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Pro-Enkephalin and Pro-Relaxin2 as Biomarkers for (Dys-)Function of Mammalian Kidney

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Abstract

Background: Our clinical understanding of acute kidney injury (AKI) has dramatically increased over the last decades, but an effective intervention for AKI is still missing. An early intervention would be desirable, since preclinical models could show a massive delay or even prevention of AKI when treated early enough, which would improve recovery and decrease mortality. New potent biomarker candidates have been identified recently: Altered plasma concentrations of pro-Enkephalin (pENK) were shown in patients with restricted renal function. Relaxin 2 (RLX2) is known to alter renal hemodynamics in pregnant women and recently the recombinant RLX2 was suggested as therapeutic intervention in several diseases involving renal dysfunction.

Methods: 3 new assay systems were developed and termed pENKrat, pENKurine and pro-RLX2. Paraphinated slices of healthy human renal tissue were examined for pENK expression using immunohistochemical procedures. $pENK_{plasma}$ concentrations were determined with the pENKrat assay in rat models of gentamycin-induced nephrotoxicity and CLP-induced sepsis. $pENK_{plasma}$ and $pENK_{urine}$ concentrations were determined in healthy kidney donors and corresponding recipients with sphingotest® penKid and the $pENK_{urine}$ assay. $pENK_{plasma}$ and pro-RLX2 plasma concentrations were determined in healthy individuals using the sphingotest® penKid and pro-RLX2. pro-RLX2 plasma concentrations were determined in pregnant women with uncomplicated and preeclamptic pregnancy as well as in non-pregnant individuals with acute and chronic heart failure with pro-RLX2.

Results: PENK expression was localized throughout the renal tubules even though results were not reproducible.

In different rat studies the association of $pENK_{plasma}$ concentration with renal dysfunction was shown by specific increase of $pENK_{plasma}$ concentration by induction of selective renal injury. $pENK_{urine}$ concentrations were associated with renal injury, too. In pregnancy, $pENK_{plasma}$ was shown to vary with gestational age.

Even though measurability of pro-RLX2 in non-pregnant adults was restricted, a weak connection of pro-RLX2 plasma concentration with renal dysfunction could be shown in patients with heart failure. In pregnancy, pro-RLX2 decreased with increasing gestational age, but was shown to be a poor marker of preeclampsia.

Conclusion: A combination of $pENK_{plasma}$ and $pENK_{urine}$ measurement could be a promising approach to achieve an earlier recognition of AKI. Nevertheless, the function pENK exhibits within the kidney remains unclear.

pro-RLX2 was shown to be less useful as biomarker, despite being directly involved in pathology of cardiorenal injury. Exogenous RLX2 (Serelaxin) is a promising approach for new therapeutic action, but monitoring of endogenous pro-RLX2 is difficult.

Abstract

Hintergrund: Unser klinisches Verständnis von akutem Nierenversagen (AKI) hat über die letzten Jahrzehnte dramatisch zugenommen, aber eine effektive Intervention für AKI fehlt noch. Eine frühe Intervention wäre erstrebenswert, da in prä-klinischen Modellen eine massive Verzögerung oder sogar Verhinderung von AKI gezeigt wurde, wenn früh genug behandelt wurde. Dies würde die Genesung verbessern und Mortalität verringern. Neue, vielversprechende Biomarker-Kandidaten wurden kürzlich entdeckt: Veränderte pro-Enkephalin(pENK)-Plasmakonzentrationen wurden in Patienten mit verringerter Nierenfunktion gemessen. Relaxin 2 (RLX2) ist dafür bekannt, bei Schwangeren die renale Hämodynamik zu verändern und wurde als therapeutische Intervention in verschiedenen Erkrankungen vorgeschlagen, die die Nierenfunktion betreffen.

Methoden: 3 neue Assaysysteme wurden entwickelt und pENKrat, pENKurine und pro-RLX2 genannt. Paraffinierte Präparate von gesundem, menschlichen Nierengewebe wurden mittels Immunohistochemie auf pENK-Expression untersucht. pENK-Plasmakonzentrationen wurden mittels pENKrat Assay bestimmt in Rattenmodellen von Gentamycin-induzierter Nephrotoxizität und CLP-induzierter Sepsis. pENK-Plasma- und Urin-Konzentrationen wurden mittels sphingotest® penKid und pENKurine-Assay bei gesunden Nierenspendern und den zugehörigen Empfängern bestimmt. pENK- und pro-RLX2-Plasmakonzentrationen wurden bei gesunden Individuen bestimmt. Pro-RLX2-Plasmakonzentrationen wurden bei Schwangeren mit unkomplizierter und preeklamptischer Schwangerschaft sowie bei Nicht-Schwangeren mit akutem und chronischem Herzversagen untersucht.

Ergebnisse: Die PENK-Expression wurde in den Nierentubuli lokalisiert, obwohl die Ergebnisse sich nicht reproduzieren ließen. In verschiedenen Rattenstudien wurde durch einen spezifischen Anstieg der pENK Plasmakonzentration durch die Induktion von selektiven Nierenschäden die Assoziation von pENK-Plasmakonzentration mit Nierendysfunktion gezeigt. Die pENK-Urinkonzentration war ebenfalls mit Nierenschäden assoziiert. Bei Schwangeren variiert pENK Plasma mit der Schwangerschaftswoche. Obwohl die Messbarkeit von pro-RLX2 in Nicht-Schwangeren limitiert ist, konnte ein schwacher Zusammenhang mit Nierendysfunktion in Patienten mit Herzversagen gezeigt werden. In Schwangeren sank pro-RLX2 mit steigender Schwangerschaftswoche, es stellte sich aber als ein schwacher Marker für Preeklampsie heraus.

Schlussfolgerung: Eine Kombination von pENK Plasma- und Urin-Messung könnte ein vielversprechender Ansatz sein, um AKI früher zu erkennen. Nichtsdestotrotz ist die Funktion von pENK in der Niere weiterhin unklar. pro-RLX2 ist als Biomarker ungeeignet, obwohl es direkt in die Pathologie kardiorenaler Schäden involviert scheint.

Exogenes RLX2 (Serelaxin) ist ein vielversprechender Ansatz für therapeutische Interventionen, aber die Messung von endogenem pro-RLX2 ist problematisch.

Contents

I.

Background

1. Kidney physiology

1.1. Functions in non-pregnant adult (physiological state)

The mammalian kidney might be one of the most specialized organs of the body. Numerous highly specialized cells work together to ensure a sufficient balance of volume and electrolytes within the body fluids. In this chapter only a brief overview of the renal function and its regulation is given.

1.1.1. Renal anatomy

For understanding the renal anatomy, it is helpful to first follow the urine flow through the segments before putting these segments into a three dimensional structure: Blood enters the kidney though the arteria renalis which is expanded to a network of so called vasae afferentes. These vasae afferentes pass through the glomeruli, the "renal sieves", becoming vasae efferentes. Within the glomerulum a high portion of the renal blood is filtered into the proximal tubules, leaving behind a relatively concentrated liquid containing and cells which cannot pass the glomerulum due to a relatively tight barrier called fenestrated or split-pore membrane formed by specialized cells (podocytes) . The filtrate, called primary urine, passes the proximal tubules which are situated in close proximity to the vas efferens and is subsequently cleared from sodium, chloride and other electrolytes which are reabsorbed by the proximal tubules and released back into the *v*as efferens while metabolites like ammonia are secreted into the urine stream. Water is subsequently forced to follow the electrolytes by osmotic pressure, so the urine remains isotonic. This reabsorption/secretion is tightly regulated through several different mechanisms depending on the current body fluid state. The filtrate now containing a relatively low concentration of electrolytes passes the intermediate tubules (Henle loop) and through osmotic pressure more water is reabsorbed by the descending Henle loop while urea is secreted. The resulting higher concentrated filtrate is subsequently purified further from sodium and calcium kations within the ascending Henle loop - which is impermeable to water - while ammonia kations and potassium anions are secreted. The distal tubules and especially the

collecting tubule then account for the fine-tuning in homeostatic fluid balance, which is again tightly regulated by hormonal systems. [43, pp. 148-166]

Fig. 1.1 shows the two dimensional positioning of the tubular system as well as its three dimensional order within the kidney. This positioning is required to obtain the osmotic balances that drive the reabsorption and secretion processes within the kidney. According to their diversified functions, renal cells differ highly from each other in shape, polarization and internal composition (e.g. ion channels expressed on their surface).

1.1.2. Hormonal regulation

As mentioned above kidney function highly depends on hormonal regulation. Even though more and more mechanisms and interactions of hormonal regulation are elucidated these days, for this thesis I would like to focus very briefly on the cardio-renal axis.

1.1.2.1. Cardio-renal axis

As our understanding of hormonal regulation of the kidney increases, more and more evidence accumulates that it would be misleading to examine the kidney without taking the heart into account. Kidney and heart function are tightly interconnected via hydrostatic pressure and volume load within the circulation. A change in cardiac output directly influences renin-angiotensin-aldosterone system (RAAS) via the atrial natriuretic peptide, vasopressor secretion and the sympathetic nervous system which directly change renal filtration and reabsorption rate [171]. On the opposite side, renal reabsorption directly impacts on cardiac preload and therefore changes cardiac function [202]. Fig. 1.2 shows a very general overview of the underlying mechanisms in case of chronic cardiac failure.

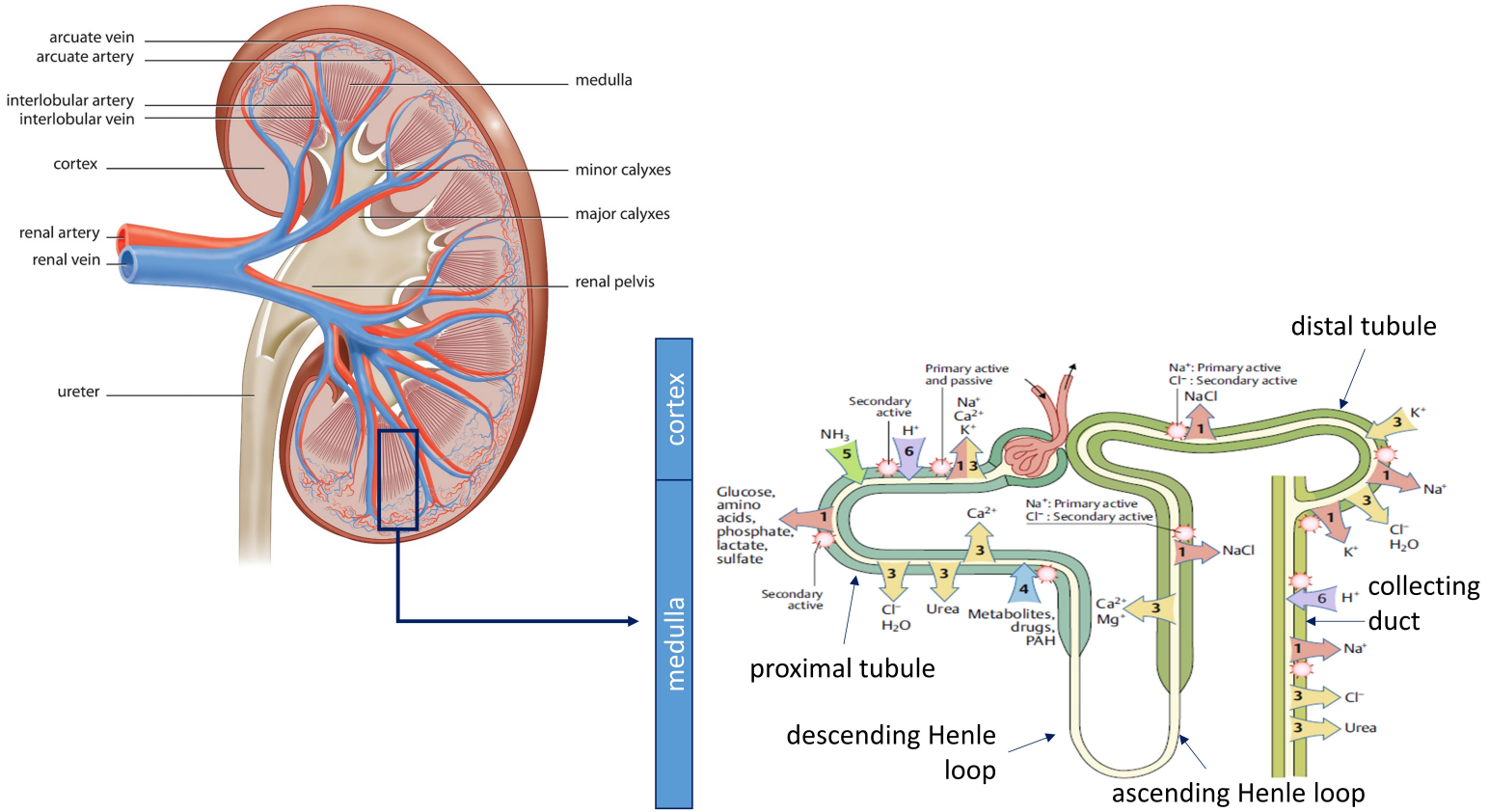


Figure 1.1.: **Renal anatomy** (figure modified from Despopoulos 2009 [43, p. 155]). The kidney is segmented into different parts by interlobular arteries and veins. Each segment consists of cortex and medulla which contain different parts of the tubule system. The primary urine is passing these different segments getting most of it's electrolytes and water reabsorbed through an tightly regulated process before leaving the collecting duct for excretion as secondary (final) urine.

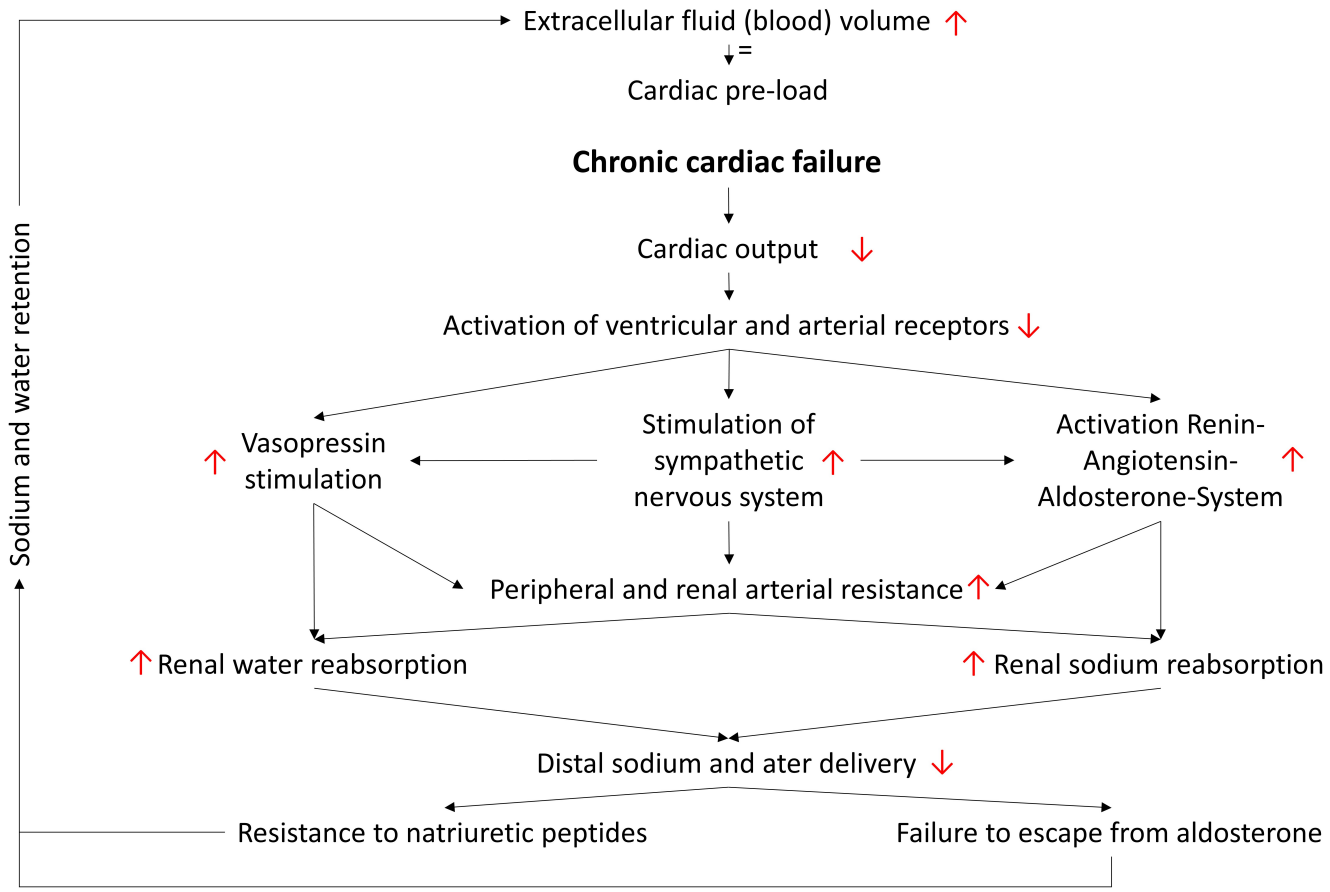


Figure 1.2.: **Cardio-renal axis** in case of chronic cardiac failure (modified from [171]). Cardiac output directly influences several systems regulating renal balance of water and sodium retention. The folllowing maintenance of arterial circulatory integrity has direct impact on the cardiac pre-load and hence the cardiac output again. Bottom-up arrows indicate an overall increase, top-down arrows a decrease.

1.2. Hormonal functions in renal and cardiac injury

1.2.1. Renal injury (pathological state)

Renal injury implements cell cycle, immunity, inflammatory and apoptosis processes which are originally meant to repair the initial injury but on the longer term may even aggravate it [80, 161]. Caused mainly by systemic problems like sepsis (47% of all acute kidney injury AKI), hypovolemia (25%), surgery (34%), cardiogenic shock (27%) and drugs (19%) [24], the injury induces structural changes within the kidney: apoptosis of tubular and glomerular cells produced directly by the injury lead to tubular loss and glomerulosclerosis. Different mechanisms also generate a "denuding" of the glomerular capillary which leads to hyperfiltration of the glomerulum. This is turn causes a hypertrophy of the tubuli which leads to further apoptosis and loss of nephrons. The glomerular injury decreases efferent blood flow which leads to a decrease in tubular blood supply. Fibrosis in the tubular interstitium further aggravates this hypoxic state especially within the distal nephron. At the same time, the per-nephron requirement of energy is increased and cannot be covered anymore by the inefficient perfusion. This leads to an anaerobic metabolism which can cause acidosis and generation of reactive oxygen species (ROS) which again deteriorate the initial injury.[161]

At the same time, inflammatory processes are triggered by necrosis within the kidney to promote repair of injured cells [143]: pro-inflammatory substances are transported to the distal nephron, reabsorbed and concentrated where they further trigger inflammatory response [161]. Macrophages are recruited to the tubulointerstitium where they remove injured cell but also promote fibrosis [161]. This again leads to loss of nephrons and reduced capillary perfusion. The overall inflammatory state also triggers ROS production which increases total peripheral resistance and in turn further decreases renal blood flow [161, 67].

While most of these processes are meant to stabilize the renal function temporarily, cell cycle mechanisms are triggered after injury to repair injured cells. After injury many terminally differentiated tubular cells re-enter cell cycle. Nevertheless it is unclear whether these processes in the long term finally lead to repair or further injury.[215]

Since the kidney plays a pivotal role in blood pressure control through the RAAS. Contrary, the RAAS is influenced as well by renal lesions inducing an exacerbated renal dysfunction and systemic hypertension. (Especially intrarenal) Angiotensin 2 (AT2) is enhanced in glomerulonephritis and acts on smooth vascular muscle cells causing vasoconstriction [193, 126]. The following development

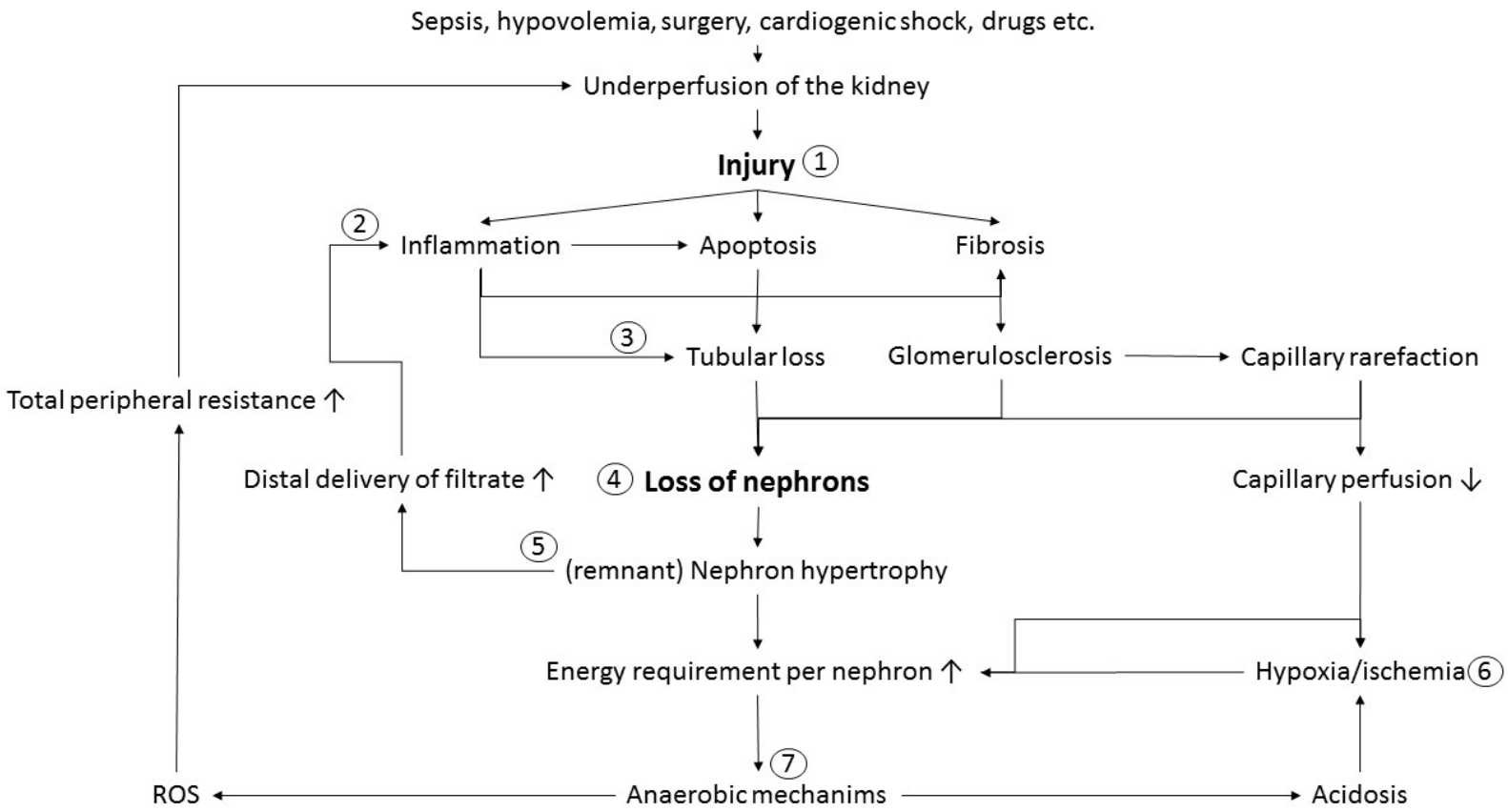


Figure 1.3.: **Mechanisms involved in renal injury and nephron loss (modified from Schnaper 2013 [161]).** Injury to the nephron affecting glomerulus and tubule (1) leads to a set of reactions (2) that in turn generate structural consequences (3) and nephron failure (4). The compensatory response involves nephron hypertrophy (5) which increases metabolic activity in the presence of reduced substrate (6) leads to ischemia/hypoxia, acidosis, and the generation of ROS (7). These factors then cause further nephron injury and loss.

of glomerular capillary hypertension and reduced renal blood flow aggravates the underperfusion of the kidney described above (point 1 and 6 in Fig. 1.3). Furthermore, AT2 increases the sensitivity of the tubulo-glomerular feedback leading to further renal blood flow and glomerular filtration reduction. [126] Another non-hemodynamic effect of AT2 is modulation of chemotaxis, proliferation and differentiation of immune cells triggering the overall inflammatory state (point 2 left side) [193, 126].

Aldosterone promotes fibrosis (point 2 right side) as well as loss of glomerular podocytes. The following increased permeability of the split-pore leads to proteinuria. Furthermore, aldosterone induces oxidative stress in tubular and interstitial tissue (point 7). [126]

Even though studies showed correlations of severity of renal damage with renin and AT2 induction as well as correlations of glomerulosclerosis with AT2 expression level, the intrarenal RAAS in renal damage might demonstrate species-specific differences even between different mammals [126].

1.3. Renal functions in pregnant women

1.3.1. In healthy pregnancy (modified physiological state)

Normal pregnancy is associated with plasma volume expansion which is extremely important for sufficient supply and normal development of the developing offspring. The physiologic changes include the neurohumoral status, systemic and renal hemodynamics and changes in water and sodium balance leading to a unique state of arterial under-filling which mirrors in several ways pathological states like the cardiorenal syndrome. Nevertheless, it shows several unique features like escape from sodium-retaining effects of aldosterone, increase in renal plasma flow (RPF) and glomerular filtration rate (GFR) and decrease in systemic vascular resistance (SVR).

Very early in gestation, the gestational hormone relaxin is released mainly by the corpus luteum and causes a relative decrease in SVR and consequently in mean arterial pressure [22]. Thus, RAAS is activated and compensatorily cardiac output increases. Simultaneously, the threshold for (non-osmotic) arginin-vasopressin (AVP) secretion and thirst is "reset" at an osmolality level by relaxin binding within the brain which increases the water reabsorption in collecting duct via aquaporins. Free water is steadily retained, the total body water rises and plasma gets hypoosmolar.

These systemic hemodynamics also influence the renal hemodynamics: normal kidney is regulated not primarily in response to total blood volume, but rather responds to the effective arterial blood volume (EABV). Systemic vasodilation decreases EABV even while cardiac output and total blood volume is increased. RAAS is activated and compensatory sodium and water increase/retention are increased. This leads to a further increase in total blood volume. Concurrently, the volume expansion increases atrial natriuretic peptide (ANP) secretion which balances the sodium reabsorption. Relaxin also causes a rise in GFR which increases distal sodium delivery and causes an escape from aldosterone. A further balancing mechanism is vascular insensitivity to vasoconstrictive action of AT2 regulation occurring in pregnancy which might be another effect of relaxin [189].

Relaxin effects and pathways are described in detail in section 4.2.2

1.3.2. In preeclampsia (pathological state)

Preeclampsia (PE) is defined as an arterial hypertension associated with proteinuria and/or edema in pregnant women and is reported as one of the major causes of maternal and fetal mortality with more than 75,000 maternal death

cases yearly [78, 128]. Usually PE does not occur before 20th week of pregnancy and affected women mostly require no dialysis and recover completely after pregnancy [195]. 3 to 6% of pregnant women develop PE and 5 to 10% of these patients develop AKI [128]. The American College of Obstetrics and Gynecology defined PE as following: "new onset-hypertension (blood pressure consistently $<$140/90mmHg) after 20 weeks of gestation accompanied by evidence of organ injury (proteinuria, thrombocytopenia, renal or liver impairment, pulmonary edema, or cerebral/visual symptoms suggesting cerebral cortical dysfunction)" [115]. Even though diagnosis of PE is quite clear, prediction and accordingly prevention is much trickier: Recent large-scale biomarker studies have not yielded any useful biomarker to predict PE [128]. To date, therapies include symptomatic treatments like anti-hypertensive drug, oral antioxidants, calcium supplementation and low-dose aspirin. In worse cases only delivery of the placenta (and thus the child) is able to really cure all symptoms [128]. Current discussions propose PE being rather a syndrome consisting of several different etiologies and pathologies than a single disease, which could explain the difficulty to define PE through a biomarker panel even in large-scale studies [128].

Basically, literature agrees on the placenta being the major cause of PE: an *impaired placentation* leads to increased utero-placental resistance which causes impaired perfusion of placenta. Following, the placenta releases several factors damaging maternal vasculature - currently angiogenic factors are proposed to be the main factors disrupting the angiogenic balance [128]. Another common view of PE is as *vascular disease*: pregnancy therefore can be seen a "stress test"[128] for the vascular system where pre-existing vascular dysfunction (e.g. chronic hypertension, pre-gestational or gestational diabetes, increased BMI) make women more susceptible to PE and subsequently to cardiovascular disease after pregnancy [128, 149].

Clearly, also *RAAS* is involved in development of PE when increased activity of the AT1 receptor due to increased decidual expression and activation by agonistic auto-antibodies against AT1 receptor lead to activation of the RAAS. In parallel, circulating AT2 is decreased in PE while vascular sensitivity to AT2 is elevated in hypertensive pregnant women which might lead to further increase in RAAS activation. Besides it's normal physiologic actions (increase in blood volume), in PE RAAS is suggested to activate release of soluble fms-like tyrosine kinase-1 (sflt-1) as well which provides a link to the angiogenic balance hypothesis [197].

In normal pregnancy, the *angiogenic factors* vascular endothelial growth factor (VEGF) promotes angiogenesis while placental growth factor (PIGF) stabilizes

endothelium in mature blood vessels, both binding to their receptor flt-1. Soluble sflt-1 is released by the placenta to antagonize VEGF and PIGF for balancing uteroplacental vascular tone and permeability[128]. In PE these processes are dysbalanced: sflt-1 production is increased by yet undiscovered unknown reasons, potentially by AT1 receptor auto-antibodies or the hypoxic placenta. Endoglin as another anti-angiogenic protein is increased in PE through unknown mechanisms as well [197].

2. Kidney Injury

2.1. Relevance to diagnose kidney injury and to forecast worsening of renal function

Even though the reported incidence of acute kidney injury (AKI) may vary in literature, there is a strong trend for increasing incidence over the last decades. Xue [213] calculated a 11% increase per year which may be mainly due to the aggravation of risk factors for AKI such as hypertension, cardiovascular disease and diabetes in an aging world population with increasing rates of obesity and metabolic syndrome [93]. Table 2.1 shows some examples for reported incidence rates for hospitalized and intensive care unit (ICU) patients. Although they vary depending on the definition of AKI and the specific sub-population, the mortality associated with AKI reported in literature is very high globally.

Table 2.1.: Incidence and mortality rates of acute kidney injury reported in literature are varying depending on the definition of AKI and the specific sub population.

Citation	incidence hospitalized	incidence ICU	mortality
Mazzone 2016 [112]	1-40%		
Wasung 2015 [204]		4-6%	60-90%
Waldum 2013 [202]	20%	>35%	
De Geus 2012[39]			60.2%
Bonventre 2011 [11]	2-7%	5-10%	>50%
McIlroy 2010 [114]	1-32%	10-90%	
Ronco 2010 [150]		12-49%	
Wan 2008 [203]		35%	
Liu 2007 [100]			40-60%
Devarajan 2007 [44]	5%	30-50%	
Rosner 2006 [152]	1-30%		
Lameire 2005 [92]		4-9%	
Schrier 2004 [163]			50%
Han 2002 [66]			50-70%

Even though our clinical understanding of AKI and renal pathology has dramatically increased over the last decades, an effective intervention for AKI is still missing [164, 98]. Especially an early intervention would be desirable since preclin-

ical models could show a massive delay or even prevention of AKI when treated early enough [164]. Furthermore, duration of AKI has been shown to be associated with mortality and an early intervention could decrease mortality [119]. Therefore the time frame of effective intervention with approximately 48 hours after AKI is pretty short [114, 203, 188]. The prediction of therapy success remains difficult as well, especially in critically ill patients where stabilization of renal blood flow is particularly difficult [163, 177]. One of the main problems of executing an effective intervention is the unacceptable delay in therapy due to delayed diagnosis [44]. Hence, an earlier recognition of AKI would improve recovery and decrease mortality [66, 52]. Within the last years there has been plenty of research in finding new biomarkers for an early and reliable diagnosis of AKI.

2.2. Status quo: Overview over current biomarkers for kidney injury and their problems

Wasung defined a biomarker as "a parameter of structural, biochemical, physiologic or genetic change that indicates the presence, severity or progress of a disease" [204]. For kidney injury there are several biomarkers currently available which should be briefly introduced in the next section regarding their mechanism of action, their benefits and problems. A list of eligible features for new biomarkers will conclude this chapter.

Serum and urine Creatinine The current gold standard in clinics is the measurement of creatinine in serum (sCrea) as well as in urine (uCrea). As a muscular metabolite it is excreted with a constant rate through urine and accumulates in blood when renal function is impaired. Creatinine is the only marker currently validated against clinically relevant outcomes [114] and able to predict worsening of AKI [87]. The concentrations are directly proportional to mortality [66]. Nevertheless, it is influenced by several factors other than renal function: As muscle metabolism is the main source of creatinine, the muscular mass strongly influences creatinine baseline concentration [8]. Coherently factors influencing muscle mass like age, sex and mobilization of the patient also influence creatinine [114, 204]. Drug metabolism, protein intake and hydration status can affect creatinine concentration, too [114]. Furthermore, sCrea is insensitive to acute changes in renal function [8]: the renal status is not reflected until a steady state is reached and approximately 50% of renal function is lost [93]. Before that, renal dysfunction

is dramatically underestimated [10]. Also type, onset, worsening or recovery of renal function cannot be predicted by measuring sCrea [172].

Another bias of sCrea appears in pregnancy: The renal hyperfiltration occurring physiologically during pregnancy decreases the accuracy of creatinine clearance estimation from serum and urine creatinine measurement. Increased GFR leads to increased filtration of sCrea and thus falsely low sCrea concentrations. Hence, renal dysfunction causing an increase in sCrea is obscured within this pregnancy-caused decrease and kidney injury is underestimated or even not diagnosed at all [85].

Cystatin C Another biomarker currently being established as potential new gold standard is serum cystatin C. Recent studies showed a definition of AKI based on cystatin C superior to one based on sCrea [221]. It is secreted by all nucleated cells and physiologically freely filtered and completely catabolized by the tubules [204]. Therefore, it is not detectable in urine [114, 48]. Tubular injury decreases normal catabolization while production is up-regulated in the proximal tubular epithelial cells and cystatin C will be measurable in urine [41, 48, 98, 114, 204]. Several studies showed its high reliability in predicting glomerular filtration rate approximately one or two days earlier than creatinine [114, 69, 95]. Also mortality, length of hospital stay, severity, worsening or recovery of renal function and need for renal replacement therapy can be predicted by cystatin C [36]. Nevertheless, the influence of age, sex, weight, smoking, thyroid function, sepsis and immunosuppression on cystatin C are still under discussion [39, 52, 6, 129, 146]. Also its predictive capacity for adverse clinical outcomes is limited [204]. Furthermore, cystatin C might be biased by pregnancy: The glomerular filtration barrier, which is normally negatively charged, looses some of these negative charges during pregnancy. Hence, filtration of the positively charged cystatin C is decreased which leads to an misinterpretable raise in serum cystatin C concentrations. Further studies have to show the impact of this effect on the applicability of cystatin C as a biomarker for AKI diagnosis [85].

Neutrophil gelatinase-associated lipocalin (NGAL) Also currently under discussion as a potential new gold standard is the neutrophil gelatinase-associated lipocalin (NGAL). NGAL is expressed mainly in secondary granules of neutrophiles which protect proximal tubules in case of injury by executing bacteriostatic function [11, 162, 212]. As a lesional biomarker NGAL is freely filtered by glomeruli and reabsorbed in tubules via receptor binding in healthy individuals [114, 162]. After injury it is highly upregulated and the urinary concentration overruns the

reabsorption capacity of the tubules which might be further diminished by the AKI itself as well [44, 95, 162, 212, 46, 136, 173]. As creatinine and cystatin C, NGAL predicts mortality, length of hospital stay, severity, worsening or recovery of renal function and need for renal replacement therapy [98, 87, 36, 136]. The detection of AKI is 1 to 3 days earlier than by sCrea concentration [173, 122] with concentration peaking 6-24 hours after initiation of the insult [162, 57]. The main bias is the sensitivity of NGAL to inflammations independent from kidney function [60, 109, 173, 146]: even smaller bacterial infections, e.g. in cystic fibrosis, hypertonia, sepsis, pneumonia, acute decompensated heart failure (ADHF) or after surgery, increase NGAL concentration dramatically [212, 60].

Kidney Injury Molecule 1 (KIM-1) A structural biomarker which is directly implemented in renal regeneration is kidney injury molecule 1 (KIM-1). Massively expressed after ischemic or nephrotoxic AKI especially in proximal tubule cells, it detects apoptotic cells by acting as a phosphatidylserine receptor and leads them into the lysosomes [66, 95, 162, 76, 194]. It promotes transformation of proximal tubular cells into semi-professional phagocytes and thereby enables clearance of obstructed tubules in the acute injury setting [11, 12]. In chronic kidney disease KIM-1 also promotes interstitial inflammation and fibrosis and thus might provide a link between acute and recurrent injury with progressive chronic kidney disease [162, 75]. Its increased concentrations are associated with mortality and hospitalization [95]. Unfortunately, there are again several factors that can influence the level: carcinoma like renal cell carcinoma or clear-cell ovarian carcinoma up-regulate KIM-1 [114]. Invasive therapy of nephroliths can increase urinary concentration [55]. Chawla [24] showed KIM-1 being reliable in a pediatric population, but not in adults. Also a linear increase of KIM-1 with age was shown in males and KIM-1 induction in various chronic proteinuric, inflammatory diseases have been shown [162].

Others Continuing along the pathophysiology of renal injury there could be several different points monitored by biomarkers:

Renal hemodynamics are critical for renal (and cardiac) function. One possible, very basal biomarker could be urine output which is depleted when renal hemodynamics are collapsing. It is less specific but more sensitive comparing to Creatinine which is why several scores like RIFLE (Risk, Injury, Failure, Loss of kidney function, and End-stage kidney disease) classification take both into account [8, 36].

The glomerular apparatus and the *filtration barrier* could be monitored by large molecules simply leaking into urine when the barrier is malfunctioning. This could be simply total protein excretion, which is highly specific for glomerular injury and allows prognosis of its worsening [48]. The renal *re-absorption capacity* describes tubular functionality and could be monitored by smaller molecules, such as ß-Mikroglobulin, which are normally freely filtered by the glomerulum and totally reabsorbed by the tubules. Another way of monitoring tubular functionality is the measurement of molecules *secreted by the injured tubular cells*. N-acetyl-ß-D-Glucosamidase (NAG) is a large molecule which cannot pass the filtration barrier. If the tubular cells are injured, they secrete NAG which is then be detectable in urine although with poor specificity [114].

The *accumulation* of harmful substances *in blood* through renal injury is another mechanism which could be monitored by biomarkers like blood urea nitrogen (BUN) which is accumulating in acute renal failure. Nevertheless it is not reliable since other factors (e.g. increased protein intake) are influencing concentration as well [10]. The differentiation between different kinds of renal injury (prerenal azotemia, acute kidney injury, obstruction of urinary tract) is poor [163].

Also the *inflammation* accompanying renal injury could be monitored, e.g. by measuring inflammatory cytokines like interleukines. IL-18 is induced and processes in proximal tubule and detectable in urine after ischemic AKI. It has been identified as a mediator of tubular necrosis [114] and predicts clinical outcomes like length of hospital stay [36, 136], severity of AKI [36], worsening of renal function [87] and allograft function [65]. Nevertheless, the specificity for renal vs other inflammations (e.g. sepsis) is questionable [39, 114, 95, 146]. For IL-6 a significant influence on AKI has been shown even after correcting for clinical parameters [100] but still it has the same limitations as IL-18.

The most promising approach in identifying new biomarkers is monitoring molecules *directly involved in (early) pathological processes* [143, 11, 170]. One example is the biomarker combination of IGFBP7 and TIMP2 [80, 88]. They are involved in G1 cell cycle arrest of injured tubular cells which is initial to repair processes [44, 13, 168] and mediate inflammation processes and regulate oxidative or toxic stress [143, 13, 168]. The FDA approval has been given in September 2014 and further studies are ongoing to demonstrate the clinical value of these markers.

2.3. Eligible features of new renal biomarkers

Already in 2005 the American Society of Nephrology ranked the development of new biomarkers for renal injury as a top priority (J Am Soc Nephr 2005).

Several authors have specified this need describing concrete features and goals of new biomarkers. Besides certain features like sensitivity and specificity, it should increase rapidly and reliably in response to kidney injury, correlate with the severity and provide risk stratification and prognostic information [204]. Its site specificity potentially identifies possible mechanisms of injury. Certainly, it should be highly stable over time and across different temperatures, have no interference with drugs and be applicable across different populations [204].

Especially the early phase detection [39, 114, 98, 127] of renal complications in high risk patients [66, 97] should be highlighted in this context. Another critical point is not only the detection but the prognosis of renal injury and its development including clinical outcomes like mortality, renal replacement therapy or recovery [114, 98, 10, 36]. Devajaran [44] furthermore illustrated several more technical features a new biomarker should possess: it should be non-invasive and simply applicable to easily accessible samples (blood, urine); the measurement should be quick and reliable, cost-effective, standardized, highly sensitive with a broad dynamic spectrum and well-described cut-off values; the clinical conclusion should be highly specific and predictive for clinically relevant outcomes. Ideally, the biomarker measurement directly implies a clinical intervention or can be used for bio-monitoring of renal function. Therefore there has to be a clearly determined time point for measurement and only clinical relevant differences should be detected. Cruz [36] summarized all this features under the term “clinically actionable”. Another eligible feature Bonventre [10] described is the applicability in human and animal for parallel investigation of clinically relevant processes.

3. Immunological methods

3.1. (Sandwich) Immunoassays

3.1.1. Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is one of the most commonly used antibody-based assay systems. It usually consists of one analyte-specific capture antibody immobilized to a solid phase and one detection antibody added in solution which is coupled to an enzyme (e.g. horseradish peroxidase, alkaline phosphatase). Both antibodies form a so called sandwich complex when reacting with the analyte. Excessive antibody is removed completely by washing before the substrate is added and the antibody bound enzyme triggers a color change which can be used for quantification of the analyte. Usually, a dose response curve with an analyte of known concentration and dilution is measured in parallel for calibration.

Variations of the system described above are possible even though less common: A direct ELISA approach is used when the analyte is binding to the multisorbent solid phase itself, so the capture antibody is omitted. The main disadvantage of this procedure is that the analyte binds to the surface in unspecific orientation so that binding of the detection antibody might be prevented only by steric problems - this occurs especially with small analytes. Another variation is the competitive approach which can be used for example when the analyte is an antibody itself: the antigen in coupled to the solid phase (potentially via capture antibody) and the enzyme-linked detection antibody equalling analyte is added in a specific amount. When the sample containing the analyte is added it competes with the already bound detection antibody and thus decreases the color signal by replacing the enzyme-linked detecting antibody with unlabeled antibody from the sample.

Also, variations in incubation can be applied: an one-step approach incubates all reagents but the substrate (detection, capture antibody and analyte) at once while in a two-step approach several washing steps are inserted between the incubation steps to completely remove the respective reagent before adding the next one. Depending on analyte and sample type, one or the other incubation variation can be favored. [102, pp. 100-103]

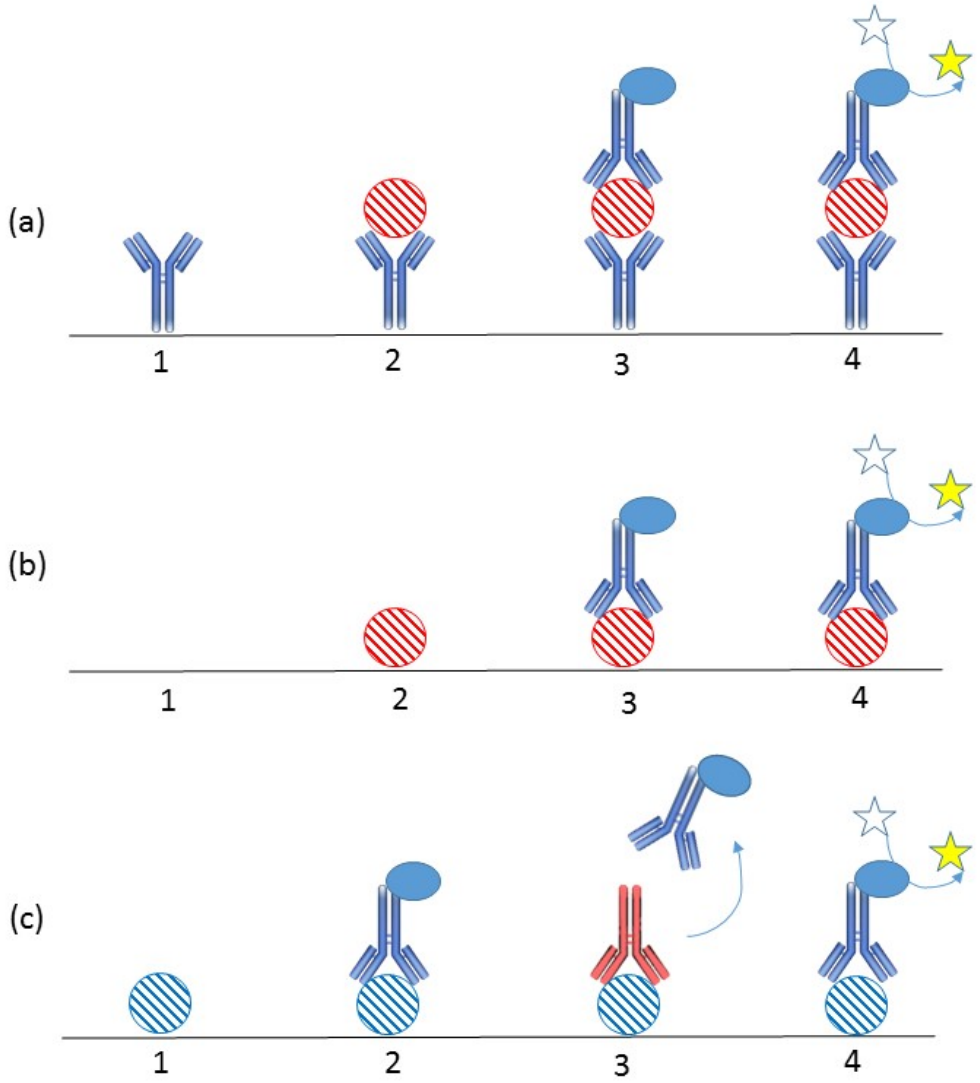


Figure 3.1.: **Schematic representation of ELISA principle.** *(a) sandwich ELISA.* Capture antibody is immobilized to the solid phase (1), analyte from the sample (shown in red) binds to the capture antibody (2). Detection antibody is added and forms the sandwich complex with analyte and capture antibody (3). When substrate is added (blue star), enzyme triggers color reaction (yellow star) (4). *(b) direct ELISA.* Analyte from the sample (shown in red) binds to a multisorbent surface (2). Detection antibody is added (3) and triggers color reaction (4). *(c) competitive ELISA.* Antigen for the analyte is coupled to the solid phase (1). Detection antibody equalling the analyte binds to this antigen (2). Analyte from the sample (shown in red) competes with bound detection antibody (3) and diminishes the color reaction (4).

3.1.2. Luminometric immunoassay (LIA)

In general, the principle of luminometric immunoassay (LIA) is very similar to the ELISA described above. In LIA, the enzymatic color reaction is substituted by a luminometric label (e.g. acridinium esters) coupled to the detection antibody which can be quantified directly in a luminometer (luminescence reader). To prevent bleaching effects, the luminometric label has to be protected from light throughout incubation. [102, pp. 100-103]

3.2. Immunohistochemistry (IHC)

In Immunohistochemistry (IHC) specific antibodies are used to stain histological tissue slices for a specific analyte. Very thin slices of tissue (e.g. organ preparations) are prepared and provide a surface containing a multitude of antigens. When the specific antibody is added, it binds only to the regions where the specific antigen is expressed while excessive antibody is removed by washing. Following, a secondary antibody targeting the Fc-term of the specific antibody (e.g. anti-mouse) is added and binds to the coupled specific antibody. The secondary antibody is linked to an enzyme which triggers the color reaction when substrate is added. Hence, the slice is stained only in regions where the specific antigen is expressed [102, pp. 106-107].

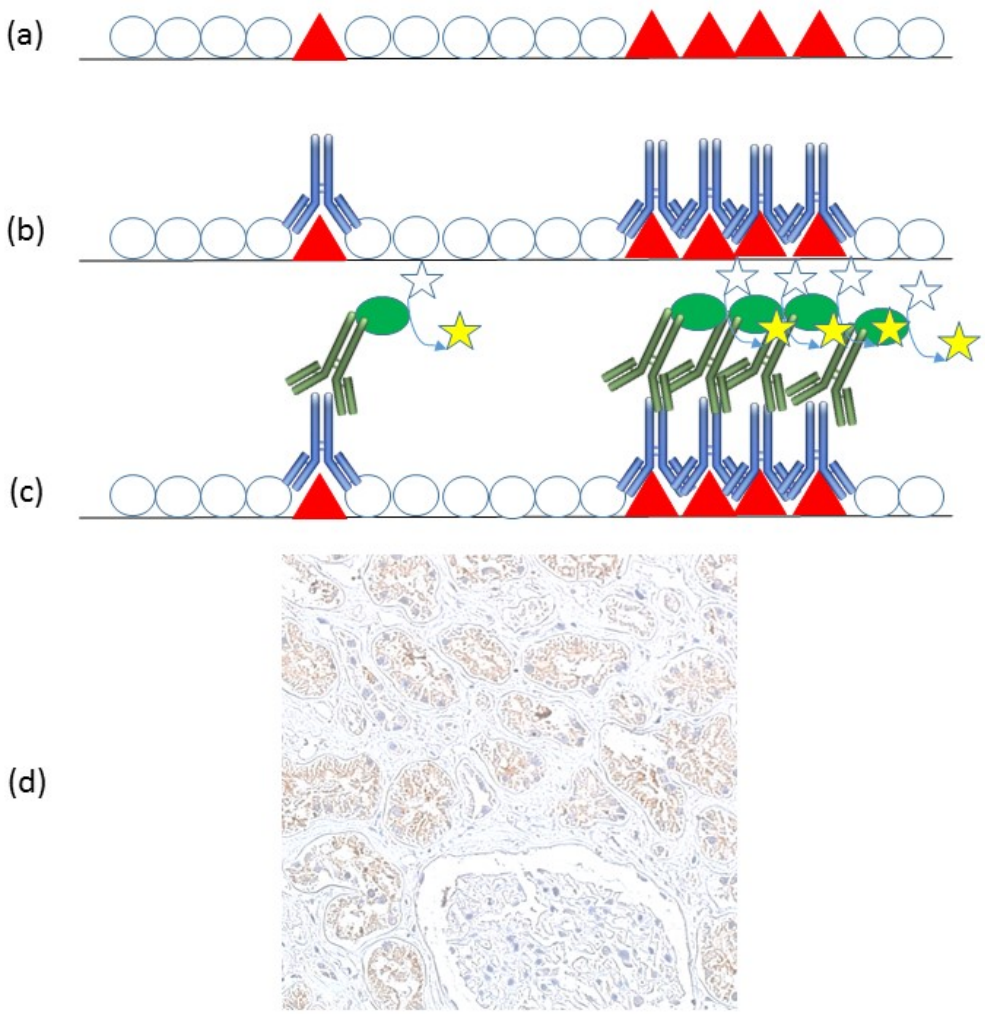


Figure 3.2.: **Schematic representation of IHC.** (a) Slice surface with multitude of unspecific antigens (blue circles) as well as the specific antigen (red triangle). (b) specific antibody binding to the specific antigen. (c) secondary (labelled) antibody (in green) binding to Fc-term of specific antibody. (d) representative immunohistochemical staining with stained (in red) and unstained areas (in blue)

4. Peptide hormones

4.1. Enkephalin and its pro-hormone

4.1.1. Structure, synthesis and biochemical properties

Human pro-Enkephalin is a 4.5kDa peptide derived from the 31kDa Preproenkephalin. After removal of the N-terminal signal-sequence the Proenkephalin precursor protein is formed, consisting of 243 amino acids. Following, this precursor is further cleaved at paired basic amino acid residues (lysin, arginine) giving rise to the active enkephalin peptides [49]. Several of them are encoded in one copy of Proenkephalin: Four copies of methionine-enkephalin (Met-Enk) and one copy each of leucine-enkephalin (Leu-Enk), methionine-enkephalin- Arg-Gly-Leu (Met-Enk-RGL), methionine-enkephalin-Arg- Phe (Met-Enk-RF) and enkelytin [86]. Cleavage furthermore unleashes several shorter peptide fragments of so far unknown biological function like the PENK A 119-159 fragment [53]. A full 3D structure of proenkephalin is not available yet, there are only three disulfide bonds reported within the very N-terminus of the molecule [94].

4.1.2. Regulatory function of Enkephalin and Pro-Enkephalin

Enkephalin was described first in 1975 as a possible endogenous ligand for the morphine receptor [74] and for several years mainly its function in pain regulation was examined. Later, an increasing number of other functions were observed as immunity, stress regulation and renal functions.

Pain management As the enkephalins act as endogenous opioids mainly on the μ and δ opioid receptor[179], they reduce nociception in several brain areas as well as in the periphery [144]. Expressed mainly by neutrophils[5] and by central and peripheral neurons [182, 140, 176, 151, 145, 186], expression levels correlate with pain treshold [27]. Thereby, longer enkephalin derivatives trigger stronger analgesia than the shorter ones [145]. In knockout mice, increased sensitivity to heat pain was shown [81].

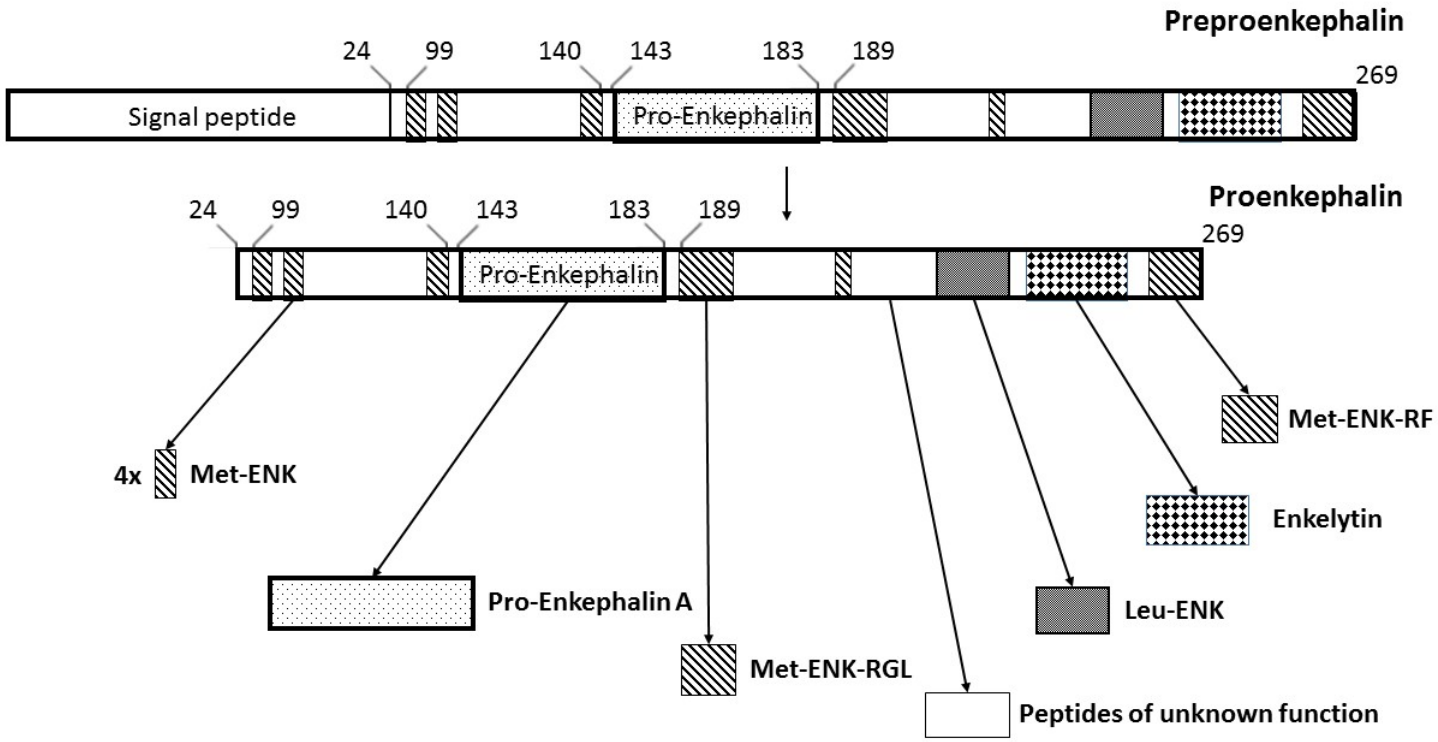


Figure 4.1.: **Schematic view of the proenkephalin synthesis.** The precursor protein preproenkephalin contains a signal peptide and several different enkephalin derivatives. After cleaving the signal peptide the generated proenkephalin is further cleaved to give rise to four copies of Met-enkephalin as well as one copy each of Leu-enkephalin, Met-ENK-RGL, Met-ENK-RF and Enkelytin. Furthermore, several other peptides located in-between these are set free through cleavage. For most of them, no is function known yet. One of them is Pro-Enkephalin A 119-159 (also knwon as pro-ENK or pENK) which is relatively stable within human plasma, serum, liquor and urine and thus applicable for biomarker assays.

Main expression regions are the striatum [182], the cerebellum [176] and local pre-synaptic interneurons within the spinal chord, the limbic system, the substantia nigra, hyphophysis and the hypothalamus [145, 186, 3]. LPS stimulates proenkephalin expression while GABA agonists act inhibitory [14]. Enkephalin release is stimulated by high potassium levels [101].

Immunity Since enkephalins are expressed by neutrophils and other immune cells as T cells and monocytes [79], immunological function was self-evident. As highly expressed in inflamed tissues [144], a dose-dependent immunomodulation [141, 79] is involving regulatory T cells as well as other T cell sub-populations[73]. The immunoexcitation is conducted via δ opioid receptors [183, 210, 155], stimulating cytokine release, chemotaxis of other immunocytes and inducing confirmational changes within the corresponding cells[81, 178]. Systemic immunological events like shock induce a massive met-ENK expression [205]. Expression of enkephalins within macrophages has been shown to act tumor suppressive by inducing M2 macrophage polarization to M1 and modulating the Th1 response [26]. Rosenberger [151] suggested a communicative role of enkephalins between the nervous and the immune system.

Regulation of cell growth Also known as opioid growth factor (OGF), Met-ENK exhibits regulatory functions on cell growth and differentiation [15] mainly in developing or renewing tissues [220]. Stimulating ζ receptors (OGF receptor), enkephalins act pro-apoptotic via p53 [47, 210] and inhibit DNA synthesis and cell replication by upregulating inhibitory kinases [220]. Inversely, blockage of the OGF receptor enhances cell proliferation [117]. Within myocytes, enkephalins are associated with the histone deacetylase controlling pro-apoptosis [118] and regulate the transition from hyperplastic to hypertrophic growth [116]. In preclinical studies enkephalin administration prevented tumor appearance in nude mice transplanted with human pancreatic cancer via a p21 cyclin-dependent pathway[219].

Kidney Zoccali et al. [223] first described a connection between the enkephalins and renal function by detecting an increased concentration of plasma met- and leu-ENK in patients with chronic kidney failure which were highly correlated with creatinine. Danno [37] and Klin [84] reported elevated plasma levels of met and leu-ENK, respectively, in patients with hemodialysis as well. In 2008, Denning [42] found proenkephalin mRNA expression within the kidney which was even higher than in brain or muscle. Consequently, a renal function was obvious.

Since plasma concentrations of the very unstable mature enkephalins are quite difficult to determine, Ernst [53] developed an immunoassay for a stable surrogate marker, the so called pro-Enkephalin (aa 119-159). Several studies have shown the reliability of this pENK in determination of renal dysfunction and prediction of adverse events: In stroke mortality, vascular events and major adverse events can be predicted by pENK plasma concentrations which are correlated with severity of stroke[50]. pENK plasma concentrations could predict major events and mortality in acute myocardiac infarct as well, correlating with age, heart rate, diastolic blood pressure and other markers for renal and cardiac function [131]. In cardiac surgery, pre-operative pENK plasma concentrations were able to predict post-operative AKI, correlating with creatinine [169]. In sepsis, pENK plasma concentrations correlated highly with creatinine clearance and severity of renal injury and showed a high prediction capacity for seven day mortality [108]. These correlations and the predictive ability of pENK was confirmed in a bigger cohort recently, where creatinine was the strongest determinant for pENK concentration also in multivariate models. Gender, age and BMI showed minor effects on pENK concentration. Elevated pENK concentrations predicted acute worsening of renal function as well as the use of renal replacement therapy (RRT) [111]. In a large cohort of critically ill patients, pENK concentrations showed comparable diagnostic capacities for AKI to cystatin C and NGAL[96]. Schulz et al. [165, 166]studied the long-term predictive capacity of pENK plasma concentrations in a population-based cohort: Patients with elevated pENK plasma concentration at baseline showed significantly greater yearly mean decline of eGFR and increased CKD incidence. Again age and BMI as well as blood pressure and glucose showed minor effects on pENK concentration. Furthermore, an association with the SNP rs1012178 (near the pENK gene) was determined which suggests a causal relationship between pENK concentration and deterioration of renal function over time [165, 166]. Another population-based study further confirmed eGFR as major determinant of pENK plasma concentration, while age and BMI showed minor influence [82]. In patients with former RRT pENK plasma concentrations was able to predict graft failure as well as mortality showing a strong correlation with eGFR. Age, BMI and glucose had minor effects on pENK [83]. An overview of renal implications is given in table 4.1.

Table 4.1.: **Overview over renal implications of (p)ENK plasma concentration in several studies.** If not indicated otherwise, pENK concentration was determined using the assay system described by Ernst et al. [53].

implication	setting	literature
diagnosis AKI vs non-AKI	CKD	Zoccali (met-&leu-ENK) [223]
	ESRD	Danno, Klin (met-&leu-ENK) [37, 84]
	critically ill	Legrand [96]
Correlation with Crea/eGFR	acute MI	Ng [131]
	cardiac surgery	Shah [169]
	sepsis	Marino, Masson [108, 111]
	population-based cohort	Kieneker [82]
	CKD	Kieneker [83]
Correlation with severity of injury	stroke	Döhner [50]
	sepsis	Marino [108]
Correlation with age	acute MI	Ng [131]
	sepsis	Masson [111]
	population-based cohort	Kieneker, Schulz [82, 165, 166]
	CKD	Kieneker [83]
Correlation with blood pressure	acute MI	Ng [131]
	population-based cohort	Schulz [165, 166]
Correlation with BMI	sepsis	Masson [111]
	population-based cohort	Kieneker, Schulz [82, 165, 166]
	CKD	Kieneker [83]
Prediction of worsening renal function/RRT	cardiac surgery	Shah [169]
	sepsis	Masson [111]
	population-based cohort	Schulz [165, 166]
Prediction of CKD	population-based cohort	Schulz [165, 166]
Prediction of mortality	stroke	Döhner [50]
	acute MI	Ng [131]
	sepsis	Marino [108]
	CKD	Kieneker [83]
Prediction of major adverse events	stroke	Döhner [50]
	acute MI	Ng [131]

4.2. Relaxin-2 and its pro-hormone

4.2.1. Structure, synthesis and biochemical properties

The relaxin subfamily of peptides within the human insulin superfamily consists of seven members including relaxin-1, -2 and -3. While relaxin-3 is encoded by chromosome 19 and differs strongly in amino acid sequence and function from the others, relaxin-1 and 2 are highly homologous probably originating from a gene duplication on chromosome 9 [72, 104]. Nevertheless, human relaxin-1 peptide has never been isolated and its function has not been explored yet, leading to the question if it might be expressed in humans at all [61, 62, 187]. Human relaxin-2 is a 6kDa peptide hormone produced mainly by the corpus luteum. Encoded by the RLN2 gene on chromosome 9, it is structurally similar to insulin with two separate polypeptide chains cross-linked by two disulfide bonds and a connecting C-peptide [31]. It exhibits its function mainly via Relaxin receptor 1 (RXFP1) which triggers a G-protein cascade regulating the adenyl cyclase as well as the phosphate-inositol-3-kinase resulting in a production of nitric oxide. Relaxin-2 is synthesized similarly to insulin as a pre-pro-hormone containing an signal peptide, the two peptide chains of the mature hormone and the connecting peptide. During synthesis, first the signal peptide and successively the C-peptide is cleaved releasing the mature hormone (Fig. 4.2).

4.2.2. Regulatory function of Relaxin

Relaxin is implicated in diverse physiological and pathophysiological processes, especially in the first half of pregnancy. It aids maternal accommodation to pregnancy including rebuilding the cardio-renal system for the specialized needs during pregnancy. Relaxin therefore causes an increase of RPF and GFR which is directly balanced by an increased heart rate and decreased systemic and arteriolar resistance, causing a hyperfiltration without hypertension. When relaxin action is diminished or even abolished it potentially leads to pathogenic processes like PE [31, 201] or preterm delivery [51, 206]. A potential therapy with relaxin is currently under discussion, but this would require an early marker to see beneficial or harmful effects of the administration [31].

Reproductive system and pregnancy While relaxin is expressed mainly by the corpus luteum, its receptors are widely distributed in the reproductive system in endometrium, decidual cells, cytotrophoblast, syncytiothrophoblast and blood vessels. Therefore its actions reach from influence on the development of mammary

gland over actions on uterine motility up to renal variations throughout pregnancy [22]. These renal variations are probably the most important function which is triggered by relaxin release in the first trimester of pregnancy and the return of relaxin concentrations to baseline within few weeks after delivery. Relaxin induces glomerular hypertrophy (increased total size), causing an increased GFR [31]. A reduction in SVR as well as a parallel decrease in afferent and efferent renal arteriolar resistance [31] accompanied by an increased cardiac output 20-40% [22, 31] increases the RPF by 40-65% [31] without elevation in glomerular hydrostatic pressure. This consequently further increases the GFR up to a total of 150-85% of non-pregnant rate.

While relaxin has been shown to be critical in first half of pregnancy [31], no correlation between serum RLX-2 & renal function could be shown postpartum.

Cardiovascular regulation Exogenous administration of relaxin-2 induces a decrease in blood pressure by induction of vasopressin secretion while reducing the vascular response to vasoconstrictors. Furthermore, relaxin reduces plasmatic osmolality by inducing a dose-dependent alteration of the threshold for thirst. Relaxin acts on heart rate, showing positive inotrope reaction in atria, but not ventricle. Studies show an improvement of myocardial perfusion and reduction in fibrosis after relaxin administration, normalizing the collagen content in the ventricles. Differently from the pregnant state, in non-pregnant adults relaxin has been shown to be secreted by the cardio-myocytes and acting in autocrine or paracrine probably compensatory mechanisms. It is assumed that relaxin secreted within the heart is not delivered into the blood stream [22].

The mechanism of action within the heart involves the PI3K, Akt and eNOS. Relaxin antagonizes angiotensin-II, endothelin-1 and catecholamines, while up-regulating endothelin type-B receptor (Ras-independent). Administration induces ET-1 clearance & endothelial release of NO as well as vascular remodeling [22] in an geometric (increased wall area) and molecular composition manner (increased muscle cell density, reduction in collagen).

Renal implications Even though the renal expression of relaxin is low, relaxin is very important in homeostasis of kidney's connective tissue thereby preserving glomerular structure [22]. Hence, the kidney is a potential therapeutic target of relaxin since preclinical studies showed relaxin administration normalizes collagen content in renal tissue and decreases interstitial fibrosis [22]. A delayed progression of kidney disease could be shown [22]. The administration caused a decrease in albuminuria , a decrease in sCrea, a decreased macrophage infiltration

and decreased TGFβ expression. Diuresis and natriuresis were induced by relaxin's interaction with ETb receptor and the inhibition of Na-K-ATPase activity in collecting ducts.

4.2.3. Therapeutic applications of Serelaxin

Serelaxin, a synthetic analogue of human relaxin-2, was able to improve multiple organ function in human and animal models [51]. Beside heart function [40, 110, 132, 45], administration showed protective effects on renal [59, 160, 217, 30], lung [153, 192] and hepatic [9, 56] function. The main mechanisms involve protection of vascular endothelial cells against inflammatory changes and oxidative stress [17]. Serelaxin was shown to repair microvascular injury through angiogenesis [167] and mobilization of bone marrow-derived stem cells [125]. Inhibition of fibrogenesis also seemed to play an important role in these processes [157, 70].

Recently, the multi-center RELAX-AHF trial has confirmed the benefit of Serelaxin administration in acute heart failure of non-pregnant adults. Serelaxin improved dyspnea, improved signs of pulmonary congestion, reduced heart function worsening, dose of diuretic use and mortality dramatically [185]. Furthermore, administration reduced further elevation of several biomarkers which have been shown to be associated with cardiac (troponin T), renal (creatinine, cystatin C) and hepatic (ALAT, ASAT) injury [121].

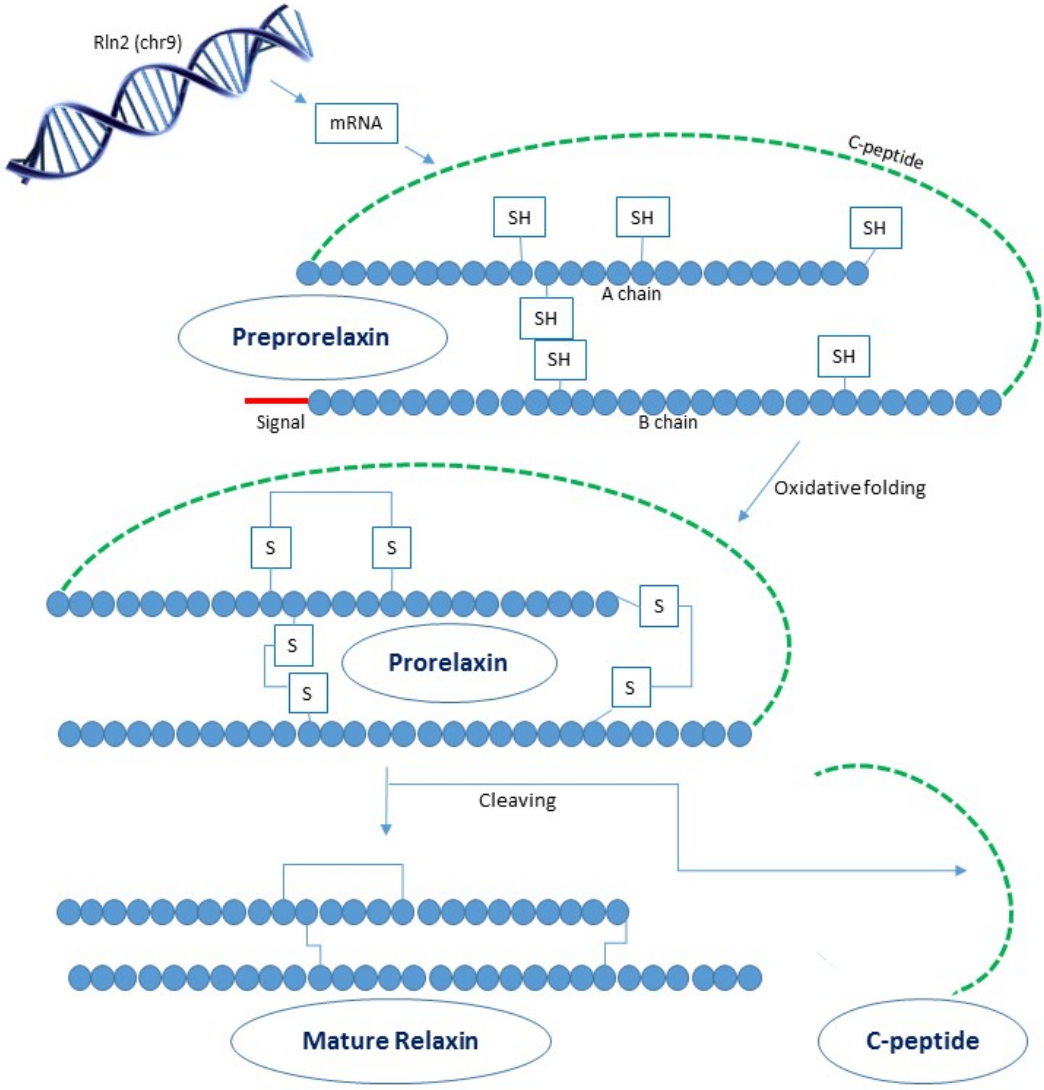


Figure 4.2.: **Schematic view of the synthesis of Relaxin-2 and its connecting peptide.** The Relaxin gene is located on chromosome 9, transcribed into mRNA which is translated at the endoplasmatic reticulum. The newly formed Preprorelaxin consists of a signal peptide, two yet unlinked peptide chains and a connecting peptide. Once translocated into the Golgi apparatus, the signal peptide is cleaved and three disulfide bonds are formed to connect the relaxin chains (Prorelaxin). Following, the C-peptide is cleaved as well and the mature relaxin is released into vesicles.

II.

Hypothesis and Goals

5. Hypothesis

AKI is a disease associated with high mortality and increasing incidence [213]. Even though our clinical understanding of AKI and renal pathology has dramatically increased over the last decades, an effective intervention for AKI is still missing [164, 98]. One of the main problems of executing an effective intervention is the inacceptable delay in therapy due to delayed diagnosis [44]. Hence, an earlier recognition of AKI would improve recovery and decrease mortality [52, 66]. Already in 2005 the American Society of Nephrology ranked the development of new biomarkers - defined as "parameter of structural, biochemical, physiologic or genetic change that indicates the presence, severity or progress of a disease" [204] - for renal injury as a top priority (J Am Soc Nephr 2005).

Neuropeptides and peptide hormones which are executing signaling and regulatory functions have been identified as valuable target for biomarker studies in several different clinical settings, e.g. breast cancer [120], bacterial infection [180] or sepsis [123]. Mostly, a reliable quantification of the mature peptide in blood, cerebrospinal fluid or urine is impossible due to instability, locally restricted release or masking through binding proteins. Therefore, measurement of the so called pro-hormones - precursor molecules of the mature peptide hormone or intermediates of hormone processing - often is a useful surrogate. Several studies have shown that both (pro)hormones - (pro-)Enkephalin as well as (pro-)Relaxin-2 - are involved in regulation of renal function of pregnant women and non-pregnant adults. Measurement of these two peptides in plasma and/or urine (alone or in combination with each other) should be a very promising approach in early detection of AKI - especially in the complicated setting of renal injury in pregnant women (preeclampsia).

6. Goals

For this thesis, we strive for several goals:

1. Technical establishment of pro-Enkephalin measurement in rat plasma samples The established assay system for human plasma measurement [53] has to be adapted to the rat peptide.

2. Technical establishment of pro-Enkephalin measurement in human urine samples The established assay system for plasma measurement[53] has to be adapted to urine.

3. Technical establishment of pro-Relaxin 2 measurement in human plasma & serum samples A *de novo* design, development and validation of an immunoassay system has to be implemented.

4. Localization of the pro-Enkephalin within renal tissue through immunohistochemistry Renal sections are stained with the antibodies used in the pENK immunoassays.

5. Proof of association of pro-Enkephalin plasma concentration with renal dysfunction in several animal studies Otherwise healthy animals are challenged with selective renal dysfunction and pENK plasma concentrations are determined.

6. Proof of pro-Enkephalin appearance in urine (association with plasma concentrations) Corresponding plasma and urinary pENK concentrations are observed.

7. Determination of the association of urinary pro-Enkephalin concentration with renal function Urinary pENK concentrations are observed within samples from healthy subjects and patients selected for renal transplantation (end-stage renal disease).

8. Determination of pro-Enkephalin appearance throughout pregnancy (healthy & preeclampsia setting) Variation analysis regarding gestational age is performed, differentiation of PE vs. non-PE through pENK serum concentrations is tested.

9. Determination of pro-Relaxin 2 in non-pregnant adults (healthy & acute heart failure setting) Measurability of pro-RLX2 plasma concentrations in non-pregnant adults with the new assay system is tested, association of pro-RLX2 concentrations with renal dysfunction in heart failure is examined (pilot study).

10. Determination of pro-Relaxin 2 expression throughout pregnancy (healthy & preeclampsia setting) Variation analysis regarding gestational age is performed, differentiation of PE vs. non-PE through pro-RLX2 serum concentrations is tested.

III.

Material and Methods

7. Material

7.1. Chemicals and substances

Table 7.1.: Chemicals and substances used for buffer preparation etc. in assay development

substance	provider
Amastatin	Biocyc GmbH, Luckenwalde, Germany
Bovine IgG	Brahms, Hennigsdorf, Germany
BSA Fraktion V (protease free)	Brahms, Hennigsdorf, Germany
Heparin	Sigma-Aldrich, Taufkirchen, Germany
Horse Serum (heat-inactivated)	Sigma-Aldrich, Taufkirchen, Germany
K_2HPO_4	J.T. Baker, Avantar, Deventer, Netherlands
Karion FP	Brahms, Hennigsdorf, Germany
KH_2PO_4	Merck, Darmstadt, Germany
Leupeptin	Biocyc GmbH, Luckenwalde, Germany
MACN (M = 1365 g/mol)	Invent diagnostica GmbH, Hennigdorf, Germany
mouse IgG	Brahms, Hennigdorf, Germany
Na_2HPO_4	Th. Geyer, Renningen, Germany
NaCl	Th. Geyer, Renningen, Germany
$Na_2 - EDTA$	J.T. Baker, Avantar, Deventer, Netherlands
NaH_2PO_4	Merck, Darmstadt, Germany
NaN_3	J.T. Baker, Avantar, Deventer, Netherlands
PBS (10x concentrated)	Invivo Biotech Services, Hennigsdorf, Germany
sphingotest® Lightning Initiator 1 & 2	sphingotec GmbH, Hennigsdorf, Germany
sphingotest® UNIWASH wash solution	sphingotec GmbH, Hennigsdorf, Germany
Tris	J.T. Baker, Avantar, Deventer, Netherlands
Triton X-100	Serva, Heidelberg, Germany
Urea	Th. Geyer, Renningen, Germany

Table 7.2.: Chemicals and substances used for buffer preparation etc. in immunohistochemistry

substance	provider
Blocking serum S3022	DAKO, Carpinteria, USA
Citric acid	Merck, Darmstadt, Germany
DAKO liquid DAB + Substrate chromogen system (DAB+ substrate buffer & DAB+ chromogen)	DAKO, Carpinteria, USA
DAKO LSAB+ System-HRP (Streptavidin-HRP)	DAKO, Carpinteria, USA
Entellan	Merck, Darmstadt, Germany
Ethanol	Herbeta Arzneimittel, Berlin, Germany
H_2O_2 30%	Merck, Darmstadt, Germany
KCl	Merck, Darmstadt, Germany
KH_2PO_4	Merck, Darmstadt, Germany
Monoclonal rat anti-mouse	DAKO, Glastrup, Denmark
Na_2HPO_4	Roth, Karlsruhe, Germany
NaCl	Roth, Karlsruhe, Germany
Paraplast embedding media	McKormick Scientific, St. Loius, USA
Sodium citrate	Serva, Heidelberg, Germany
Triton-X	Roth, Karlsruhe, Germany
Xylene	J.T. Baker, Avantar, Deventer, Netherlands

7.2. Buffers

Table 7.3.: Buffers for antibody immobilization and assay preparation

buffer	ingredients
Coupling buffer	50mM TRIS pH 7.8
Blocking solution	6.5mM KH_2PO_4 3.5mM $Na_2HPO_4x2H_2O$ 3% Karion [w/v] 0.5% BSA [w/v] pH 6.5-6.8
Assay buffer pro-RLX2	200mM Phosphate 20mM Na_2-EDTA 0.5% BSA [w/v] 0.1% bovine IgG [w/v] 0.02% mouse IgG [w/v] pH 6.5
Assay buffer pENK urine	150mM TRIS 0.1% Triton-X [v/v] 0.5% BSA [w/v] 0.1% bovine IgG [w/v] 0.02% mouse IgG [w/v] pH 6.8
Assay buffer pENK rat	150mM TRIS 20mM NaCl 0.5% BSA [w/v] 0.1% bovine IgG [w/v] 0.02% mouse IgG [w/v] pH 6.8

Table 7.4.: Buffers for immunohistochemistry

buffer	ingredients
PBS	137mM NaCl 2.7mM KCl 10mM $Na_2HPO_4x2H_2O$ 1.8mM KH_2PO_4 pH 7.4
PBS-T	0.5% Triton-X in PBS [w/v]
Citrate buffer	0.1M citric acid 0.1M Sodiumcitrate pH 6.0

7.3. Peptides and antibodies

7.3.1. Peptides

Table 7.5.: Peptides for immunization

pro-hormone	peptide	sequence
Pro-Relaxin 2	$RLX\,2_{69-82}$	C-AEIVPSFINKDTE
	$RLX\,2_{88-110}$	C-SEFVANLPQELKLTLSEMQPALP
Pro-Enkephalin A (human)	$PENK\,A_{143-152}$	DAEEDDSLAN-C
	$PENK\,A_{153-168}$	C-SSDLLKELLETGDNRE
	$PENK\,A_{176-183}$	C-SDNEEEVS
Pro-Enkephalin A (rat)	$PENK\,rat_{176-183}$	C-STNNDEDS

7.3.2. Antibodies

Table 7.7.: Antibodies

pro hormone	peptide	antibody label
Prorelaxin	$RLX2_{69-82}$	AB2156
	$RLX2_{88-110}$	AB2356
Proenkephalin (human)	$PENK\,A_{153-168}$	AB2334
	$PENK\,A_{176-183}$	AB1879 & 2077
Proenkephalin (rat)	$PENK\,rat_{176-183}$	AB2480

Table 7.6.: Standard peptides

pro hormone	peptide	type	sequence
Pro-Relaxin 2	$RLX\,2_{56-157}$	recombinant	SLSQEDAPQT PRPVAEIVPS FINKDTETIN MMSEFVANLP QELKLTLSEM QPALPQLQQH VPVLKDSSLL FEEFKKLIRN RQSEAADSSP SELKYLGLDT HS
Pro-Enkephalin A (human)	$PENK\,A_{143-183}$	synthetic	DAEEDDSLAN SSDLLKELLE TGDNRERSHH QDGSDNEEEV S
Pro-Enkephalin A (rat)	$PENK\,rat_{143-183}$	synthetic	DADEGDTLAN SSDLLKELLG TGDNRAKDSH QQESTNNDED S

7.4. Sample material

7.4.1. Development of immunoassays

Serum and EDTA plasma as well as urine of healthy (human) control subjects for the development of the immunoassays were taken from serum bank of the Sphingotec GmbH. EDTA plasma samples of healthy rats were collected by the Preclinics GmbH.

7.4.2. Renal tissue for immunohistochemistry

Renal tissue for immunohistochemistry was provided as frozen biopsies from healthy parts of cancerous kidneys by Dr. Hans Krause (Charité Berlin). Tissue slices were prepared from biopsies as given in section 8.4.

7.4.3. Clinical cohorts

All clinical procedures described below were performed by cooperation partners previously to this thesis without the intention to assess pro-RLX2 or pro-Enkephalin concentrations. Residual samples stored at -80°C could be recruited for assessment of the pro-hormones and clinical databases were provided (as far as so far existing) for comparison. Methods of measurement and analysis performed by the author will be described in the methods section.

Ethical approvals Ethical approvals for all clinical studies were filed by cooperation partners and accepted by the ethical boards of the correspondent clinics. According to German law (§ 2 (5) 3. Absatz Medizinproduktegesetz) residual samples from laboratory routine can be used for technical development (here: for development and validation of the assay system) as long as they have been anonymized and retraceability back to the patient is not given. Agreement of the patient as well as duty of disclosure at the responsible authority is not needed in this case.

Gentamycin-induced nephrotoxicity (rat) The study was conducted by order of the Adrenomed AG and executed by thze Ricerca Biosciences Taiwan Ltd. Groups of 8 male Sprague-Dawley rats weighing $250 \pm 20g$ were employed. Animals were challenged with gentamycin at 120 mg/kg i.m. for seven consecutive days (Groups 1 and 2). Test compounds and control compound (aliquoted in vials) were injected intravenously 5 min. before gentamicin on day 0, followed by further injections on day 2, 4 and 6. Body weights and clinical signs of nephrotoxicity were

monitored daily. Twenty-four (24) hour urine collections on ice were performed on Days 0, 2, and 6. Concentrations of Na^+ and K^+ and creatinine as well as NAG activity (N-acetyl- ß-glucosaminidase; a marker of impaired proximal tubule function) and NGAL were detected within urine specimens. Blood samples for clinical chemistry have been collected on Day 1(before gentamicin), 3 (before gentamicin) and 7. All animals were sacrificed on Day 7.

Serum electrolytes (Na^+ and K^+), creatinine and BUN as well as NGAL and NAG activity were measured by Ricerca Biosciences. Creatinine clearance was calculated and provided by Ricerca Biosciences as well. Additional plasma samples were collected in EDTA tubes and shipped to Adrenomed AG on dry ice. Kidneys were weighed and shipped to Adrenomed AG on dry ice as well.

Measurement of pENK was performed within residual EDTA-plasma samples stored by the Adrenomed AG.

CLP-induced sepsis (rat) The study was conducted by order of the Adrenomed AG, approved by the local animal research ethics committee (Lariboisière-Villemin, Paris, France) (77-2014 -ceea9) and executed by the Department of Anesthesiology and Critical Care and Burn Center (GH St-Louis- Lariboisière, APHP, University Paris Diderot). Male Wistar rats (2-3 months, 300 to 400 g) from the Centre d'élevage Janvier (France) were allocated randomly to one of seven groups. All the animals were anesthetized using ketamine hydrochloride (90 mg/kg) and xylazine (9 mg/kg). For induction of polymicrobial sepsis, Cecal ligation and puncture (CLP) was performed using Rittirsch's protocol with minor modification. A ventral midline incision (1 cm) was made to allow exteriorization of the cecum. The cecum then was ligated just below the ileocecal valve and punctured once with an 18-gauge needle. The abdominal cavity was closed in two layers, followed by fluid resuscitation (3ml/100g body of weight of saline injected subcutaneously).

After surgery an anti-adrenomedullin antibody called HAM8101 (2 mg/kg) or placebo was injected (IV, 5 minutes after surgery) and norepinephrine or saline infusion was started. Hemodynamics points (BP and echocardiography) were registered 15 minutes and at further timepoints (1, 2, 3 h) after HAM8101 or placebo injection. A blood draw and subsequent EDTA-plasma generation was only done at the end of each experiment (27 h after CLP). There were 3 control groups (Sham, Sham + Norepinephrine, Sham + HAM8101 + Norepinephrine) and 4 CLP groups (CLP, CLP + HAM8101, CLP + Norepinephrine, CLP + HAM8101 + Norepinephrine). At the end of the experiment, the animal was euthanized, blood was drawn for EDTA-plasma generation and organs harvested for subsequent analysis.

Measurement of pENK was performed within residual EDTA-plasma samples (taken at the end of study) of CLP groups and only 1 control group (CLP). Since other studies showed no difference between treated an untreated CLP groups, for analysis these groups were merged and compared to the control group.

Renal transplantation therapy (Dragun) The study has been published elsewhere previously [107]. Briefly, a prospective observational pilot study with 28 living donor recipient pairs have been conducted. Blood and urine were collected on days -1, +1 and +7 pre and post transplantation. Plasma creatinine were measured daily between day -1 und +8. Samples were retrieved from freezer storage (-20°C) and tested for pENK concentrations without knowledge of outcome.

Healthy pregnant women Routine blood withdrawal was performed in healthy pregnant women in all three trimesters and EDTA-plasma samples stored anonymized at -80°C within the serum bank of the Sphingotec GmbH. For analysis, only gestational and biological age were recorded for each sample. A small cohort of pregnant women with consecutive blood samples (one for each trimester) was collected by the invent Diagnostica GmbH by order of the Sphingotec GmbH.

Preeclampsia Patient records in the Division of Maternal Fetal Medicine were reviewed during 2010-2014 to find women who received care for preeclampsia in pregnancy. Diagnostic criteria for preeclampsia were based on ACOG [115]: "new onset-hypertension (blood pressure consistently >140/90mmHg) after 20 weeks of gestation accompanied by evidence of organ injury (proteinuria, thrombocytopenia, renal or liver impairment, pulmonary edema, or cerebral/visual symptoms suggesting cerebral cortical dysfunction)". In 2013, the Hypertension in Pregnancy task Force [2] modified the criteria and eliminated the dependence of the diagnosis on proteinuria. Severe preeclampsia (now referred to as preeclampsia with severe features included any of the following: systolic blood pressures of 160 mmHg or higher, or diastolic blood pressure of 110 mg Hg or higher on two occasions at least 4 hours apart, thrombocytopenia, impaired liver function tests, progressive renal insufficiency, pulmonary edema or new-onset cerebral or visual disturbances. Early onset preeclampsia refers to those cases with symptoms requiring delivery of the baby before 34 weeks of gestation. Cases of preeclampsia were matched to prenatal screening records, resulting in 51 women with residual second trimester serum available for study. Residual second trimester serum samples from women who had developed preeclampsia in pregnancy were retrieved from freezer storage, along with 5 control samples matched to each case. Controls were matched

for duration of freezer storage within 3 months, completed week of gestation at sample collection (15-22 weeks), and African American or Caucasian race. This retrospective study was conducted with the approval of the Institutional Review Board at Women and Infants Hospital (Rhode Island, USA).

Samples were retrieved from freezer storage (-20°C) and tested without knowledge of outcome. All samples were tested for levels of endoglin (R&D Systems, MN), PlGF (provided by Perkin Elmer) and pro-RLX2 [148]. All assay coefficients of variation were less than 20%. Furthermore, second trimester levels of maternal serum alpha-fetoprotein (AFP), unconjugated estriol (uE3), human chorionic gonadotropin (hCG) and inhibin A were determined at the time of sample receipt using an automated chemiluminescent immunoassay method (DxI, Beckman Coulter Inc., Chaska, MN).

PROTECT A total of 301 patients hospitalized for acute heart failure (AHF) with an estimated creatinine clearance of 20 to 80 mL/min and elevated natriuretic peptide levels were enrolled within 24 hours of presentation to placebo or rolofylline 10, 20, or 30 mg administered as 4-hour infusions for three days in addition to intravenously administered loop diuretics. The study was approved by the relevant ethics committees, institutional review boards, and regulatory authorities. It was conducted under Good Clinical Practice and International Conference on Harmonization guidelines and was registered with www.clinicaltrials.gov as NCT00328692 (PROTECT-1) and NCT00354458 (PROTECT-2).

Consenting patients who met all inclusion and exclusion criteria (see below) were randomized to receive placebo or rolofylline 10, 20, or 30 mg in double-blind fashion. Before enrollment, evaluations of medical history, concomitant medications, and full physical examination with emphasis on heart failure (HF) symptoms and signs were performed. Laboratory samples (including safety chemistry and hematologic variables, electrolytes, serum creatinine, and urea) were drawn before study drug administration. Patients were randomized on day 1 to receive IV rolofylline or placebo as a 4-hour daily infusion. Study drug was administered daily for 3 consecutive days or until discharge.

Before study drug administration and up to day 7 or discharge, patients were evaluated daily for symptoms and signs of HF. In addition, blood samples for laboratory evaluation by the central laboratories were drawn daily to day 7 or discharge. Patients discharged before day 6 were asked to return for evaluation at an outpatient visit at day 7. During this visit, symptoms and signs of HF were evaluated and laboratory samples were collected. Symptoms and signs of HF were evaluated and laboratory samples were collected at day 14. Patients discharged

before day 14 were asked to return at that time for an outpatient visit. Patients were contacted by telephone at day 60, and hospital readmissions and deaths were recorded [35].

A randomly taken subset of 100 (residual) baseline samples were examined in pro-RLX2 assay and R&D relaxin2 assay.

Inclusion criteria

- aged $>$ 18 years
- AHF (defined by dyspnea at rest or minimal exertion accompanied by fluid overload manifested by jugular venous pressure $>$8 cm, pulmonary rates $\geq$ 1/3 up the lung fields not clearing with cough, $\geq$2+ peripheral edema, or presacral edema)
- Impaired renal function (defined as an admission estimated creatinine clearance of 20 to 80 mL/min using the Cockcroft-Gault equation)
- elevated screening natriuretic peptide levels (brain natriuretic peptide [BNP] $>$250 pg/mL or N-terminal pro-BNP [NT pro-BNP] $>$ 1000 pg/mL)

Exclusion criteria

- systolic blood pressure $<$ 95 or $>$ 160 mm Hg
- fever $>$ 38°C
- acute contrast-induced nephropathy
- resistant hypokalemia
- ongoing or planned intravenous (IV) therapy with positive inotropic agents, vasopressors, vasodilators with the exception of IV nitrates, or mechanical support (intra-aortic balloon pump, endotracheal intubation, or ventricular assist device)
- severe pulmonary disease
- significant stenotic valvular disease; previous heart transplant or admission for cardiac transplantation
- clinical evidence of acute coronary syndrome $<$ 2 weeks before screening; and AHF caused by significant arrhythmias
- high risk for seizures

BENEFICIAL This study is a double-blind, randomized, placebo-controlled, parallel design trial enrolling 100 patients (2x50) with stable CHF. Patients were randomized to either 200 mg Alagebrium twice daily or placebo for a period of 9 months. Efficacy measurements were performed at baseline and at the end of the study, and included aerobic capacity (VO2max) exercise testing, echocardiography, Minnesota Living with Heart Failure score, advanced glycation end product (AGE) measurements in blood and skin, New York Heart Association (NYHA) heart failure class, patient's and physician's global assessment, and levels of NT-pro-BNP. Safety visits were performed at intervals of three months. In addition, one safety visit was performed 2 weeks after the randomization visit and 1 month after the last treatment visit. A total of 8 visits were performed during the entire study [208]. (Residual) baseline samples were examined in the pro-RLX2 assay.

Inclusion Criteria

- age >18
- NYHA II-IV heart failure
- Echocardiographic ejection fraction $\leq 40\%$
- Duration of heart failure $>$ 3 months
- Stable heart failure medical therapy for $>$ 1 months

Exclusion Criteria

- History of myocardial infarction in previous 6 months
- History of stroke in previous 6 months
- Clinically significant renal, liver, pulmonary,or hematological disease
- Active and or treated malignancies within 12 months
- Uncontrolled diabetes mellitus

7.5. Equipment

Table 7.8.: Equipment used for assay development experiments

equipment	provider
analysis scale ABJ 320-4NM	Kern, Balingen, Germany
Centro-Pure P25 columns	emp Biotech, Berlin, Germany
combitips advanced	Th. Geyer, Renningen, Germany
EP T.I.P.S. 2-200µL	Sarstedt, Nümbrecht, Germany
EP T.I.P.S. 50-1000µL	Sarstedt, Nümbrecht, Germany
EP T.I.P.S. Standard 0.1-20 µL	Th. Geyer, Renningen, Germany
EP T.I.P.S. Standard 20-300 µL	Th. Geyer, Renningen, Germany
Filtropur S (0.2µm)	Th. Geyer, Renningen, Germany
graduated cylinders (50-2000mL)	Labsolute, Renningen, Germany
HPLC Smartline	Knauer, Berlin, Germany
luminometer Centro LB 952 T/16	Bertold, Bad Wildbad, Germany
luminometer Centro LB 960	Bertold, Bad Wildbad, Germany
lyophilizator Alpha 1-4 Ldplus	Christ, Osterode am Harz, Germany
microtiter plates Lumitrac 600	greiner bio-one, Frickenhausen, Germany
multipette Research 0.5-5mL (Multistep)	Eppendorf, Wesseling-Berzdorf, Germany
multipette research plus 30-300µL (Multichannel)	Eppendorf, Wesseling-Berzdorf, Germany
multipette Research pro 1200 (electrical Multichannel)	Eppendorf, Wesseling-Berzdorf, Germany
pH-Meter SevenEasy	Mettler Toledo, Gießen, Germany
Pipet Assistant	Labsolute, Renningen, Germany
Pipette Research plus 1 - 10 µL	Eppendorf, Wesseling-Berzdorf, Germany
Pipette Research plus 10 - 100 µL	Eppendorf, Wesseling-Berzdorf, Germany
Pipette Research plus 100 - 1000 µL	Eppendorf, Wesseling-Berzdorf, Germany
Pipette Research plus 2- 20 µL	Eppendorf, Wesseling-Berzdorf, Germany
polytubes (15 ml)	Sarstedt, Nümbrecht, Germany
polytubes (50 ml)	Sarstedt, Nümbrecht, Germany
precision scale 440-49A	Kern, Balingen, Germany
SafeSeal reaction tubes (1.5 ml)	Sarstedt, Nümbrecht, Germany
SafeSeal reaction tubes (2 ml)	Sarstedt, Nümbrecht, Germany
serological pipettes 5-50mL (single use)	Th. Geyer, Renningen, Germany
shaking device KS 250	Ika-Labortechnik, Staufen, Germany
shaking device Titramax 101	Heidolph, Schwabach, Germany
vortexer IKA Vortex 1	Ika-Labortechnik, Staufen, Germany
washer Wellwash Versa	Thermo Scientific, Dreieich, Germany

Table 7.9.: Equipment used for immunohisto experiments

equipment	provider
humid chamber	self-made
microscope	Zeiss, Göttingen, Germany
microtom (RM2125RT)	Leica, Nussbach, Germany
staining jar	A. Hartenstein GmbH, Würzburg, Germany

7.6. Software

Table 7.10.: Software

Software	provider	purpose
GraphPad Prism 7	GraphPad Software	simple statistical analysis design of figures
SPSS 11	IBM	advanced statistical analysis
Powerpoint	Microsoft	simple image editing design of figures
Microwin 10	MSE	measurement of luminescence standard curve plotting
LyX	Open Source project	word processing
Zotero	Roy Rosenzweig Center for History and New Media	literature management

8. Methods

8.1. Peptide synthesis

Peptide sequences of a length of 14-20 amino acid (aa) were selected from the full-length pro-hormone using protein blast (blast.ncbi.nlm.nih.gov) to check for cross-reactivities with other peptides. All peptides were synthesized by the Peptides & Elephants GmbH.

8.2. Production of specific antibodies

All antibodies were produced by the InVivo BioTech Services GmbH according to their GLP-certified standard operating procedures. All antibodies are monoclonal and protein G purified.

Protein quantification Protein concentration of antibody solutions is determined photometrically at the InVivo BioTech Services GmbH by default and reported to the customer.

Quality control (SDS-PAGE) For quality control, purified antibodies are applied to an SDS-PAGE at InVivo BioTech Services GmbH by default. A quality report containing a picture of this SDS-PAGE - proofing the antibody consisting of only one type of heavy and light chain each - is given at delivery of the antibody to the customer.

Labeling with acridinium ester (MACN) Proteins and antibodies can be conjugated easily to the chemiluminescent dye MACN, a so called acridinium ester. Light emission of these conjugates is triggered by the application of acetous hydrogen peroxide solution followed by adding sodium hydroxide. Here, the acridinium ester dissociates to N-Methyl-Acridon which emits light of 442nm wavelength [23].

Purified antibodies were labeled with MACN 1:5 as follows: The corresponding volume of MACN was added to the antibody solution and incubated for 30±5 min. in the dark. Incubation was stopped by adding 1M TRIS solution. Labeled antibody was purified from free MACN by gel filtration (using gel filtration columns).

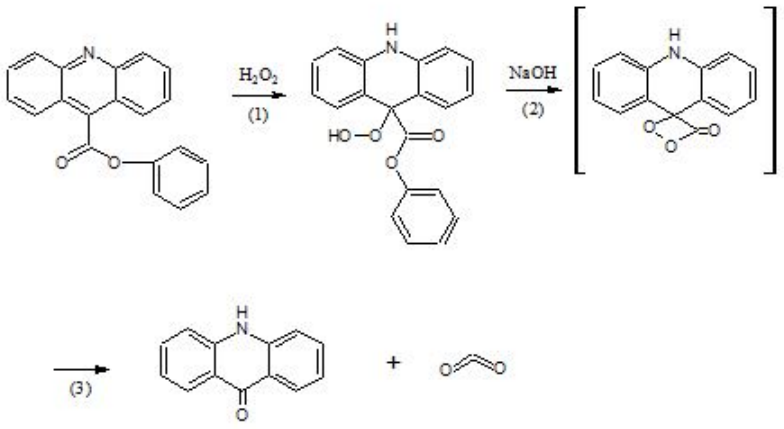


Figure 8.1.: **Schematic view of the acridinium ester reaction.** The acridinium ester is oxidized by hydrogen peroxide (1). Sodium hydroxide triggers the dissociation via dioxetanone (2) to the excited acridone (3) the fluorescence of which is blue (442nm).

By using HPLC (0.5mL/min. flow rate) the labeled antibody was purified, further separating the antibody peak from free MACN and unlabeled antibody. The purified tracer concentrate was quantified (in an appropriate dilution) with the luminometer and stored until further use at -20°C.

Immobilization of antibodies to microtiter plates Wells were filled with coupling buffer (containing the antibody), covered and incubated overnight (approx. 19h) at room temperature. After removal of coupling buffer, the wells were washed with blocking solution (BSA-containing) once and then incubated with 300µL blocking solution for 1h at room temperature. Afterwards, blocking solution was removed again. Plates were dried and used immediately or stored at 2-8°C.

8.3. Establishment of immunoassay methods

Technical documentation of the established immunoassay methods is given in the appendix Section A-C.

8.4. Immunohistochemical procedures

Fixation of frozen kidney For immunohistochemical staining, frozen kidney biopsies needed to be fixed in paraffin. Therefore, biopsies were de-frozted, fixated in 4% formalin for 24 hours and transferred to PBS. Then biopsies were dehydrated

in a specialized fixation facility through chloroform, different concentrations of ethanol and paraffin. Finally, biopsies were embedded in paraffin.

Preparation of slides Paraffin-fixed kidney biopsies were sliced in sections of 2µm which were placed on microscope slides.

Deparaffination Microscope slides were deparaffinated before staining since antibodies need accessible epitopes for binding. Therefore, slides were incubated in solutions of decreasing ethanol concentration as follows: 2 x 5 min in Xylol, 2 x 5 min in 100 % Ethanol, 2 x 2 min in 96 % [v/v] Ethanol, 1 x 2 min in 70 % [v/v] Ethanol, 1 x 2 min in 40 % [v/v] Ethanol and 2 x 5 min in Aqua dest.

Heat demasking Epitopes were made accessible by heat demasking: 0.01 M citrate buffer was brought to the boil at 800 W (approx. 3 – 6 min). Slides were boiled at 450 W for 2 x 5 min and cooled down to room temperature in the citrate buffer. Slides were rinsed and washed for 2 x 5 min in Aqua dest.

Blocking of endogenous peroxidases To block endogenous peroxidases, slides were incubated 10 min at room temperature in 3 % [v/v] H_2O_2 in Aqua dest. Afterwards, slides were again washed 2 x 5 min in Aqua dest and quickly rinsed in PBS-T and PBS. Following, slides were blocked in blocking serum DAKO S3022 for 30 min at room temperature within a humid chamber.

Primary and secondary antibody Primary antibody was diluted in DAKO S3022. Slides were incubated with primary antibody overnight at 4°C within a humid chamber. On the next morning, slides were quickly rinsed in PBS, then washed 3 x 5 min in PBS-T and quickly rinsed in PBS again. Slides were incubated with secondary antibody (anti-mouse) for 30min at room temperature within a humid chamber. Afterwards, slides were quickly rinsed in PBS, then washed 3 x 5 min in PBS-T and quickly rinsed in PBS again.

Staining: Streptavidin complex and DAB chromogen Slides were incubated with Streptavidin-HRP K0690 for 30min at room temperature within a humid chamber. Following, slides were quickly rinsed in PBS, then washed 3 x 5 min in PBS-T and quickly rinsed in PBS again. DAB-Chromogen (DAKO K3466) was diluted 1:50 in DAB buffer. Staining development (1 – 15 min) was cross-checked under microscope. Slides were cross-stained with hemalm.

Covering of slides Slides were dehydrated within solutions of increasing ethanol concentrations as followed: 2 x 2 min 96 % [v/v] Ethanol, 2 x 2 min 100 % Ethanol and 2 x 5 min Xylol. Slides were covered using Entellan.

8.5. (Pre-)Clinical study measurement

8.5.1. Immunoassay protocols

General protocol for immunoassays developed at the sphingotec GmbH Most immunoassays developed at the sphingotec GmbH follow the same basic procedure:

Pre-preparation All kit components and samples are equilibrated to room temperature. All liquid components are gently mixed prior to use. Plate layout is noted on a form. The luminometer is prepared for operation by injecting lightning reagents. Detection antibody is diluted in assay buffer to an appropriate concentration (determined by luminescence in relative light units).

Assay preparation If not indicated otherwise, calibrators, controls and samples are assessed in double determination. To avoid carry-over new pipet tips for each sample are used. After application of standards/controls/samples to the cavities, detection antibody diluted in assay buffer is applied to the cavities. The assay is incubated at temperature, shaking frequency and timespan noted in the specifications of the assay. The plate needs to be covered and protected from light while incubating. After incubation time, all cavities are aspirated and washed with sphingotec washing solution . All cavities necessarily need to be fully aspirated and re-filled with washing solution to ensure reliable measurement. For 2step assay preparation, assay buffer is applied instead of diluted detecting antibody in step 1. In step 2 diluted detecting antibody is added to the washed cavities, incubated and removed by washing after incubation again.

Measurement After washing cycles well contents are fully aspirated and inserted into the luminometer. Lightning reagents ($H_2O_2 + NaOH$) are added automatically and luminescence signals detected (recommended measurement time: 1s/cavity).

pro-RLX2 assay Table 8.1 shows the incubation scheme for the pro-RLX2 assay system applied to all clinical study cohorts reported in this thesis.

Table 8.1.: **Incubation scheme for pro-RLX2 assay system.** All variable parameters of the general scheme described above are listed.

	action	material	volume	standard	controls	samples
1.	pipetting	standard	µL	100	-	-
		controls	µL	-	100	-
		samples 1,...n	µL	-	-	100
2.	pipetting	diluted detecting AB	µL	150	150	150
3.	incubation	20 ± 2 hours at 4-8°C with 600rpm				
4.	pipetting aspiration	washing solution	µL	350	350	350
5.	washing step	repeat 5x				
6.	measurement					

pro-Enkephalin (human): plasma/serum Table 8.2 shows the incubation scheme for the human pENK assay system for measurement within plasma/serum to all clinical study cohorts reported in this thesis.

Table 8.2.: **Incubation scheme for pENK assay system for measurement within plasma/serum.** All variable parameters of the general scheme described above are listed.

	action	material	volume	standard	controls	samples
1.	pipetting	standard	µL	50	-	-
		controls	µL	-	50	-
		samples 1,...n	µL	-	-	50
2.	pipetting	diluted detecting AB	µL	150	150	150
3.	incubation	20 ± 2 hours at room temperature without shaking				
4.	pipetting aspiration	washing solution	µL	350	350	350
5.	washing step	repeat 4x				
6.	measurement					

pro-Enkephalin (human): urine Table 8.3 shows the incubation scheme for the human pENK assay system for measurement within urine to all clinical study cohorts reported in this thesis.

Table 8.3.: **Incubation scheme for pENK assay system for measurement within urine.** All variable parameters of the general scheme described above are listed.

	action	material	volume	standard	controls	samples
1.	pipetting	standard	µL	50	-	-
		controls	µL	-	50	-
		samples (prediluted) 1,...n	µL	-	-	50
2.	pipetting	assay buffer	µL	150	150	150
3.	incubation	4 hours at room temperature without shaking				
4.	pipetting aspiration	washing solution	µL	350	350	350
5.	washing step	repeat 4x				
7.	pipetting	diluted detecting AB	µL	200	200	200
8.	incubation	16 ± 2 hours at room temperature without shaking				
9.	pipetting aspiration	washing solution	µL	350	350	350
10.	washing step	repeat 4x				
11.	measurement					

pro-Enkephalin (rat) Table 8.4 shows the incubation scheme for the rat pENK assay system for measurement within plasma/serum to all clinical study cohorts reported in this thesis.

Table 8.4.: **Incubation scheme for rat pENK assay system for measurement within plasma/serum.** All variable parameters of the general scheme described above are listed.

	action	material	volume	standard	controls	samples
1.	pipetting	standard	µL	50	-	-
		controls	µL	-	50	-
		samples 1,...n	µL	-	-	50
2.	pipetting	diluted detecting AB	µL	150	150	150
3.	incubation	20 ± 2 hours at 4-8°C without shaking				
4.	pipetting aspiration	washing solution	µL	350	350	350
5.	washing step	repeat 4x				
6.	measurement					

8.5.2. Statistical analysis of results

In general, the level of significance was two-tailed $p < 0.05$.

Gentamycin-induced nephrotoxicity (rat) pENK concentrations are shown as means with standard deviation. Differences between the days were compared by

multiple Wilcoxon signed-rank tests applying Bonferroni correction for α error accumulation. A day by day analysis for every treatment group was performed using Mann-Whitney test. Comparison of treatment groups regarding day to day differences was performed using Wilcoxon tests without Bonferroni correction. Correlation with other renal biomarkers was performed using Spearman correlation.

CLP-induced sepsis (rat) pENK concentrations are shown as means with standard deviation. Multiple Mann-Whitney tests were performed comparing different treatment groups. No Bonferroni correction was applied since the overall difference between CLP and Sham remained significant . Correlations with absolute and relative organ weight, total body weight, heart rate, mean arterial pressure and body temperature were performed using Spearman correlation.

Renal transplantation therapy Summary data are shown as Median with Interquartile Range. Correlations were performed using Spearman correlation. Mann-Whitney tests with Bonferroni correction were applied to determine differences between non-connected groups (donors vs recipients). For comparison of differences between connected groups (pro-RLX2 conc. at different days) Wilcoxon test was performed.

Healthy pregnant women Summary data are shown as Means with Standard Error Mean. Correlations were performed using Spearman correlation. Mann-Whitney tests with Bonferroni correction were applied to determine differences between non-connected groups (trimester differences between different individuals, pregnant vs non-pregnant, male vs female). Distribution was tested for normality using D'Agostino-Pearson omnibus test. For comparison of differences between connected groups (pro-RLX2 conc. at different gestational age within single individuals) the Wilcoxon test was performed.

Preeclampsia Marker concentrations were compared between cases and controls using matched rank analysis (summary data shown as Median and Interquartile range). The expected mean case rank for no association is 3.5, with lower ranks indicating lower levels in cases. These ranked analyses were also performed for each outcome subtype (e.g., severe/early preeclampsia). Data from control pregnancies were combined to create gestational age specific reference data (medians). All data, including cases, were converted to multiples of the median (MoM). Spearman correlation coefficients for pro-RLX2 with each marker were also calculated.

PROTECT Correlations between assay methods and biomarkers were calculated using Spearman correlation. For analysis patients were grouped according to their pro-RLX concentration (undetectable vs low vs high) and renal markers tested for significant differences using Mann-Whitney for skewed variables and T-test for normally distributed variables (such as age etc.). A multivariable linear regression was performed to determine strongest predictors for pro-RLX2 concentration.

BENEFICIAL Data from the beneficial study were analyzed in parallel to data from PROTECT (see above).

IV.

Results

9. Establishment of immunoassay methods and further characterization of analyzed pro-hormones

9.1. Pro-Enkephalin

9.1.1. Measurement in plasma

The method of measuring pENK in plasma has been established by Dr. Andrea Sparwaßer (neé Ernst) and described elsewhere [53]. The assay is commercially available under the designation of sphingotest® penKid.

9.1.2. Measurement in urine

9.1.2.1. Rationale

Since for other biomarker even a stronger association of urine concentration with renal function was reported than for plasma concentrations, we analyzed the possibility to measure pENK in urine [57]. Therefore, the assay system had to be tested for the use with urinary matrix. Biological variance had to be determined since high fluctuations in urinary concentrations over daytime would make a possible urinary marker useless for clinical routine. The correlation with plasma values and renal function was investigated to evaluate a possible benefit in measuring urinary pENK concentrations in addition to plasma values.

9.1.2.2. Processing of assay conditions

The established system for quantification of pENK in human plasma samples was taken as a basis and further optimized for detection in urinary matrix. Buffer system, standard resuspension matrix and incubation setting were adjusted to a two-step incubation at 6°C. For details about assay development also compare appendix Section A.

9.1.2.3. Pilot analysis of biological variance

To estimate biological variance of pENK during daytime, two healthy individuals (male & female) were examined over 12 hours with regularly taken urine samples as very small pilot study. As expected, urinary pENK and creatinine concentration showed a relatively high variation due to changing urine concentrations (Fig. 9.1 a & b). When urinary pENK values were normalized to creatinine, this effect was balanced and the ratio was more or less stable with a median ratio of 0.09 [IQR 0.05-0.125] over 12 hours (Fig. 9.1c). A Friedman test for repeated measures showed no significant differences ($p = .1333$). Nevertheless, statistical analysis of two individuals remains problematic and bigger studies are required before drawing any conclusions.

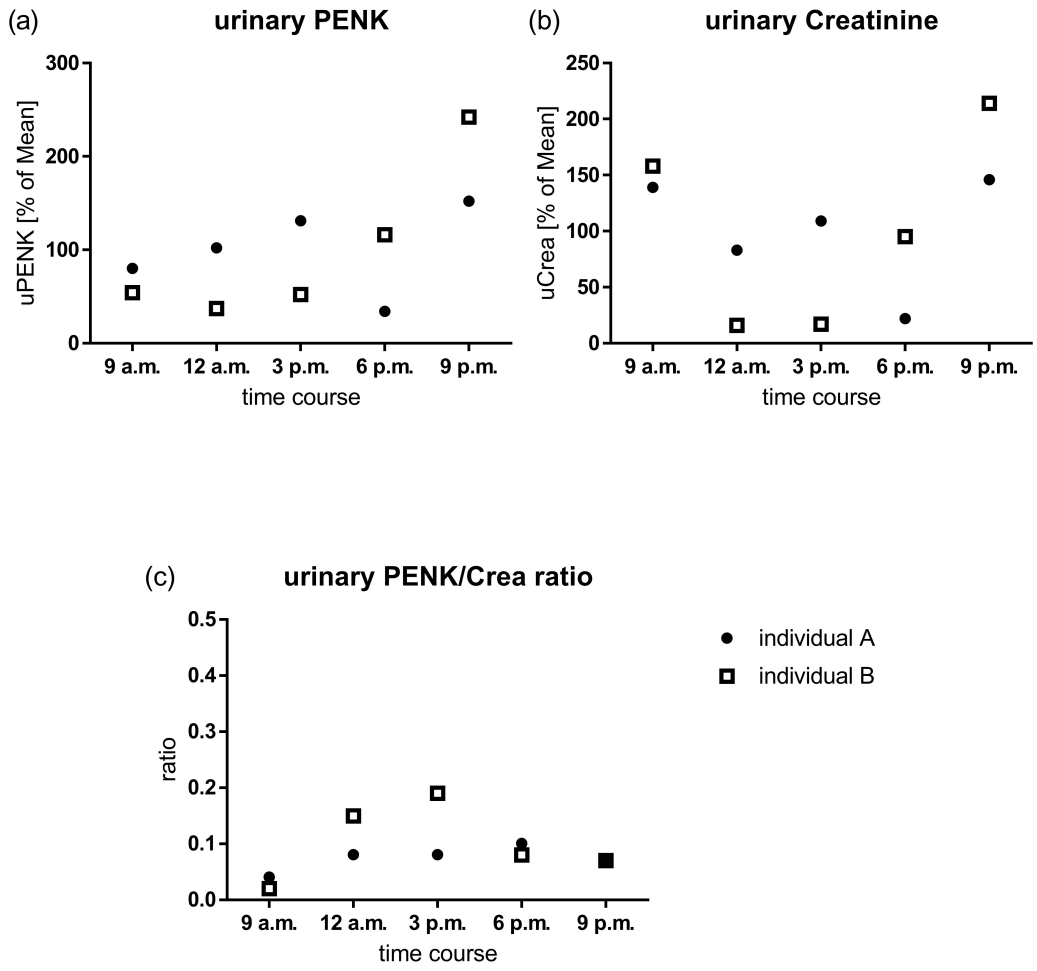


Figure 9.1.: **Pilot study of urinary pENK and uCrea in two healthy individuals.** (a) urinary pENK concentration shows relatively high variation during daytime (range 37-242% of mean). (b) uCrea concentration shows high variation, too (range 16-214%). (c) urinary pENK normalized to uCrea shows a ratio varying between 0.02 and 0.19 over 12 hours.

9.1.2.4. Correlation with Pro-Enkephalin plasma values

Healthy kidney donors and their recipients with ESRD were examined before and after transplantation for urinary and plasma pENK concentrations. Fig. 9.2a shows a strong, highly significant correlation between plasma and urinary pENK ($\rho = .56$) in healthy as well as in injured patients. Healthy donors can be easily discriminated from injured recipients. On 1st day after transplantation, correlation is still significant, but weaker ($\rho = .37$). Discrimination between healthy and

injured is only possible in pENK plasma concentrations, urinary concentrations are more or less comparable. 7 days after transplantation, no significant correlation can be shown anymore, potentially indicating (at least partially) separated regulatory mechanisms after transplantation for plasma and urinary pENK.

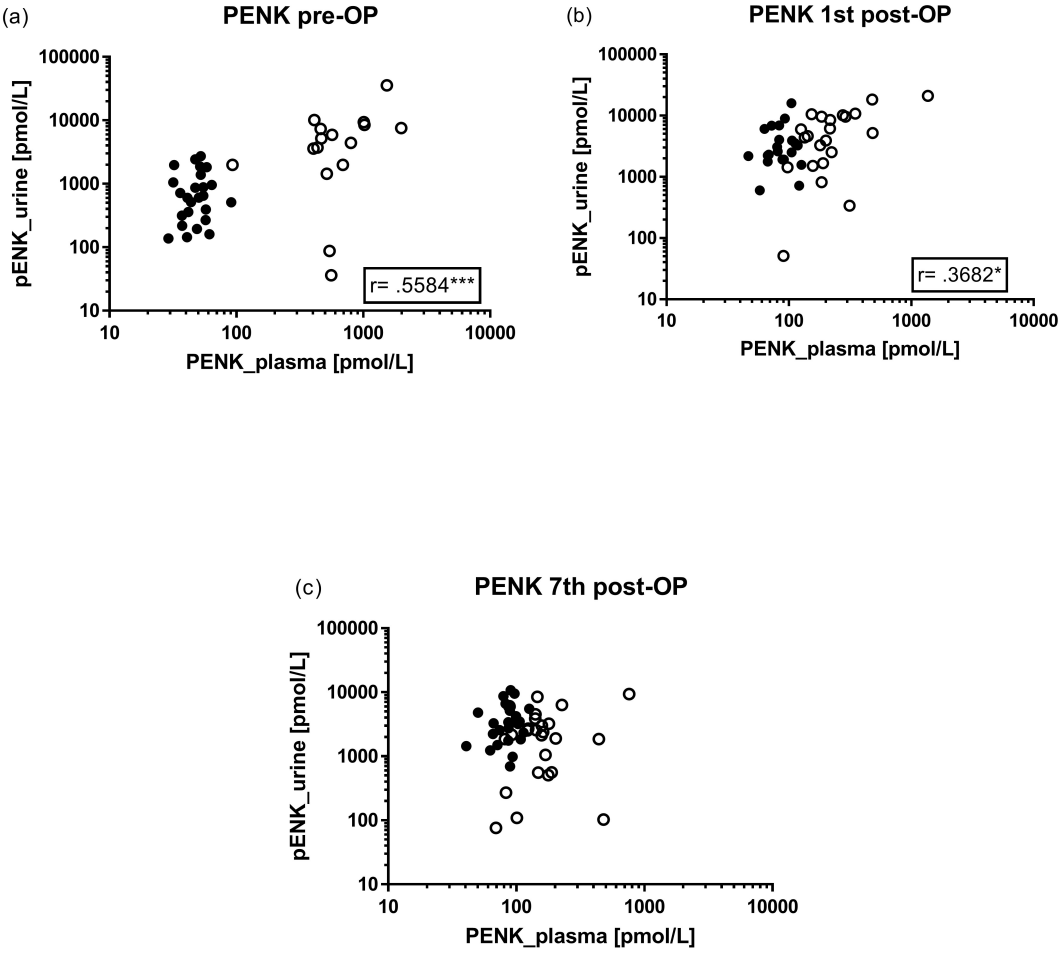


Figure 9.2.: **Correlation of pENK concentration in plasma and urine.** (a) pENK concentration in healthy donors (filled circles) and renally injured recipients (empty circles) before transplantation. Correlation is strong and highly significant ($\rho = .5584$, $p = .0001$) (b) 1 day post-OP in healthy donors (filled circles) and renally injured recipients (empty circles). Correlation is still significant, but weaker ($\rho = .3682$, $p = .0151$) (c) 7 days post-OP in healthy donors (filled circles) and renally injured recipients (empty circles). No significant correlation can be shown.

9.1.3. Measurement in animal model

9.1.3.1. Rationale

When analyzing human study data there is always a problem of confounding factors which might lead to misinterpretation in disease development. Therefore it can be helpful to investigate in animal models where one can directly control confounding factors. Thus, we investigated the technical possibility to measure pENK plasma concentrations in rat. Following, we measured plasma concentrations in several renal injury models in the assay for proof of concept (comp. Section 10.1.1).

9.1.3.2. Analysis of cross-reactivity with rat of antibodies against human epitopes

13 antibodies obtained from mice immunized against six different, partially overlapping epitopes within the human pENK sequence were tested for binding to the corresponding rat pENK sequence. Fig. 9.3 shows the sequence homology between human, rat and mouse. Human and rat share 27/41 amino acids (66%), rat and mouse sequence are nearly completely homologous (40/41, 98%). The rat sequence is prolonged by two amino acids inserted within the C-terminal sequence, while the murine sequence contains one insertion. Still the assay is very likely to detect murine pENK even though designed for the rat sequence. The human sequence differs in three N-terminal amino acids, the N-terminal to mid-regional amino acids are homologous or similar, the C-terminus contains greater differences (10/15 aa). As expected, N-terminally to mid-regionally binding antibodies showed moderate to good binding (antibodies C-H). Highest crossreactivity was observed for the mid-regional antibodies (antibodies G,H,I). Antibodies against the very N-terminus of the human peptide showed no binding (antibodies A&B) as well as these against human C-terminus (antibodies J-M). These epitopes showed a big overlap, so antibodies against these regions might not be able to form a sandwich complex. Also antibodies C-F were not able to form sandwich complexes with antibodies G-H (data not shown). Therefore, an immunization against the C-terminal rat sequence was ordered. Immunization peptide was selected in a version reduced by the two very C-terminal amino acids (tyrosine and lysine) for two reasons: Since the rat and murine sequence differ in the very C-terminal aa so that with the selected epitope antibodies against it should be more likely to detect also the murine pENK. Furthermore, if reducing the immunization peptide by two aa, the corresponding antibodies should not only detect the cleaved form of pENK A, but also longer fragments.

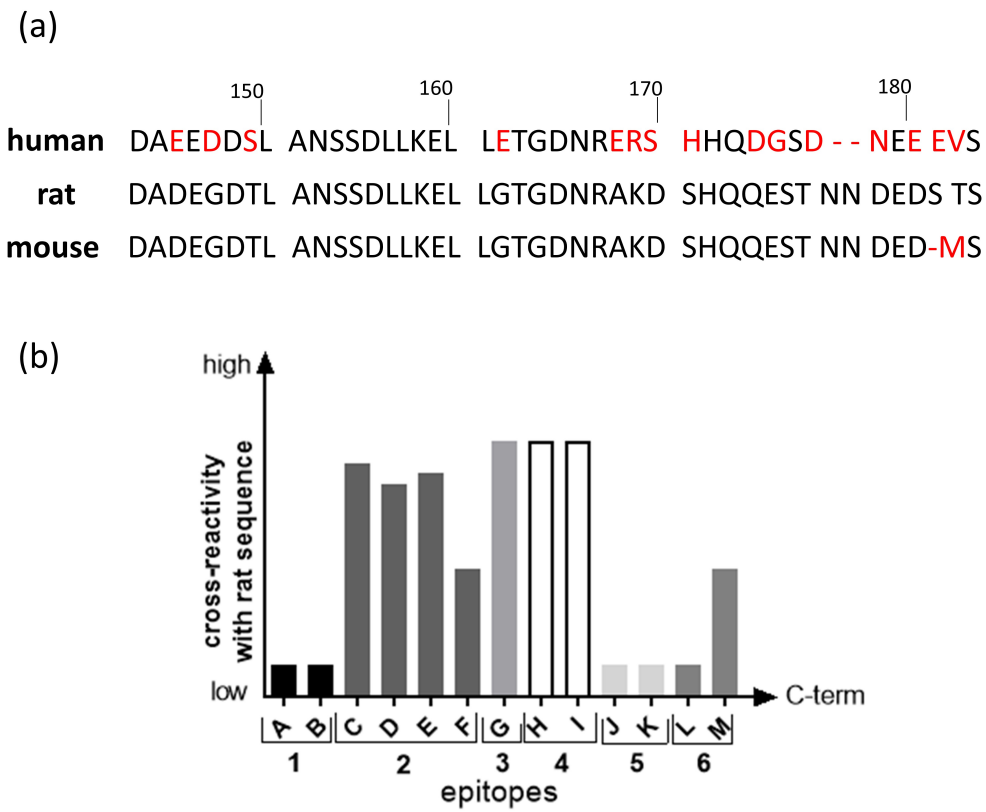
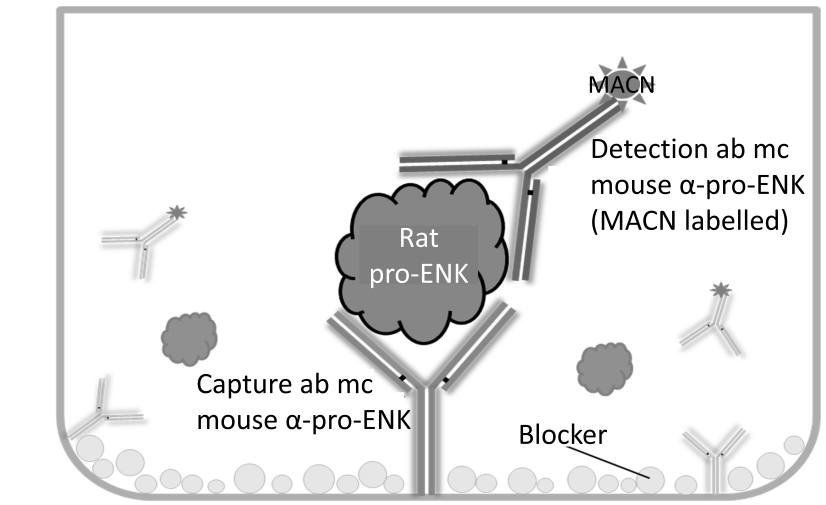


Figure 9.3.: **Cross-reactivity with rat of antibodies against human epitopes.** (a) sequence homology reveals a homologous N-terminal to midregional region between human and rat (aa 150-160) while the C-term contains greater differences (aa 168-182). Rat and mouse sequence is full homologous exept one amino acid at the very C-term (aa 183). Amino acids differing from rat sequence are highlighted in red. Numbering follows the human sequence and is of course shifted in rat and murine sequence due to the insertions. (b) shematic representation of the binding of antibodies to rat pENK sequence sorted by epitopes from N- to C-term. Cross-reactivity is grouped in five categories from low to high. As exptected, there is a moderate to good binding to N-terminal and midregional epitopes. Antibodies G, H and I show highest binding.

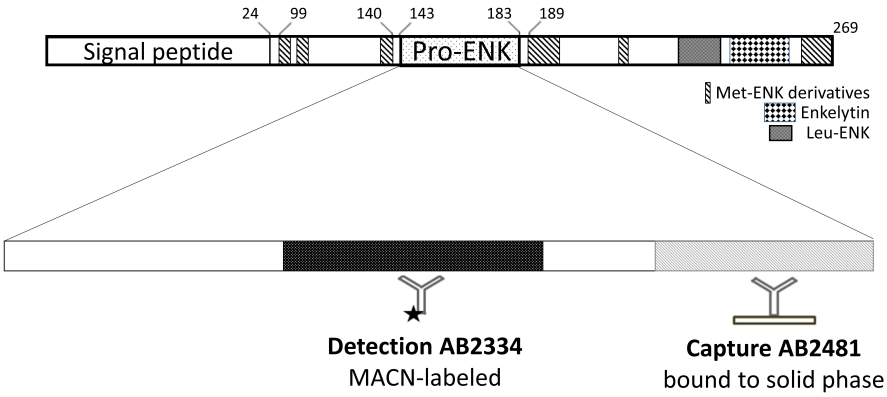
9.1.3.3. Development of the assay system

Assay principle We designed a one step sandwich immunoassay system based on two monoclonal antibodies targeting two different regions within the connecting peptide. Two antigen-specific antibodies are used in excess. One of them is labeled with a luminescent substance (detection antibody), the other one is immobilized to cavities of a 96well plate (capture antibody). As the incubation is ongoing, both antibodies are reacting with the pENK molecules of the sample forming so called sandwich complexes. After reaction is finished, excessive detection antibody is removed completely by washing. Bound detection antibody remains bound throughout the washing steps and can be detected in the luminometer. The relative light units (RLU) are directly proportional to the pENK concentration of the sample and are calibrated with synthetic rat pENK fragment of known concentration.

Figure 9.4 shows the general assay principle of one capture antibody targeting the pENK C-terminal amino acids and one MACN-linked detection antibody targeting the mid-region. 96well plates are coated with capture antibody, saturated with BSA solution and lyophilized. 50µl of sample are added to each well and incubated with detection antibody diluted in assay buffer over night at 4-8°C. After washing, signal is detected using luminometric immunoassay (LIA) technology. For details about assay development also compare appendix Section B.



(a)



(b)

Figure 9.4.: **Assay principle of pENKrat assay.** (a) a sandwich complex is formed by two antibodies and the analyte in a well saturated with a blocker (BSA) (b) representation of the common peptide structure of Enkephalin. The illustrated sequence includes the signal peptide, the (bio-active) Met- and Leu-Enkephalins as well as the pro-hormones Enkelytin and pENK. The capture antibody is targeting the C-terminal amino acids. The MACN-linked detection antibody is targeting the midregion.

9.1.4. Localization of pro-hormones within renal tissue (pilot experiment)

9.1.4.1. Rationale

We investigated the specific localization of the measured pro-hormone pENK within renal tissue via immunohistochemistry. Denning et al. [42] showed that pENK mRNA expression in rats is widely distributed in non-neuronal tissues, especially in the kidney. Nevertheless, the global detection of mRNA expression in a complex organ like the kidney does not allow deductions about the distribution within the tissue which is highly important to understand the functional context. De Falco et al. [38] stained the anuran kidney for PENK expression as well but with different expression patterns in two different frog species - therefore the results might not be transferable to human as well.

9.1.4.2. Expression of pro-Enkephalin in healthy renal tissue

In a first experiment in August 2014 sections of healthy renal tissue were stained with an antibody targeting the C-terminal region of pENK. This antibody targets the same epitope as the catching antibody of the sphingotest® penKid. Since no "real" negative control like knockout rats was available, the antibody was pre-incubated with synthetic pENK peptide as surrogate for a negative control. A strong immunopositivity could be shown in the tubules while no immunopositivity was detected in glomeruli (glomerulus: upper right corner of figure 9.5a). As expected, sections stained with the pre-incubated antibody show no immunopositivity (Fig. 9.5b). The experiment was repeated three years later and no distinct immunopositivity could be shown (Fig. 9.5c). Interestingly, some of the negative controls showed a nuclear immunopositivity (Fig. 9.5d). Furthermore, sections were stained with the detecting antibody of the sphingotest® penKid targeting a midregional epitope. This staining showed no distinct immunopositivity as well (Fig. 9.5e). As control of the negative control, the antibody was pre-incubated with a foreign peptide (recombinant pro-RLX2) as well which is supposed not to interfere with the binding between antibody and section. Again, no distinct immunopositivity could be detected.

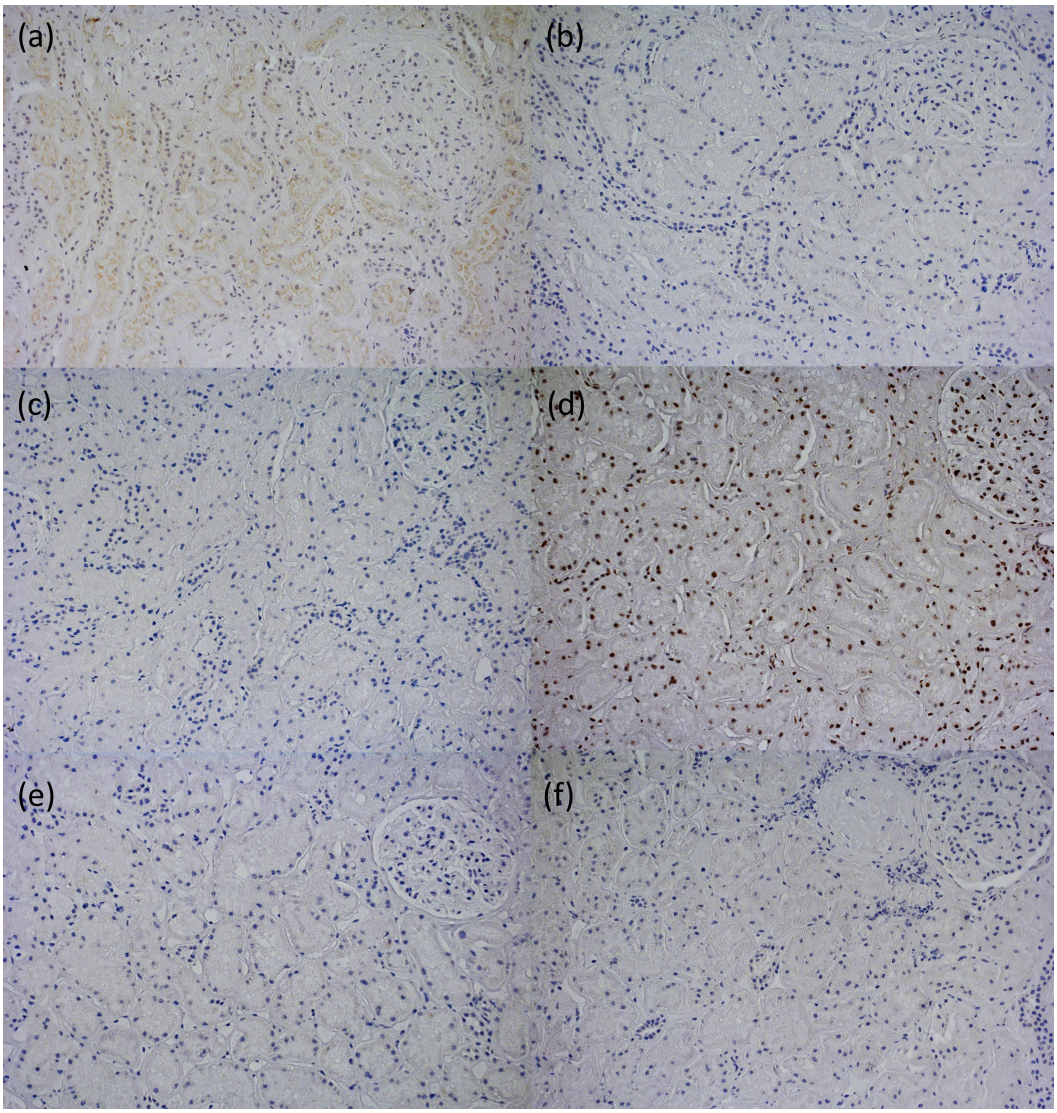


Figure 9.5.: **Immunohistochemical staining of healthy renal tissue sections (representative figures).** (a) sections were stained with c-terminal anti-pENK antibody (AB2077) in August 2014. Immunopositivity can be detected in tubules while no immunopositivity is found in glomeruli. (b) sections were stained with AB2077 pre-incubated with synthetic pENK peptide as negative control. As expected, no immunopositivity can be detected. (c) sections were stained with c-terminal anti-pENK antibody (AB2077) in March 2017. No distinct immunopositivity can be detected. (d) sections were stained with AB2077 pre-incubated with synthetic pENK peptide as negative control. While several sections show no immunopositivity, some sections show nuclear immunopositivity. (e) sections were stained with midregional anti-pENK antibody (AB2334) in March 2017. No distinct immunopositivity can be detected. (f) sections were stained with AB2077 pre-incubated with recombinant pro-RLX2 peptide.

9.2. Pro-Relaxin 2

9.2.1. Assay development

9.2.1.1. Rationale

Several studies showed a short half-life for mature relaxin-2 *in vitro* varying between 0.5 and 4 hours in serum [71, 51, 25]. Therefore, a reliable quantification of mature relaxin-2 might be difficult to achieve. A few assays for quantification of the mature human relaxin-2 are commercially available (e.g. by R&D systems, Phoenix pharmaceuticals, Immundiagnostik AG etc.), though none of these assay methods are published in detail regarding sensitivity and reliability. Furthermore, a quantification of its connecting peptide (pro-RLX2) might be beneficial even if the peptide itself might be without function: Analogous to insulin, the measurement of the connecting peptide could show the actual synthesis rate while the mature relaxin-2 might be already metabolized or degraded [34]. The level of the connecting peptide is also independent of exogenous therapeutic administration of recombinant relaxin and could therefore be used for monitoring the endogenous function during therapy.

9.2.1.2. Selection of antibody epitopes and immunization

For immunization several different epitopes were selected from the pro-RLX2 sequence. Each had a length of 12 to 15 amino acids which had been previously shown in our routine as an appropriate length to ensure sufficient specificity. Prior to immunization, selected epitopes were tested *in silico* for cross-reactivity with other peptides in the whole proteome sequence of human (NCBI protein blast). The cross-reactivities for the two antibodies used for assay development are shown in table 9.1.

The Expect value (E) is used as a convenient way to create a significance threshold for reporting results. (E) describes the number of hits occurring only by chance when searching a database. It decreases exponentially as the Score of the match increases. This basically describes the model fit of the analysis. Essentially, the E value describes the random background noise. The lower the E-value, the more significant the match. The calculation of the E value also takes the length of the query sequence into account, so short alignments have relatively high E values.

Because the detecting antibody binds to the homologous region within the pro-RLX2 sequence, it shows the highest score for both isoforms 1 and 2. Basically, E values are p-values multiplied by the database size. As the human proteome contains approximately 70,000 proteins, the Expect value of 2e-6 is equivalent

with a p-value of 7×10^{-8}, which is highly significant. Only reporting results with a p-value lower than 0.0001 (highly significant), we determine a threshold Expect of 7. For the detection antibody, only the CCR4-associated factor 1 shows significant identity.

For the capture antibody highest identity (beside the pro-RLX2 itself) is reached - as expected - with pro-RLX1. The specificity of the antibody for pro-RLX1 vs. pro-RLX2 is tested experimentally to estimate the relevance in the assay system (Fig. 9.6). Three other peptides show high identity scores as well, but to a much smaller extent. Taking into account, that a putative peptide biasing the assay result has to bind to both antibodies, the chances have to be multiplied to find a real cross-reactivity. Therefore, it can be nearly excluded that the assay is measuring anything else than RLX2.

Immunization was performed by the InVivo Biotech Services GmbH. Different antibody combinations were tested for their ability to form a sandwich complex which is not only depending on their epitope specificity but also on biophysical properties of the antibody which can hardly be predicted but have to be tested experimentally. After identifying the most sensitive sandwich, detection antibody was tested for cross-reactivity to the pro-RLX1 isoform to clearly examine the specificity of the assay system for pro-RLX2 only. Figure 9.6 shows the results of a competitive assay where the antibody binds to pro-RLX bound to solid phase and is displaced by adding increasing concentrations of free peptide. As expected the binding of antibody decreases exponentially when adding increasing concentrations of pro-RLX2 and reaches a minimum at 1µg/well which is equivalent to the coupled amount of peptide - a steady state is reached there. The antibody does not bind to the pro-RLX1 peptide, so added amounts of free pro-RLX1 show no influence at all. The basal signal level is below that of the binding to pro-RLX2 since no steady state can be reached.

Table 9.1.: **Cross-reactivities of capture and detection antibody of pro-RLX assay system**. Only results with an Expect value <10 are shown for (a) detecting antibody (b) catching antibody

(a)

protein	Score	Expect	Identities	Positives	Gap
preproRelaxin 2	44.8 bits	2e-6	13/13 (100%)	13/13 (100%)	0/13 (0%)
preproRelaxin 1	44.8 bits	2e-6	13/13 (100%)	13/13 (100%)	0/13 (0%)
CCR4-associated factor 1	25.7 bits	3.9	9/14 (64%)	10/14 (71%)	2/14 (14%)

(b)

protein	Score	Expect	Identities	Positives	Gap
preproRelaxin 2	46.9 bits	3e-07	14/14 (100%)	14/14 (100%)	0/14 (0%)
preproRelaxin 1	31.6 bits	0.052	9/11 (82%)	10/11 (90%)	0/11 (0%)
early placenta insulin-like peptide precursor	26.5 bits	2.2	8/12 (67%)	9/12 (75%)	0/12 (0%)
zink finger protein 792	26.1 bits	3.1	8/9 (89%)	8/9 (88%)	0/9 (0%)
hydroxysteroid (11-beta) dehydrogenase 2	25.2 bits	5.8	7/7 (100%)	7/7 (100%)	0/7 (0%)

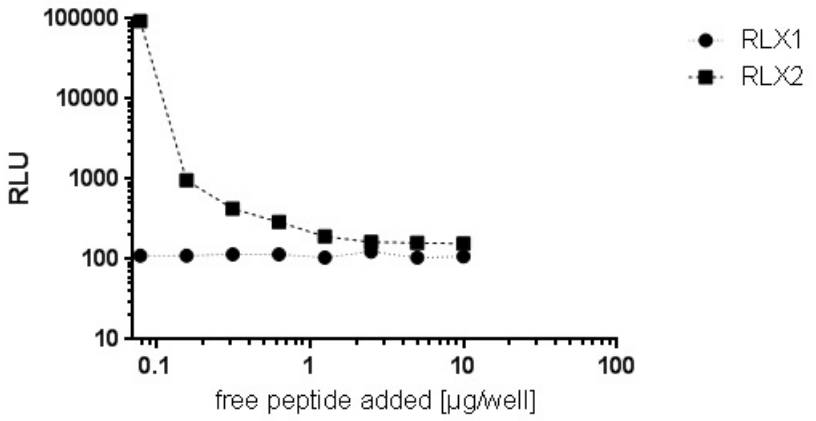


Figure 9.6.: **Competitive assay to determine specificity of detection antibody.** The antibody shows no cross-reactivity with pro-Relaxin 1 (pro-RLX1). Binding to pro-RLX2 shows a typical exponentially decreasing displacement curve with increasing concentrations of free peptide added, while there is constantly no binding to pro-RLX1.

9.2.1.3. Development of the assay system

Assay principle We designed a one step sandwich immunoassay system based on two monoclonal antibodies targeting two different regions within the connecting peptide (comp. Section 9.1.3.3).

Figure 9.7 shows the general assay principle of one capture antibody targeting the Relaxin-2 specific mid-regional amino acids and one MACN-linked detection antibody targeting the homologous N-terminal region. 96well plates were coated with capture antibody, blocked with BSA solution and dried. 100µl of sample were added to each well. Detecting antibody was diluted in assay buffer, added to the wells and the assay was incubated over night at 4-8°C. After washing, signal was detected using luminometric immunoassay (LIA) technology. For details about assay development also compare appendix Section C.

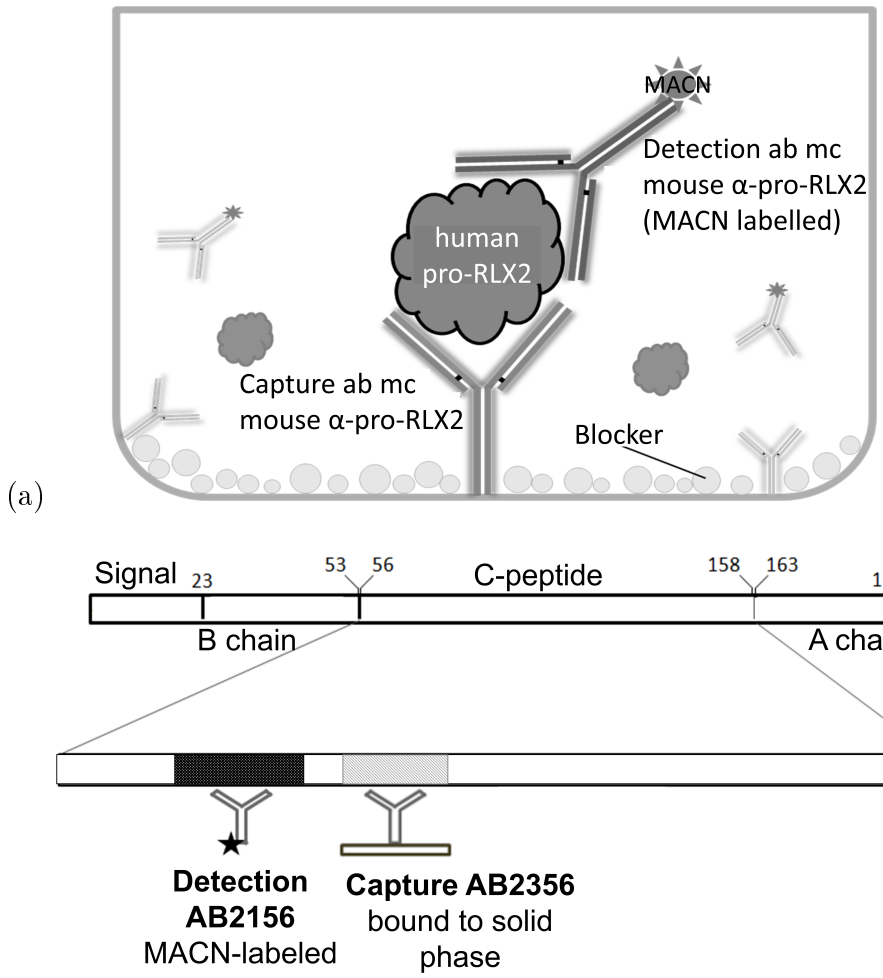


Figure 9.7.: **Assay principle of pro-RLX2.** (a) a sandwich complex is formed by two antibodies and the analyte in a well saturated with a blocker (BSA) (b) representation of the common peptide structure of RLX. The illustrated sequence includes the signal peptide, the (bio-active) relaxin A and B chain and the connecting C-peptide (pro-RLX1/2) in-between them. The capture antibody is targeting the midregional amino acids (aa 88-110), which are specific for the pro-Relaxin 2 variant. The MACN-linked detection antibody is targeting the homologous N-terminal region (aa 69-82) within the pro-RLX1 and 2.

A functional assay sensitivity of approximately 1.8pmol/L was determined by intra-assay and inter-laboratory coefficient (for details compare appendix Section C).

Correlation to existing assay system There are several immunoassay systems available for the measurement of mature bio-active Relaxin2. The Human

Relaxin-2 Quantikine ELISA Kit has been established with good sensitivity in several published as well as in unpublished studies from our cooperation partners [4, 175]. 32 EDTA-plasma samples were measured with pro-RLX2 and Human Relaxin-2 Quantikine ELISA Kit in parallel. Of 32 measured samples, 26 were measurable with the pro-RLX2 assay while only 20 were measurable with the R&D assay. Fig. 9.8b shows that concentrations determined by the two assays for the two peptides correlated significantly (Spearman's $\rho = .95$, $p < .0001$). Apparently, the concentration of the connecting peptide pro-RLX2 was 4.5-fold higher than that of the mature relaxin-2, provided that calibrators used have a 1:1 stoichiometric relationship. Hence, the measurability of samples with extremely low concentrations (non-pregnant) was improved with the sphingotec assay due to the higher concentrations of connecting peptide in plasma.

Since R&D systems is not providing a functional assay sensitivity (FAS) in their specifications, we estimated it as doubled unspecific binding. This would lead to a FAS of approximately 1.64 pmol/L which is similar to the one determined for our assay system (comp. Fig 9.8a). Nevertheless, the effective measurability is improved with the sphingotec assay due to the higher concentrations of connecting peptide in plasma in comparison to the mature peptide.

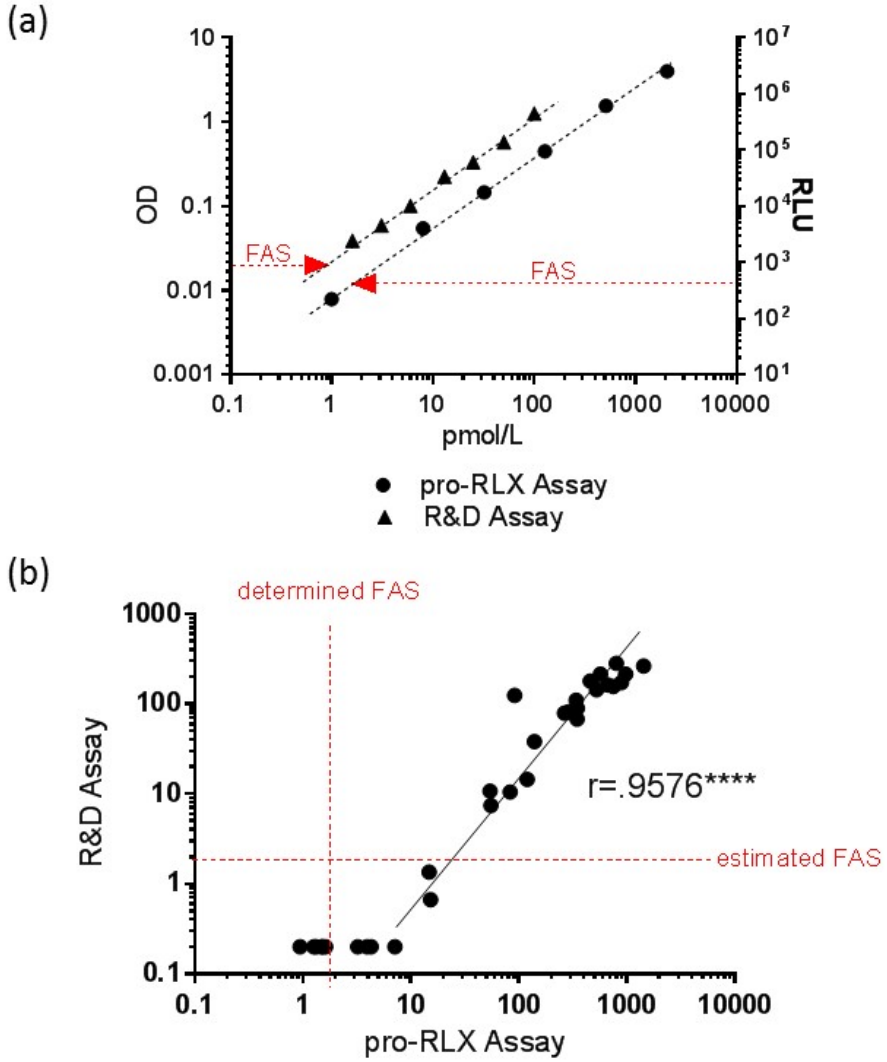


Figure 9.8.: **Correlation of bio-active relaxin-2 and its connecting peptide** (detected by R&D systems assay and sphingotec proRelaxin assay, respectively). (a) concentration-response curves of both assay systems. Both assays generate concentration-dependent signals which are increasing linearly. The functional assay sensitivities are comparable. (b) correlation of the concentration of 32 samples measured in both assay systems. The correlation is excellent ($Spearman's\ \rho = .9576$, $p < .0001$), even though several samples are not detectable with the R&D system.

10. (Pre-)Clinical application of biomarkers

10.1. Pro-Enkephalin (pENK)

10.1.1. pENK and (non-pregnant) renal function

10.1.1.1. Reference range

Reference ranges of pENK plasma concentrations have been determined in rat (Fig. 10.1a). A median concentration of 57.9 pmol/L was detected in rat. The upper 99th percentile - which is defined as the treshold to distinguish healthy from injured renal function - is 62.6 pmol/L in rat.

10.1.1.2. Gentamycin induced nephrotoxicity (rat)

Healthy rats have been challenged with gentamycin treatment and treated with therapeutic adrenomedullin antibodies (injection prior to gentamycin administration) for six consecutive days. Plasma samples were collected on day 1, day 3 and 7. HAM1101 treated animals showed significantly decreased baseline pENK concentrations (day 1) compared to the other treatment groups (data not shown), nevertheless all groups showed slightly increased concentrations compared to the healthy cohort. Established-gentamycin induced nephrotoxicity was reflected in significantly increased pENK plasma concentrations in all treatment groups on day 7 (Fig. 10.1b), while the median pENK concentrations were decreased on day 3. Treatment with both antibodies showed a slightly increasing trend on day 3 even though all groups (especially the HAM1101 treated group) showed great variation (Fig. 10.1c). pENK plasma concentrations were comparable in all three groups on day 3 (data not shown). On day 7, increases seemed comparable between all groups even though the variations are sizeable again (Fig. 10.1d). On day 7 pENK concentrations were slightly decreased in HAM1101 treated group, while concentrations in HAM2801 treated animals differ greatly (Fig. 10.1e).

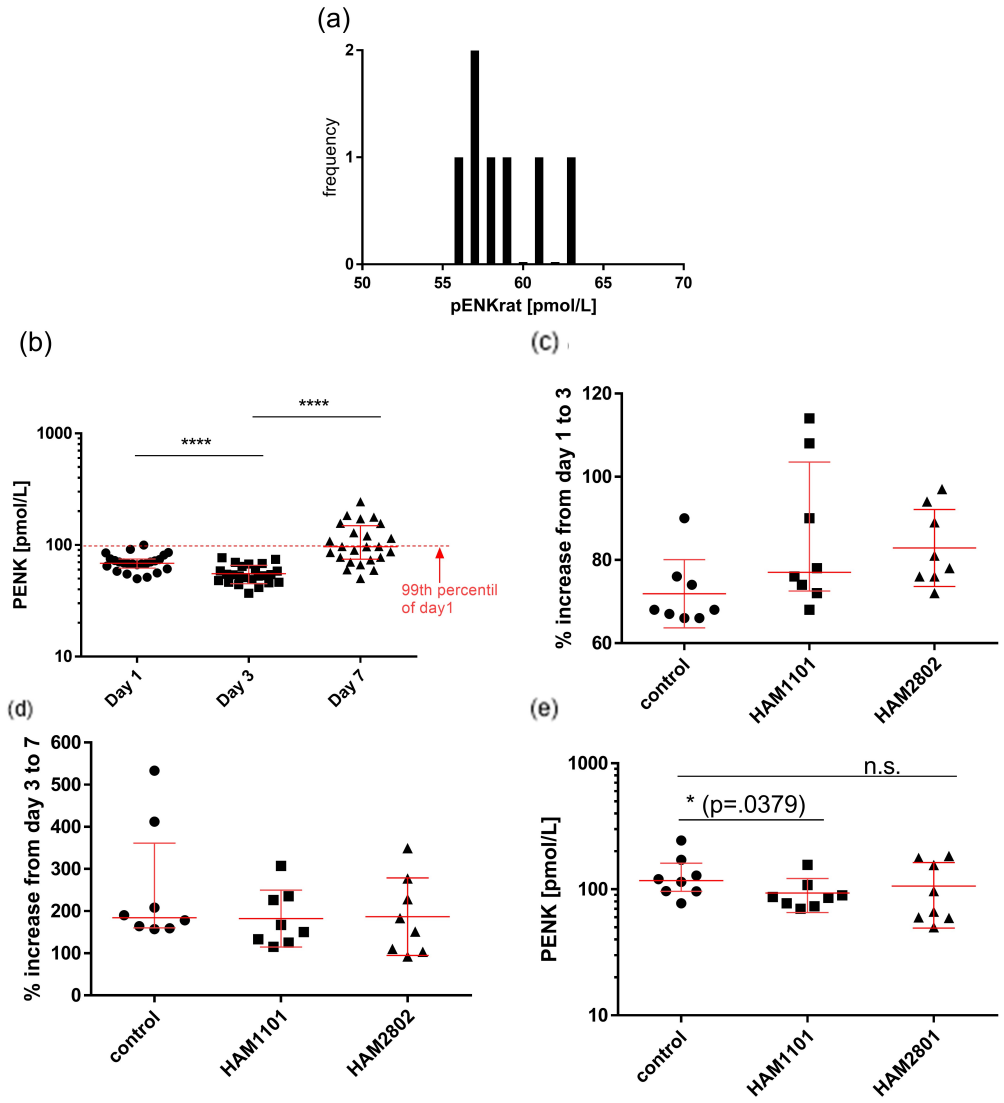


Figure 10.1.: **Gentamycin-induced nephrotoxicity in rat.** Error bars indicate median and IQR. (a) distribution of plasma pENK concentration in healthy rat ($n = 7$). (b) pENK after gentamycin-induced nephrotoxicity. (c) relative change in pENK concentration from day 1 to day 3. (d) relative change in pENK concentration from day 3 to day 7. (e) absolute pENK concentration on day 7 in control vs. treatment groups.

Correlation with other renal biomarkers was examined to cross-check the connection of pENK with renal function. On day 7 we observed strong and highly significant correlations of pENK with BUN, sCrea, Creatinine clearance, NGAL and NAG (Fig. 10.2a-c). Interestingly, on day 1 and 3 all correlations were non-significant.

Established gentamycin-induced nephrotoxicity was reflected in significantly increased sCrea concentrations in all treatment groups on day 7 (Fig. 10.2d), while

the sCrea concentrations were stable on day 3. sCrea concentrations stayed stable in all three groups on day 3 compared to day 1 (Fig. 10.2e). On day 7, both treatment groups showed a slightly decreasing trend compared to control group even though variation is great (Fig. 10.2f). pENK concentrations were slightly decreased on day 7 in HAM1101 treated group, while concentration in HAM2801 treated animals differed greatly (Fig. 10.2f).

10.1.1.3. Septic renal injury

Rats were treated with vehicle (20 mM His/HCl, pH 6.0) or CLP compound to induce sepsis. 24 hours after CLP surgery animals were treated with therapeutic adrenomedullin antibody (ADM) and/or Norepinephrine for four days. pENK concentrations were significantly increased in all septic groups compared to rats treated with vehicle (Sham), while no differenced in pENK concentrations could be observed between treated and untreated (CLP group) animals (Fig. 10.3a). When comparing pENK concentration with organ weight and cardiac parameters, pENK concentrations correlated significantly with the relative kidney weight (kidney/body weight) and the body temperature (Fig. 10.3b & c). Correlations with absolute and relative organ weight of heart, lung and liver were non-significant as well as correlations with total body weight, heart rate and mean arterial pressure.

(a)

Spearman's ρ (p-value)	BUN	sCrea	uCrea	Creatinine clearance	NGAL	NAG
day 1	.127	.120	.136	.098	.117	.117
	(.56)	(.58)	(.53)	(.65)	(.58)	(.58)
day 3	.003	-.121	.053	-.060	.355	.355
	(.99)	(.57)	(.81)	(.78)	(.09)	(.09)
day 7	**.867**	**.906**	-.383	**-.901**	**.415**	**.415**
	(<.0001)	**(<.0001)**	(.06)	**(<.0001)**	**(.04)**	**(.04)**

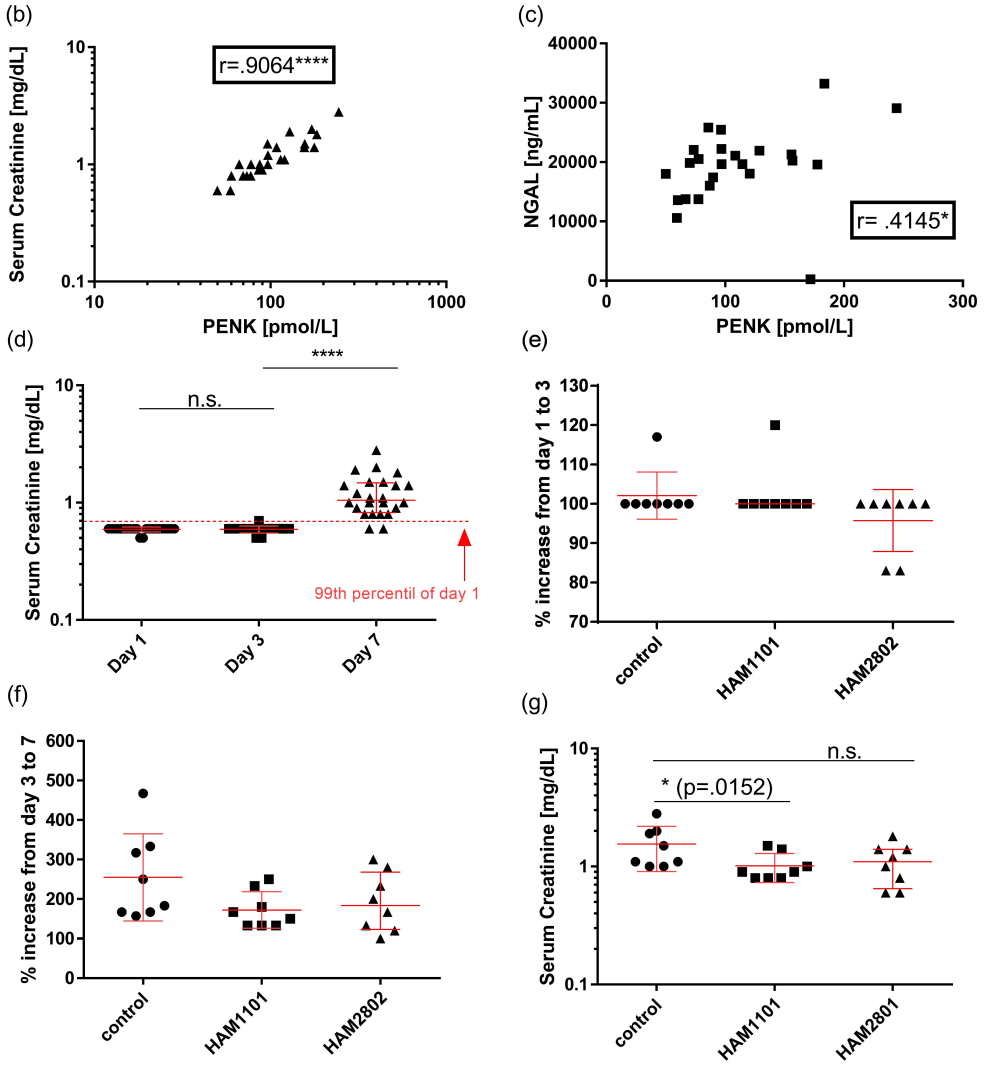


Figure 10.2.: **Correlation of pENK with other renal biomarkers**. (a) matrix of pENK correlations on day 1, 3 and 7. pENK strongly correlates with other renal biomarkers on day 7, but not on day 1 and 3. (b) correlation plot of pENK with sCrea on day 7. (c) correlation plot of pENK with NGAL on day 7. (d) sCrea after gentamycin-induced nephrotoxicity. (e) relative change in sCrea concentration from day 1 to day 3. (f) relative change in pENK concentration from day 3 to day 7. (g) absolute pENK concentration on day 7 in control vs. treatment groups.

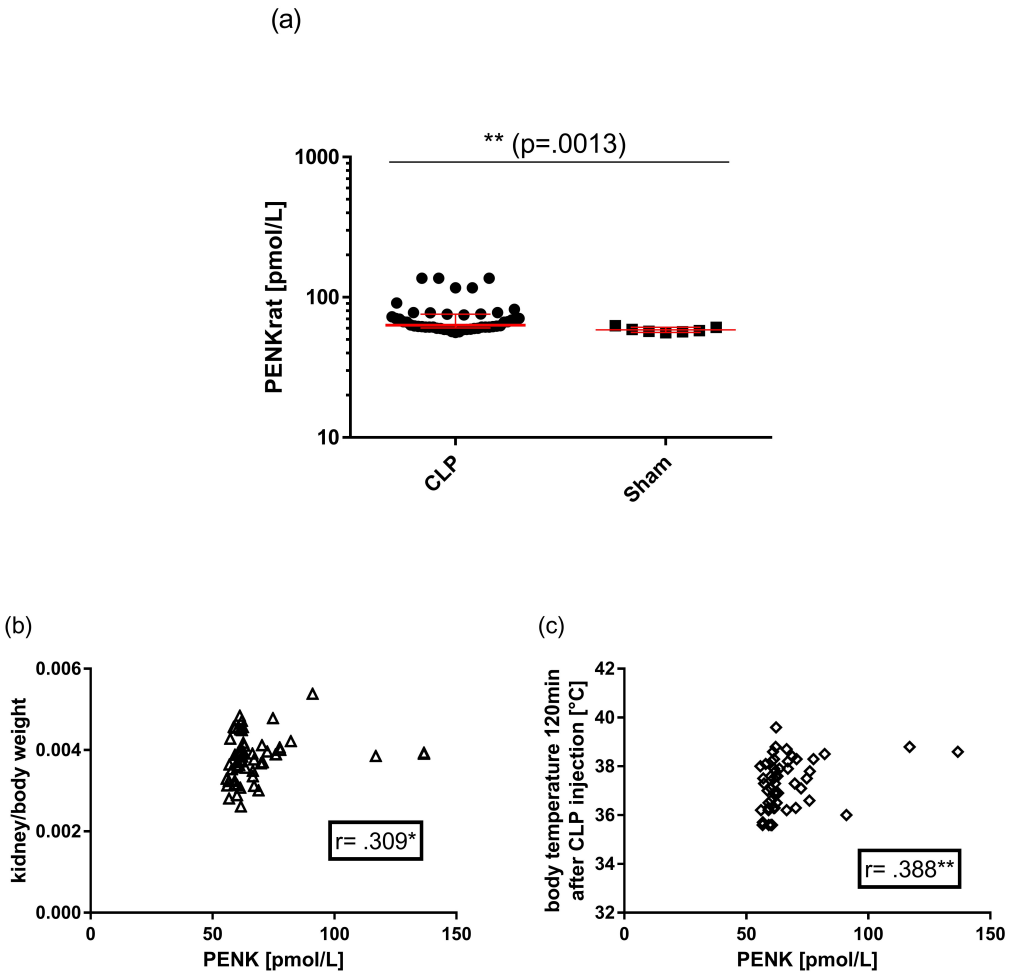


Figure 10.3.: **pENK plasma concentration in septic renal injury.** (a) pENK concentrations were significantly increased in CLP-treated animals. Error bars indicate median with IQR. (b) correlation of pENK with relative kidney weight. (c) correlation of pENK with body temperature 120 min. after CLP injection.

10.1.2. pENK concentration in urine after renal transplant therapy in non-pregnant

28 matched pairs of kidney transplant recipients and their healthy donors were examined before and up to 7 days after transplantation. Plasma, serum and urinary samples were collected before as well as 1 and 7 days after transplantation. SCrea as well as plasma and urinary pENK concentrations were determined for each day.

In section 9.1.2.4 a strong, highly significant correlation for plasma and urinary pENK concentration was shown before transplantation which was decreased after transplantation, potentially due to (at least partially) separated mechanisms regulating plasma and urinary pENK. When comparing healthy donors and injured recipients for each day separately, highly significant differences could be found in all three markers with a median concentration of 0.805 mg/dL [IQR 0.7125-0.8925] vs. 7.68 mg/dL [5.55-8.81] for sCrea in healthy and injured, respectively, 47 pmol/L [39.0-51.7] vs. 640 pmol/L [430.3-1039] for plasma and 384 pmol/L [279.1-816.2] vs. 3375 pmol/L [1562-5110] for urinary pENK (Fig. 10.4a-c). Donors mostly showed concentrations within the established normal range for sCrea (<1.1-1.2mg/dL [103]) and $pENK_{plasma}$ (<80pmol/L [165]), while recipients measured above. Since no established cut-off concentration has been determined for$pENK_{urine}$, the 99th percentile of the healthy donor group before transplantation (<1437pmol/L) was used as reference. Hence, before operation most transplant recipients measured above this reference value.

1 day after transplantation, healthy and injured individuals still showed highly significant differences in sCrea and $pENK_{plasma}$ concentrations with a median concentration of 1.21 mg/dL [1.07-1.40] vs. 4.36 mg/dL [3.03-5.16] for sCrea and 77 pmol/L [61.9-93.0] vs. 165 pmol/L [116.1-259.4] for $pENK_{plasma}$. Compared to concentrations before transplantation, sCrea and $pENK_{plasma}$ concentrations were elevated in donors and decreased in recipients. Hence, 46% and 43% of donors measured above the cut-off in sCrea and $pENK_{plasma}$, respectively. All recipients still measured above the cut-off. For urinary pENK concentrations, no significant difference between donor and recipient could be shown on day 1 after transplantation with a median concentration of 1717 pmol/L [1126-2610] vs. 3264 pmol/L [1377-5316]. Compared to concentrations before transplantation, urinary pENK concentrations were strongly elevated in donors and only slightly decreased in recipients. 71% of donors measured above the cut-off defined above, while 21% of recipients now measured below.

7 days after transplantation, again healthy and injured showed highly significant differences in sCrea and $pENK_{plasma}$ concentrations with a median concentration of 1.2 mg/dL [1.053-1.268] vs. 1.73 mg/dL [1.33-2.29] for sCrea and 76 pmol/L [64.3-83.3] vs. 136 pmol/L [97.9-166] for $pENK_{plasma}$. Compared to concentrations on day 1, sCrea and $pENK_{plasma}$ concentrations were more or less constant in donors and further decreased in recipients. 54% of donors measured above the cut-off in sCrea and $pENK_{plasma}$, while 14% of recipients measured below the cut-off. For urinary pENK concentrations, no significant difference between donor and recipient could be shown on day 7 after transplantation as well with a median concentration of 1661 pmol/L [1208-3242] vs. 1235 pmol/L [527.9-2075]. Compared to concentrations on day 1, urinary pENK concentrations were more or less constant in donors, but strongly decreased in recipients. 61% of donors measured above the cut-off defined above, while 57% of recipients now measured below.

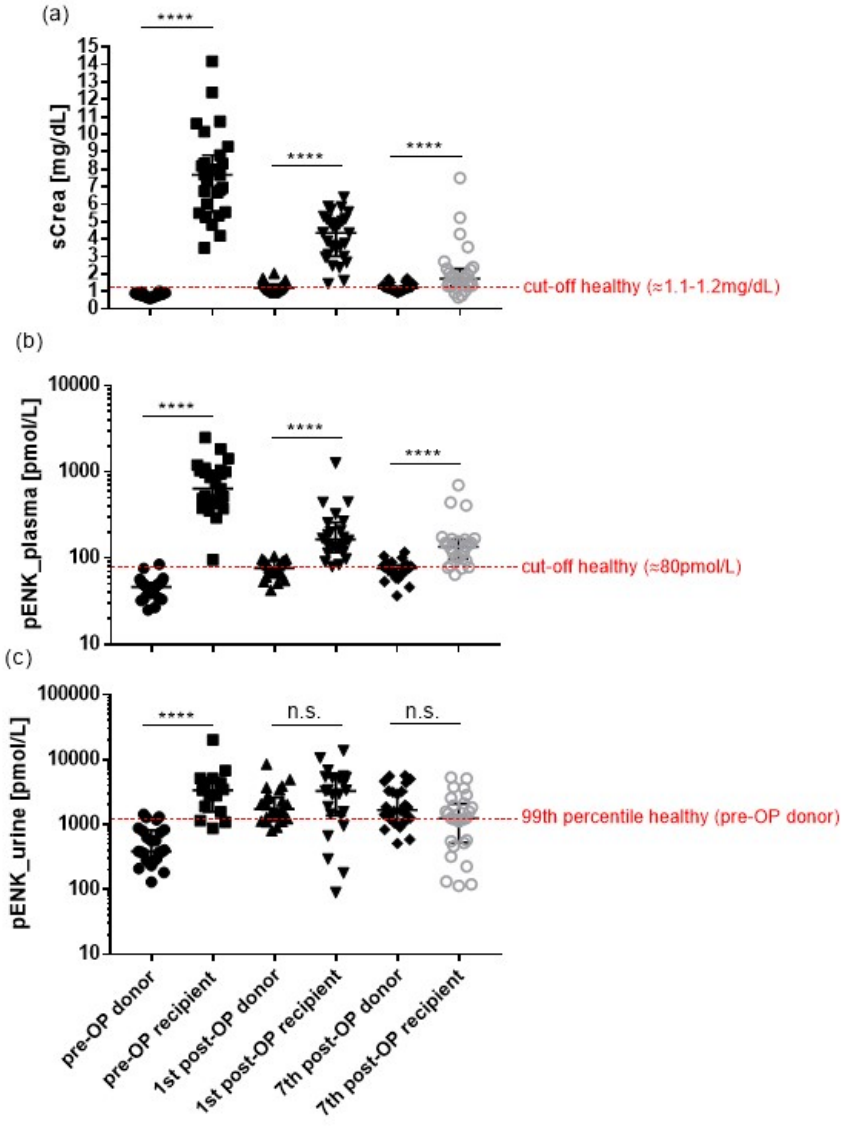


Figure 10.4.: **Healthy kidney donors as well as recipients with ESRD were examined regarding their concentrations of sCrea and plasma and urinary pENK concentrations before (pre-OP) and after transplantation (post-OP).** (a) sCrea concentration is significantly increased in recipients compared to donors over the observed period. After transplantation, concentration is increased above the normal level in approximately half of the donors, while concentration in recipients is decreasing to only slightly increased levels. (b) pENK plasma concentration mirrors sCrea concentration. (c) While urinary pENK concentration is significantly increased in recipients compared to donors before transplantation, no significant differences could be observed afterwards. The majority of donors measures above the normal range after transplantation while an increasing portion of recipients measure below with increasing time after transplantation.

As the parallel changes described above already indicate, $pENK_{plasma}$ concentration and sCrea correlated with high significance on all three days observed ($\rho = .48 - .79$, $p < .001$) (Fig. 10.5a-c). While urinary pENK concentration correlated with sCrea highly significant before transplantation as well ($\rho = .66$, $p < .0001$), correlation directly after transplantation was decreased ($\rho = .30$, $p = .04$) and no significant correlation on day 7 could be observed anymore. This again might indicate separately regulated mechanisms for urinary pENK after transplantation, while $pENK_{plasma}$ concentration and sCrea seem to be regulated similarly.

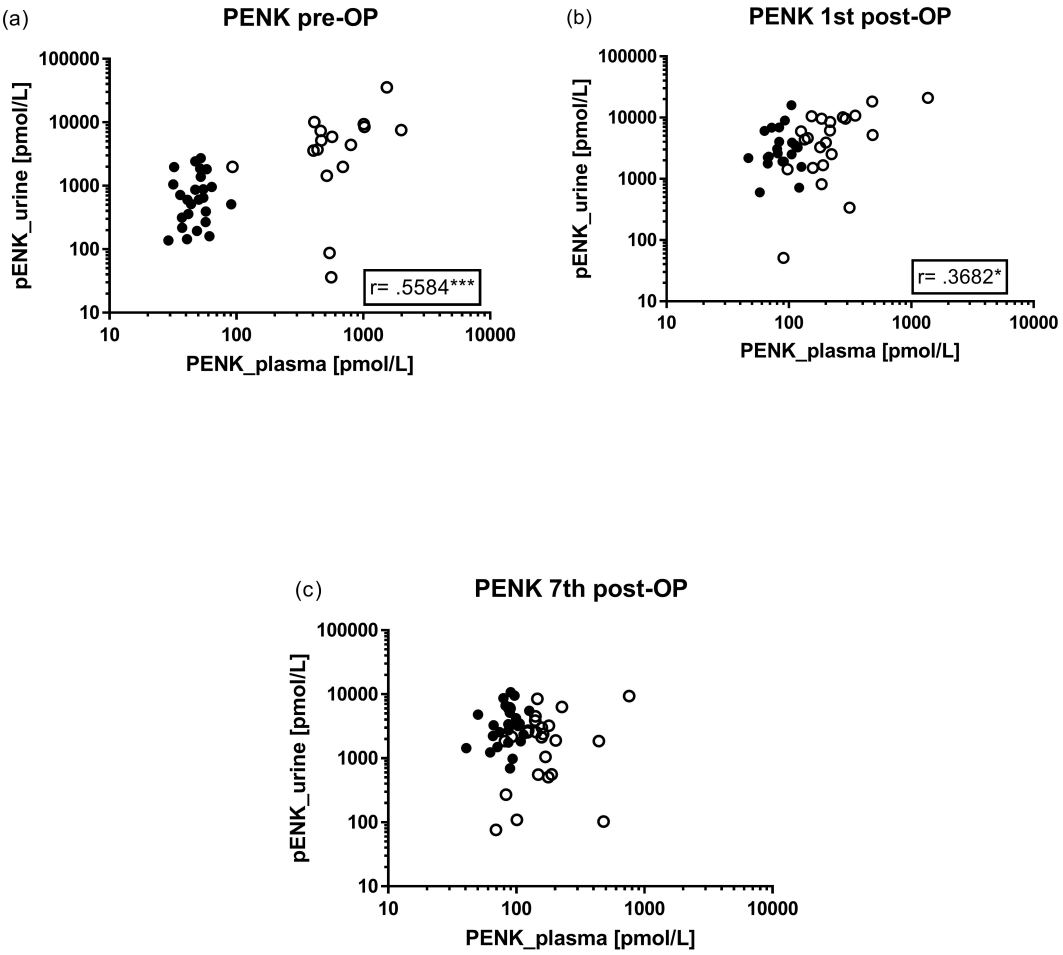


Figure 10.5.: **Correlation of pENK and sCrea concentration before and after transplantation.** (a) before transplantation plasma and urinary pENK concentration correlated significantly with sCrea ($\rho = .7417\, and\, .66\, for\, pENK_{plasma}\, and\, pENK_{urine}$, $respectively$; $p < .0001$). (b) 1 day after transplantation, plasma pENK concentration still correlated significantly with sCrea ($\rho = .7946$, $p < .0001$). Urinary pENK concentration correlated significantly with sCrea, but much weaker ($\rho = .3026$, $p = .04$). (c) 7 days after transplantation, plasma pENK concentration still correlated significantly with sCrea ($\rho = .4843$, $p = .0004$). Urinary pENK concentration showed no significant correlation with sCrea anymore ($p = .3144$).

As shown previously, baseline sCrea and $pENK_{plasma}$ concentration depend on age in this study as well: sCrea shows a significant correlation to age especially in kidney transplant recipients ($\rho = .44$, $p < .001$). The correlation of $pENK_{plasma}$ and age lied in a similar range ($\rho = .42$, $p < .01$). Baseline urinary pENK concentration showed no significant dependence on age ($p = .17$).

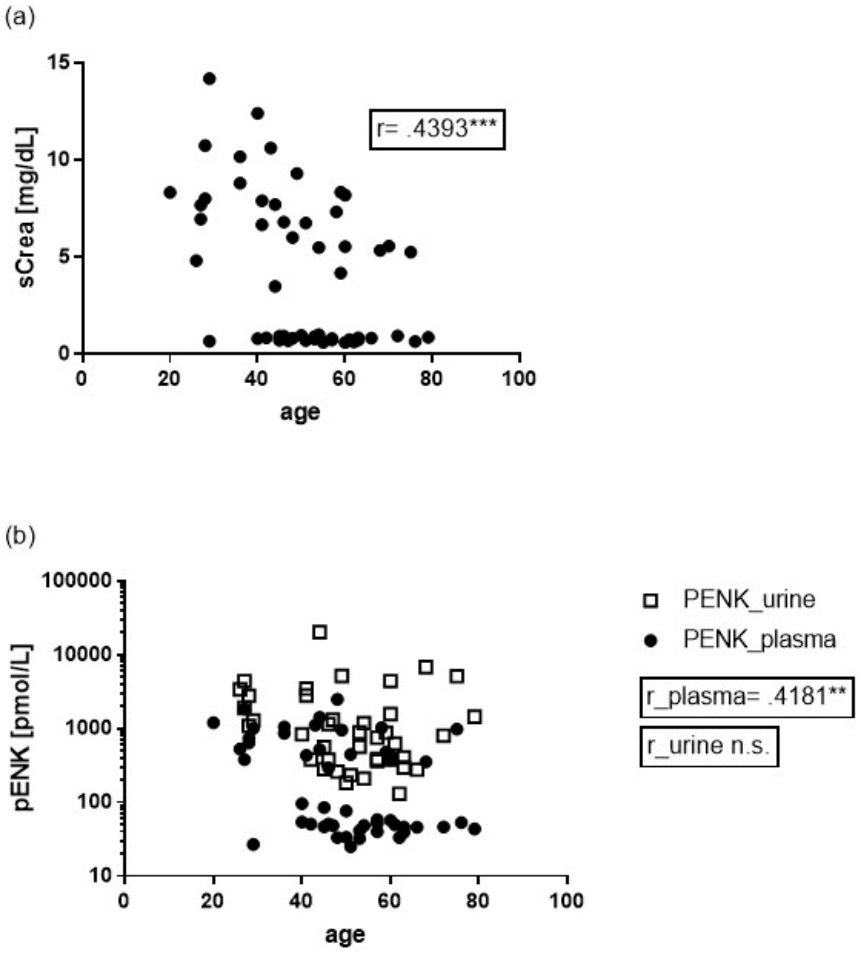


Figure 10.6.: **sCrea and pENK plasma concentration depend on age, while urinary pENK concentration shows no significant dependence.** (a) Correlation of sCrea with age ($\rho = .44$, $p = .002$). (b) Correlation of sCrea with plasma and urinary pENK concentration ($\rho = .4181$, $p < .0001\ for\ pENK_{plasma}$; $non-significant\ for\ pENK_{urine}$, $p = .1678$).

10.1.3. Dependence of pregnant pENK plasma concentration on gestational age

To our knowledge it has never been tested if pENK exhibits any special function in pregnancy and therefore shows varying plasma concentrations. Hence, we determined $pENK_{plasma}$ concentration in 99 healthy pregnant women of different gestational age. Fig. 10.7a shows highly fluctuating $pENK_{plasma}$ concentration over gestational age which may peak around week 9 and 21/22. Beside these peaks, all concentrations were determined below the cut-off for the reference range of 80pmol/L which was determined in other studies [108, 165, 169]. When looking at the scatter-plot three individuals with concentrations higher than 80 pmol/L can be identified who account for the putative peaks while there is an overall negative correlation between pENK and gestational age ($Spearman's\, \rho = -.4091, p < .0001$). Non-parametric testing revealed significant differences between trimester 1 and the other two trimesters while there is no difference between trimester 2 and 3. $pENK_{plasma}$ concentration seems to be slightly increased by the end of the first trimester but then quickly felt back to normal concentrations.

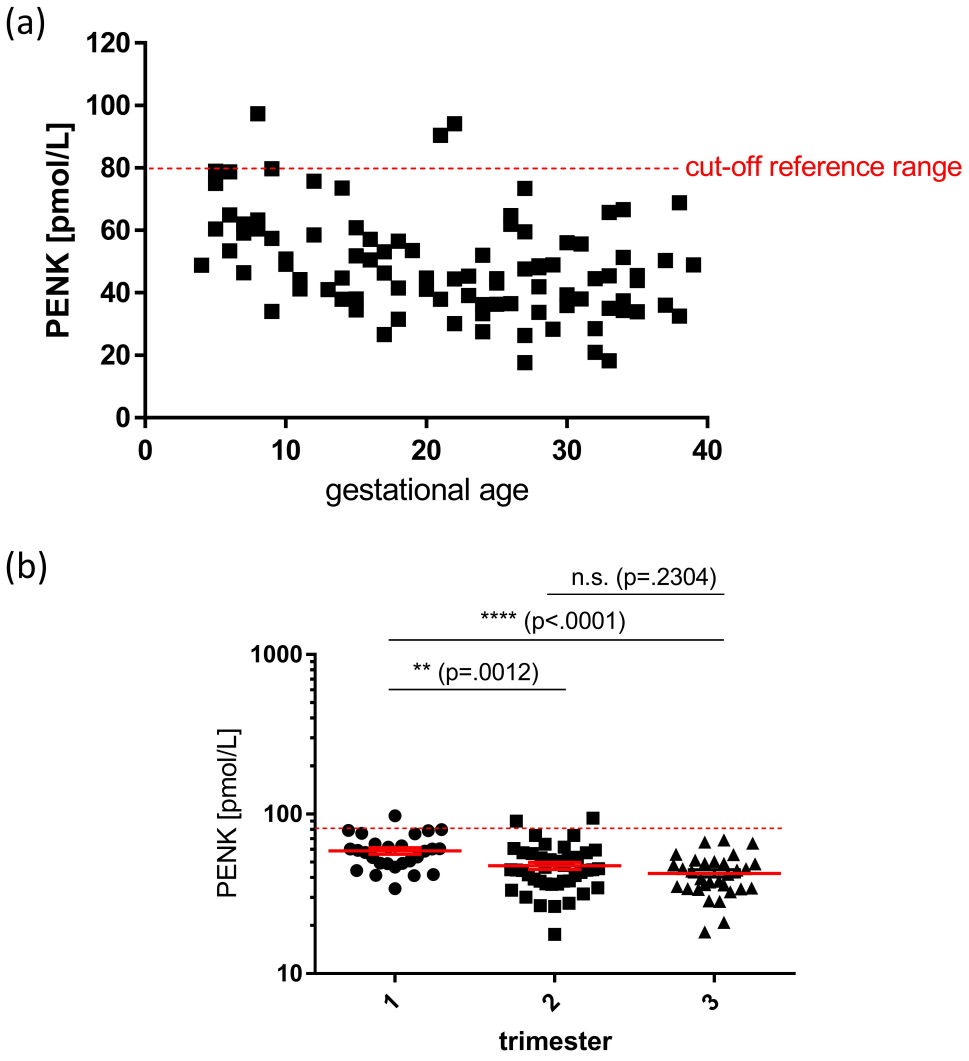


Figure 10.7.: **pENK plasma concentration depends on gestational age.** (a) gestational age negatively correlates with pENK concentration ($Spearman's\ \rho = -.4091, p < .0001$). Dotted red line indicates cut-off of reference range. (b) Mean concentrations are significantly different between trimester 1 vs 2 and 3, respectively (Mann-Whitney test). Differences between trimester 2 and 3 are non-significant. Error bars indicate Mean with SEM. Dotted red line indicates cut-off of reference range.

10.1.4. pENK in preeclampsia

Surprisingly, no pENK was detectable in a cohort of preeclamptic serum samples and corresponding cohorts (compare Section 7.4.3). Since the analytical procedure of assay measurement was cross-validated, a degradation of pENK must have occurred probably due to the process of transport and storage. Unfortunately,

further information about the transport and storage procedure is not available to clarify the specific reasons for degradation.

10.2. Pro-Relaxin2 (pro-RLX2)

10.2.1. Comparison pro-RLX2 plasma concentration in pregnant vs non-pregnant adults

Pro-RLX2 concentrations were determined in plasma samples from healthy, fasting non-pregnant adult individuals of both genders with the newly established pro-RLX2 assay ($n = 28$ and 53 for male and female, respectively). 80 % of these measured below the FAS with a median concentration of 1.49 pmol/L (IQR 0.925 to 2.14 pmol/L). There was no significant difference in plasma pro-RLX2 concentration between males and females ($p = .75$). The median concentration was 1.53 pmol/L (IQR 1.04 to 2.44 pmol/L) in male and 1.48 pmol/L (IQR 0.89 to 2.01 pmol/L) in female, respectively (Fig. 10.8a). When comparing healthy non-pregnant ($n = 81$) to pregnant women of different gestational age ($n = 100$), we found pro-RLX2 concentrations significantly increased in pregnant women with a median concentration of 562 pmol/L (IQR 341 to 789 pmol/L), $p < .0001$ (Fig. 10.8b). Both, plasma concentrations of pregnant women and non-pregnant of both genders were normally distributed even though plasma concentrations of non-pregnant required log transformation due to left skewedness. No significant correlation with age was shown, neither for samples from pregnant women ($r = .034$, $p = .734$) nor for those from non-pregnant healthy individuals ($r = .49$, $p = .66$).

10.2.2. Dependence of pro-RLX2 plasma concentration on gestational age

As Relaxin-2 is a well know pregnancy hormone, the pro-RLX2 plasma concentration is highly dependent on gestational age. In 99 healthy pregnant women of different gestational age the pro-RLX2 concentration peaked at the end of the first trimester (around week 10/11) and then declined till end of pregnancy (Fig. 10.9a). Mean concentrations were significantly different between the trimesters (Fig. 10.9b).

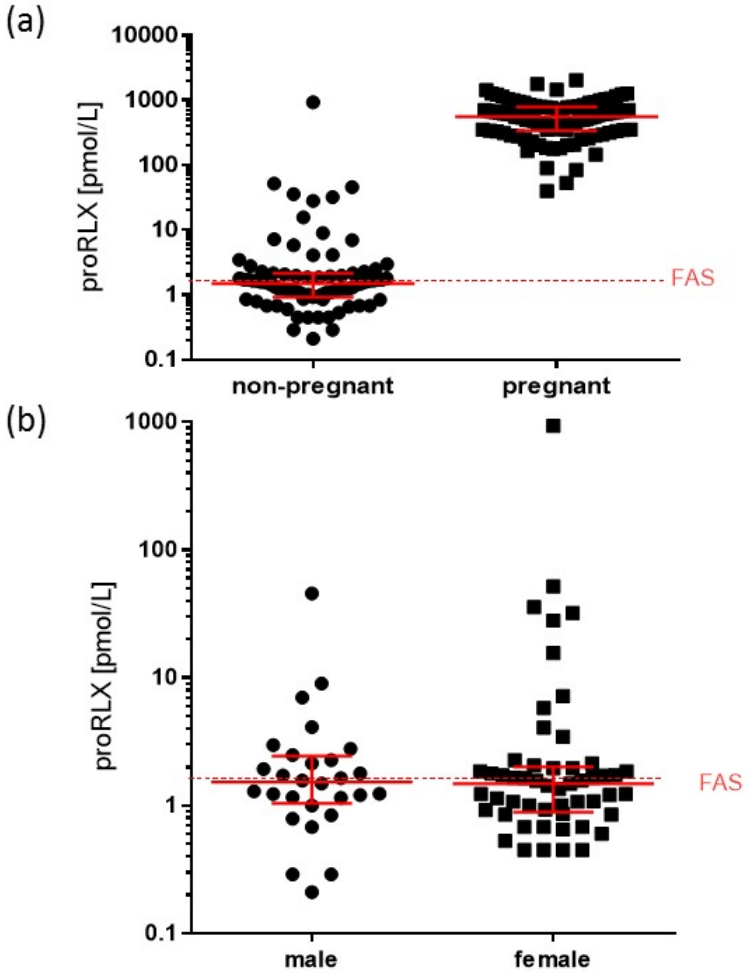


Figure 10.8.: **pro-RLX2 plasma concentration in pregnant and non-pregnant adults.** (a) pro-RLX2 concentration was dramatically increased in pregnant women ($n = 81$ in non-pregnants, $n = 99$ in pregnant, respectively), while 80% of non-pregnant adults measured below the functional assay sensitivity (FAS). Error bars indicate median with inter-quartile range. (b) There was no significant difference in pro-RLX2 concentration between male and female non-pregnant adults ($n = 28$ and 53, respectively). Again the highest portion of patients measured below the functional assay sensitivity (FAS).

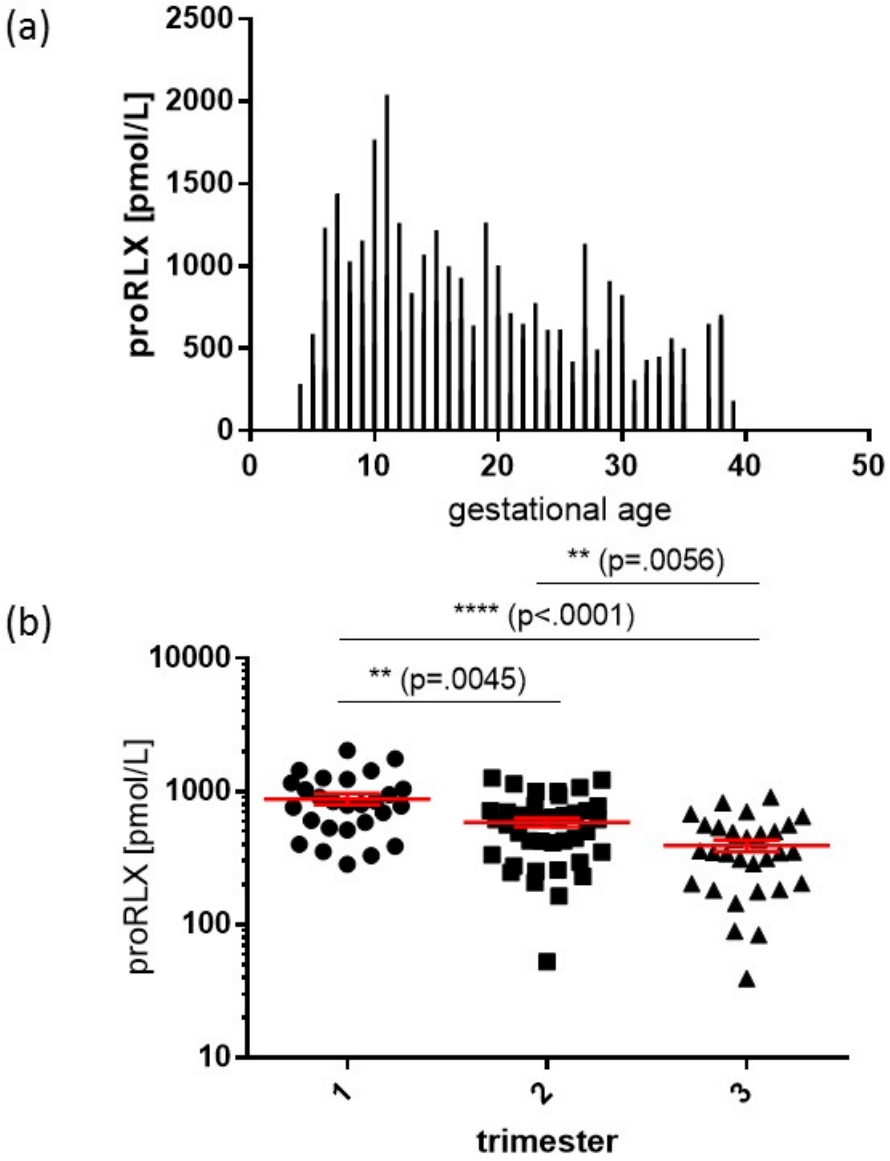


Figure 10.9.: **pro-RLX2 plasma concentration depends on gestational age.** (a) pro-RLX2 concentration peaked at the end of the first trimester. (b) Mean concentrations were significantly different between the trimesters (Mann-Whitney test). Error bars indicate Mean with SEM.

Ten patients had consecutive blood withdrawals in all three trimesters. Fig. 10.10 shows that there was greater inter-individual differences and the formally shown trend was not that obviously shown anymore. Again there were differences between the trimesters in mean concentration, but they do not reach significance (Fig. 10.10c).

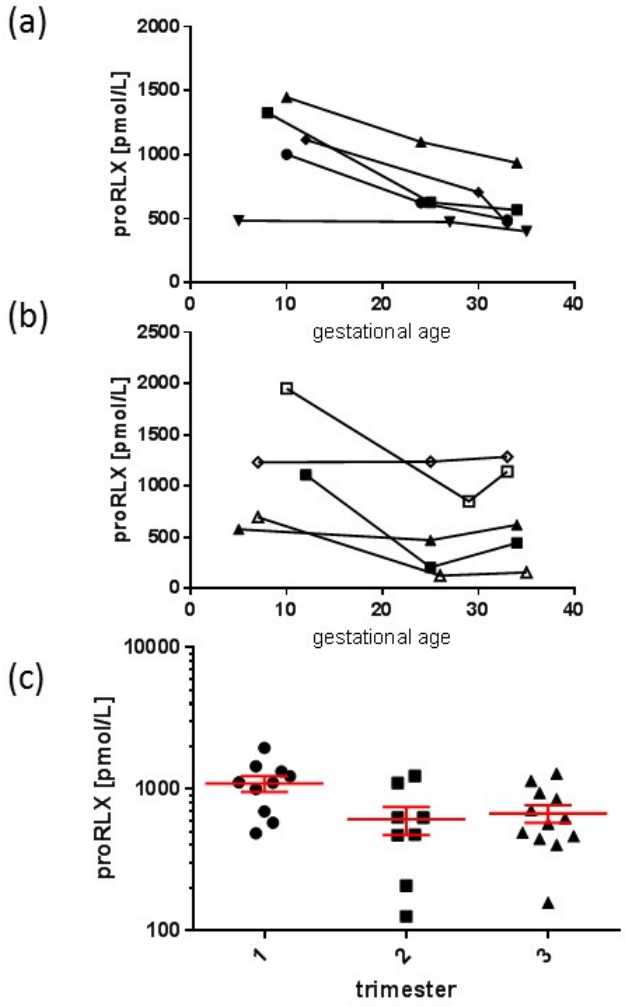


Figure 10.10.: **Consecutive blood withdrawals in all three trimesters.** (a)&(b) For better readability, study group is separated into two figures. (c) differences between trimesters are non significant. Error bars indicate mean with SEM.

10.2.3. pro-RLX2 plasma concentration is independent of biological age

For pregnant and non-pregnant adults the relationship between pro-RLX2 plasma concentration and biological age was examined. We could show no significant correlation in two different cohorts of healthy pregnant and non-pregnant adults (n=99 and 81, respectively).

10.2.4. pro-RLX2 in preeclampsia

The following study will be published in detail in Rehfeldt et al. [147]:

51 pregnant patients diagnosed with PE were matched with 5 healthy controls each (255 in total) according to their gestational age. Of the 51 PE patients, 44 were diagnosed with severe and 7 with mild PE. Serum samples were collected between 15th and 23th week of pregnancy and examined in pro-RLX2 assay.

No significant differences in pro-RLX2 serum concentration were observed: median pro-RLX2 concentration was 1032 pmol/L (IQR 718-1451) in control pregnant women, 1176 pmol/L (IQR 766-1552) in patients with severe and 715pmol/L (IQR 414-715) with mild PE, respectively. Figure 10.11a shows weighted regression analysis for calculating the median corrected for gestational age. Figure 10.11b shows the calculated multiples of the median (MoM) for PE and control pregnancies. Median MoM is 1.12 in PE and 1.02 in control pregnancies, respectively, the difference is not significant ($p = .5032$). Patients with a pro-RLX2 concentration in the lowest quartile did not develop preeclampsia significantly more often (odd's ratio 0 95, $p = .89$).

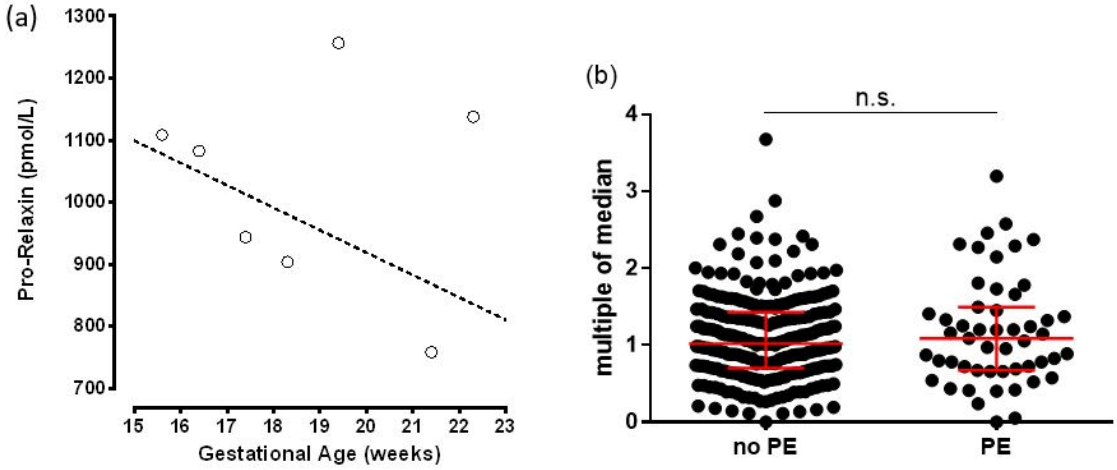


Figure 10.11.: **Pro-RLX2 serum concentration in pregnant controls vs. patients with PE.** (a) regression analysis to compute median pro-RLX2 concentration corrected for gestational age (b) no significant differences between preeclamptic patients and control pregnancies could be observed (median MoM 1.116 [0.69-1.54] vs. 1.019 [0.70-1.43],$p = .5032$).

Another method for correction is an analysis of the matched ranks: each sample set is ranked by their pro-RLX2 concentration and the median rank of the PE patient is determined. In a sample set of six samples each, an overall median of 3.5 indicates no effect. In our cohort the median rank was 3 (IQR 2-5) which indicates a non-significant reduction in relaxin levels in cases of PE relative to matched controls.

Correlations of pro-RLX2 serum concentrations with other biomarkers and parameters were determined. Significant correlations were found for proRLX2 and gestational age, weight, inhibin A and sEng levels (Table 10.1 and 2). Multivariate analysis revealed inhibin A MoM as the strongest predictor for pro-RLX2 serum concentration (adjusted R^2 for model fit= .151; β for Inhibin A = .400, $p < .0001$; excluded variables: gestational age, age, weight, sEng, PAPP-A).

Table 10.1.: **Correlations of pro-RLX2 serum concentration with biological parameters.** Non-significant correlations are given in gray letters.

biomarker	*Spearman's ρ*	p-value
gestational age	-.12	.0416
biological age	n.s.	.9106
weight [lbs]	-.2023	.0005

Table 10.2.: **Correlations of pro-RLX2 MoM and other biomarkers.** Non-significant correlations are given in gray letters.

biomarker	*Spearman's ρ*	p-value
maternal serum alpha-fetoprotein [iu/mL]	n.s.	.1852
unconjugated estriol 3 [ng/mL]	n.s.	.9301
total hCG [iu/mL]	n.s.	.2749
Inhibin A [pg/MI]	.1472	.0121
Nuchal translucency [mm]	n.s.	.8037
PAPPA [miu/MI]	-.1624	.0290
soluble Endoglin [ng/mL]	.2654	<.0001
PIGF [pg/mL]	n.s.	.2092

10.2.5. pro-RLX2 in non-pregnant adults with heart failure

10.2.5.1. renal implications in acute heart failure (PROTECT)

301 patients hospitalized for acute heart failure (AHF) with an estimated creatinine clearance of 20 to 80 mL/min and elevated natriuretic peptide concentrations were enrolled within 24 hours of presentation to placebo or rolofylline. A subset of 100 randomly chosen baseline samples were examined in pro-RLX2 assay and R&D relaxin2 assay. 55% out of these 100 samples were undetectable in pro-RLX2 assay (concentration below 1.8pmol/L). Interestingly, pro-RLX2 assay and R&D relaxin2 assay only showed a low, non-significant correlation ($\rho = .126$, $p = .169$). This might be caused by the presumably higher detection limit of the R&D relaxin2 assay system (comp. section 9.1.2.3). Therefore it is possible most of the samples in this cohort were not reliably measurable in the R&D relaxin2 assay.

When comparing detectable to undetectable samples in pro-RLX2 assay, renal function was significantly decreased in the detectable samples: median sCrea increased from 1.2 to 1.4 µmol/L ($p = .001$), median Creatinine clearance was decreased from 58.5 to 45.9mg/mL ($p = .001$). Median BUN was increased from 24.5 to 28.5 mg/dL ($p = .019$), while median NGAL was increased from 63.1 to 89.8 ng/mL ($p = .001$). Correlation of pro-RLX2 with sCrea (Fig. 10.12) and creatinine clearance were significant (Spearman's $\rho = .207$ with $p = .025$ and $-.243$ with $p = .008$, respectively). Multivariable linear regression analysis revealed sCrea as the strongest predictor for pro-RLX2 concentration. No association of pro-RLX2 concentration with death or re-hospitalization could be found.

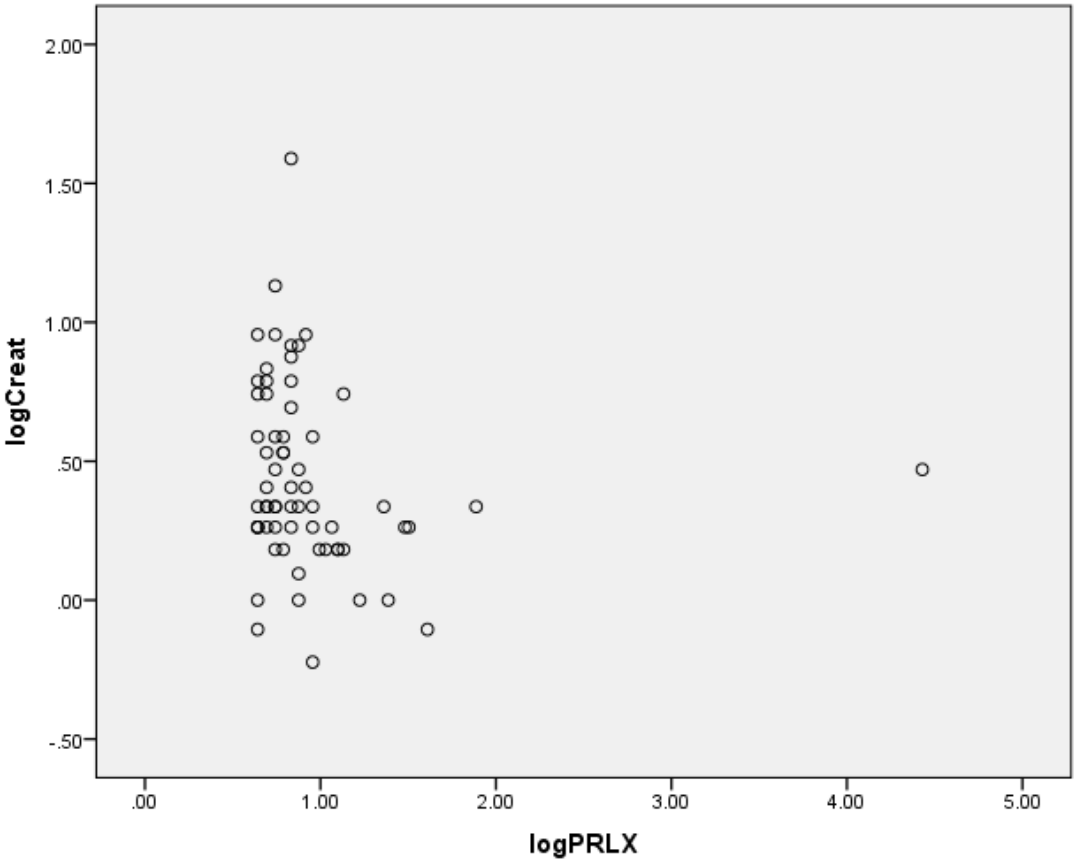


Figure 10.12.: **Correlation plot of logarithmic pro-RLX2 vs. logarithmic sCrea (Spearman's $\rho = .207$, $p = .025$).**

Interestingly, when dividing the sample-set into three groups according to their pro-RLX2 concentration (undetectable <1.8pmol/L, low 1.8-2.1pmol/L, high >2.1pmol/L), associations of pro-RLX2 concentration with renal impairment seemed to be lost: While all markers were increased in undetectable compared to low pro-RLX2

group, sCrea, BUN and NGAL were decreased in high pro-RLX2 group (even though still elevated compared to undetectable group). Table 10.3 shows median concentration and interquartile range in all three groups.

Table 10.3.: **Renal markers grouped according to their pro-RLX2 concentration** (undetectable <1.8pmol/L, low 1.8-2.1pmol/L, high >2.1pmol/L).

Marker	Undetect. (n = 55)	Low (n = 30)	High (n = 35)	P-value
pro-RLX2 (pmol/L)	≤ 1.8	1.8-2.1	>2.1	
sCrea (µmol/L)	1.2	1.6	1.4	.142
	(1.0 – 1.5)	(1.3 – 2.1)	(1.2 – 1.9)	
Crea clearance	58.5	45.9	45.0	.979
(mg/mL)	(44.6 – 71.0)	(32.1 – 58.4)	(28.2 – 57.4)	
BUN (mg/dL)	24.5	32.0	28.0	.572
	(19.8 – 32.0)	(23.5 – 51.3)	(20.0 – 45.0)	
NGAL (ng/mL)	63.1	93.3	75.6	.660
	(43.4 – 91.1)	(64.2 – 148.5)	(62.7 – 155.2)	

10.2.5.2. renal implications in chronic heart failure (BENEFICIAL)

100 patients with stable chronic heart failure were recruited for double-blind, randomized, placebo-controlled, parallel design trial with alagebrium. Baseline samples (before treatment) were examined in pro-RLX2 assay. 64% out of these 100 samples were undetectable (concentration below 1.8pmol/L). Therefore performed analyses did not reach significance even though there is an indication that increased pro-RLX2 concentration might be associated with decreased renal function: When dividing the sample-set into three groups according to their pro-RLX2 concentration similar to analysis in the PROTECT study (undetectable <1.8pmol/L, low 1.8-2.0pmol/L, high >2.0pmol/L), urea concentration as well as eGFR slightly decrease with increasing pro-RLX2 concentration (urea: 7.4 vs 7.4 vs. 7.2mmol/L; eGFR: 76.7 vs 72.1 vs 71.0mL/min/1.73m^2).

V.

Discussion

11. Establishment of immunoassay methods and further characterization of analyzed pro-hormones

11.1. Immunoassay methods

11.1.1. Measurement of prohormones as surrogates for the bio-active molecule

Synthesis of many neuropeptides and peptide hormones involves one or more precursor proteins which are processed by convertases to release the mature bio-active peptide [7, 137]. Quantitative and reliable measurement of these neuropeptides and peptide hormones in many cases is difficult since most bio-active peptides act in an auto- or paracrine way and do not even enter circulation in measurable amount [34]. Furthermore, circulating peptides might be masked by binding proteins or bind to blood cells [139, 142]. Another more technical problem for routine measurement is the short half-life of many neuropeptides and peptide hormones which can be only minutes - *in vivo* as well as *in vitro*. Hence, quantification of the more stable precursor fragment has been shown as sufficient substitute [53, 54, 105, 123, 135, 180]. Another advantage of measuring prohormones instead of the bio-active molecule is to represent the actual synthesis rate being independent from administration of exogenous peptide (e.g. as drug). Of course, representing the actual synthesis rate includes the disadvantage of not reflecting other mechanisms that might change the action of bio-active molecules like altered degradation, recycling processes or altered bioavailability [28, 29]. Nevertheless, in many cases the instability of the bio-active molecule renders the direct measurement all but impossible in clinical routine since immediate measurement of the instable molecule would be extremely laborious [34].

Generally, (sandwich) immunoassays including immunofluorescence/ immunoluminescence (LIA), radioimmunoassays and enzyme immunoassays (ELISA) are

a common, easy to handle quantification method which requires few conditioning steps (extraction of peptides from blood/urine sample, HPLC etc.) [154] and is applicable on many laboratory automats (KRYPTOR by B·R·A·H·M·S, VIDAS® by bioMérieux, ADVIA® by Siemens etc.). Disadvantageously, all immunoassays might be less sensitive than other methods like mass spectrometry [196], immunosensors [222] or luminescent oxygen channeling assay (LOCI) [191]. While mass spectrometry requires extensive conditioning steps [196], the portability of immunosensors might enable the development of point-of-care devices in the future [222]. Unfortunately, to date the method is not as common as immunoassays and therefore expensive [222]. LOCI is a homogeneous immunoassay method capable of rapid, quantitative determination of a wide range of analytes with approximately 20fold lower detection limits than commercially available immunoassays but to date it is not as well established [191]. Enzyme labeled immunoblot assays might be able to detect biomarkers in blood/urine samples as well, but are definitely less sensitive than immunoassays and furthermore difficult to quantify reliably [154].

11.1.2. (pro-) Enkephalin

In 2006 Ernst [53] introduced an immunoassay system to quantify the precursor fragment of met- and leu-Enkephalin, the so called pENK. In this thesis, this immunoassay has been successfully advanced for the application in rat models and human urine which in fact led to the development of two new assays derived from the original one. The rodent assay (pENKrat) is a one-step immunoassay based on two monoclonal antibodies against the midregional human $PENK\,A_{153-168}$ and the rat C-terminal $PENK\,A_{176-183}$ for detection of pENK in rat (or murine) plasma. The assay system presented in this thesis shows acceptable linearity, the estimated functional assay sensitivity is 25 pmol/L which is adequate for the intended use. Preliminary studies also showed detectable pENK concentrations in murine samples (data not shown).

The urine assay (pENKurine) is a two-step immunoassay based on two monoclonal antibodies against for the N-terminal human $PENK\,A_{143-152}$ and the midregional human $PENK\,A_{153-168}$ for detection of pENK in human urine. The assay system shows sufficient linearity and pool recovery in a specific dilution range, the estimated functional assay sensitivity is 7.2 pmol/L which is adequate for the intended use. Nevertheless, necessity of sample pre-dilution makes the assay system quite unhandy for routine application. Furthermore, urine samples are less readily accessible in clinical routine than plasma or serum samples. While

blood samples can be collected at any time, urine samples are difficult to obtain especially in critically ill patients who are many times oligo- or even anuric [58]. Hence, the advantages of pENK measurement in urine over measurement in blood samples (e.g. better predictive capacity etc.) need to be studied in detail.

Since a reliable detection of enkephalins is not possible due to stability reasons (half-life in blood <15min. [124]) other fragments of the pENK prohormone could be measured alternatively. Salzet et al. [156] introduced an ELISA assay system in 1995 for the enkelytin peptide which showed increased blood concentrations in patients undergoing CABG [184]. Unfortunately, description of the assay system in literature is lacking detailed information regarding sensitivity, specifity and reliability. Since polyclonal sera are used instead of monoclonal purified antibodies there is a high probability that the assay system is biased by cross-reactions with other peptides. Hence, the pENK assay systems provided by the sphingotec GmbH are the first reliable possibility to determine pENK concentration in specimens from patients.

11.1.2.1. Localization of pENK within healthy renal tissue (pilot experiment)

First experiments showed a tubular immunopositivity for the pENK molecule. Unfortunately, these results could not be reproduced in a second run. Therefore, results should not be interpreted before immunohistological experiments have been repeated. Even discussions why the two runs showed different results would be highly speculative. Unfortunately, conducting more experiments was beyond the temporal possibilities of this thesis.

11.1.3. (pro-) Relaxin 2

The pro-Relaxin2 assay (pro-RLX2) is a one-step immunoassay based on two monoclonal antibodies against the N-terminal human $RLX\,2_{69-82}$ and the midregional human $RLX\,2_{113-125}$ for detection of pro-RLX2 in human plasma and serum. The assay system was developed according to the guidelines for CE certification (ISO 13485) which means all quality criteria (linearity, pool recovery, recovery of added exogenous analyte, determination of FAS etc.) are fulfilled. The determined assay sensitivity is 1.7 pmol/L: In a sample volume of 100µL this assay is able to detect 0.17fmol - equating to approximately 10^8molecules. This is close to the highest achievable sensitivity with this kind of immunoassay. For higher sensitivity other methods like mass spectrometry are required.

Other assays for quantification of the mature human relaxin-2 (e.g. by R&D systems, Phoenix pharmaceuticals, Immundiagnostik AG etc.) are commercially

available, though none of these assay methods are published in detail regarding sensitivity and reliability. Nevertheless, some of them have been successfully used for clinical studies with different questions than renal dysfunction (e.g. birthweight regulation and other pregnancy complications [4, 175, 214], peripartum cardiomyopathy [133], vascular dysfunction in type 1 diabetes [130], cirrhosis [174]).

12. (Pre-)Clinical application of the biomarkers

12.1. Application of pENK in rat

Two independent studies impairing renal function in rat were analyzed for pENK plasma concentration: Both showed the assay being able to distinguish healthy from injured animals which was confirmed by several other established renal biomarkers as creatinine, NGAL etc.

In animals challenged with Gentamycin, pENK plasma concentrations were significantly elevated after 7 days of treatment. Differential effects of treatment with antibodies could not be shown due to high inter-individual variations within the relatively small study groups ($n = 8$). In several animals the increase in pENK concentration from day 1 to day 3 wass much greater than in others. Further studies need to be carried out to elucidate potential reasons for that. Median pENK plasma as well as sCrea concentrations were significantly decreased in the animals treated with HAM1101 while HAM2801 treated animals showed great variation. Therefore, no final conclusion about a potential beneficial effect of HAM2801 can be drawn from this study.

While correlation with BUN and creatinine was very high ($\rho > .8$) in established (gentamycin-induced) renal injury, correlation with NGAL and NAG was only intermediate ($\rho > .4$). Interestingly, in developing renal injury (day 3), correlation of NGAL and NAG with pENK is close to significant ($p = .06$) while it shows no correlation to creatinine at all at this stage. This could indicate that pENK is able to detect already developing renal injury and therefore rises earlier than creatinine - as was shown for NGAL previously [57, 122, 173].

Nevertheless, the kinetics and mechanisms controlling the increase in plasma concentration need to be studied in detail. A lesional mechanism (up-regulated secretion in the tubules overruns reabsorption capacity) similar to that one of NGAL is a possibility even though data supporting this hypothesis are currently lacking [44, 173, 212]. Nevertheless, also pENK being up-regulated in tissues

different from the kidney itself (extrarenal) due to the injury might play a role in the systemic rise in pENK plasma concentration.

In animals with induced sepsis, pENK plasma concentrations were significantly elevated in animals challenged with CLP compared to the Sham group. Even though the overall median was significantly different between both groups, it was remarkable that not all animals treated with CLP showed elevated pENK plasma concentrations. Taking the 99th percentile of the Sham group as treshold (63 pmol/L), in only 50% of the animals the pENK plasma concentration was increased. This effect seemed independent of the treatment with therapeutic antibody and/or Norepinephrine: 50% of animals showed elevated concentrations in untreated group as well as in groups treated with ADM and NA as well as NA only. In the group treated with ADM only, 58% of animals showed increased concentrations. Therefore, the efficiency of the induction of sepsis by CLP injection might be questionable in this study. Consequently, interpretations about treatment effects of the therapeutic antibody should not be drawn from this study.

Furthermore, an intermediate correlation ($\rho > .3$) with relative kidney weight and intensity of sepsis (monitored via body temperature of the animal) could be shown in this study. In other studies it was shown that sepsis is accompanied by increased renal perfusion (hyperemic AKI) [203]. Nevertheless, afferent and efferent arterioles are dilated due to the sepsis and the GFR is decreased. At the same time, the blood flow is internally redistributed in favor of the cortex, the medulla (and thus the tubules) are ischemic [203]. This causes apoptosis of tubular cells and following leads to AKI. The correlation of pENK plasma concentration with body temperature (as an indicator for intensity of sepsis) might be interpreted that decreased GFR leads to accumulation of (potentially extrarenal) pENK in the blood stream. It could be possible as well that intra-renal pENK is upregulated due to the tubular cell apoptosis and thus is also increased in plasma.

Usually, AKI is accompanied by a reduction in renal weight due to loss of nephrons. Hence, we would expect a negative correlation of renal weight with pENK plasma concentration similar to that one shown e.g. for NGAL [218]. Interestingly, in this study pENK shows a positive correlation to relative kidney weight. Since the experiment was ended 27 hours after induction of sepsis we would hypothesize that in this relatively early stage, the increased renal weight might indicate a hypertrophic kidney that tries to compensate for decreased renal function. Also the increased renal perfusion might increase the renal weight (which is wet mass including water captured within the tissue). The fall in renal weight potentially occurs much later when tubular loss due to AKI aggravates - Zager et

Table 12.1.: Eligible features for new biomarkers applied to pENKrat/ sphingotest®penKid.

feature	fulfilled	not fulfilled	need to be tested
high sensitivity	x		
high specificity	x		
correlation with severity	x		x
risk stratification			x
highly stable	x		
no interference with drugs	(x)		x
detection in early phase	(x)		x
prognosis of worsening renal function	x		
prognosis of major adverse events	x		
non-invasive application	x		
quick measurement		x (24h)	
reliable, cost-effective & standardized measurement	x		
well-described cut-offs	x		
applicability in human and animal	x		

al. [218] examined the renal weight three weeks after the insult. This hypothesis would support the idea that especially the fall in GFR leads to pENK accumulation while tubular loss is not (yet) pronounced enough to cause an increased expression which is measurable at systemic level. As new refined methods of measurement of renal perfusion with imaging techniques are emerging, one approach to clarify the connection of renal blood flow and pENK plasma concentration could be to monitor both simultaneously in close-mesh intervals [106, 113].

If eligible features and goals of new biomarkers (compare section 2.3) are applied to the new assay system, we can assert that several of the features are fulfilled while others still need to be tested (overview given in table 12.1).

Both studies proved pENK is detectable in rat plasma as well - interestingly even at levels similar to that in human. Furthermore, pENK seems to be up-regulated in case of renal injury in rat as was shown for human previously [83, 108, 111, 165, 166, 169]. Therefore, it would be extremely interesting to examine multiple animal models of renal injury in the pENK (pENKrat) assay to characterize kinetics of its concentration and regulation in detail.

12.2. Application of pENK in human samples

12.2.1. Application in urine

PENK is expressed in a detectable concentration in urine and seems to be relatively stable during daytime. Extended studies need to confirm this stability since in this thesis only two individuals (1 male, 1 female) could be tested for a 12 hour period.

Interestingly, pENK concentration changes in urine differ from plasma measurement in the setting of acute adaptation to renal transplantation: while $pENK_{plasma}$ concentration (similar to sCrea) needs several days to reach a steady state (donor and recipient with comparable concentration), urinary pENK concentration is already balanced on day 1 after surgery to an intermediate (above normal) level in both, donors and recipients. This potentially indicates different regulatory mechanisms for plasma and urinary pENK: While urinary pENK seems to directly reflect the actual renal function - which is identical directly after transplantation since both, donor and recipient, got one healthy kidney -, $pENK_{plasma}$ seems to reflect the systemic status which needs several days to adapt to the new situation (clearance of pENK/Crea from blood by the new kidney in recipients and accumulation due to the reduced renal function by loss of one kidney in donors, respectively). Both can be clinically relevant: As already shown, $pENK_{plasma}$ concentrations are perfectly able to predict long-term outcomes such as mortality. Those reflect the systemic status also including cardiac and other functions [50, 83, 108, 131]. Especially the cardiorenal interaction might be reflected by $pENK_{plasma}$ concentrations. Further studies need to prove if urinary pENK concentrations are able to reflect and especially predict short-term (intrarenal) events as acute worsening of renal function.

In our study, absolute, uncorrected values for urinary pENK concentrations were compared in donors and recipients. Generally, correction for urinary volume or other urinary markers (uCrea, BUN, total urinary protein etc.) is often used to normalize urinary concentrations to the differing concentration process of primary to secondary urine. The benefit and reliability of this normalization is currently under discussion for other markers[57]. In this retrospective ancillary study, none of these methods was applicable since data other than sCrea were missing. Other studies should show if corrected values might be even more reliable regarding clinical relevance.

12.2.2. Application in pregnant women (serum/plasma)

We showed pENK plasma concentration being slightly increased by the end of the first trimester but then quickly falling back to normal concentrations (in-between the 99th and 1st percentile of normal cohort). During pregnancy renal hyperfiltration is occurring physiologically starting around week 6 [31]. Increased GFR leads to increased filtration of molecules like serum creatinine and thus low serum creatinine levels that might obscure an injury-caused increase [85]. In this study, seemingly we found the opposed effect that early pregnancy increases $pENK_{plasma}$ concentration - even though to a level still within the normal range except for very few cases. Hence, we could discuss different hypotheses:

One hypothesis would be this increase being an artifact caused by single individuals and there is no real increase. Nevertheless, there is still an overall negative correlation between $pENK_{plasma}$ concentration over the whole pregnancy which indicates that there is definitely an impact of pregnancy on $pENK_{plasma}$ concentrations. Importantly, $pENK_{plasma}$ changes are rather small and values never drop below the lower cut-off of reference range (approx. 25pmol/L). Therefore, a second hypothesis could be this correlation simply being an effect of plasma volume changes occurring in pregnancy [207]. If the kidney (or whatever other organ) produces a constant amount of pENK while the overall plasma volume increases, the measured pENK plasma concentration drops. This hypothesis could also explain the slight increase shown in early pregnancy: Relaxin causes renal hyperfiltration already very early in pregnancy while the compensatory increase of water retention shows a little delay causing an increase in plasma volume not before week 12 [190]. Hence, plasma volume could be slightly decreased in week 10 and following pENK plasma concentration increased (as shown in our study) due to increased GFR.

Altogether, since pENK concentration changes are small, gestation should not impair the potential of pENK to predict AKI. Correction for gestational age however is recommended in further studies to confirm this hypothesis.

Unfortunately, this study includes no baseline values of very early (or Pre-) pregnancy which could be used to check the hypothesis of artifacts (see above). Furthermore, interpretation of this study should be handled with some care since samples are taken from different individuals at different weeks of gestation and inter-individual differences might overlap with the effects of gestational age. Bigger studies with consecutive samples taken from the same individuals should clarify this question.

12.3. Application of pro-RLX2 in human

12.3.1. Application in pregnant women

As previously shown for mature relaxin, pro-RLX2 also peaks at the end of first trimester (around week 10/11) and then declines until the end of pregnancy [22, 32]. In a consecutive cohort, this trend was reproducible even though differences were non-significant due to cohort-size. Furthermore, two subgroups might be identified based on their pro-RLX2 expression: One showing constantly declining pro-RLX2 concentration (Fig. 10.10a), the other with constantly increased pro-RLX2 or concentrations declining from 1st to 2nd and increasing again from 2nd to 3rd trimester (Fig. 10.10b). The constant decline could be interpreted as physiological pro-RLX2 expression by the corpus luteum which is constantly degraded along gestation after the 1st trimester. Increased relaxin concentration in later pregnancy (between 18th and 30th week of gestation) has previously been discussed as a predictor of pre-term labor previously [138, 198, 199, 200]. Nevertheless, the origin of relaxin in late pregnancy is still under discussion as well as its physiological function. Unfortunately, in this study no more clinical data are available for the included cases, so no conclusions can be made about what is causing the differing pro-RLX2 pattern. Expanded clinical trials are needed to characterize the expression of pro-RLX during gestation in detail. Nevertheless, correction for gestational age is obligatory when examining the influence of pro-RLX2 plasma concentration on other pathologies as PE while biological age does not influence relaxion concentrations .

Second trimester levels of pro-RLX2 were not associated with the development of preeclampsia in pregnancy. This differs from other biomarkers like inhibin A, PAPP-A and sEng MoMs. Elevated serum inhibin A and sEng levels as well as decreased PAPP-A levels have been associated with preeclampsia in pregnancy in numerous publications [89, 91, 211]. PE is associated with impaired perfusion of placenta [128], leading to altered secretion of markers of angiogenesis. Tumor growth factor β3 (TGFβ3) is known to be upregulated by low oxygenation and overexpressed by preeclamptic placentas [20, 19, 216]. It inhibits the invasive capabilities of the trophoblast [19]. Endoglin is a co-receptor for TGFβ3 that is highly expressed on the syncytiotrophoblast and vascular endothelium and functions as a negative regulator of trophoblast differentiation [21]. Endoglin in its soluble form circulates in increased concentrations throughout the blood stream in PE, thereby inhibiting angiogenesis [99, 18]. Another molecule, placental growth factor (PIGF) binds to its endothelial receptor, flt-1 and promotes endothelial

blood vessels in normal pregnancy [128]. Levels of PlGF are significantly reduced in PE, as corroborated presently. Deficient relaxin bioactivity was proposed in preeclampsia based on the notion that renal vasodilatory effects and increased nitic oxide (NO) production should occur to compensate for hypertension occurring in PE [197]. In addition, previous studies showed that a lack of corpus luteum (and relaxin) in women undergoing assisted reproductive technology and conceiving through donor eggs had an increased incidence for PE [22]. Other data supporting a role for relaxin in PE include those showing that relaxin antagonists (asymmetric dimethylarginin etc.) are increased in preeclampsia, and there is reduced placental expression of the relaxin receptor [22]. Serelaxin (a recombinant analogue of human Relaxin-2) administration reduces mean arterial pressure (MAP) and improves uterine artery resistance index (UARI) and NO bioavailability in the Reduced Uterine Perfusion Pressure (RUPP) model of preeclampsia. Furthermore, serelaxin decreases prepro-endothelin-1 (PPET-1), soluble fms-like tyrosine kinase-1 (sFlt-1) and tumor necrosis factor alpha ($TNF\alpha$) plasma concentrations, which are established biomarkers for vascular function in PE [158]. Lastly, decreased relaxin-2 serum concentrations in early pregnancy are associated with an increased risk of PE [16, 77]. Both studies used a nested case-control set of first trimester samples. Sample size was comparable to our study or bigger (n(PE)=37 and 151, respectively). Absolute serum concentrations of mature relaxin did not differ significantly in both studies, but they were able to show an increased odd's ratio for patients with reduced relaxin concentration. In contrast, several recent studies challenge this hypothesis. For example, in relaxin knockout mice, evidence of proteinuria, but no other characteristics of PE (e.g. hypertension, increased PIGF plasma concentration) could be found [134]. Furthermore, administration of Serelaxin could not ameliorate hypertension and proteinuria in an Angiotensin-2-based model of preeclampsia [63]. The finding of relaxin-2 not being altered in PE is also supported by human studies showing that circulating levels of relaxin-2 were not altered in PE compared to normal pregnancies within second and third trimester [16, 90, 181]. Recently, Zoet et al. showed pro-RLX2 levels being comparable between women 10 years after preeclampsia and women with an uncomplicated pregnancy [224]. Since our study showed no difference between preeclamptic and healthy women as well, the option may be excluded that the lack of discrimination is a problem of relaxin molecule stability. The hypothesis that PE does not influence relaxin-2 as well as pro-RLX2 concentration – at least after the end of first trimester - seems rather likely. An open question relates to the relationship of pro-RLX2 and mature relaxin-2, especially in the setting

of PE. While pro-RLX2 plasma concentrations are closely associated with relaxin in healthy pregnant as well as non-pregnant individuals [148], PE may influence the synthesis rate and secretion of prepro-relaxin-2, the metabolism, half-life and proteolytic activation of the prohormone. Decreased synthesis in PE could, for example, be compensated by decreased activation of the prohormone convertases leading to an overall unchanged pro-RLX2 plasma concentration. To prove this in study, mature relaxin and metabolites would have to be measured in the same set of healthy and preeclamptic samples. In summary, second trimester serum concentrations of proRLX2 are not predictive of the development of preeclampsia in pregnancy. Other markers of angiogenesis, such as soluble endoglin, Inhibin A and PlGF are stronger predictors with potential for clinical use. Nonetheless, local regulation of relaxin in the kidney or altered circulating relaxin-2 and pro-RLX2 at other times in pregnancy (especially in the first trimester) warrant further study to fully assess the pathophysiology of PE.

12.3.2. Application in non-pregnant males and females

While Relaxin as well as pro-RLX2 is increased and reliably measurable in pregnant women, expected plasma concentration in non-pregnant is rather low. Although our assay has been shown to be very sensitive (compare Chapter 12), in a cohort of healthy non-pregnant individuals 80% of samples measured below the FAS. In cohorts of acute heart failure this portion is slightly decreased to 55 and 64%, respectively, but still the major portion is undetectable. Useful application of the assay system in non-pregnant individuals therefore is questionable in general. Nevertheless, pathologies that increase pro-RLX2 plasma concentration to an intermediate level (between healthy non-pregnant and pregnant) are imaginable. As therapy with Serelaxin (synthetic human relaxin-2) was shown to have beneficial effects in heart failure [121, 185], we hypothesized relaxin to be involved in pathogenesis and therefore expected changed pro-RLX2 plasma concentration in patients with acute and/or chronic heart failure.

Since a high portion of samples was undetectable in acute heart failure (PROTECT study), we grouped samples as up or below detection limit and compared clinical and laboratory outcomes especially regarding renal function. A relatively weak correlation could be found between pro-RLX2 and sCrea concentration. Established biomarkers for renal function (sCrea, Crea clearance, BUN, NGAL) showed significant differences between the low and high pro-RLX2 concentration groups. Differences in the BENEFICIAL study did not reach significance. It can be hypothesized that renal injury might increase pro-RLX2 (and relaxin equally)

plasma concentration, potentially as an early rescue mechanism which can be supported by application of synthetic relaxin [30, 59, 159, 217]. Also the inversed connection could be hypothesized that pro-RLX2 is increased by changes in cardiovascular hemodynamics occuring during heart failure and thus pro-RLX2 causes renal injury. Potentially, pro-RLX2 is not even involved in the renal changes directly but the shown correlation is not causal but only an effect of statistical mediation of cardiovascular hemodynamics (cardiorenal syndrome) on both, pro-RLX2 and renal function.

Nevertheless, when grouping patients of the PROTECT study into three groups (undetectable, low and high), we find bigger variations within renal biomarker concentrations. Extended studies need to show if this is only an artifact of variations within a relatively small cohort or if there is a more complex regulatory mechanism of pro-RLX2, potentially with an optimal range of beneficial effects and disadvantageous effects below and above this range. That this effect could not be reproduced in BENEFICIAL study might support the hypothesis of artifacts.

12.4. Potential mechanisms of pENK and pro-RLX2 actions in renal dysfunction

12.4.1. pENK regulation in renal dysfunction

Previous studies showed pENK being present in detectable amount in healthy individuals within the blood stream [83, 165, 166]. In this thesis, detectable amounts of pENK were found within urine of healthy individuals as well. Furthermore, we know that pENK is synthesized in relatively high amount (comp. Denning et al. 2008 [42]) but also in other non-neuronal tissues like heart, lung etc. Hence, we could discuss several different mechanisms how pENK concentration within blood and urine is regulated in different settings (Fig. 12.1):

healthy individuals In healthy individuals, relatively low but detectable (further called "normal") concentration of pENK within blood was determined. Whether this originates from renal or other cells is still questionable. pENK with a molecular mass of 4.8k Da is small enough to be (more or less) freely filtered by the glomerulus like inulin, creatinin, cystatin C, NGAL, KIM-1 or other renal biomarkers. Hence, pENK should be concentrated similarly (normally) in primary urine as in blood and final urinary concentration could potentially depend only on glomerular filtration and tubular water retention- this hypothesis could be easily tested

by normalizing blood and urine concentration to volume. This would be a similar mechanism as shown for creatinine.

Nevertheless, since renal tubular cells seem to express pENK it is probable that they are involved in pENK metabolism as well: pENK urine concentration could be mainly influenced by catabolism/resorption by the tubular cells (as shown for cystatin C or $\beta - Microglobulin$) or by secretion of pENK from the tubular cells (as shown for KIM-1) or even both (as shown for NGAL). As studies showed the presence of κ opioid binding sites on renal tubular epithelial tissue whereas δ and μ opioid receptors were absent [42, 64, 68, 209], renal action of pENK and also resorption might be conducted via κ receptors.

Injury In renal injury, several things happen at the same time: First, the GFR drops which could explain the elevated pENK plasma concentrations. If no additive pENK is secreted by tubular cells, urinary pENK concentration would thus be decreased (like shown for sCrea). Nevertheless, we could show increased urinary pENK concentrations in patients with ESRD. Therefore, renal tubular cells must be involved: when injured, potential resorption of pENK would be decreased. Depending on the portion which is normally resorbed by the tubular cells different scenarios are imaginable: if normally a very high portion is resorbed, decreased resorption might lead to an increased urinary concentration compared to normal even if urinary concentration in primary urine is decreased (as shown in our study). If normally only a smaller portion is resorbed pENK concentration in secondary urine would be still decreased. PENK release by injured cells would aggravate the increase in urinary pENK concentration

Simultaneously, injured renal as well as other (e.g. cardiac) cells could secrete pENK into the blood stream to elevate pENK plasma concentration even more.

Pregnancy Pregnancy is a very interesting state to clarify the question if pENK plasma concentration is determined by GFR alone since in pregnancy GFR is increased without having impaired tubular cells involved. Hence, if pENK concentration is determined by GFR and water retention, pENK plasma concentration shortly increases and then drops again (as shown in this thesis). Still, in injury additional secretion of pENK from other organs might aggravate the increase in pENK plasma concentration.

Furthermore, measurement of urinary pENK might clarify the question of tubular resorption of pENK: If resorption is involved, the increased GFR leads to an increase in available pENK within primary urine which might overrun resorption capacity and urinary concentration would be increased. If only secretion is

involved, this would have no impact on urinary concentration compared to the healthy status - pENK concentration in urine would be normal.

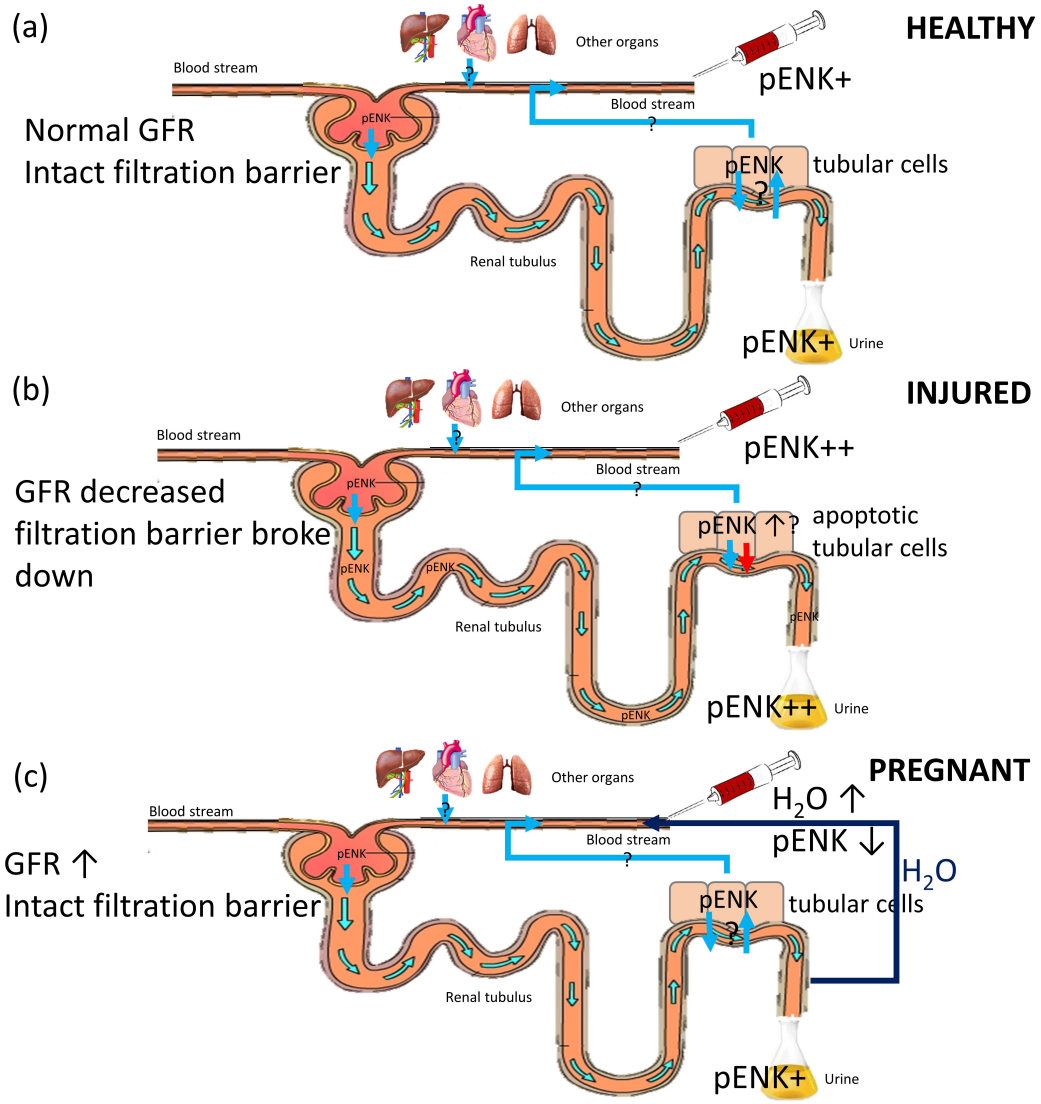


Figure 12.1.: **Schematic view of potential mechanisms regulating pENK plasma and urine concentration.** (a) in healthy, non-pregnant individuals. (b) in patients with renal injury. (c) in pregnant women.

12.4.2. Involvement of pro-RLX2 in renal dysfunction

Human relaxin-2 has been shown to have numerous beneficial effects on renal function: Administration of Serelaxin (or other exogenous forms of relaxin-2) in animal models was shown to reduce blood pressure and improve (uterine) artery vascular resistance, decrease plasma $TNF\alpha$ levels, $TGF\beta$ expression, decrease macrophage infiltration, plasma sFlt-1 levels and renal and placental preproET-1.

Plasma nitrate-nitrite concentration was increased [158, 22] and collagen content in renal tissue normalized to decrease interstitial fibrosis [22]. As a consequence of these beneficial actions, animals supplied with exogenous relaxin-2 showed a decrease in albuminuria and sCrea and delayed progression of kidney disease [22].

Figure 12.2 shows a schematic view of the potential mechanisms by which relaxin-2 improves renal function: In renal injury, inflammatory processes are triggered by tubular necrosis (caused by the injury) to promote repair [67]. While this is meant to stabilize renal function temporarily, inflammation can also promote further injury in the long term. Relaxin could help to control of these adverse effects, e.g. by reducing plasma $TNF\alpha$ and $TGF\beta$ levels as well as decreasing macrophage infiltration. Since renal inflammation is known to trigger fibrosis, this would be in concordance with the hypothesis that relaxin-2 is very important in homeostasis of kidney's connective tissue [161]. Balance of fibrosis leads to improved perfusion of the kidney directly as well as via acting on the RAAS (especially AT2).

A second beneficial mechanism involves the cardiovascular balance influenced by relaxin-2: As relaxin-2 decreases ET-1, vasoconstriction is decreased which leads to decreased systemic blood pressure as well as renal capillary resistance. Both improve perfusion of the kidney. Furthermore, reduced blockade of angiogenic factors like VEGF and PIGF by reduced sFlt-1 plasma concentration triggers angiogenesis and further improves renal perfusion.

In this thesis a slight increase in pro-RLX2 concentration could be shown in patients with heart failure and accompanying renal injury. Even though this difference was barely detectable on systemic level (plasma), it can be hypothesized that probably locally increased relaxin-2 expression might be a mechanism by which kidney triggers to compensate for the injury.

12.5. Neuronal effects of the prohormones

Recent studies showed that sympathetic hyperactivity is a major factor in the etiology of resistant hypertension and other inflammatory processes within the kidney [1]. Hence, renal sympathetic ablation (renal denervation) has been suggested as a possible treatment [33]. Since enkephalins are known to be extensively expressed by central and peripheral neurons [140, 145, 151, 176, 182, 186] within the striatum [182], the cerebellum [176] and local pre-synaptic interneurons within the spinal chord, the limbic system, the substantia nigra, hyphophysis and the hypothalamus [3, 145, 186], neuronal effects of the enkephalins on the kidney are possible as well. Hence, the increase of pENK plasma concentrations in renal

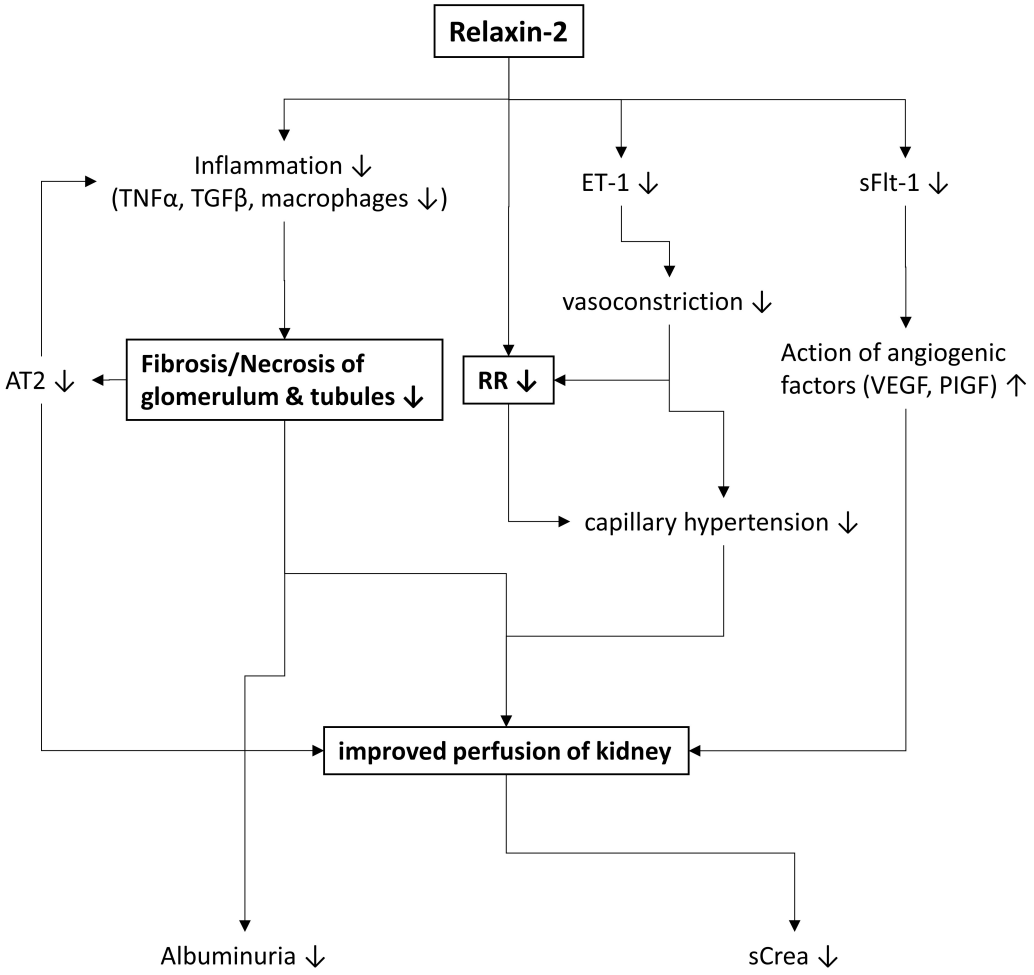


Figure 12.2.: **Schematic view of renoprotective actions of human relaxin-2.** Relaxin-2 decreases renal fibrosis/necrosis as well as blood pressure (RR) by inhibiting inflammation, ET-1 and sFlt-1. The following improved perfusion of kidney leads to decreased symptoms of renal injury.

injury might reflect neuronal effects rather than differential expression within the kidney. Examination for instance of cerebrospinal fluid in AKI patients regarding pENK concentrations might elucidate these possible processes further. Even though for relaxin-2 no neuronal expression has been documented yet, potential neuronal effects should not be ruled out completely.

VI.

Conclusion and Outlook

In this thesis, the technical establishment of several new assay systems for pro-Enkephalin (pENK) and pro-Relaxin2 (pro-RLX2) was described: The established assay system for $pENK_{plasma}$ measurement in human samples [53] was adapted to the rat peptide and to urine *(goal 1&2 of this thesis)*. A de novo design, development and validation of an immunoassay system for measurement of pro-RLX2 in human serum and plasma was conducted *(goal 3)*.

Renal sections of healthy kidney were stained with the antibodies used in the human pENK immunassay. PENK expression was localized throughout the renal tubules even though results were not reproducible *(goal 4)*. Further examination, especially in sections from injured kidney, might elucidate the mechanisms by which pENK is implicated in renal injury. Staining of renal sections with pro-RLX2 antibodies might be interesting as well to clarify renal implications of pro-RLX2.

In different animal studies in rat association of $pENK_{plasma}$ concentration with renal dysfunction was shown by specific increase of $pENK_{plasma}$ concentration by induction of selective renal injury *(goal 5)*. Even though $pENK_{plasma}$ still might at least partially originate from other sources than the kidney, these studies support previous results indicating renal function as the main determinant for $pENK_{plasma}$ concentration. Furthermore, detectable urinary pENK concentration *(goal 6)* was shown to be associated with renal injury, but potentially being regulated by different mechanisms than $pENK_{plasma}$ *(goal 7)*. Since urinary pENK seems to adapt quicker to acute renal changes (e.g. transplantation), it might be an earlier marker for impaired kidney function than the established gold standards. $pENK_{plasma}$ seems to behave similar to sCrea, but still might be clinically important to forecast long-term adverse events and mortality. Further studies need to confirm these differential clinical implications of $pENK_{plasma}$ and $pENK_{urine}$ in comparison to sCrea.

In pregnancy, $pENK_{plasma}$ was shown to vary with gestational age, but unfortunately no analysis regarding influence of PE could be performed due to pre-analytical problems *(goal 8)*. Therefore, extensive study of $pENK_{plasma}$ in different pregnancy complications (especially involving renal function) is still needed.

Pro-RLX2 was shown to increase in parallel with the mature relaxin-2 molecule in healthy pregnant and non-pregnant individuals. Even though measurability of pro-RLX2 in non-pregnant adults was restricted (50-80% of plasma samples measure below the detection limit), a weak connection of pro-RLX2 plasma concentration with renal dysfunction could be shown in patients with heart failure *(goal 9)*. To assess the usefulness of pro-RLX plasma measurement in non-pregnant individ-

uals bigger cohorts would be required to achieve a sufficient portion of detectable samples. Determination of pro-RLX2 concentration within samples linked more directly to the kidney than the systemic plasma (e.g. urine, renal biopsies) might be advantageous as well.

In pregnancy, pro-RLX2 variates with gestational age as expected, but was shown to be a poor marker of PE compared to established markers (PIGF, Endoglin) *(goal 10)*. Nevertheless, relaxin-2 still might be implicated in the pathogenesis of PE on a local level. Again, determination of urinary pro-RLX2 concentration might be advantageous.

In summary, a combination of $pENK_{plasma}$ and $pENK_{urine}$ measurement could be a very promising approach to achieve an earlier recognition of AKI to improve interventions and decrease mortality. If earlier recognition is possible, new effective interventions could be designed as well. Therefore, $pENK_{plasma}$ and $pENK_{urine}$ are new biomarkers - defined as "parameter of structural, biochemical, physiologic or genetic change that indicates the presence, severity or progress of a disease" [204]. Still the implication of pENK in renal pathology needs to be examined in more detail since the function pENK exhibits within the kidney still remains unclear.

In contrast, plasma pro-RLX2 was shown to be less useful as biomarker. Nevertheless, it seems to be directly involved in pathology of cardiorenal injury. Exogenous relaxin-2 (Serelaxin) is a promising approach for new therapeutic action, but monitoring of endogenous pro-RLX2 is difficult.

Appendix

Publications

All publications the author was involved in and contributing to during this dissertation work are listed below with details regarding contributorship (papers submitted but not yet accepted are written in grey letters):

- Marino, R., Struck, J., Hartmann, O., Maisel, A.S., **Rehfeldt, M.**, Magrini, L., Melander, O., Bergmann, A., and Di Somma, S. (2015). Diagnostic and short-term prognostic utility of plasma pro-enkephalin (pro-ENK) for acute kidney injury in patients admitted with sepsis in the emergency department. J. Nephrol. 28, 717–724: *M. Rehfeldt worked on the interpretation of data and contributed to the writing and editing of the manuscript and approved its content.*

- Shah, K.S., Taub, P., Patel, M., **Rehfeldt, M.,** Struck, J., Clopton, P., Mehta, R.L., and Maisel, A.S. (2015). Proenkephalin predicts acute kidney injury in cardiac surgery patients. Clin. Nephrol. 83, 29–35: *M. Rehfeldt worked on the interpretation of data and contributed to the writing and editing of the manuscript and approved its content.*

- **Rehfeldt, M.,** Sparwasser, A., Funk, E., Köhrle, J., and Bergmann, A. (2017). Quantification of Relaxin-2 connecting peptide (pro-RLX2) in human blood samples. JALM, unpublished (article accepted by the journal): *M. Rehfeldt worked on conception and assay design, the acquisition of laboratory data, the analysis and interpretation of data. M. Rehfeldt contributed to the writing and editing of the manuscript and approved its content.*

- **Rehfeldt, M.,** Eklund, E.E., Struck, J., Sparwasser, A., O'Brien, B., Palomaki, G.E., Köhrle, J., Bergmann, A., and Lambert-Messerlian, G. (2017). Relaxin-2 connecting peptide (pro-RLX2) levels in second trimester serum samples to predict preeclampsia. Submitted to Pregnancy Hypertens., currently under review: *M. Rehfeldt worked on conception and study design, the acquisition of laboratory data, the analysis and interpretation of data. M. Rehfeldt contributed to the writing and editing of the manuscript and approved its content.*

- Zoet, G., van Rijn, B., **Rehfeldt, M.**, Franx, A., Maas, A. (2017). Similar pro-NT and pro-RLX2 levels after preeclampsia and after uncomplicated pregnancy. Submitted to Maturitas, currently under review: *M. Rehfeldt developed the pro-RLX2 assay system and performed the laboratory analysis of samples. M. Rehfeldt contributed to the writing and editing of the manuscript and approved its content.*

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Validation of circulating Wnt1 inducible signaling pathway protein 1 (WISP1) as a novel marker of insulin resistance and associated diseases. Submitted to J Cell Commun Signal, currently under review: *M. Rehfeldt advised on design for assay validation, and worked on the analysis and interpretation of data. M. Rehfeldt contributed to the writing and editing of the manuscript and approved its content.*

Features of established assay systems (technical documentation)

A) Features of pENK urine assay

Typical standard curve Signal (RLU) linearly increases with increase of analyte amount. Figure 12.3 shows repeated measurements of the concentration response curve with the respective RLU values.

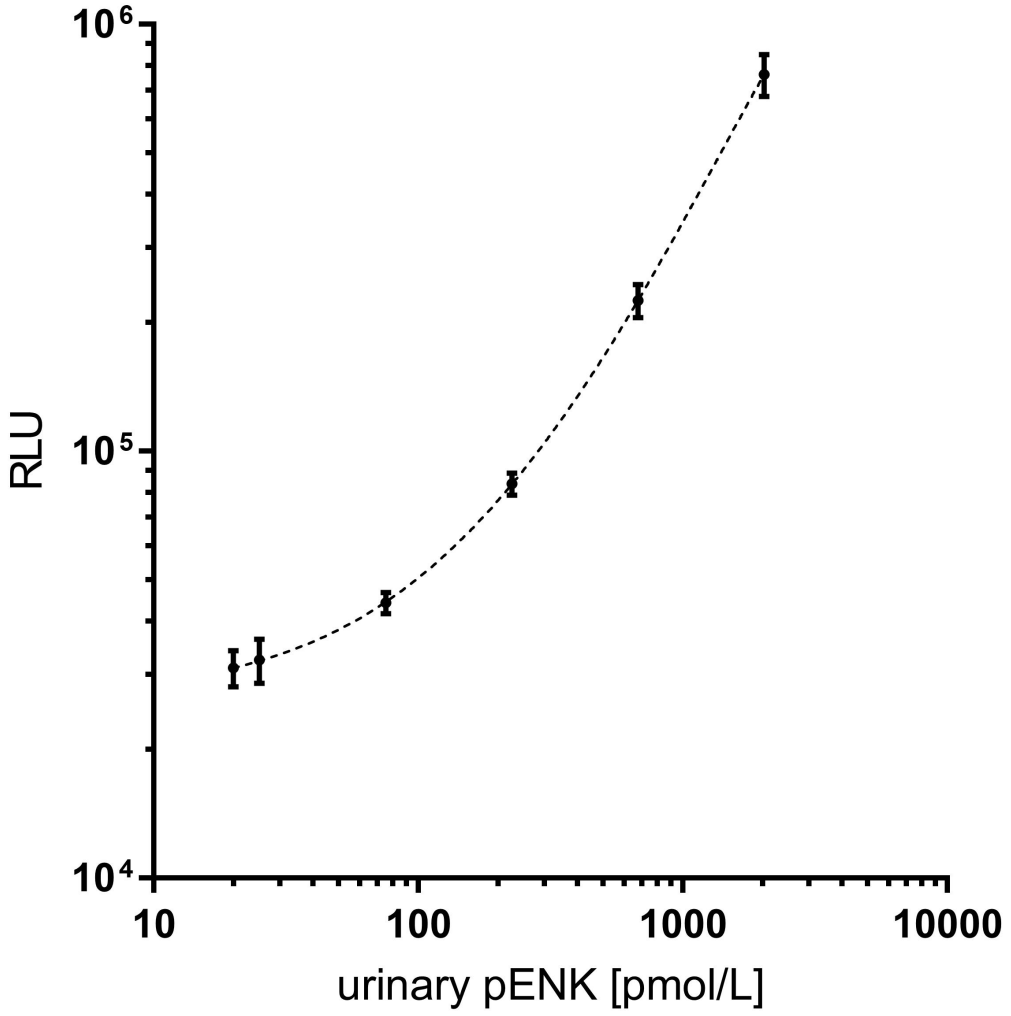

Figure 12.3.: **Concentration-response curve for the detection of pENK immunoreactivity in human urine.** There is a linear increase in signal (RLU) with increase of analyte concentration. Error bars represent mean and SD of 10 repeated measurements.

Robustness Since development of this assay system was designed as a proof of principal, no exact determination of variation thresholds was performed. Variation of ±10% was assumed as accepetable.

Stability In clinical routine short storage of samples could occur. Also repeated freeze-thaw-cycles could happen. Both was tested previously for blood samples, but repeated for urine since other enzymatic processes cleaving pro-Enkephalin might occur in urine. Standard stability was shown previously within the plasma system and can be accepted as given for this approach.

Stability of samples Samples have been stored at 4-6°C (cold room), room temperature and 37°C (incubator) for 24 hours with only slight signs of degradation (Fig. 12.4a-c). After storage at 4°C for 24 hours only one out of eight samples measures below the treshold of 20% variation. Mean recovery is 95% with a range from 86-106%. At room temperature and 37°C samples are slightly less stable, a storage of more than 4 hours is not reccomended. Samples have been frozen at -80°C and re-thawed up to four times with no sign of degradation, mean recovery is 100% with a range from 81-111% (Fig. 12.4d).

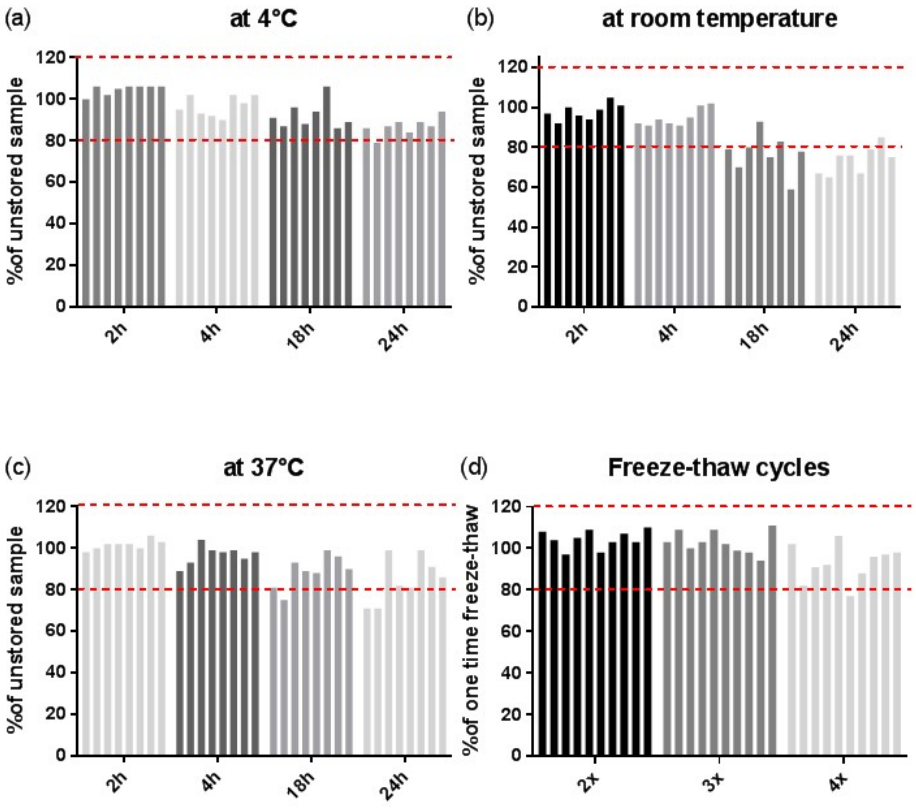

Figure 12.4.: **Stability of pENK within urin.** (a) *stability of samples* at 4-6°C (b) at room temperature and (c) at 37°C. The analyte is stable within samples for at least 4 hours at room temperaure and 37°C, respectively, and up to 18 hours at 4°C. (c) *stability of samples* during repeated freeze-thaw-cycles. The analyte is stable within samples during at least 3times repeated freeze-thaw cycles.

Precision Since development of this assay system was designed as a proof of principle, no detailed determination of precision was performed. A functional assay sensitivity of 2xBlank was assumed to determine measurability of samples. Hence, the estimated FAS is approximately 3410 RLU which resembles a concentration of 7.215 pmol/L.

Correctness

Linearity Samples were diluted within a range of 1:2 to 1:128, revealing a matrix effect of the urinary matrix slightly distorting the measurement within the less diluted samples (Fig.12.5a). This effect decreases to an acceptable range in a dilution of 1:8 and below. Around a dilution factor of 32/64, most samples measure below detection limit (Fig. 12.5b). A dilution of 1:10 is therefore recommended for routine clinical study measurement. For higher accuracy, measurement of up to three different dilutions (e.g. 1:8, 1:16, 1:32) can be applied.

Pools Sample can be pooled without restrictions in recovery (Fig. 12.5c). The mean deviation is 3.3% with a range from -9.1 to 19.1%.

Interference Protein (albumin), bilirubin, hemoglobin, urea and triglycerides show now influence within the tested concentrations (Fig. 12.5d). As shown previously for the plasma assay as well synthetic heparin diminishes the recovery dramatically.

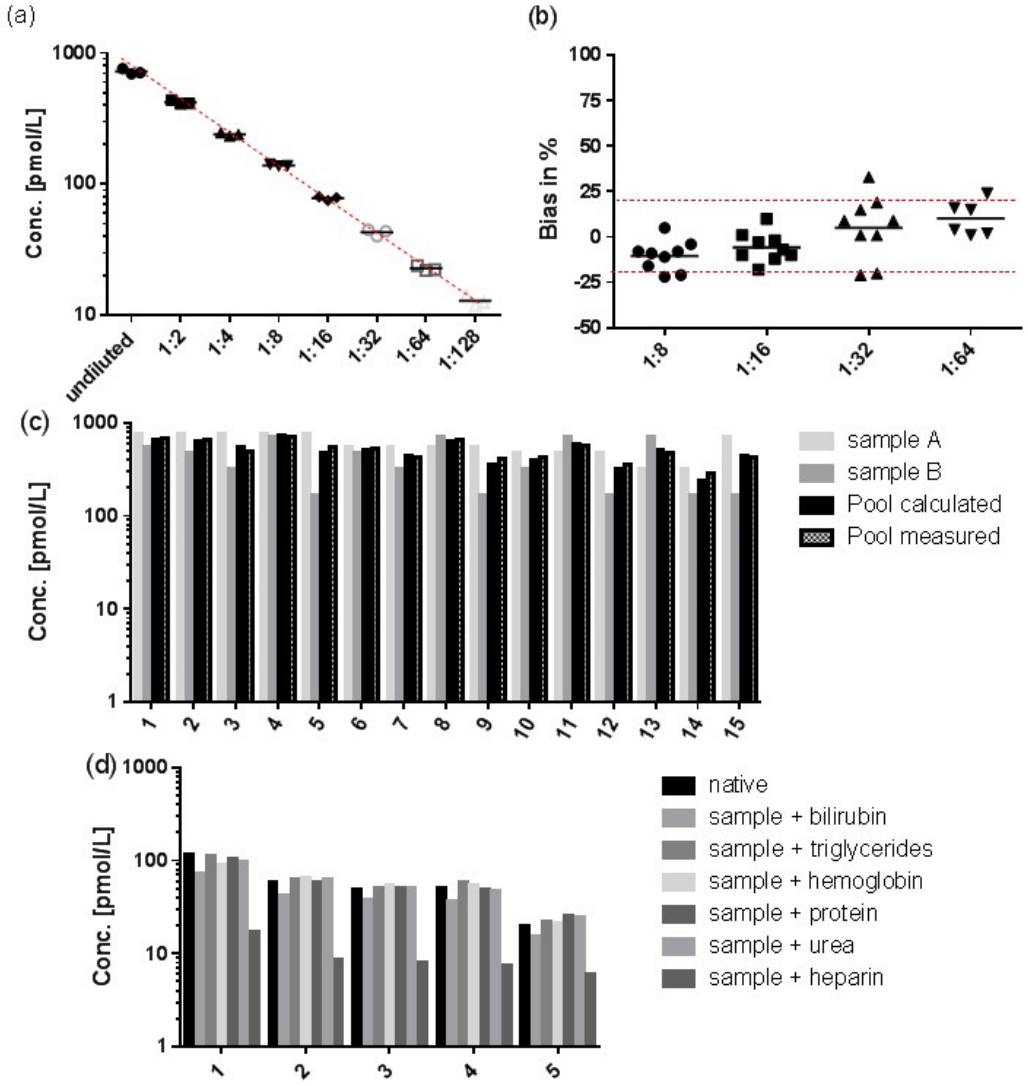

Figure 12.5.: **Correctness & interference of the pENK assay sytem within urine.** (a) samples dilute with sufficient linearity below a dilution of 1:8. (b) linearity bias from expected concentration calculated from acceptable in 1:8 and 1:16 dilution. Most samples measure below detection limit when diluted below 1:16. (c) expected concentrations are sufficiently recovered in fifteen different pooled samples (d) Five samples were supplemented with (patho)physiologic concentrations of potentially interfering substances (4mg/L bilirubin, 50mg/L hemoglobin, 600mg/L albumin, 300mg/L triglycerides, 0.5µg/L urea, 200U/L heparin). No interference could be shown exept by heparin which was expected from experiments with the plasma assay system.

B) Features of pENK rat assay

Typical concentration response curve Signal (RLU) linearly increases with increase of analyte concentration. Figure 12.6 shows repeated measurements of the concentration response curve with the respective RLU values. Even though there is a high variation in the background signal, plasma concentrations of above 25pmol/L should be detectable reliably.

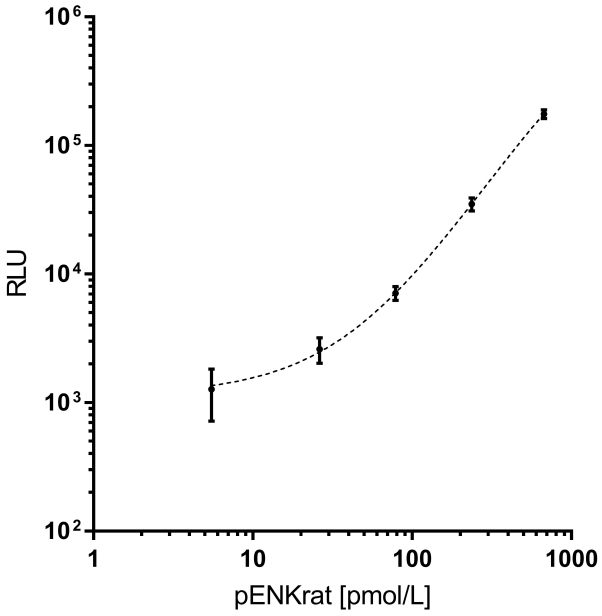

Figure 12.6.: **Concentration-response curve for the detection of rat pENK immunoreactivity.** There is a linear increase in signal (RLU) with increase of analyte amount. Error bars represent mean and SD of 10 repeated measurements.

Kinetics of incubation Signals in standards and samples are increasing over time. Standards incubated 4 hours show 53-76% binding of the incubation for 20 hours (Fig.12.7a). Hence, the assay needs over night incubation for completed reaction. A variation of the incubation time $\pm 2h$ should have no further impact on recovery (tested for human system).

Temperature of incubation Binding of antibodies is extremely depended on temperature, there is a signal loss of about 5% for every degree (°C) of increasing incubation temperature. This effect could be shown for both, standards and samples. Even though the unspecific background is decreasing as the signal decreases with increasing temperature, there is a massive loss of differentiation when increasing temperature (Fig. 12.7b).

Following, recovery rates in samples are dramatically decreased at higher temperatures (Fig. 12.7c). An incubation temperature of 6°C is recommended.

Rotation throughout incubation Rotation of the 96well plate diminishes binding of the antibodies. Even though this causes a reduction in non-specific background, differentiation of the system is decreased due to decreased specific binding (Fig. 12.7d). Hence, incubation without rotation is recommended.

Sample volume A reduction of sample volumen would be desirable since in animal models usually samples of smaller volumes are available than in human. Unfortunately, the assay sensitivity is decreased by decreasing the sample volume below 50µL. Thus, a sample volume of 50µL is recommended, if available (Fig. 12.7e).

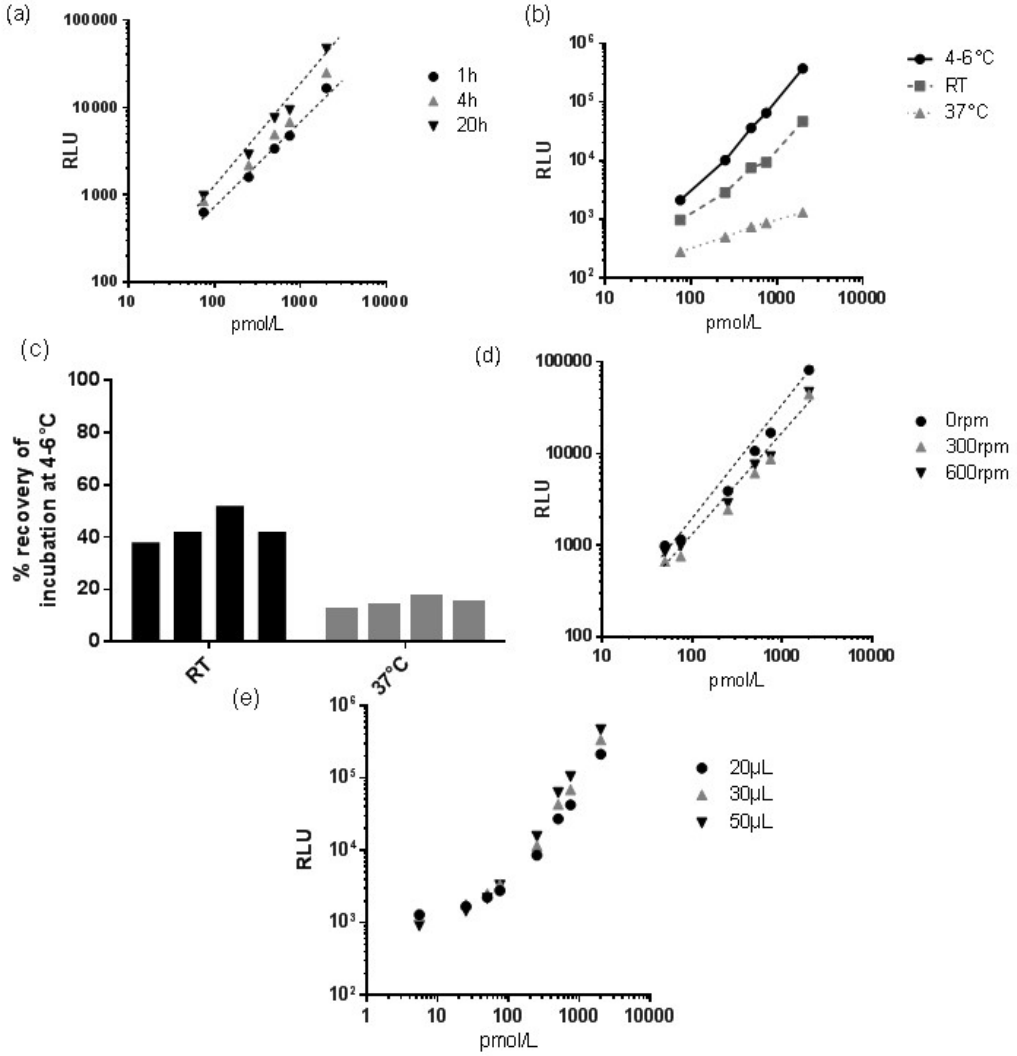

Figure 12.7.: **Assay characteristics of pENKrat assay system.** (a) decreased incubation duration deteriorates assay sensitivity (b) increased incubation temperature reduces antibody binding to the analytes (c) sample recovery is dramatically decreased at increased incubation temperature (d) reduction of rotation speed slightly increases assay sensitivity (e) assay sensitivity is reduced when sample volume is decreased.

Stability Stability has not been tested in detail. Since the stability of pENK has been tested sufficiently in human, we assume a satisfying stability in rodents as well.

Precision Since the development of this assay system was designed as a proof of principle, no detailed determination of precision was performed. A functional assay sensitivity of 2xBlank was assumed to determine measurability of samples.

Correctness

Linearity Samples were diluted within a range of 1:1.5 to 1:5.06. (Fig. 12.8a). Even though there were greater deviations (especcially within the 1:2.25 dilution and below 1:38), linearity was defined as acceptable for several proof of principal clinical studies (Fig. 12.8b).

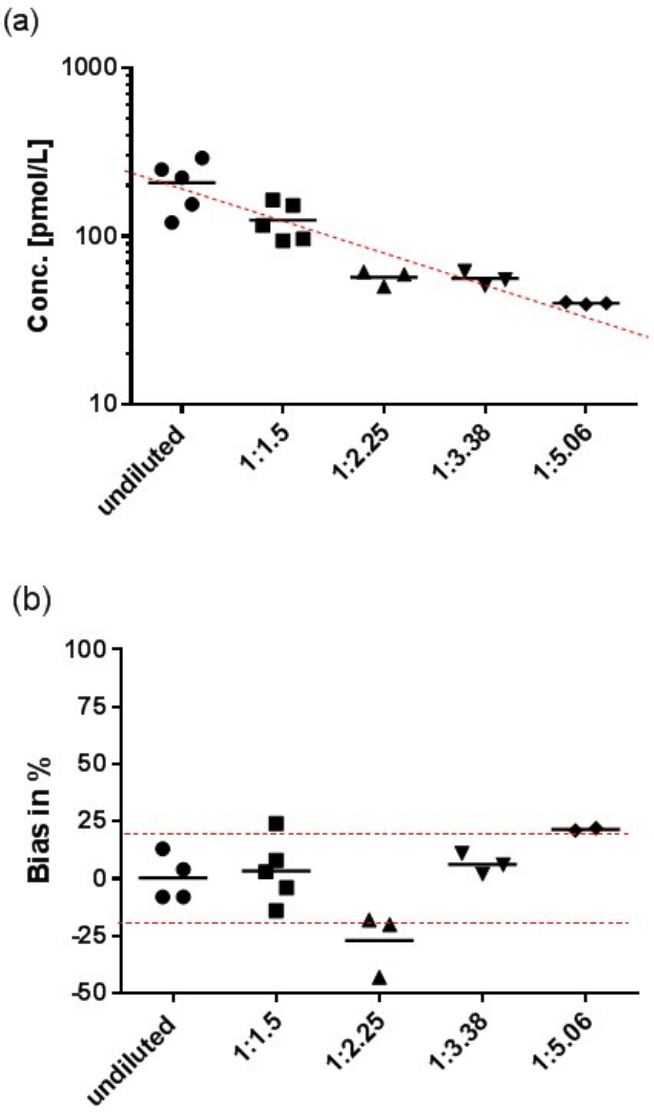

Figure 12.8.: **Dilution linearity of assay system.** (a) Serial dilution of rat plasma samples. Given are only concentrations above detection limit. (b) deviations, linearity was defined as acceptable for several proof of principal clinical studies.

C) Features of pro-RLX2 assay

The technical details of the assay system are published in Rehfeldt et al. [148].

Typical concentration response curve Signal (RLU) linearly increases with increase of analyte amount. Figure 12.9 shows repeated measurements of the concentration response curve with the respective RLU values.

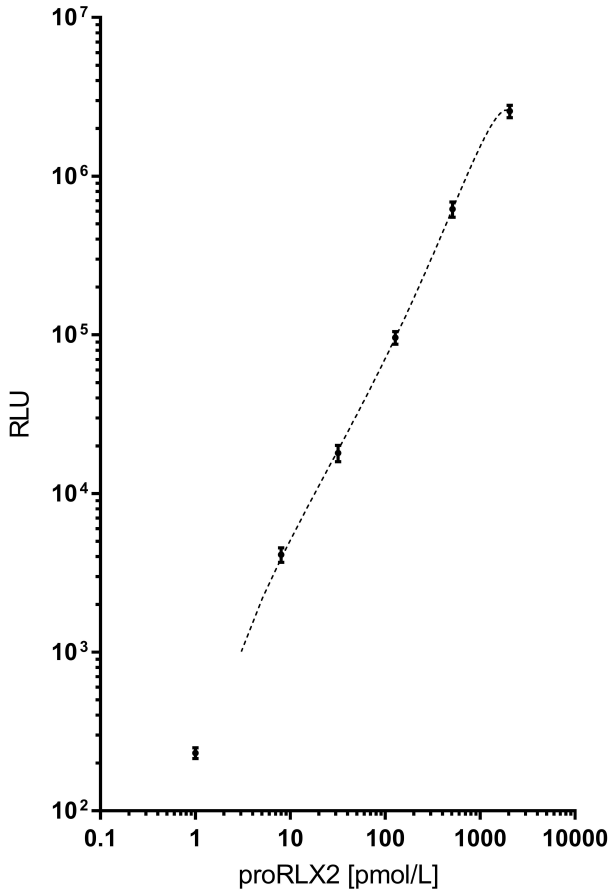

Figure 12.9.: **Concentration-response curve for the detection of pro-RLX2 immunoreactivity.** There is a linear increase in signal (RLU) with increase of analyte amount. Error bars represent mean and SD of 15 repeated measurements.

Robustness The assay system is robust to smaller changes in assay preparation. There is no influence of incubation shift (delayed incubation start for several samples due to time-consuming manual pipetting), kinetics, temperature, rotation, sample, total volume or number of washing steps if keeping limits specified in table 12.2. Order of pipetting

shows a significant influence on measured concentrations, so it is recommended to always pipette sample first.

Table 12.2.: Limits of incubation robustness

parameter	recommended value	tolerance
Shift	0'	0-40'
Incubation time	20h	$\pm 2h$
Incubation temperature	6°C	2°C to 10°C
Rotation	600rpm	<300rpm
Sample volume	100µl	75-125µl
total volume	250µl	125-175µl
washing steps	5x	4-6x

Shift After 40 minutes of shift still there is only a slight decrease in recovery of the sample concentration found without any shift. After 60 minutes two of four tested samples are recovered under 85% which we state as a threshold (Fig. 12.10a).

Kinetics of incubation Signals in standards and samples are increasing over time. Standards incubated 4 hours show approximately 40% binding of the incubation for 20 hours. Samples show only 30%, so the time the native analyte takes for complete binding differs from that one the recombinant standard material takes. Following, the recovery after 4h is increased by 10-26%. The shorter the incubation time, the lower recovery rates could be found (Fig. 12.10b). Hence, the assay needs over night incubation for completed reaction. A variation of the incubation time $\pm 2h$ has no further impact on recovery (Fig. 12.10e).

Temperature of incubation Binding of antibodies is extremely depended on temperature, there is a signal loss of about 5% for every degree (°C) of increasing incubation temperature. This effect could be shown for both, standards and samples. Since the unspecific background is not decreasing as the signal decreases with increasing temperature, there is a massive loss of differentiation when increasing temperature (Fig. 12.10c). Following, recovery rates in samples are decreased in higher temperatures (Fig. 12.10d). An incubation temperature of 6°C is recommended. A variation of within 2 to 10°C is acceptable (Fig. 12.10e).

Rotation throughout incubation Complete binding of antibodies requires rotation of the 96well plate throughout incubation. If shaking frequency is decreased, binding is diminished especially in the higher standards and samples. Reduction of the shaking frequency by half shows up to 20% reduction in signal, if assay is not shaken at all signals decrease by up to 50%. Since standards decrease to a lesser degree than samples,

a higher recovery rate is found at lower shaking frequencies (Fig. 12.11f). This reflects the result from kinetics analysis where the binding antibodies to standards is quicker than to the native analyte. Obviously, one or both antibodies have a higher affinity to the recombinant standard material than to the native analyte. This might be due to folding reasons.

Sample and total volume Sample volume variation of up to ±25% has no influence on recovery (Fig. 12.11g). Tracer volume may be varied by up to ±17% (±10% of total volume) as well (Fig. 12.11h).

Order of pipetting Pipetting order shows no influence on standard or sample signal (Fig. 12.11i).

Washing steps Varying the number of washing steps has no impact on recovery as long as a minimum of 4 times is preserved (Fig. 12.11j). Less washing steps increase specific as well as unspecific binding with slightly decreasing differentiation.

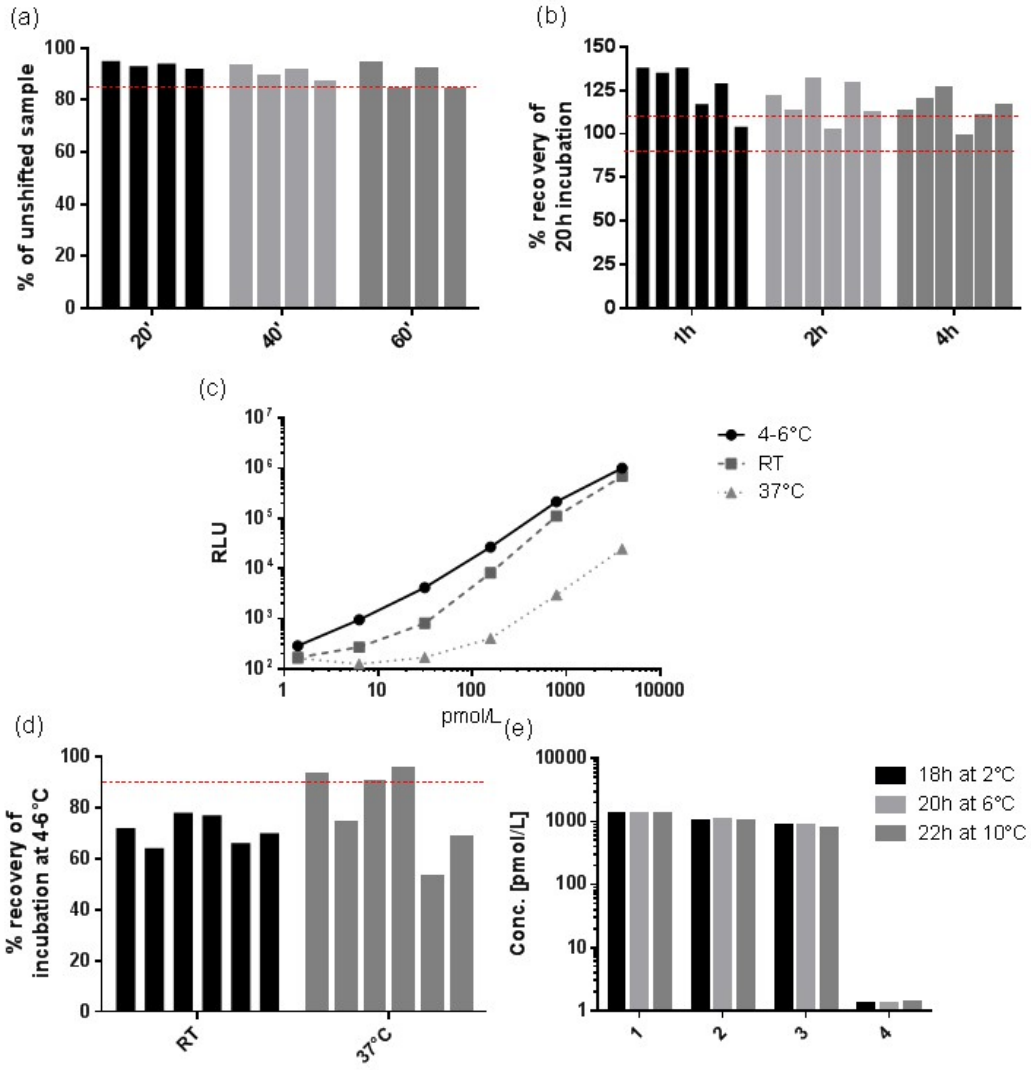

Figure 12.10.: **Robustness characteristics of pro-RLX2 assay system I.** (a) shift (b) kinetics (c) standard curves at different temperatures (d) sample recovery at different temperatures (e) threshold scenario

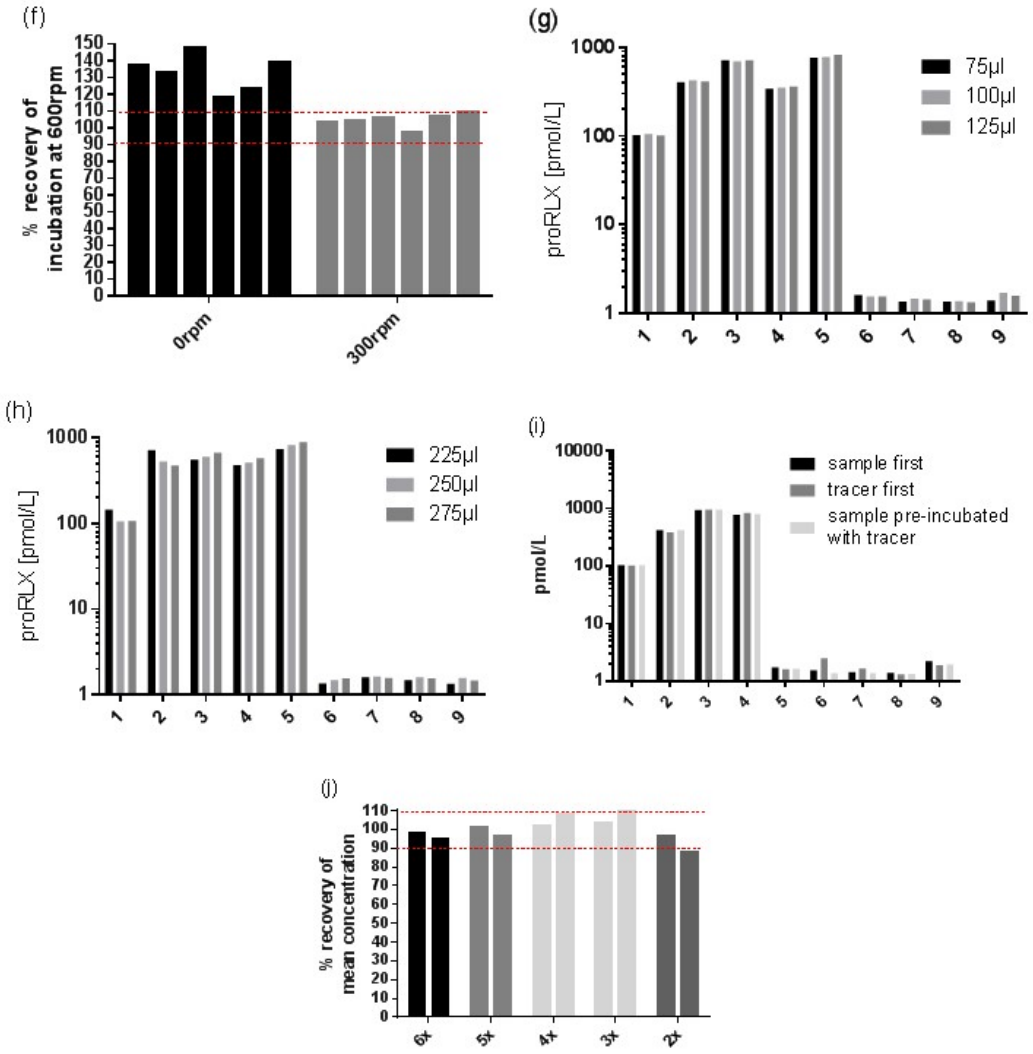

Figure 12.11.: **Robustness characteristics of pro-RLX2 assay system II.** (f) reduction of rotation speed changes recovery of sample concentration. (g) smaller changes in sample volume show no influence on recovery (h) smaller changes in total volume show limited influence on recovery (i) order of pipetting shows no influence on measurement (j) repeated washing steps of minimum 3 washing steps is required for sufficient recovery.

Stability

Stability of standards Stability of recombinant standard material after reconstitution has been tested for 24 hours. There is a slight decrease (up to 20%) in recovery for a storage time of 4 hours at room temperature (Fig. 12.12a). When storing longer, signals decrease by about 30-50%. Hence, it is recommended to avoid longer storage than 4 hours after reconstitution.

Stability of samples We evaluated the stability of the analyte in EDTA-plasma samples at 4°C, room temperature and 37°C. All 8 samples for stability studies contained only endogenous pro-RLX2 and no added peptide. The analyte stability was tested for 24h at 4°C and 37°C to prove stability during short-term storage, e.g. transport on ice etc. (Fig. 12.12b & d). Furthermore, samples were shown to be stable up to 8 days at room temperature (Fig. 12.12c). Mean measured concentrations were between 81 and 109% of the original value at 4°C (over 24 hours), 85 and 122% at 37°C (over 24 hours) and 85 and 117% at room temperature (over 8 days), respectively.

Samples have been frozen at -80°C and re-thawed up to four times with no sign of degradation (Fig. 12.12e). Again we see a difference between native analyte and recombinant material. This could be due to folding reasons and therefore higher susceptibility of the recombinant material to proteases. Another possibility is plasma matrix containing factors inhibiting degradation of pro-RLX2 that are not contained in horse serum (standard matrix).

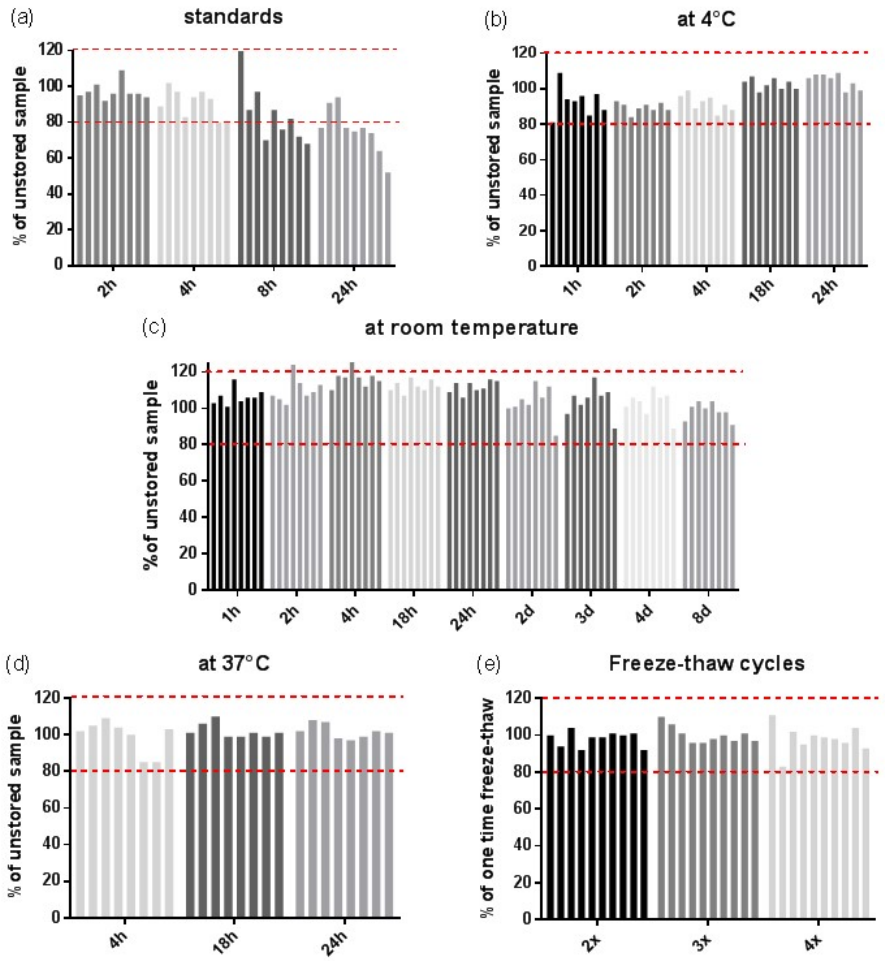

Figure 12.12.: **Stability of pro-RLX2.** (a) *Stability of standards* after reconstitution. The stability is limited. (b) *stability of samples* at 4-6°C (c) at room temperature and (d) at 37°C. The analyte is stable within samples for at least 24 hours at 37°C and up to 8 days at room temperature. (e) *stability of samples* during repeated freeze-thaw-cycles. The analyte is stable within samples during at least 4times repeated freeze-thaw cycles.

Precision

Intra-assay variation coefficient The intra-assay precision is defined by multiple determination (10times) of at least 20 samples covering the whole detection range. It represents the random measuring error which statistically afflicts the measurement result within an observed measurement series. Is is specified by the variation coefficient.

By calculating the mean blank value and the respective standard deviation (SD), the 1s, 2s and 3s limit can be determined. The 1s limit characterizes the lower limit above which a measurement will be found with 68% probability. The 2s shows 95%, the 3s

99% probability. The limit of detection (analytical assay sensitivity) is defined as the range where a measured concentration is greater zero with 95% probability.

The FAS represents the range where a sample is measured with a specific precision. Above and below this range random errors possibly exceed a precise designation of measurement result. For the intra-assay precision, the FAS is defined by the concentration where the mean variation coefficient exceeds 10%.

For the pro-RLX2 assay system we determined an 1s limit of 1.50 pmol/L, a 2s of 1.59 pmol/L and a 3s of 1.67 pmol/L. Hence, the analytical assay sensitivity is approximately 1.6 pmol/L. Interpolating a curve for the intra-assay precision, a FAS of approximately 1.9 pmol/L is determined (Fig. 12.13b).

Inter-laboratory variation coefficient The inter-laboratory precision is defined by multiple determination of at least 20 samples covering the whole detection range. It represents the random measuring error which statistically afflicts the measurement result under changing conditions (different days, operators, batches of reagents, equipment). It is specified by the variation coefficient.

The FAS represents the range where a sample is measured with a specific precision. Above and below this range random errors possibly exceed a precise designation of measurement result. For the inter-laboratory precision, the functional assay sensitivity is defined by the concentration where the mean variation coefficient exceeds 20%. It should reflect the FAS determined by the intra-assay precision (Fig. 12.13c).

(a)

	Blank+1SD	Blank+2SD	Blank+3SD
RLU	368	416	464
pmol/L	1.50	1.59	1.67

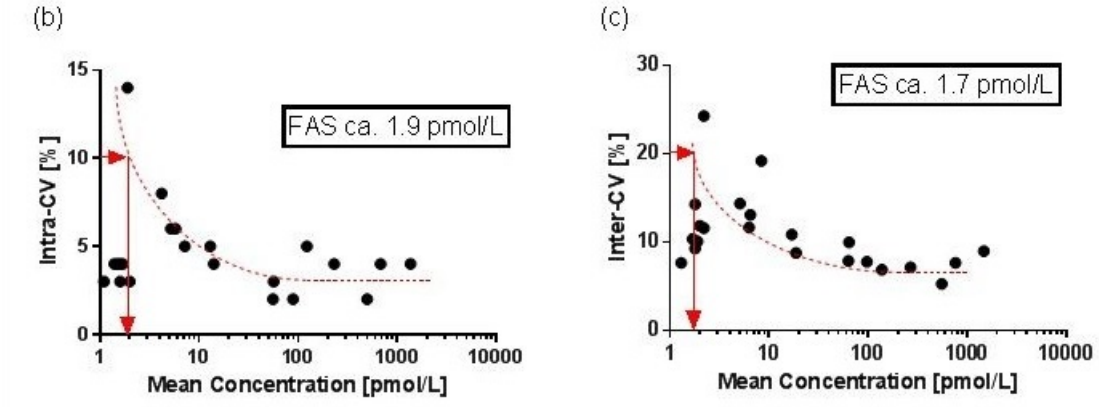

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Figure 12.13.: **Precision of pro-RLX2 assay system.** (a) analytical assay sensitivity is 1.59 pmol/L. (b+c) functional assay sensitivity is approximately 1.7-1.9pmol/L.

Correctness

Linearity Dilution of samples (up to 1:16) is ideal as tested in 5 EDTA-plasma samples. Measured concentrations were multiplied by the dilution factor and compared with the original undiluted concentrations. None of the 5 samples showed a deviation during dilution >20% of the original value (correlation of expected vs measured concentration Spearman r = 0.9923, p<0.0001) (Fig.12.14a).

Starting from a dilution factor of 1:32, the dilution recovery exceeds the accepted threshold of 20% (Fig. 12.14b).

Pools Sample can be pooled without restrictions in recovery (Fig. 12.14c). Pooling of 4 plasma samples with low pro-RLX2 concentrations with 6 plasma samples of intermediate and 4 plasma samples with high pro-RLX2 concentrations in 7 different combinations gave a mean measured concentration that was 103% of the expected concentration (range 89-114%)..

Recovery We performed recovery experiments by adding the calibrator peptide in 5 concentrations (40, 110, 390, 1,400 and 3,600 pmol/L) to 5 different plasma samples (endogenous conc. 2, 4, 370, 450 and 800 pmol/L). The deviation in recovery rate is <20% in 19/25 samples (mean bias -5%) (Fig. 12.14d).

High Dose Hook A high-dose hook effect was seen when pro-RLX2 concentrations >8,000pmol/L were added to plasma samples. When measuring a solution of 8,192 pmol/L, the determined concentration was deviating 38% of the expected value. This effect increases up to recovery of 99.7% at concentrations of 2,000,000 pmol/L. However, 8,000 pmol/L is 4-fold higher than the highest calibrator and would still be found as highly positive in the assay. Fig. 12.14e shows the assay system reaching a saturation around 4,000pmol/L which starts declining slightly at concentrations over 320.000 pmol/L.

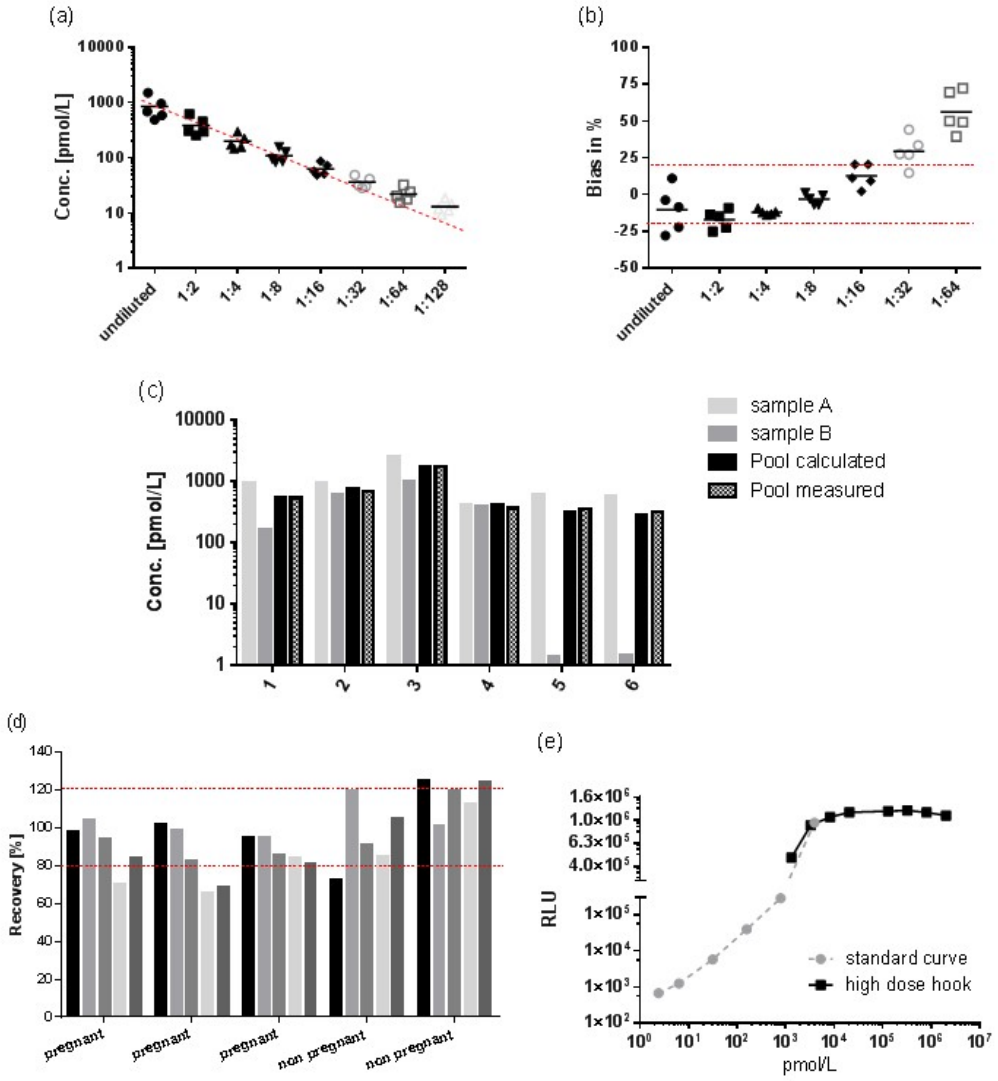

Figure 12.14.: **Correctness characteristics of pro-RLX2 assay system.** (a) samples dilute with sufficient linearity up to a dilution factor of 1:16. (b) linearity bias from expected concentration calculated from mean exceeds 20% with a dilution factor of 1:64 (c) expected concentrations are sufficiently recovered in six different pooled samples (d) recovery of recombinant material within samples is acceptable even though several samples show recovery rates lower than 80%. (e) high dose hook is far beyond normal sample measurement range.

Interference We measured the interference of several biological substances by adding the potential interference factor to 7 human plasma samples of different concentrations, according to the protocol recommended by the Clinical and Laboratory Standards Institute. The assay was not influenced by albumin concentrations up to 6mg/dL, bilirubin up to 40mg/dL, hemoglobin up to 500mg/dL, triglycerides up to 3g/L, or heparin up to

200 IU/L. The deviation from the values for samples without added interference factor was always $\leq$<20% (data not shown). For albumin the maximum deviation from native samples was +12% (range -8 to +12%), for bilirubin -13% (-17 to -7%), for hemoglobin -20% (-20 to +13%), for triglycerides -16% (-16 to +7%) and heparin +16% (-3 to +16%) (Fig. 12.15a).

Other isoforms of relaxin could potentially interfere with the assay system as well. Especially isoform 1 (pro-RLX1) is structurally similar to pro-RLX2 and possesses 86.4% (89 out of 103) identical amino acids within the connecting peptide. We therefore tested for possible interference with the pro-RLX1 peptide. As the detecting antibody is binding a homologous region within the peptide and accordingly pro-RLX1 competes with pro-RLX2 for binding, we see the expected dose-dependent decrease in detectable pro-RLX2 concentration (Fig. 12.15b). If we design the assay as a 2step system, the effect is abolished since the pro-RLX1 is not binding to the capture antibody and thus removed before the detecting antibody is added. Since in the literature there is no hint if the relaxin-1 isoform is expressed at all in human, we decided to continue with the 1step assay for better sensitivity.

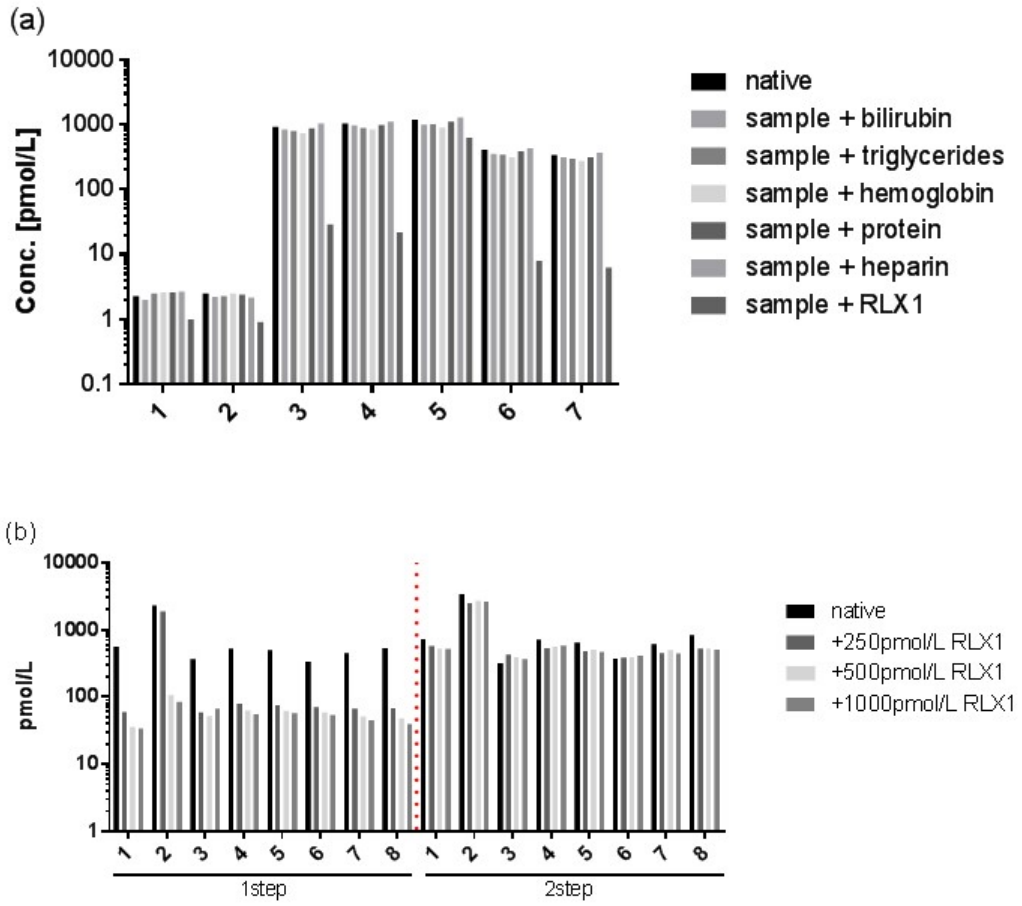

Figure 12.15.: **Interference of bilirubin, triglycerides, hemoglobin, protein and pro-RLX1 with pro-RLX2 assay system.** (a) Seven samples were supplemented with (patho)physiologic concentrations of potentially interfering substances (4mg/L bilirubin, 50mg/L hemoglobin, 600mg/L albumin, 300mg/L triglycerides, 0.5µg/L urea, 200U/L heparin, 500pmol/L pro-RLX1). No interference could be shown exept for pro-RLX1. (b) Eight samples were supplemented with different concentrations of pro-RLX1 (250-1000pmol/L), incubated as 1 and 2step assay. A concentration-dependent interference in the 1step could be shown while there is no interference in the 2step assay.

Sample type Serum samples were tested with 38 matched EDTA-plasma samples obtained from both, pregnant and non-pregnant (male and female) healthy controls. A correlation of $>.9$ with plasma concentrations and a deviation of $<20\%$ in accuracy ex-

periments (dilution experiments, pooling and recovery studies) were set as quality goal for acceptance of the assay as suitable for serum measurement. Spearman correlation was 0.969 (p<.0001) even though ratios serum/plasma were highly varying (range 60-190%) and the serum/plasma ratio is correlating with EDTA plasma concentrations as well (Fig. 12.16 a & b). Thus, the assay was tested for accuracy (dilution experiments, pooling and recovery studies) in serum samples and all values were in acceptable range (data not shown). Hence, the assay is suitable for measurement of pro-RLX2 in serum samples. Nevertheless, before transfer potential cut-off values etc. from one matrix to another, further analyses might be required.

Other matrices (heparin/citrate plasma) were not tested.

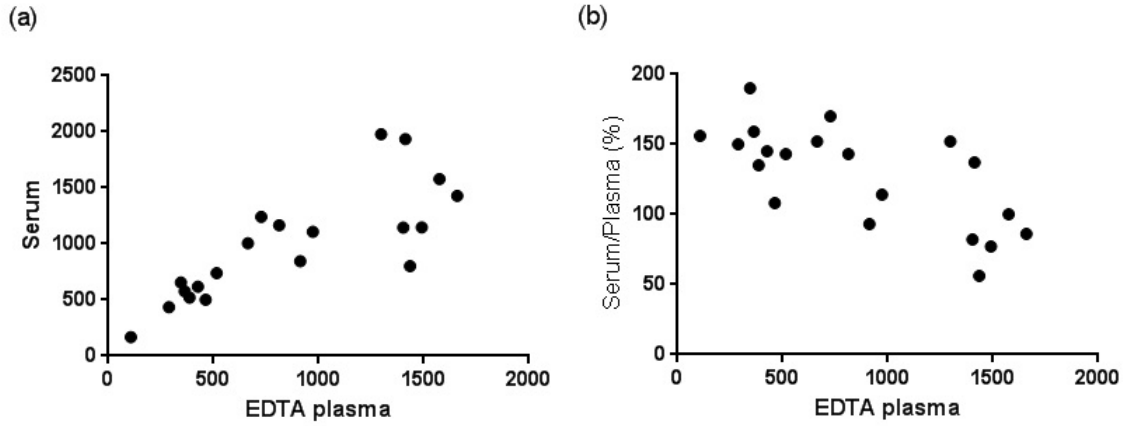

Figure 12.16.: **Measurability of different sample types.** (a) measured concentration in plasma and serum samples highly correlate ($r = 8241, p < 0001$) (b) strong negative correlation of coefficient serum/plasma to absolute plasma conc. ($r = -7148, p = 0004$)

List of abbreviations

Table 12.3.: List of abbreviations I (A-G)

Abbreviation	full length
aa	amino acid
ACOG	American Congress of obstetricians and gynecologists
ADHF	acute decompensated heart failure
AFP	alpha-fetoprotein
AG	Aktiengesellschaft
AGE	advanced glycation end product
AHF	acute heart failure
AKI	Acute Kidney Injury
Akt	Protein kinase B
ALAT	Alanin amino transferase
ANP	atrial natriuretic peptide
Aqua dest.	distilled water
ASAT	Aspartate amino transferase
AT1	Angiotensin 1
AT2	Angiotensin 2
AVP	arginine vasopressin
BMI	body mass index
BNP	brain natriuretic peptide
BP	blood pressure
BSA	bovine serum albumin
BUN	blood urea nitrogen
C-peptide	connecting peptide
DAB	3,3'-Diaminobenzidine
(E)	Expect
e.g.	exempli gratia (for example)
EABV	effective arterial blood volume
EDTA	ethylene diamine tetraacetid acid
eGFR	estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ESRD	end stage renal disease
ET-1	Endothelin-1
ETb	endothelin b
Fc-term	constant region of the immunoglobulins
FDA	Food and Drug Administration: US american authority for medical and pharmaceutical products
GABA	gamma-aminobutyric-acid
GFR	glomerular filtration rate
GLP	good laboratory practice
GmbH	Gesellschaft mit beschränkter Haftung
G-protein	guanosin-triphosphate binding protein

Table 12.4.: List of abbreviations II (H-P)

Abbreviation	full length
HAM1801	human adrenomedullin antibody 1801: subtype of ADM antibody
hCG	human chorionic gonadotropin
HF	heart failure
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
ICU	intensive care unit
IGFBP7	Insulin-like growth factor-binding protein 7
IgG	Immunoglobuline type G
IL-18	Interleukin-18
IL-6	Interleukin-6
IQR	interquartile range
IV	intravenous
J Am Soc Nephr	Journal of the American Society of Nephrology
kDa	kiloDalton
KIM-1	Kidney injury molecule - 1
Leu-ENK	Leucine-Enkephalin
LIA	luminometric immunoassay
LOCI	luminescent oxygen channeling assay
LPS	Lipopolysaccharide
LSAB	labeled (strept)avidin biotin
M2 macrophage	macrophage subtype 2
MACN	N-Methyl-Acridon
MAP	mean arterial pressure
Met-ENK	Methionine-Enkephalin
Met-ENK-RF	Methionine-Enkephalin-Arginine-Phenylalanine
Met-ENK-RGL	Methionine-Enkephalin-Arginine-Glycine-Leucine
MoM	Multiples of median
mRNA	messenger ribonucleic acid
NAG	N-Acetylglucosamine
Na-K-ATPase	Natrium-Kalium-Adenosin-triphosphate converting enzyme
NGAL	Neutrophil gelatinase-associated lipocalin
NO	nitric oxide
NYHA	New York Heart Association functional classification: classification of heart failure
OGF	opioid growth factor
PAPPA-A	Pregnancy-associated plasma protein A
PBS	phosphate buffered solution
PE	preeclampsia
pENK	pro-Enkephalin
PENK A	Pro-Enkephalin A: fragment of the Proenkephalin (aa 119-159)
PI3K	phosphoinosid 3 kinase
PIGF	placental growth factor

Table 12.5.: List of abbreviations III (P-V)

Abbreviation	full length
PPET-1	prepro-endothelin-1
prepro-ET	prepro-endothelin
PROTECT	Placebo-controlled Randomized study of the selective A1 adenosine receptor antagonist KW–3902 for patients hospitalized with acute HF and volume Overload
RAAS	Renin-Angiotensin-Aldosterone System
Ras	rat sarcoma: proto-oncogen
RELAX-AHF	Relaxin-Acute heart failure: clinical trial of serelaxin treatment in patients with AHF
RIFLE	Risk Injury Failure Loss End stage renal disease: classification score for renal disease
RLU	relative light unit
RLX2	Relaxin 2
ROS	reactive oxygen species
RPF	renal plasma flow
RRT	renal replacement therapy
RUPP	Reduced Uterine Perfusion Pressure: model of preeclampsia
RXFP1	relaxin receptor 1
sCrea	serum creatinine
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sflt-1	soluble fms-like tyrosine kinase-1
SNP	single nucleotide polymorphism
SVR	systemic vascular resistance
T cells	T lymphocytes: immune cells maturing within the thymus
TGFβ	tumor growth factor beta
Th1	T helper cells type 1
TIMP2	Tissue inhibitor of metalloproteinases 2
TNF α	tumor necrosis factor alpha
TRIS	Tris-aminomethane
UARI	uterine artery resistance index
uCrea	urinary creatinine
uE3	unconjugated estriol type 3
VEGF	vascular endothelial growth factor
VO2max	maximal oxygen adsorption

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Curriculum Vitae

The CV is not included in the online version due to data privacy protection.

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Declaration of Authorship

I hereby certify that this thesis has been composed by me and is based on my own work, unless stated otherwise. No other person's work has been used without due acknowledgment in these pages. All references and verbatim extracts have been quoted, and all sources of information, including graphs and data sets, have been specifically acknowledged.

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