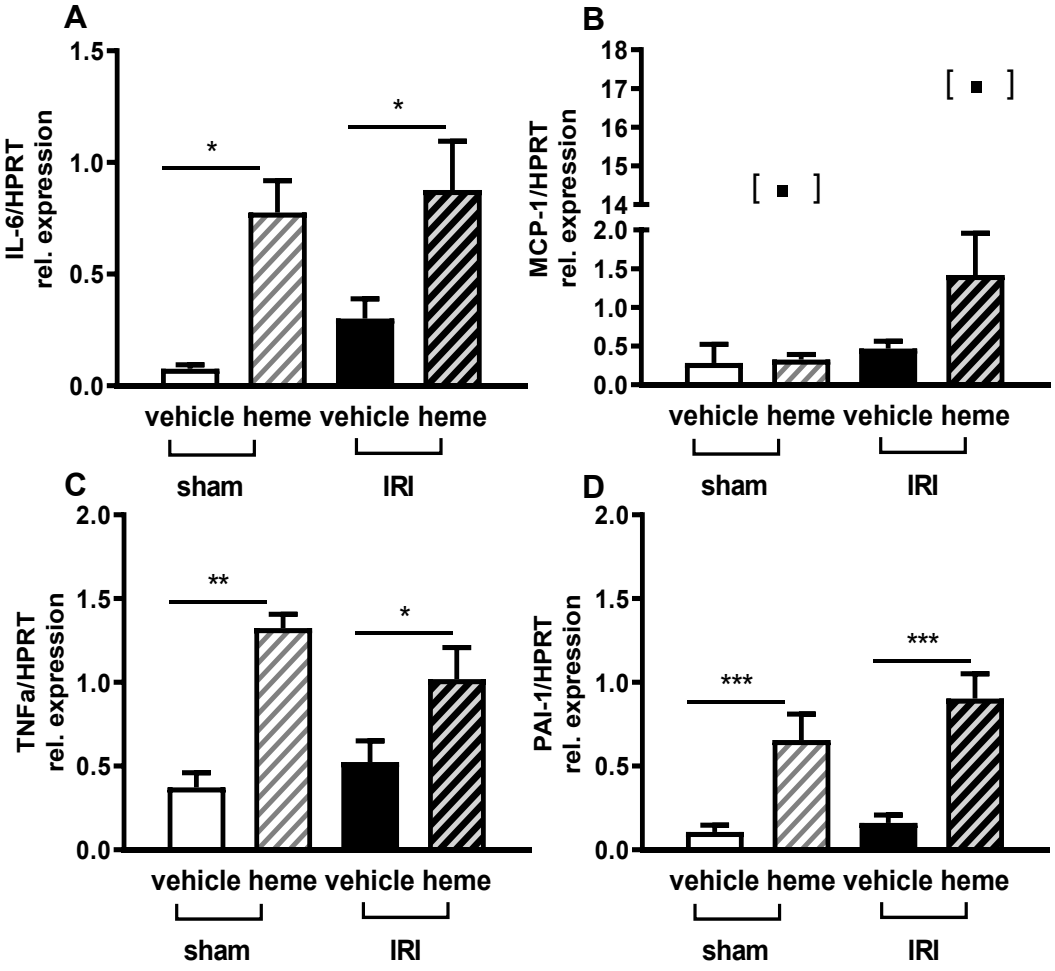

Figure 16: mRNA expression of pro-inflammatory cytokines
The mRNA expression of pro-inflammatory cytokines in renal tissue was measured by qPCR. Labile heme induced significant upregulation of the pro-inflammatory cytokines IL-6, MCP-1, TNFα and PAI-1 compared to the IRI vehicle group. Labile heme injection to the sham group also caused significant increase of IL-6, TNFα and PAI-1 compared to sham surgery alone. (mean ± SEM. *$p < 0.05$, *** $p < 0.001$).

4.3.2 Labile heme aggravated AKI and enhanced neutrophil cells infiltration

PAS staining revealed normal renal morphology in the kidneys of the sham groups with vehicle and heme treatment (Fig.17 first and second row). The kidney of IRI vehicle group displayed mild signs of AKI with mild tubular injury after IRI with minimal loss of brush border on tubular epithelial cells and detachment of these cells. (Fig.17G and H). Due to labile heme infusion, the signs of AKI were even more severe with many protein casts obstructing the damaged tubuli and enhanced inflammatory cell infiltration (Fig.17J and K). The first inflammatory cells after IRI are

neutrophils. Therefore, Gr-1 staining was done and showed that Gr-1$^+$ neutrophils accumulated in the outer medulla in the kidney after IRI. Labile heme infusion caused the most severe GR-1$^+$ neutrophil infiltration compared to all the other groups (Fig.17 L and O).

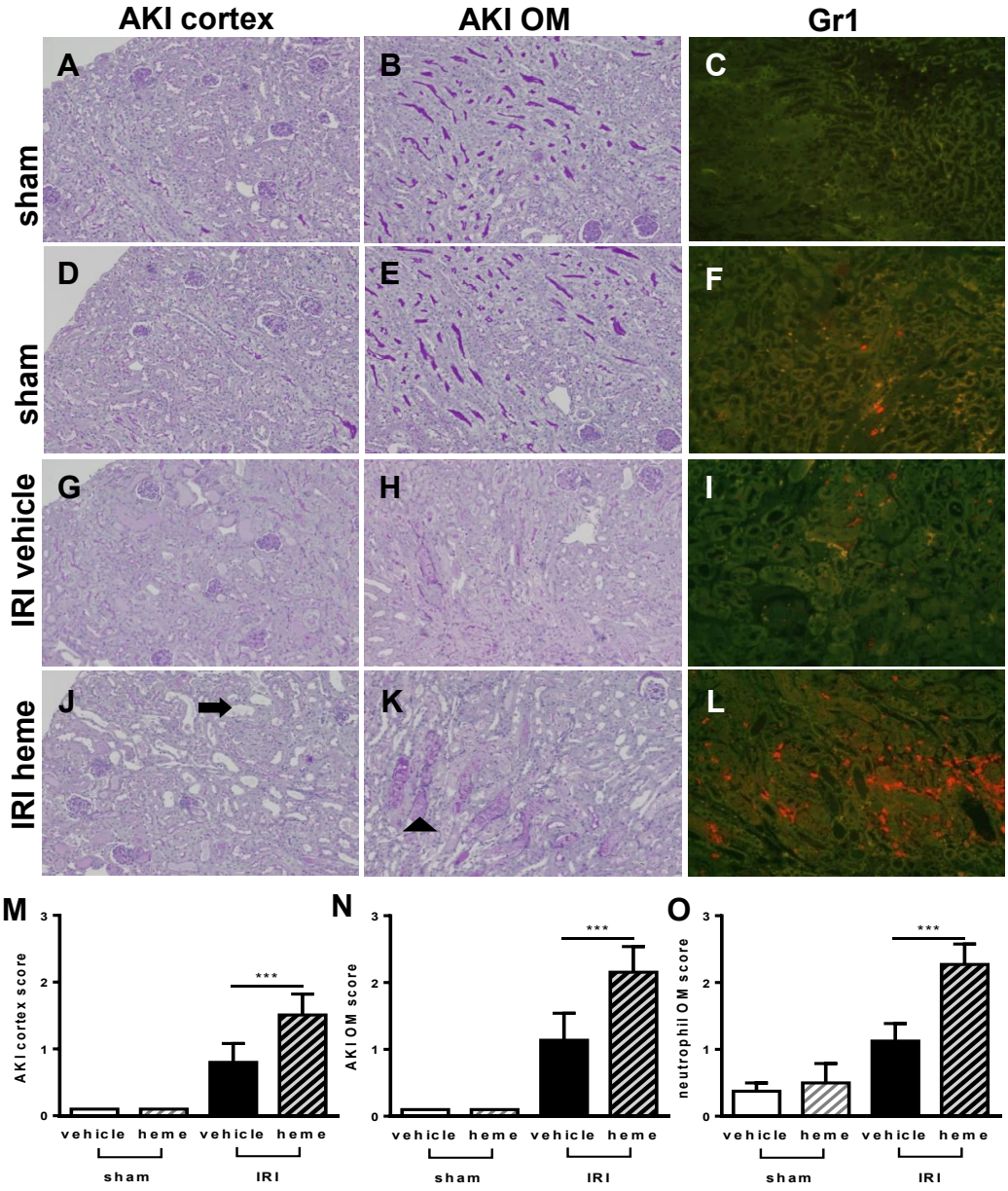

Figure 17: Effect of labile heme on AKI and the neutrophil infiltration
AKI at d1 after IRI was aggravated by labile heme injection in the cortex (left row) and even more pronounced in the outer medulla (middle row). The arrow shows tubuli with necrotic debris and the arrowhead shows cast formation. GR-1 positive neutrophils accumulated in the outer medulla and were significantly more in the IRI heme group (mean ±SEM, n=9 IRI vehicle, n=11 IRI heme, ***p < 0.001, magnification 200x).

4.3.3 Labile heme caused deterioration of kidney function and enhanced the expression of NGAL, heme oxygenase 1 and haptoglobin

NGAL is a kidney damage marker expressed in proximal tubular epithelial cells after kidney injury[83]. Immunohistochemistry of NGAL showed that the expression of NGAL was almost absent in the kidney of the sham group. However, infusion of labile heme after IRI significantly enhanced the expression of NGAL compared to the IRI vehicle group (Fig.18J and M, ***$p < 0.001$).

Heme oxygenase-1 (HO-1) is a renoprotective protein and is induced by oxidative stress and by heme. HO-1 catabolizes the conversion of heme into carbon monoxide (CO), biliverdin and iron[84]. In the sham group HO-1 expression was almost absent, upregulated after IRI and significantly increased in the IRI heme group (Fig.18H and K). Haptoglobin (hp) is an acute-phase protein and is the scavenger of free hemoglobin. Furthermore, hp is up-regulated in the tubuli of the outer medulla due to IRI. The sham group did not express hp. IRI caused an up-regulation of hp which was significantly enhanced after IRI heme (Fig.18I, L and O).

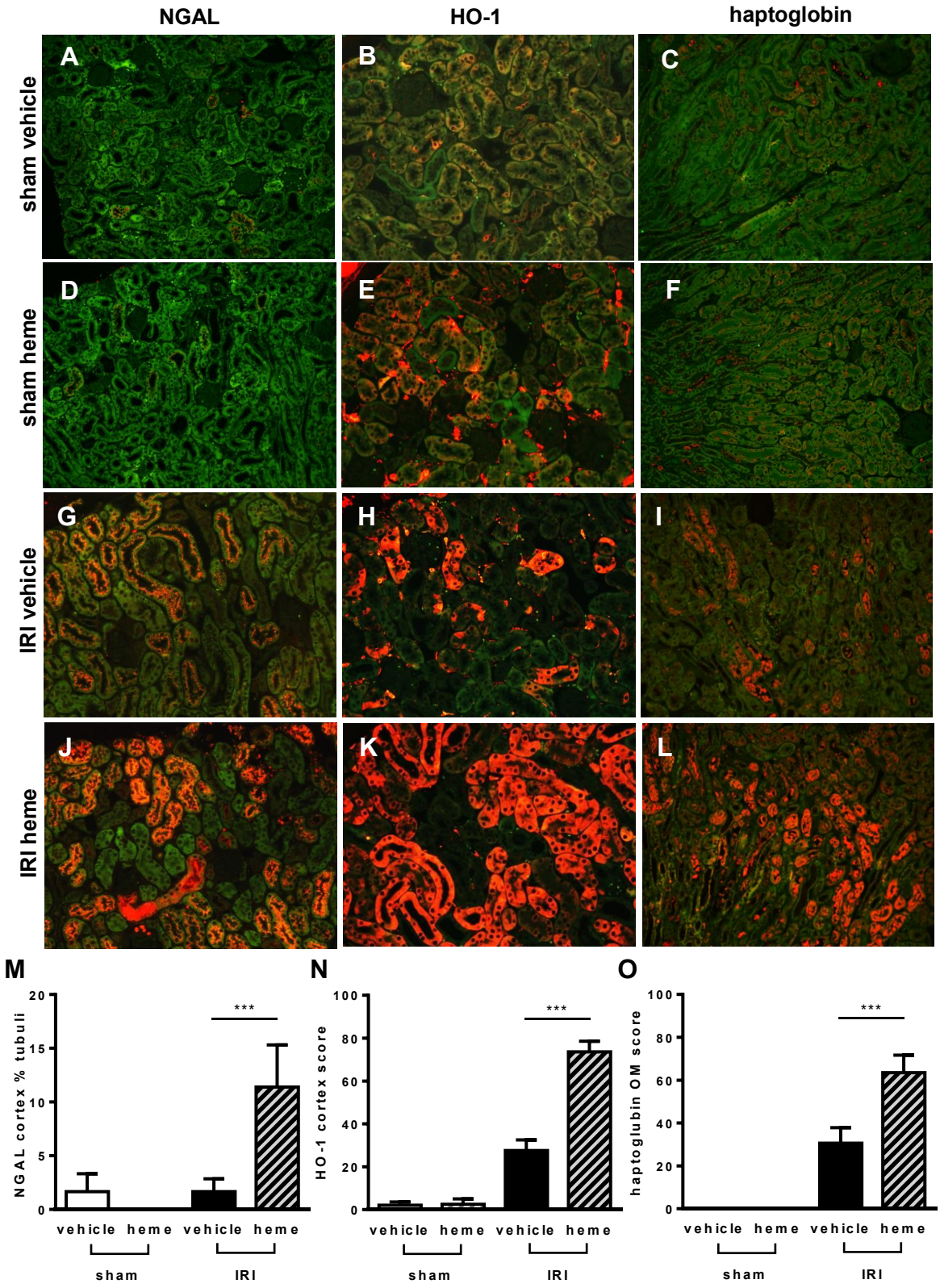

Figure 18: Effect of labile heme on the expression of NAGL, HO-1 and haptoglobin
The tubular damage marker NGAL (left column) was up-regulated in the IRI heme group. In addition, HO-1 which is strongly induced by heme was significantly higher expressed in the proximal tubuli of the IRI heme group (middle row). Haptoglobin was also significantly higher expressed in the outer medulla of the IRI heme group (left row). (mean ±SEM. ***p < 0.001, magnification 200x, , IRI vehicle n=7, IRI heme n=11).

4.3.4 Functional magnetic resonance imaging to measure renal perfusion

Renal perfusion can be quantified by functional magnetic resonance imaging (fMRI) by using arterial spin labeling (ASL). At day one day after IRI the renal perfusion impairment was compared between sham-heme mice, IRI vehicle and IRI-heme mice. The IRI vehicle group and the sham heme group exhibited similar impairment of renal perfusion. However, after labile heme infusion the renal perfusion renal perfusion deteriorated and was significantly decreased compared to the other groups. The renal perfusion decrease of the IRI labile heme group was in line with impairment of renal function and s-creatinine elevation (Fig.19, **p<0.01, ***p<0.001).

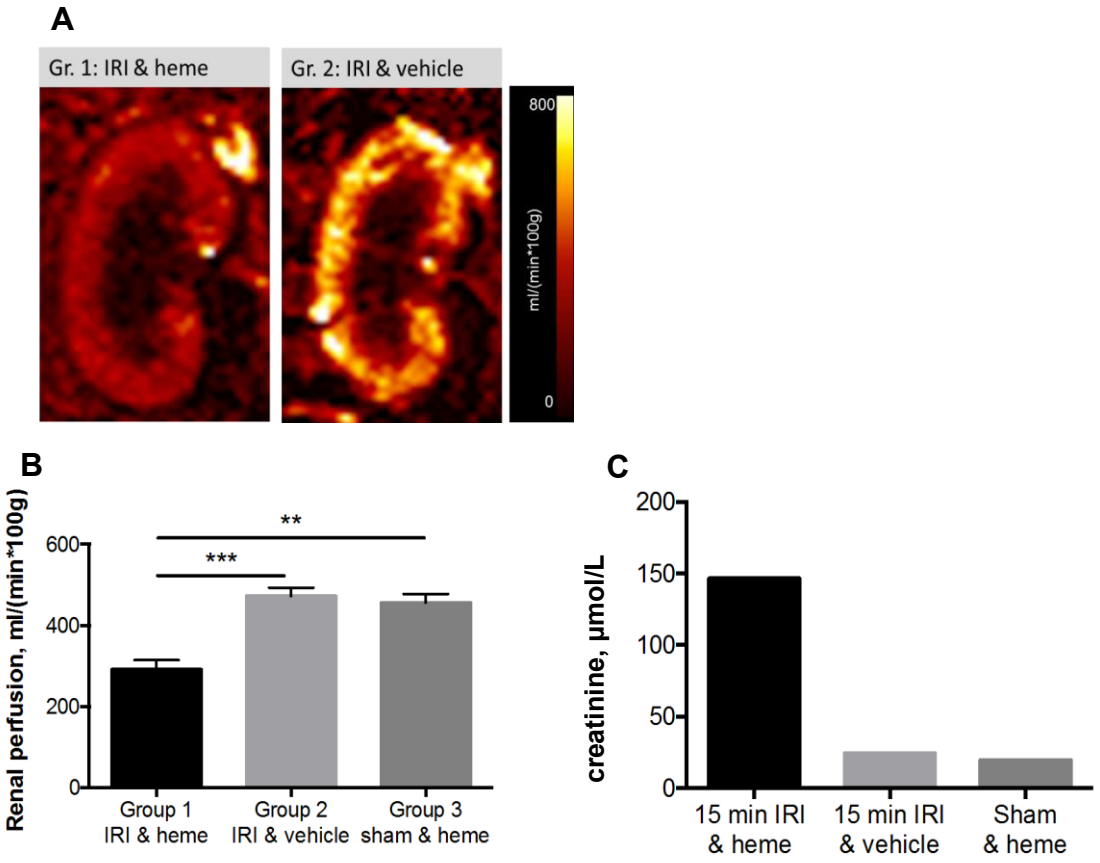

Figure 19: Functional MRI to measure renal perfusion impairment
Renal perfusion was measured by arterial spin labelling (ASL) and revealed significant impairment of renal perfusion after injection free heme at d1 after 15min IRI (A, B). Renal function measured by s-creatinine was normal in the sham and the IRI vehicle group and deteriorated in the IRI heme group (C, mean ±SEM, B: quantification in ml/min*100g kidney tissue, **p<0.01, n=7 mice per group).

4.4 Albumin treatment to bind labile heme in the IRI model

4.4.1 Effect albumin treatment on cytokine release after IRI

Prolonged IRI causes heme release locally in the renal tissue. In a model with prolonged IRI of 45min a potential treatment strategy to bind labile heme was tested. Albumin (4mg/mouse) was injected 10min prior IRI 45min iv and after 2h organ retrieval was done to investigate cytokine release. In the albumin treated IRI group, TNF-α mRNA up-regulation was significantly less compared to vehicle treatment. In addition, similar tendencies were seen in IL-6 and CXCL2/MIP2 mRNA expression (Fig.20, *p<0.05, n=6).

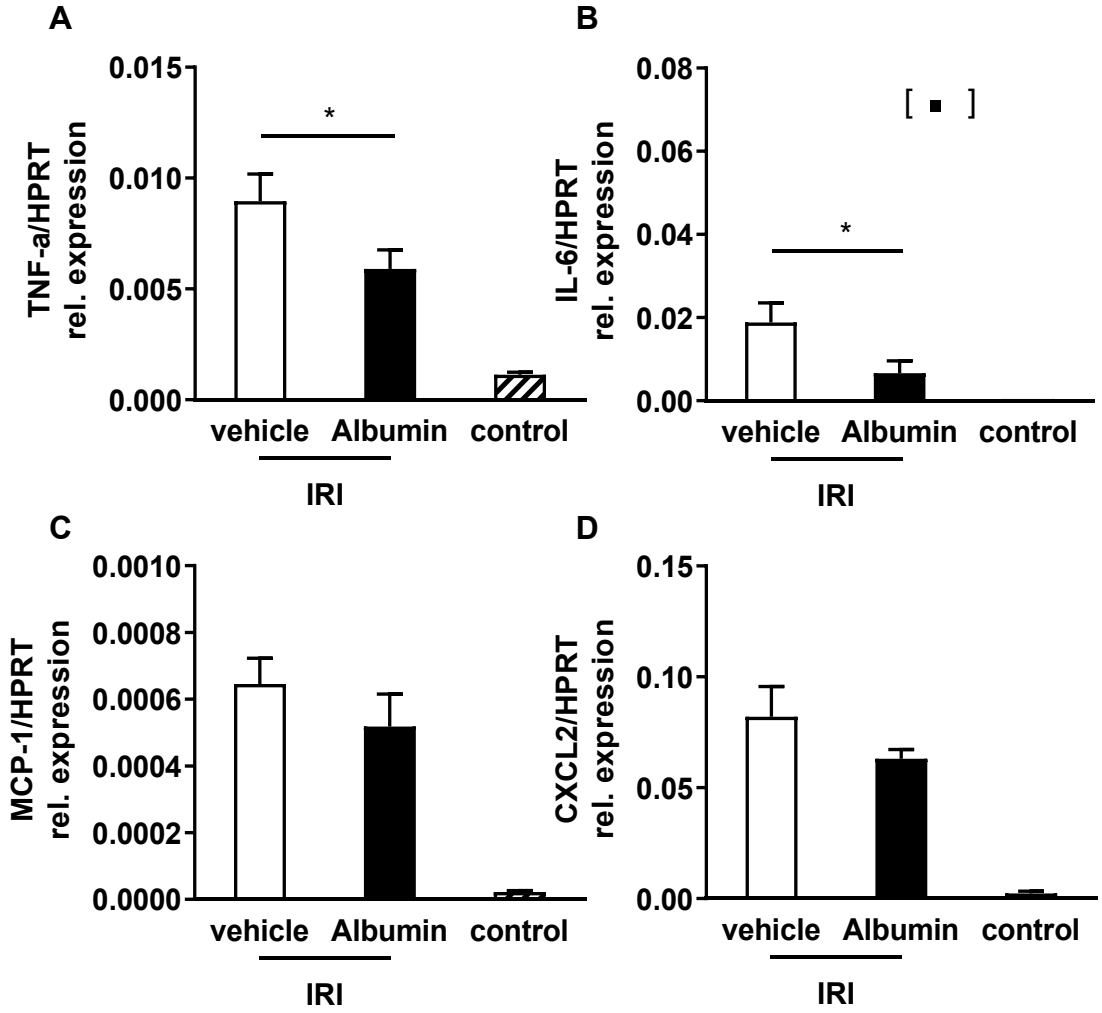

Figure 20: Albumin treatment attenuated renal IRI
Albumin pre-treatment prior to 45min IRI resulted in significantly less TNFα mRNA up-regulation in the renal tissue compared to IRI vehicle (A, *p < 0.05). IL-6, CXCL2 and MCP-1 mRNA expression was also lower after albumin pre-treatment but did not reach significance (B-D, n=6 mice per group).

4.4.2 Renal morphology

Renal morphology showed that despite pre-treatment with albumin renal damage was similar in both IRI groups. A1M expression was markedly reduced in the IRI vehicle group and was less affected in the IRI+albumin group (Fig.21 C, F, I).

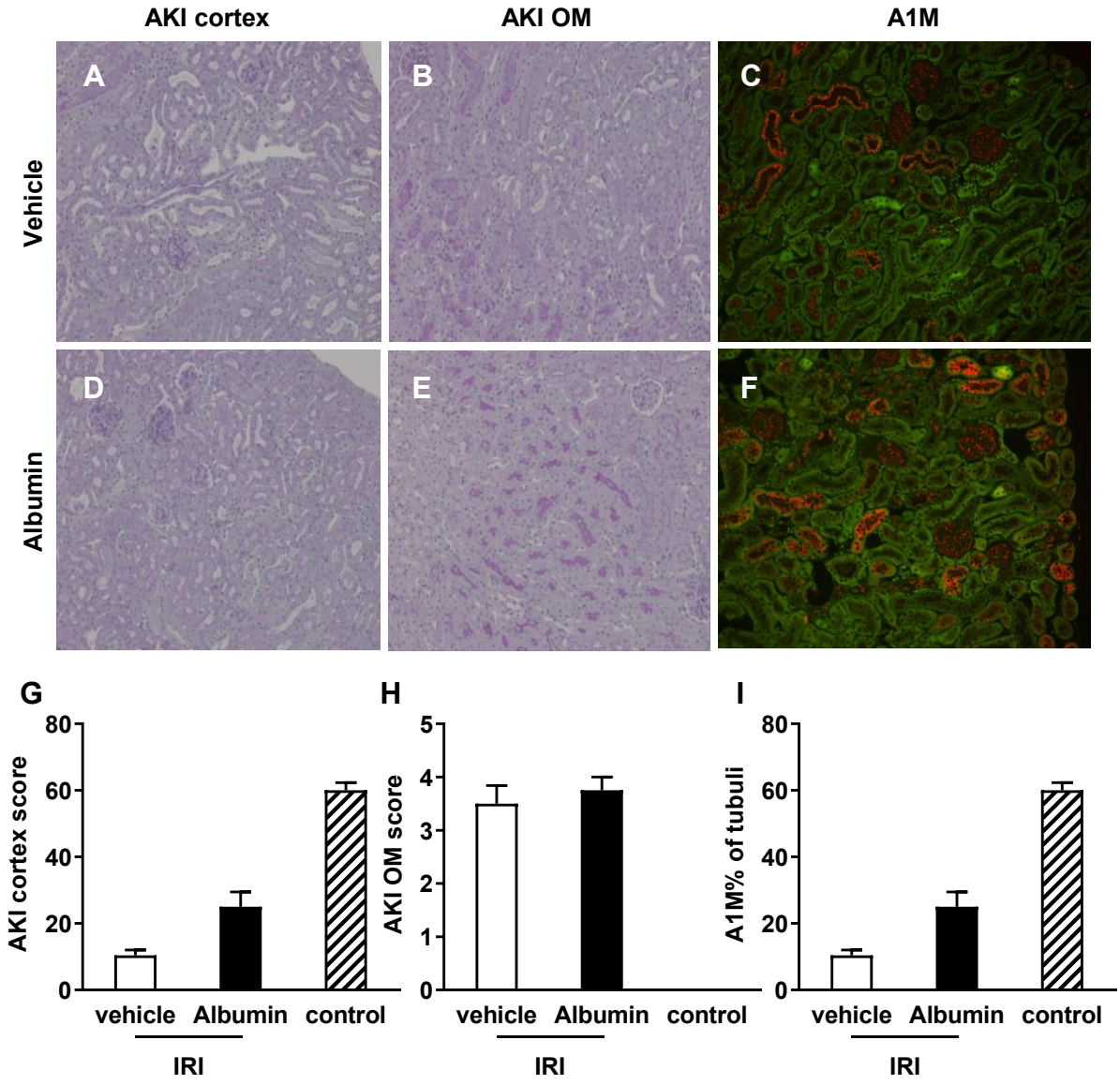

Figure 21: Albumin treatment attenuated renal IRI
Albumin pre-treatment prior to IRI 45min had a similar extent of mild AKI as IRI vehicle (left and middle row). However, A1M reabsorption as a tubular function marker was improved in IRI+albumin compared to IRI vehicle (right row, n=6 mice per group).

5 Discussion

In this study, we established a new model for free hemoglobin and labile heme induced aggravation of renal ischemia reperfusion injury leading to severe acute kidney injury (AKI). In addition, molecular mechanisms were studied and a potential treatment strategy to bind labile heme was tested. Clinically, surgery associated AKI is a relevant complication after major surgeries such as solid organ transplantation and cardiac surgery with the need of heart and lung machine. During major surgeries blood products are life-saving but can also induce inflammation. pRBCs are used widely for the surgical patients however there is no test system in clinical practice to test for RBC degradation and aging. As varying amounts of degradation products (i.e. labile heme, free iron) are released during storage testing pRBCs before transfusion would be a reasonable approach to avoid adverse effects. There are a large number of studies[38-41, 85] that describe the issue of potential consequences from transfusion of stored pRBC with a series of controversial issues. To investigate the effects of Hb degradation products on the course of AKI this new mouse model is valuable and will be used in further studies.

First, a subclinical AKI model was established to mimic the clinical situation of hypotension during surgery and temporary reduction of renal perfusion. In a pilot study 15, 20 and 25min renal ischemia time was tested in CD1 mice. This mouse strain has been shown to be very sensitive towards renal IRI[86]. The 15min IRI model caused a subclinical renal injury without elevation of serum creatinine but with mild transient signs of AKI in the renal tissue. In the next step, free Hb isolated from human pRBC (kind gift of Dr. Magnus Gram, Lund, Sweden) was injected directly after IRI when releasing the clamps. Free Hb injection resulted in a significant impairment of renal function within 24 hours after IRI. In addition, free Hb also led to

enhanced neutrophil infiltration into the renal tissue as measured by immunohistochemistry and flow cytometry showing that free Hb also promoted $CD11b^+$ leucocyte infiltration. Interestingly, neutrophil infiltration was prolonged in the IRI+ free Hb group. Normally, the neutrophils invade the kidney within 3-4 hours after injury and reach a maximum at 24h. Later they are replaced by macrophages and after 48h normally $Gr\text{-}1^+$ neutrophils are not present in the tissue any more[24]. In the free Hb IRI model Gr-1 positive neutrophils persisted in the tissue and were still detectable 7 days after IRI. In addition, free Hb caused significant up-regulation of pro-inflammatory cytokines IL-6, MCP-1 and PAI-1. To exclude uncontrolled effects based on the human source free Hb the degradation product labile heme which is highly conserved during evolution[87] was tested in further experiments. Dose dependent effects (15, 20, 30, 40 and 50 mg/kg) were investigated in two different commonly used mouse strains: CD1 and C57BL/6 (B6) mice. Renal Function impairment was more pronounced in B6 compared to CD1 mice. Similarly, the distant organ injury as observed by elevation of liver enzymes was pronounced. It has been reported previously that IRI of solid organs induces a systemic inflammatory reaction with neutrophil invasion in different organs causing organ dysfunction[21-24, 88]. In renal IRI the liver enzyme elevation correlates with the severity of AKI and was pronounced after labile heme injection. This might be in part due to the distant organ injury effects but could also be mediated by direct effects of labile heme on the liver. As seen in the free Hb IRI model also labile heme caused an upregulation of the pro-inflammatory IL-6 expression in B6 mice undergoing sham surgery. Increasing doses of labile heme were correlated with higher mortality after IRI within hours after injection. Most likely, cardiorespiratory distress was enhanced by systemic labile heme injection[89, 90]. Our data revealed that additional iv. injection of 20 mg/kg labile heme after 15 min IRI in B6 mice resulted in a stable and reproducible AKI model with elevation of pro-

inflammatory renal cytokine expression, morphological signs of AKI and increase of the tubular damage marker NGAL. In further experiments, we investigated local labile heme concentration in the renal tissue which showed that there was 3-fold increased after systemic application compared to control mice and also to sham experiments in which mice received heme after midline abdominal incision without IRI. We showed previously, that renal IRI causes impairment of renal perfusion[91 74, 92] thus functional MRI was performed to study the additional effects of free heme injection. Subclinical AKI had similar renal perfusion values compared to mice receiving sham surgery. By additional labile heme injection after IRI significant reduction of renal perfusion was measured and correlated with the serum-creatinine elevation.

This finding strongly emphasizes that labile heme promotes microcirculation disturbances which aggravate tissue hypoxia and renal damage. Results from earlier studies demonstrate a strong and consistent association, that heme and bilirubin oxidation end products are involved in the development of vasospasms by depletion of endothelial nitric oxide, oxidative stress and inflammation[93-95]. In the clinical context, even though doctors try to minimize the blood loss and are using patient blood management protocols to optimize preoperative hemoglobin status[96], certain surgeries have the risk of major bleeding and the need of pRBC transfusions. The pRBC transfusion is lifesaving especially for patients losing > 40% of the circulating blood[97-99]. However, the storage lesion still occurs during storage due to aging and individual donor specific factors which are largely unknown. While each of these storage lesions has been studied for the potential toxic effects, heme still is the major inducer of inflammatory tissue injury in many studies[100-102].

Another important finding in the study was that labile heme without IRI in sham control mice caused mild changes in pro-inflammatory cytokines. Although, the mechanism is still not fully understood, previous research has established that heme

causes reactive oxygen generation (ROS), activation of cells of the innate immune system and cell death[103]. In the labile heme exposed IRI kidneys strong up-regulation of heme oxygenase-1 (HO-1) was observed by immunostaining and on mRNA level. HO-1 is a stress-responsive enzyme that catabolizes excessive heme into CO, biliverdin and iron. HO-1 has renoprotective effects as has been demonstrated in renal allografts[84]. According to our results, the local labile heme generation in the kidney increases after IRI depending on the duration of ischemia time. In addition, IRI in combination with iv labile heme injection caused enhanced HO-1 expression in the proximal tubular epithelial cells. It has been shown that HO-1 is strongly induced by heme and this finding might also reflect protective mechanisms of the kidney after tissue injury[104].

Based on the previous results, we think labile heme plays an important role in the severity and progression of acute kidney injury. It indicates that transfusion of aged pRBC might be harmful for patients who have already impaired renal function due to CKD and who undergo major surgery or solid organ transplantation with high risk of AKI. To further prove that labile heme was responsible for the severity of AKI a rescue experiment was done. Albumin is established in clinical practice and has the potential to bind labile heme. Therefore, an experiment with prolonged IRI of 45min was done and albumin pre-treatment 10min prior surgery was tested. This experiment revealed that albumin treatment caused decreased pro-inflammatory cytokine mRNA expression in the renal tissue and better integrity of the tubular function parameter A1M corresponding to the ability to reabsorption of proteins. Further experiments will be done to test further free hb and labile heme scavengers and their potential benefit to reduce AKI. Candidates are haptoglobin as free hb scavenger and hemopexin as labile heme scavenger.

Taken together, this study showed that blood degradation products such as free

hemoglobin and labile heme have the potential to aggravate renal IRI in mice. The molecular mechanisms were enhanced neutrophil invasion into the kidney and prolonged persistence of neutrophils with enhanced production of pro-inflammatory cytokines. Furthermore, we could show by functional MRI studies that renal perfusion was significantly reduced by additional labile heme infusion after IRI. HO-1 which is strongly induced by labile heme was significantly up-regulated on macrophages and on proximal tubular epithelial cells after heme exposure. Finally, we showed that additional treatment with albumin which is one of the heme scavenging proteins has the potential to reduce inflammation in the given context. In conclusion, this study added important insights into the new role of hemoglobin degradation products and the molecular mechanisms of heme induced aggravation of renal IRI. Further studies to develop preventive treatment strategies have already been planned and will be conducted in our group.

6 Overview

Acute kidney injury (AKI) is a frequent complication after many major surgeries, especially solid organ transplantation such as lung-, heart- and liver-transplantation are associated with substantial blood loss and the need of packed red blood cell (pRBC) transfusions. Although pRBC are beneficial and in many cases lifesaving, blood products also have adverse effects. Release of toxic extracellular hemoglobin (Hb) and labile heme has been shown to contribute to acute organ (especially kidney and lung) injury. In this study, a translational mouse model was developed to investigate molecular mechanisms of free Hb and labile heme induced aggravation of a renal ischemia reperfusion injury causing AKI.

Our study was divided into three parts. First, we established a sublcinical AKI model by testing different warm ischemia times in the IRI model. Next, a combination of short IRI of 15min with additional injection of free Hb model was tested and analyzed. To optimize the model further, free Hb from human origin was replaced by the highly conserved degradation product labile heme. Two different mouse stains (CD1 and B6) were tested in the IRI – heme model and different dose response experiments were done. Finally, we established a stable and reproducible IRI-heme model and investigated renal perfusion impairment by functional MRI which revealed that additional heme injection did not only cause enhanced neutrophil influx but also potentiated renal perfusion impairment. In rescue experiments albumin treatment prior IRI+heme was tested and showed that the heme scavenger caused reduced pro-inflammatory cytokine expression and better integrity of tubular function. Taken together, we developed a new model for heme induced renal organ injury which can be used to develop better treatment strategies to overcome AKI during major surgery.

7 Abbreviations

A	alpha
A1M	Alpha-1 microglobulin
ALT	alanine aminotransferase
APC	antigen-presenting cell
apo-HRP	Apo horse radish peroxidase
AP	alternative pathway
APPs	acute-phase proteins
ATP	Adenosine triphosphate
AKI	acute kidney injury
AKIN	Acute Kidney Injury Network
Bcl-2	B-cell lymphoma 2
BP	blood pressure
BSA	Bovine Serum Albumin
BUN	blood urea nitrogen
col 1a1	Collagen, type I, alpha 1
DC	dendritic cell
CD	cluster of differentiation
cDNA	Complementary DNA
CP	Classical pathway
CIT	cold ischemia times
CKD	chronic kidney disease
CTGF	connective tissue growth factor
CXCL2	Chemokine (C-X-C motif) ligand 2
DAPI	4',6-diamidino-2-phenylindole
DGF	delayed graft function
DNA	Deoxyribonucleic acid
eF780	eFluor 780
ERK	extracellular signal–regulated kinases
ESRD	end stage renal disease
EU	European Union
FACS	Fluorescence-activated cell sorting
FAIR	flow-alternating inversion-recovery
Fe	ferrous
Fig.	figure
FITC	Fluorescein isothiocyanate
fMLP	N-Formylmethionine-leucyl-phenylalanine
fMRI	functional magnetic resonance imaging
g	gram
GFR	glomerular filtration rate
Hb	hemoglobin
HBSS	Hank's Balanced Salt Solution
HCT	Hematocrit
HIC	high-income countries
HO-1	Heme Oxygenase 1
Hp	haptoglobin
Hx	hemopexin
IV	intravenous
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFR	glomerular filtration rate

Gr-1	Ly-6G/Ly-6C
h	hours
hb	hemoglobin
HPRT	hypoxanthine guanine phosphoribosyl transferase
ICAM-1	Intercellular Adhesion Molecule 1
ICU	Intensive Care Units
IFNγ	Interferon gamma
IHC	Immunohistochemistry
IL-6	interleukin-6
LP	lectin pathway
JNK	c-Jun N-Terminal kinase
LD	life dead
LDH	lactate dehydrogenase
LMIC	low- and middle-income countries
IRI	renal ischemia reperfusion injury
MAP kinase	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
mmHg	millimeter of mercury
NF-κB	nuclear factor-κB
NGAL	neutrophil gelatinase-associated lipocalin
NK	natural killer
PAI-1	Plasminogen activator inhibitor-1
PAS	Periodic acid Schiff staining
PBS	phosphate buffer saline
PFA	paraformaldehyde
PH	potential of hydrogen
PO2	partial pressure of oxygen
pRBC	packed red blood cell
qPCR	quantitative polymerase chain reaction
RBCs	red blood cells
RNA	Ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
SAG-Manitol	saline-adenine-dextrose-mannitol
SD	standard deviation
TMB	tetramethyl benzidine
TNF-α	tumor necrosis factors-alpha
VPR	volume-pressure recording
VPR-cuff	volume–pressure recording cuff
WHO	World Health Organization
WT	Wild type

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Poster presentations at national conferences

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Poster presentations at international conferences

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International Meeting on Ischemia reperfusion Injury in Transplantation, 21-2017, Poitiers, France: Acute kidney injury (AKI) after solid organ transplantation - circulating free heme as a contributing risk factor authors

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Declaration (Erklärung)

Ich erkläre, dass ich die der Medizinischen Hochschule Hannover zur Promotion eingereichte Dissertation mit dem Titel
Molecular mechanisms of surgery associated acute kidney injury (AKI) by blood degradation products in experimental mouse models
in der Klinik für Nieren- und Hochdruckerkrankungen
unter Betreuung von Prof. Dr. med. Faikah Güler

und der Ko-Betreuung von Prof. Dr. Stephan Immenschuh

ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Die Gelegenheit zum vorliegenden Promotionsverfahren ist mir nicht kommerziell vermittelt worden. Insbesondere habe ich keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

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