NEW PHARMACOLOGICAL TREATMENT TO PRESERVE/IMPROVE RENAL FUNCTION AFTER PARTIAL NEPHRECTOMY

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DISSERTATION SUBMITTED 2023

AALBORG UNIVERSITY
DENMARK

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PREFACE

This PhD study was conducted during my employment as a PhD fellow at the Department of Clinical Medicine at Aalborg University and the Department of Urology at Aalborg University Hospital Nord, Denmark, in the period from September 2018 to March 2022.

The research was supervised by Senior Registrar, Clinical Associate Professor Brian Kloster from Aalborg University Hospital and Professor Lars Lund from the Department of Urology at Odense University Hospital. The content of the thesis is based on three papers written during this period:

1. Protection of kidney function and tissue integrity by pharmacologic use of natriuretic peptides and neprilysin inhibitors. Brignone J, Assersen KB, Jensen M, Jensen BL, Kloster B, Jønler M, Lund L. Pflugers Arch. 2021 Apr;473(4):595-610. doi: 10.1007/s00424-021-02555-w. Epub 2021 Apr 12. PMID: 33844072.

2. Protective effect of sacubitril/valsartan (Entresto®) on kidney function and filtration barrier injury in a porcine model of partial nephrectomy. Brignone J, Jensen M, Jensen BL, Assersen KB, Goetze JP, Jødal L, Andersen TB, Magnusdottir SO, Kloster B, Jønler M, Lund L. Nephrol Dial Transplant. 2022 Jun 15:gfac200. doi: 10.1093/ndt/gfac200. PMID: 35704678.

3. Kidney volume increase following unilateral nephrectomy relates to plasma natriuretic peptide. Juan Brignone, Boye L Jensen, Mia Jensen, Claus Bistrup, Jens P. Goetze, Nessn Azawi and Lars Lund ‹submitted to BMC Nephrology Journal)

TABLE OF ABBREVIATIONS

AKI: Acute kidney injury

ANG: Angiotensin

ANOVA: Analysis of varianceANP: Atrial natriuretic peptide

ARNI: Angiotensin-renin-neprilysin-inhibitors

AS: Active surveillance

AUC: Area under the curve

BMI: Body Mass Index

BNP: Brain natriuretic peptide

CI: Confidende interval

cGMP: Cyclic guanosine monophosphate

CKD: Chronic kidney disease

CN: Cytor-reductive nephrectomy

CNP: C-type natriuretic peptide

CT: Computed tomography

DPTA: Diethylenetriaminepentaacetic acid

EDTA: Ethylenediaminetetraacetate

ESKD: End-stage kidney disease

Fr: French

GFR: Glomerular filtration rate

HU: Houndsfield units

KIM: Kidney-injury molecule 1

MAP: Mean arterial pressure

MBq: Million Becquerels

MRI: Magnetic resonance image

MR-proANP: Mid-regional pro-hormone atrial natriuretic peptide

NEP: Neutral endopeptidase

NO: Nitric oxide

NP: Natriuretic peptide

NPR-A: Natriuretic peptide receptor type A

NPR-C: Natriuretic peptide receptor type C

NRP-B: Natriuretic peptide receptor type B

NT proBNP: N-terminal pro-hormone brain natriuretic peptide

LCZ696: Sacubitril/Valsartan or Entresto®

PN: Partial nephrectomy

RAAS: Renin angiotensin-aldosterone system

157 RBF: Renal blood flow

158 RCC: Renal cell carcinoma

159 RN: Radical nephrectomy

160 SD: Standard deviation

161 SE: Standard Error

162 Tc: Techtenium

163 TN: Total nephrectomy

164 TNM: Tumour-Node-Metastasis

165 VHL: Von-Hippel-Lindau

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ENGLISH SUMMARY

The prevalence of renal cancer is increasing, which may, among others, be due to a rise in the number of radiological investigations performed for other reasons, e.g., to determine the origin of abdominal pain or weight loss. Recent years have therefore seen an increase in the number of surgical interventions involving the kidneys. This trend and the rapid aging of the general population command careful considerations of possible treatments and strategies to protect the renal function during and after kidney surgery. Searching for a feasible therapeutic option that avoids or at least minimizes post-surgery loss of kidney function, we conducted studies to evaluate the role of natriuretic peptides and blockage of their degradation as this approach has shown promising results in the cardiovascular area.

The group of NPs counts three vasoactive peptides with a molecular weight below 10 kDa. that are structurally interrelated; they are atrial NP (ANP), B-type NP (BNP), and C-type NP (CNP). NPs are synthetised, stored and secreted at myocardiocyte level.

Aims:

a) To perform a review to determine the potential benefits of the use of NPs and angiotensin-receptor-neprilysin inhibitor (ARNI) drugs to protect kidney function.

b) To determine the feasibility of single kidney glomerular filtration rate (GFR) measure using 99m TC diethylenetriaminepentaacetic acid (DPTA) as radiotracer in a pilot study, using pigs as an animal model.

c) To perform a randomised pre-clinical study investigating the action of LCZ696 at the kidney level after laparoscopic partial nephrectomy with prolonged ischemia time.

d) To elucidate the role of NPs in the mechanism of kidney hypertrophy after partial or total nephrectomy.

Results and conclusions

a) Having conducted an extensive review, we conclude that the main functions of these peptides at kidney level are to promote water and sodium excretion and to increase renal blood flow. Continuous ANP infusion above physiological levels induces vasodilatation of arcuate and afferent vessels and constriction of efferent arterioles, maintaining oxygen supply at the medullary level. In addition, NP administration increased GFR and diuresis, but with a stable filtration fraction. This effect is of vital importance since the concomitant increase of GFR and filtration fraction are

interpreted as detrimental effects in acute kidney injury (AKI) scenarios because the increased transport of filtered sodium is linearly correlated with oxygen usage, dramatically increasing oxygen consumption.

b) Six pigs were injected with a known quantity of [Tc-99m] Tc-DTPA, with 12 blood samples drawn from 5 to 240 minutes after injection. GFR was determined as injected activity divided by area under area under a bi-exponential fit to the plasma concentration curve. Additionally, we determined GFR by total and single kidney clearance of [Tc-99m] Tc-DTPA by collection of urine from the bladder and single kidney respectively. The results were within +/- 2 standard deviations (SD) = +/- 6% of the value from the bi-exponential fit and had only a single outlier. Based on these finding, our conclusion is that in pigs, GFR can be reliably determined with a simplified procedure similar to the one used for humans.

c) We performed a prospective randomised animal study and tested the possible protective effect of NPs at kidney level using angiotensin II receptor- neprilysin inhibitor LCZ696 = Entresto® before, during and after partial nephrectomy. In the group of PN+ Entresto® treatment for 15 days, one of the main findings was a GFR increase in the non-injured kidney and protection of the kidney filtration barrier in both kidneys.

d) In our observational sub-study with follow up in patients subjected to partial or radical nephrectomy, we evaluated NPs in plasma and kidney hypertrophy association in the compensatory post-operative kidney hypertrophy. Patients undergoing radical nephrectomy experienced an average increase of 27% on the remnant kidney volume one year after surgery. The NPs, especially *mid regional-proANP* (precursor of ANP) showed significant association to this phenomenon through direct positive correlation with volume gain after total nephrectomy.

Concluding perspective

In perspective, combined AT1 receptor and neprilysin inhibition is a promising approach for long-term renal protection in addition to AT1 receptor blockers in AKI and chronic kidney disease. Therapy with a focus on NPs seems to be auspicious, and our studies could represent a starting point for future investigations. We are aware that larger clinical trials are needed for definitive conclusions.

DANSK RESUME

Antallet af nyrekræfttilfælde er stigende. Dette skyldes bl.a. de mange radiologiske undersøgelser, der udføres af andre grunde, f.eks. for at afdække årsagen til mavesmerter eller vægttab. Dette har medført et stigende antal kirurgiske nyreindgreb. Tendensen med hyppigere kirurgisk behandling af nyretumorer har sammen med aldringen af den generelle befolkning affødt et behov for at evaluere mulige behandlinger og strategier for at beskytte nyrefunktionen under og efter nyrekirurgi. Tidligere forskning har vist, at naturiuretiske peptider og blokering af deres nedbrydning har lovende perspektiver i forhold til behandling af kardiovaskulære lidelser. Vi ønskede derfor at undersøge, om disse perspektiver også findes for nyresygdomme, specifikt om tab eller reduktion af nyrefunktion kan undgås.

Gruppen af natriuretiske peptider (NP) består af tre vasoaktive peptider med en molekylvægt på mindre end 10 kDa, som er strukturelt forbundne; atrielt natriuretisk peptid (ANP), B-type natriuretisk peptid (BNP) og C-type natriuretisk peptid (CNP). NP'er syntetiseres, lagres og udskilles på myokardiocytniveau.

Mål:

a) At foretage et systematisk review og på baggrund af dette bestemme potentielle fordele ved brugen af natriuretiske peptider og angiotensin-renin-neprilysin-hæmmere (ARNI).

b) At bestemme gennemførligheden af målinger af nyrens glomerulære filtrationshastighed (GFR) ved hjælp af 99m TC-DPTA som radiotracer i en grisedyremodel.

c) At udføre en randomiseret præklinisk undersøgelse af virkningen af LCZ696 på nyreniveau efter laparoskopisk partiel nefrektomi med forlænget iskæmitid.

d) At belyse NP mekanismernes rolle for nyrehypertrofi efter delvis eller total nefrektomi.

Resultater

a) Efter en omfattende litteraturgennemgang kan vi konkludere, at på nyreniveau er disse peptiders hovedfunktion at fremme vand- og natriumudskillelse, samt at øge nyregennemblødningen. Kontinuerlig infusion af ANP over fysiologiske niveauer inducerer vasodilatation af de afferente arterioler og indsnævring af efferente arterioler, der opretholder ilttilførslen på medullært niveau. Derudover fører administration af NP'er til en stigning i GFR og diurese, men med stabil filtreringsfraktion. Denne effekt er af afgørende betydning, da den samtidige stigning af GFR og filtreringsfraktionen tolkes

som en skadelig effekt i scenarier med akut nyresygdom, fordi den øgede transport af filtreret natrium er lineært korreleret med iltforbruget, hvilket øger iltforbruget dramatisk.

b) I alt blev 6 grise injiceret med en kendt mængde [Tc-99m]Tc-DTPA, hvorefter der blev taget 12 blodprøver fra 5 til 240 minutter efter injektionen. GFR blev bestemt som injiceret aktivitet divideret med areal under en bi-eksponentiel tilpasning til plasmakoncentrationskurven. Resultaterne lå inden for +/- 2 standarddeviationer (SD) = +/- 6% af værdien fra den bi-eksponentielle tilpasning og med kun en enkelt outliner.

Konklusion: Hos grise kan GFR bestemmes pålideligt med en forenklet procedure svarende til proceduren, der anvendes hos mennesker.

c) Vi udførte en prospektiv randomiseret dyreundersøgelse og testede den mulige beskyttende effekt af NP'er på nyreniveau baseret på brugen af angiotensin-renin-neprilysin-hæmmere (ARNI-lægemidler = Entresto®) før, under og efter partielle nefrektomier. Et af hovedfundene i gruppen af PN+ Entresto® i løbet af 15 dage var en stigning af GFR i den ikkeskadede nyre og beskyttelse af nyrefiltreringsbarrieren fra begge nyrer.

d) I vores observationsstudie med patienter udsat for partiel eller radikal nefrektomi ønsker vi at evaluere rollen af NP'er i den kompensatoriske mekanisme for postoperativ nyrehypertrofi. I gennemsnit oplevede de patienter, der blev udsat for radikal nefrektomi, en stigning på 27% på den resterende nyre et år efter operation. De natriuretiske peptider, især mid-regional-proANP (forløber for ANP), synes at være forbundet med dette fænomen og havde en stærk positiv korrelation med volumenforøgelse efter total nefrektomi.

Konklusioner

Ud over AT1-receptorblokkere synes kombineret AT1- og neprilysinhæmning at være en lovende tilgang til langsigtet nyrebeskyttelse ved akut nyreskade og kronisk nyresygdom. Terapien med fokus på NP'er ser ud til at være gunstig, og vore undersøgelser kan være et udgangspunkt for fremtidige undersøgelser. Vi er klar over, at der er behov for større kliniske forsøg, før der kan drages endelige konklusioner.

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INTRODUCTION

KIDNEY PHYSIOLOGY

The nephron is the functional unit of the kidney. It consist of a glomerulus, formed by a renal corpuscle (within Bowman's capsule), a proximal tubule, the loop of Henle, a distal tubule and a collecting duct system[1, 2]. The glomerular unit receives the blood supply from the afferent arteriole, and the product of the filtration returns into the efferent arteriole. Each kidney counts approximately 1 million functional units, but this amount decreases dramatically with age, e.g. only 50% of the functional units remain at the age of 70 years[1]. The primary function of the glomerulus is to ultrafiltrate the blood plasma at an average rate of 125 ml/min in healthy young people. This ultrafiltration process refers to a passive mechanism of fluid movement with fewer proteins than in plasma[1]. The filtration barrier is formed by the capillary endothelium, the basement membrane and the foot processes of the podocytes (thick endothelial cell in contact with Bowman's capsule)[1]. This barrier allows the free passage of water, Na^+, urea and glucose. However, it is not permeable to large proteins such as albumin, red blood cells, white blood cells or platelets[1]. The membrane avoids the permeability of proteins not only by size, but also as a charge-selective filter[1]. In addition to this filtration function, the endothelial cells play an important role in the homeostasis of renal plasma flow by synthetising vasoactive substances (nitric oxide, endothelin)[1]. Another important area is the juxtaglomerular apparatus. At the level of the afferent arterioles, this region contains granular cells that manufacture, store and release renin, which is involved in the formation of angiotensin (ANG) II and, as a last instance, in the release of aldosterone, playing a determining role in the regulation of the circulating volume and blood pressure[1].

RENAL CLEARANCE

In an effort to simplify the process of renal clearance, we can say that for substances that are not synthetised or metabolised by the kidneys, the only input to the kidney will be from the renal artery, and the only two outputs will be the renal vein and the ureter. This principle focusses on the excretion function into the urine in an amount of time (volume/time) and allows determination of the glomerular filtration rate (GFR) which, in other words, is the sum of the filtration function of all the nephrons in the kidney[1].

The dynamics of ultrafiltration at glomerulus are driven by Starling forces, pressure changes at this level will signify changes also in the GFR. Thus, a decrease in the resistance of the afferent arteriole will increase the pressure at the glomerular capillary and therefore the GFR. Contrary to this, a decrease in the resistance of the efferent arteriole will reduce the hydrostatic pressure at the glomerular capillary level, and, in addition, reduce the GFR [1].

REGULATION OF RENAL BLOOD FLOW AND GLOMERULAR FILTRATION RATE

The regulation of the blood flow that the kidneys receive, and the effectiveness of the glomerular filtration rate are essential to maintain metabolic and electrolytic stability.

The stability of renal blood flow (RBF) and GFR could be affected by diverse pathways and regulated back to initial level by endogenous counter-regulatory mechanisms.

Basicaly, there are two mechanisms that plays a determinant role on RBF and GFR regulation: tubuloglomerular feedback(TGF) and myogenic response. The TGF can alter the tone of the afferent arteriole by a pathway that involves adenosine and/or ATP; in the other hand the myogenic response produces direct vasoconstriction of the afferent aretriole based on the transmural pressure. Both mechanisms have the aim to stabilize renal function and protect the kidney from hypertensive injury[3].

Within this autoregulation mechanism there would be some modulators that play an important role in the success of the process, among them the most important ones are:

- ***Sympathetic nerves***: when the external fluid volume falls, the sympathetic nerves release norepinephrine and epinephrine, which causes vasoconstriction by union at the level of the afferent arteriole causing reduction of the RBF and GFR[1].
- ***Nitric oxide*** (NO): the increase in the blood flow could determine the release of NO, which causes vasodilatation of the afferent and efferent arterioles in the kidney[1].
- ***Bradykinin***: its vasodilatory actions are explained by the release of NO and prostaglandins, increasing RBF and GFR[1].
- ***Prostaglandins*** : in response to situations of ischemia or vasoconstriction (for example after binding of ANGII at the afferent and efferent arteriole) the kidney has the ability to produce prostaglandins (PGE_2 and $PGF_{2\alpha}$) that will contribute to vasodilate the preglomerular vessels contributing in that way to increase or maintain the blood flow and GFR[4-6]
- ***Natriuretic peptides*** (NPs): released by the atria and the ventricle (atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), these compounds have a vast number of receptors at the kidney level.

The interaction of these compounds with the receptor Natriuretic Peptide Receptor A (NPRA) generates, among others, the paradoxical effect of dilate the afferent arteriole and constrict the efferent arteriole, producing an increase in GFR[1].

Angiotensin II: Ang II is determinant in the regulation of the vascular resistance, GFR and tubular reabsorption at kidney level.

The efferent arteriole is more sensitive to ANG, and at low concentrations, ANG causes vasoconstriction of the efferent arteriole. Thus, this action causes an increase in GFR. At higher concentrations and due to the capacity to act in both afferent and efferent arterioles, the administration of ANGII produces and opposite mechanism decreasing the RBF, GFR and increasing the filtration fraction[1, 6, 7].

On the other hand mechanisms that intend to block ANG II generates an increase in RBF and pressure related changes in GFR [7].

INTRODUCTION TO KIDNEY CANCER AND PARTIAL NEPHRECTOMY WITH THE USE OF WARM ISCHEMIA

EPIDEMIOLOGY

There are basically two types of kidney cancer :

1. Urothelial (15-20%)
2. Renal cell carcinoma (70-80 %)

The remaining types are Wilms tumour (children 4-6%), lymphoma, sarcoma, and metastasis[8].

Renal cell carcinoma (RCC) is the most common solid kidney tumour[8], worldwide it is estimated that RCC accounts for almost 3% of all cancers, with a clear disposition for western countries, and a predominance of 1.5:1 of men over woman and a higher incidence and diagnosis in elderly population (>75 years of age)[8, 9].

The incidence is increasing due to the more frequent use of imaging studies such as ultrasound, computed tomography (CT)-scanning or magnetic resonance image (MRI)[8-10]. Therefore, the average diameter of the tumors has decreased over the last decades from 12 cm to 5.5 cm, and the total number of small renal masses (SRM) (2-4 cm) has increased[9, 10]. It is estimated that around 300.000 patients are diagnosed every year of kidney cancer worldwide[9].

Even though the incidence remains with a stable growth over the years, is it to note that the rates of mortality due to renal cancer in most of Europe, United States and Australia are in continuous decrease since 1990[8, 9]. This trend could be explained because the diagnosis is made in early stages of the cancer, but also due to a change of life habits, such as decreased smoke rates, better treatment options, and better access to the health system[9].

In Denmark around 1000 new RCC are diagnosed every year, responding also to 2-3% of all the newly diagnosed cancers. It is shown, as in the rest of the world, an increase in incidence from 12,8/100000 to 16,8/100000 habitants when the registries from the period 2011-2012 are compared with the period from 2017-2018[11].

ETIOLOGY

High body mass index (BMI), smoking, and hypertension are risk factors linked to RCC[8, 9, 11]. In addition, end stage kidney disease (ESKD), with the concomitant use of dialysis, represents a risk factor based on the chronic changes that the disease produces on the renal system[8]. Recent studies were able to find a correlation between diabetes and RCC, representing a matter of public health concern due to continuous increase of diabetes in the population[12].

Some genetic conditions predispose to the development of RCC e.g. Von Hippel-Lindau (VHL), hereditary papillary renal carcinoma, Birt-Hogg-Dube, hereditary leiomiomatosis, succinate dehydrogenase deficiency RCC, tuberous sclerosis, and PTEN hamartoma tumor syndrome (Cowden syndrome)[9]. Most of these syndromes are associated with the diagnosis of tumors in other parts of the human body[9].There is no strong correlation between RCC and chemical exposure, with the exception to chlorinated solvents and asbest[9].

It is shown that moderate alcohol consumption, and a diet rich in fruit and vegetables, plus regular physical activity has a protective effect[8, 9, 11].

TYPES OF RCC

Based on the classification of the World Health Organization (2016) the most frequent kidney cancers are: clear cell renal carcinoma (ccRCC), papillary RCC, and Chromophobe RCC[8, 9, 11, 13]

Clear cell carcinoma is by far the most frequent type, representing 65-70% of all RCC, papillary tumors represents around 10-20%, and chromophobe only 5-7% of newly diagnosed RCC[11].

BIOLOGY AND GENETICS OF KIDNEY CANCER

The discovery of different genetic pathways in the last decades helped to understand the biology, development, and behavior of many cancers. With particular interest in kidney cancer, it has been of enormous interest to understand the VHL-HIF (hypoxia inducible factor)[14]. Under normal conditions the VHL gene has, among others, to trigger the degradation of HIF by the activation of different enzymatic processes. Malfunction of the VHL gene lead to overaccumulation of HIF and subsequent activation of HIF targets such as VEGF 1 (Vascular Endothelial Growth Factor) and PDGF (Platelet-Derived Growth Factor) resulting in uncontrolled growth, increased neovascularity, survival, and development of several cancer hallmarks[14].

The understanding of the VHL-HIF pathway allowed the comprehension of renal cancer as an entity which could be triggered by different genetic malfunctions. In the last decades the genes responsible for hereditary papillary renal carcinoma (HPRC), Birt-Hogg Dube, BAP1-tumor predisposition syndrome, Microphthalmia Transcription Factor (MiTF) family translocation renal cell carcinoma, familial mixed epithelial and stromal tumor (MEST) were all described[14].This allowed a more personalized treatment for hereditary conditions that may not fit in the general algorithms of diagnostic, treatment and follow up of kidney cancer.

SYMPTOMS AND DIAGNOSIS

The classical triad of hematuria, upper flank pain and abdominal mass described in some textbooks is infrequent. This combination is only present in less than 5% of the newly diagnosed RCC, and represents most of the times an advanced stage of RCC[9].The implementation of CT and MRI for multiple diseases has increased considerably the number of incidental diagnosis of kidney tumors, of those, the vast majority in early and asymptomatic stages and with diameter of less than 4 cm[8-11]. Obviously, none of the abovementioned imaging diagnosis method can with a certainty of 100% demonstrate if the renal mass is either malign or benign. In the last decades consensus about IV (intra venous) contrast uptake and image characteristics has been done with the aim to increase the accuracy of the diagnose of RCC and avoid unnecessary surgery. Usually the renal masses will enhance more than 15-20 Hounsfield units (HU) without fat density should be consider highly suspicious of RCC[9]. In order to provide more information, the tumor biopsy has, in the last years, gained an important role in the diagnosis of small renal masses, and in some urological departments it has become the gold standard in the path to guide the patient for further treatment[9, 15].

CLASSIFICATION

It is based on the Tumor Node Metastasis classification system (TNM)[16].

Which evaluates the size of the tumor in cm (T1, T2, T3, T4), the presence of positive lymph nodes (N0, N1) , and metastasis (M0, M1) at the moment of diagnosis.

TREATMENT

After an MDT (multidisciplinary conference) where the individual case is presented and discussed a treatment is decided and later presented to the patient and relatives.

The treatment for RCC it is based in 4 modalities:

1) ACTIVE SURVEILLANCE (AS): is a method to follow an incidental renal mass between 2 cm and 4 cm . It requires, as all the AS protocols, a close follow up with the use of images and biopsies, a clear definition of when to change to active intervention and the assumption, by the patient and the doctor, of a risk of evolution into a more aggressive cancer and the possibility of metastasis. Normally, the AS protocol is used in elderly patients with comorbidities, where the risk of a surgical intervention, per se, is higher than the risk of progression[9]. Active surveillance should be only offered if an eventual progression would be treated.

2) NEPHRON-SPARING treatment could be either partial nephrectomy or ablation therapy.

A) PARTIAL NEPHRECTOMY (PN): it consists in the removal of the tumor and only a minor portion of healthy tissue to ensure safe margins[8, 17]. The first partial nephrectomy with oncological purposes dates from 1921 and it was performed by Rosenstein[18]. The way to gain acceptance in the medical community was slow, and the consolidation of the technique waited until the publications of Licht and Novick in 1993[18-20]. They were able to demonstrate good results concerning oncological outcomes, peri postoperative complications, and mortality rates in 241 patients with tumors of mean size of 3,5 cm[18-20]. Normally it requires to clamp the renal artery and vein for a suggested period of less than 30 minutes in order to allow the surgeon to work in a dry space to safely remove the tumor[21]. Nowadays for the treatment of certain tumors, with for example favorable locations, some surgeons have developed techniques that avoid the clamping of the artery or perform super-selective clamping of the branch or branches of the renal artery that supplies blood into the tumor location[21].

Overall , partial nephrectomy represents an improved way to save kidney functionality in patients with a non-optimal kidney function, and so, a probable way to avoid the serious complications that renal insufficiency could carry.

Partial nephrectomy is a challenging technique and is not exempt of complications, which can be divided in intraoperative and postoperative .

Among the first ones, the most common are bleeding, pleural or surrounding organ injuries and tumor violation[22].

The most common postoperative complications are: bleeding, pseudo aneurism, urine leakage, pneumothorax and renal failure[22].

To achieve the postoperative trifecta of negative margins, no urological complications and maintenance of 90% of the preoperative renal function there are non-modifiable and modifiable factors. Whitin the last ones it seems that the duration and clamp of the renal artery and vein plays a determinant role, because the clamp of this structures lead to a period of temporary ischemia. of the nephrons[22, 23].

During the period of warm ischemia, a process of cellular suffering under norm thermic conditions begins[24]. The mechanism of cellular injury occurs at vascular level, where vasoconstriction and inflammation play a determinant role. There is mobilization of proinflammatory components such as interleukins, neurohumoral activation of angiotensin II and release of free oxygen radical, giving to the process the characteristic of a vicious cycle named as *ischemia- reperfusion syndrome*[23, 25].

The syndrome could occur in all the organs who experience sudden blockage of the irrigation for some minutes, but then is re**e**stablish. With focus on the kidney, several studies arrived to the conclusion that human kidneys could tolerate no more than 30 minutes of warm ischemia at 37 C. Keeping the insult bellow 30 minutes, only acute tubular necrosis with slow but predictable recovery (3-4 weeks) is expected[24, 26, 27]. Situations that require between 30 and 60 minutes of warm ischemia often results in severe injuries, with no reversible loss of function and elevated mortality[24, 27]. Moreover, preclinical studies with rats as subjects of study demonstrated that more than 90 minutes of ischemia in the kidneys will result in a complete loss of function of the kidney with mortality rates of more than 80 %[24, 27].

In addition to the ischemia-reperfusion syndrome, the patients undergoing partial nephrectomy, and the patients exposed to other types of major surgery, such as major intrabdominal or intrathoracic surgery, are in serious risk to develop acute kidney injury (AKI) with an abrupt decrease in GFR[10].

Acute kidney injury is defined by the RIFLE score (risk-injury-failure-loss-end stage) as a reduction => than 25% in baseline eGFR (estimated glomerular filtrate rate) or >1.5-fold increase in baseline creatinine at discharge from hospital[28]

Specifically, postoperatively AKI is a common complication in patients with background of cardiovascular disease. Among them the risk of AKI varies in a proportion between 15- 35%, addressing hypovolemia, loss of perfusion pressure and hemodilution as main causes[10, 29, 30].

In patients undergoing partial nephrectomy the risk of AKI should not be underestimated as 20% of the patients will suffer this complication after the procedure[28].Several reports remark that the duration in days of AKI would play a determinant role in de development of CKD or worsening of the preoperative stage. Those patients with AKI for more than three days had a lower probability to recover 90% of the preoperative eGFR and had 6 fold risk to upstage CKD in comparison with those without postoperative AKI[28].

The link between temporary ischemia of the kidney and the development of postoperative AKI is consistent, showing that more than 20 minutes of warm or 35 minutes of cold ischemia increased the risk of AKI (24.4% vs. 6.4% and 32.0% vs. 13.0%, respectively)[31]. In the same direction Lane and Simmons et al. associated that the risk of AKI increases 1%per minute of ischemia and decreases with the percentage of kidney tissue preserved.[14, 32].

Acute kidney injury is often followed by inflammation, with infiltration of neutrophil granulocytes and macrophages at renal level. In addition, there is a release of cytokines that accelerate the process of kidney damage[10, 33]. This population is therefore in a higher need for temporary use of hemodialysis, which increases the risk of postoperative mortality in more than 50%.and elevates also the risk to develop chronic kidney disease (CKD) and subsequently end stage kidney disease[10, 29, 30, 34].

Ischemia time also plays a determinant role in death donor kidney transplants; it has been noted that the success rate of the intervention has a strong relationship with the cold ischemia time from ablation to transplantation[35, 36].The outcomes concerning renal function and rate of rejection are considerably better when cold ischemia time remains below 12 hours[35, 36].

B) THERMAL ABLATION: it consists in the application of different sources of energy are used to achieve cellular death of the tumor by different techniques: open surgery, laparoscopic or percutaneous. It is reserved for tumors of less than 4 cm. The techniques most used are cryoablation and radiofrequency (RFA). It is shown that they could be effective for tumors of less than 4 cm compared with the oncological and functional outcomes of partial and radical nephrectomy (PN, RN), but the benefits decrease dramatically when compared with tumors between 4 and 7 cm[8, 9, 37]. However, there is no sufficient data to recommend these techniques as standard of care, and they

should only be offered when radical or partial nephrectomy are not an option due to the patient e.g. comorbidity, age, poly pharmacy or a solitary kidney[8, 9, 11].

3) RADICAL NEPHRECTOMY (RN): the first described radical nephrectomy to treat a tumor was performed by Langenbuch in 1875, but it was in 1903 that Gregoire reported the "en bloc" resection of the tumors as we know nowadays[18]. The procedure could be performed by different approaches such as open surgery, laparoscopic or robot assisted. The cancer specific survival after RN is outstanding, and the rate of postoperative complications seems to be better when compared with PN; but in order to preserve as much renal function as possible, RN is nowadays a technique, which has been relegated to the tumors that cannot be removed by partial nephrectomy, commonly those of more than 7cm or the tumors located in close relationship with the renal hilum[8, 9, 11, 38].

4) CYTOREDUCTIVE NEPHRECTOMY(CN): from all newly diagnosed patients with RCC, 20% will present metastasis at the moment of diagnosis[39, 40]. In this scenario, the standard of care for patients with good performance status has been CN with concomitant oncological medical treatment[40]. The CN improves overall survival in metastatic RCC, and helps to control symptoms derived from the disease, such as hematuria, flank pain and paraneoplastic syndrome[40, 41] .

ADAPTIVE CHANGES FOLLOWING PARTIAL OR TOTAL NEPHRECTOMY

Following total unilateral nephrectomy (TN) or PN, the contralateral kidney/remaining kidney mass undergoes adaptive changes. This phenomenon is well known and was first reported in 1672, when Roomhuysen, Zambecarious and Blanchard, among others, discovered that a dog, in which TN was performed, could live with only one kidney without any problem. In addition, they noticed that the remaining organ went through a compensative hypertrophy process.[19, 42, 43].

The process of compensatory kidney hypertrophy can occur after tumour resection with PN or TN and has also been observed after elective nephrectomy in healthy kidney donors. Most studies conclude that the adaptive phase of the contralateral kidney starts some hours following the surgery[44]. After one year, the remnant kidney may have achieved around 70% of the aggregate function of two fully functional kidneys and may be able to keep levels of plasma creatinine and GFR at normal levels[43-45].

We still await firm scientific evidence or causality of how the process of compensatory hypertrophy begins but several factors modulate or affect the mechanism, such as age, gender and pre-operative GFR.

Young male patients with better preoperative GFR respond better than other patients to the adaptive changes following total nephrectomy. In young male patients, the contralateral kidney could undergo compensatory hypertrophy, increasing its volume up to 39%[43, 44, 46-50]. In patients undergoing PN, as expected, the adaptive changes are of lesser magnitude but still present. They may experience hypertrophy in the contralateral kidney of around 9% after the first year of surgery[44]. This discrepancy in the relative extent of hypertrophy may be explained by the simple fact that that in PN as opposed to total nephrectomy, less parenchyma is excised, and the hypertrophy stimulus is perhaps only temporal.

Hence, postoperative changes may be linked to the size of the tumour and the extent to which overall kidney volume is lost [43, 50-53].

Compensatory hypertrophy and GFR changes are important to avoid ESKD. Indeed, patients who experienced no compensatory changes have been reported to be at higher risk of developing CKD than patients who develop compensatory hypertrophy[46]. A similar effect has been observed in live healthy kidney donors; and those who had no early compensatory hypertrophy and GFR changes within the first month of surgery had a tenfold higher risk of developing cardiovascular and CKD than the general population[54-56].

At the humoral level, certain changes induced by surgery may help explain the mechanism of post-operative compensatory hypertrophy. Thus, levels of circulating of nitric oxide are elevated following nephrectomy[51]. In experimental studies it has been demonstrated that the implication of a circulating factor could be implied in the post operatory compensatory kidney hypertrophy after total nephrectomy, and blood transfusion of nephrectomised rats induced changes in GFR of healthy recipients[57]. Moreover the incubation of atrial natriuretic peptide and epidermal growth factor was effective to induce cell hypertrophy at the proximal renal tube during compensatory kidney growth[58].

In humans it has been noted that after TN, patients experience a significant increase in N-terminal pro-hormone brain NP (NT-proBNP). A similar mechanism occurs with mid-regional pro-hormone atrial NP (MR-proANP), remaining this particular peptide significantly increased after one year of surgery, probably supporting the role of natriuretic peptides in the mechanism of renal adaptation[43, 57].

Natriuretic peptides

The group of NPs (natriuretic peptides) consists of three structurally interrelated vasoactive peptides with a molecular weight below 10 kDa. They are known as ANP, B-type NP (BNP) and C-type NP (CNP)[10, 59-61].

NPs are synthetised, stored and secreted at the level of myocardiocytes[62]. Their main functions are to promote water and sodium excretion, but also to increase RBF, lower blood pressure and suppress renin and aldosterone secretion and increase lipolysis[10].

The expression of the NP receptors is widespread in the body. Hence, NP receptors are plentiful in the myocardium, brain, endothelial and smooth muscle of vessels, adipocytes, kidney epithelium and glomerular podocytes[10, 63-66]. In the human kidney, NPR-A expression was observed in the outer medulla and the pericytes and endothelial cells of the descending vasa recta[10, 67]. NPR-C was also abundantly expressed in the kidney as opposed to NRP-B which was not found in the kidney[10, 67].

After ligand binding with NPR-A, guanylyl cyclase is activated and a concomitant rise of cyclic guanosine monophosphate (cGMP) is observed[10, 68-70]. Thus, in preclinical studies in mice, genetic deletion of NPR-A resulted in hypertension, hypervolemia, cardiac hypertrophy, fibrosis, and impaired salt excretion[10, 71].

A different mechanism is seen after ligand binding by NPR-C. Thus, ligand binding by NPR-C internalises the receptor-ligand complex and takes the NP out of circulation, facilitating its degradation[10, 72, 73]. In addition to the mechanism of degradation by NPR-C, the NPs are also degraded by the neutral endopeptidase neprilysin (NEP) and excreted by glomerular filtration in approximately 5 minutes[10, 72]. The mechanism of inactivation of NPs by NPR-C or neprilysin sparked the hypothesis that catabolic degradation inhibition may increase circulating level of peptides and hence exploit their beneficial effects[10].

EFFECTS ON SODIUM/VOLUME REGULATION AND BLOOD PRESSURE OF NATRIURETIC PEPTIDES

The average half-life of circulating NP is around 5 minutes. Based on that feature several studies investigated the effects of ANP or BNP by continuous infusion, showing a reduction of mean arterial pressure (MAP) of around 30 mmHg[10, 74]. Even after administration of ANG II, which stimulates the concentration of aldosterone, ANP attenuated the hypertensive effect[10, 75].

In terms of sodium/volume regulation, no direct relation seems to exist between the dose of ANP and its effect, indicating that the effect may not occur after receptor interaction. In fact, no expression of NPR-A at tubular level was observed[76]. A probable explanation for this is that sodium handling could be indirectly affected by sympathetic nerve activity and inhibition of the renin-aldosterone-angiotensin-system (RAAS)[10, 67, 77].

EFFECTS OF NATRIURETIC PEPTIDES AT KIDNEY LEVEL.

Mechanism that could enhance oxygen supply, blood flow and maintain or increase GFR could be of vital importance to avoid or antagonize threatening AKI, which is a common diagnosis among severely ill patients and/or patients having undergone major surgery[10, 29, 30].

Accordingly, infusion of ANP above physiological levels could induce vasodilatation of vasa recta and constrict efferent arterioles maintaining GFR[10, 78-80].

In addition to the maintenance of oxygen supply at the medullary level, ANP infusion could led to an increase of GFR and diuresis, but with a stable filtration fraction and RBF[10, 81-83].

The continuous infusion of atrial natriuretic peptide was effective to increase GFR and sodium extraction after total nephrectomy in rats[84]. Expression of the natriuretic receptors at the kidney level under this conditions was not overexpressed at the papilla, medulla or cortex but the infusion of the ANP lead to increases of GFR and FeNA greater than in controls[84]

The ability of natriuretic peptides to increase or maintain the GFR but without increasing the filtration fraction could be interpreted as of vital importance during AKI scenarios because that will imply a stable consumption of O_2 which is critical specially for delicate areas of the organ as the renal medulla. [10, 85, 86].

ANIMAL MODELS TO EVALUATE KIDNEY FUNCTION

The most used animal models to evaluate kidney function and kidney disease are rodents. Such models represent a convenient option to conduct research but has the disadvantage that does not reproduce the human kidney anatomy and function in an accurate way and therefore the results of the investigations could be difficult to translate to the human field.

Pigs represent an opportunity to study the effects in vivo in a large animal setting. The use of a model that is similar to the human anatomy and function allows to practice surgeries, blood- and urine test determinations in similar ways as in patients[87].

Pigs were chosen in the present study for several reasons: Pharmacodynamic and -kinetic aspects; the possibility to do precise kidney tissue reduction; the ability to perform separate timed collections of urine from ureters and thus each kidney; the possibility to apply same methods of precise GFR determination by tracer methods and finally a metabolism similar to humans.

The investigations at the research institute of Aarhus University Hospital served as a basis to inspire our approach. In their studies of kidney donation and effects of kidney ischemia in pigs they were able to demonstrate the feasibility of the methods and the reproducibility of the technique of measurement of GFR using 51^{CR} EDTA as radiotracer. Another important consideration is these investigations is to address the resistance to ischemic injury that the pigs have[87, 88]. Therefore, we decided to adopt a similar experimental strategy and use 1h ischemia time by clamping of the renal artery and vein to investigate if the treatment with Entresto® for a period of 15 days could play a protective role at the kidney level in the immediate postoperative phase.

The use of natriuretic peptides or the inhibition of their degradation has been extensively used with rodents as animal model. The results of these studies showed that the natriuretic peptide pathway could have substantial implications in the maintenance of GFR and fractional excretion of Na after total nephrectomy, but as explained above this model could represent a lack of transferability to a clinical setting. [58, 84].

The use of a model with a similar kidney structure and function could help us to address the answer of the role that Entresto® or similar drugs could play in kidney protection after the addition of another detrimental factor represented by the ischemia-reperfusion scenario after partial nephrectomy and prolonged warm ischemia.

HYPOTHESIS AND AIMS:

The endogenous postoperative increase of natriuretic peptide levels could relate to the magnitude of compensatory postoperative hypertrophy of the kidney and its increase in single kidney GFR after total nephrectomies. We speculate that the natriuretic peptide pathway may also have a role in the maintenance of kidney function after partial or total nephrectomy. Therefore, approaches to prolong the half-life of the peptides would be beneficial.

To address these proposals, a series of hypotheses were postulated:
Overall, we hypothesize that the treatment with a pharmacologic drug to enhance in vivo the half-life of NPs by LCZ696 before, during and after elective kidney mass reduction by partial nephrectomy using an animal model will contribute to:

1) maintain preoperative levels of total and single kidney GFR.
2) reduce post operative glomerular barrier injury measured as proteinuria and tissue injury, and
3) increase ANP/BNP plasma concentrations more than sham.

We also hypothesize that constant elevations of natriuretic peptides could play a potential role on postoperative kidney hypertrophy after total or partial nephrectomy.

Aims:
a.1 To perform an invited review to obtain an overview of the Natriuretic peptide pathway and elucidate the effects that they might have in kidney function. We aimed to determine the protective effect of the use of NPs and ANG-renin-neprilysin-inhibitors (ARNI) drugs at kidney level, in different pathological scenarios such as ischemia reperfusion syndrome, major cardiovascular and thoracic surgeries, acute kidney injury and chronic kidney disease.

a.2ATo determine the feasibility of a single kidney GFR measurement using 99^{m} TC-DPTA as a radiotracer by ureter sampling of urine in a pilot study, using pigs as an animal model and

a.2B to perform a randomised placebo-controlled open-label pre-clinical study investigating the protective action of LCZ696 at kidney level after laparoscopic PN with prolonged ischemia time.

a.3To elucidate the role of NPs in the mechanism of postoperative kidney hypertrophy after PN or TN.

ANIMAL LEGISLATION AND ETHICS

The study was approved by the Board of Animal Experiments, the Danish Animal Experiments Inspectorate under the national Ministry of Environment and Food (license 2018-15-0201-01576).

Before, during and after the operative procedures, the pigs were housed at the Biomedical Laboratory at Aalborg University Hospital, in accordance with national guidelines regarding the care and use of laboratory animals.

All interventions were conducted under general anaesthesia using the same protocol for all groups. In the postoperative period, the animals were given analgesics on the first day as planned and analgesics were continued if needed. The animals were controlled daily; and if unforeseen events occurred per-/postoperatively and the three stress criteria - pain, suffering or discomfort - could not be controlled, they were scheduled for euthanasia with a lethal dose of intravenous phenobarbital.

MATERIAL AND METHODS

STUDY 1

To elucidate the potential protective effect of the use of NPs after kidney level in different pathological scenarios such as ischemia-reperfusion syndrome, major surgeries, acute kidney injury and chronic kidney disease, an invited review was performed.

The review was entitled “Protection of kidney function and tissue integrity by pharmacologic use of NPs and neprilysin inhibitors”[10].

Four main investigators conducted a systematic search. The search terms were ((((((natriuretic peptides) OR Entresto) OR sacubitril) OR valsartan) OR chronic kidney disease) OR acute kidney injury) OR ischemia reperfusion syndrome. We included articles in English describing investigations performed in preclinical or clinical setting[10].

STUDY 2

Due to the lack of information about methods with which to measure single kidney GFR in pigs using 99^{m} TC-DPTA as a radiotracer, we performed an initial pilot study.

A total of six female pigs with a mean weight of 32 kg (27-38) were enrolled to 1) determine the reproducibility of repetitious GFR measurement, 2) make a comparison of plasma decay-based vs bladder-collection-based GFR determination and 3) at termination, appraise the feasibility of measurement of single kidney GFR using 99^{m} TC-DPTA in a pig model (CONSORT diagram) (Fig. 1).

For the procedures, general anaesthesia was induced using zoletid 150 mg/10 kg body mass as pre-anaesthetic induction followed by continuous infusion of fentanyl 8.5 µg/kg/hr and propofol 2mg/kg/hr to maintain enough depth of anaesthesia. Continuous measurements of blood pressure, oxygen saturation, respiratory curves, temperature and heart rate were conducted during the procedures[89].

GFR determination in pigs: Ten million Becquerels (MBq) of 99^{m}Tc diethylenetriaminepentaacetic acid (DTPA) were given as a bolus injection in a peripheral venous catheter, and blood samples were collected, using a central venous catheter, at different time points (5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 minutes) after injection. At baseline, the complete amount of urine was collected from the bladder using a 10-French (Fr.) Foley silicone catheter. After 15 days, plasma and single-kidney GFR determinations were again performed. For determination of single-kidney GFR, a 5 Fr. catheter was inserted into each ureter, placed and fixed in the renal pelvis to allow collection of urine during 4 hours[89].

Blood samples and a sample of the collected urine were counted in a Wizard2 2480 gamma counter (PerkinElmer, Mass., US), and the plasma concentration curve was determined as a bi-exponential fitting to the plasma data. The GFR was calculated by the principle that clearance (GFR) equals excreted activity divided by the corresponding area under the curve (AUC) for the plasma concentration curve[90, 91]. From the fitted curve, the AUC can easily be calculated, either for a specific time interval (AUCurine = area from injection to end of urine collection) or until infinity (AUCinfinity). Based on activity excretion from a single kidney, Qurine-R or Qurine-L, single-kidney renal function was calculated as GFRurine = Qurine/AUCurine, with Qurine for the relevant kidney. If time is stretched to infinity, all the injected tracer (Q0) will also have been excreted, giving the standard formula GFRtotal = Q0/AUCinfinity[89].

With the positive results about feasibility of measurement of GFR using 99^{m} TC-DPTA in pigs we followed to the phase 2 of the study that consisted in an experimental trial to test possible positive effects that the treatment with LCZ696 could have on

single kidney GFR, proteinuria and blood levels of natriuretic peptides after partial nephrectomy and prolonged ischemia time of 60 minutes.

Before initiation of the study, we made a power calculation to determine the minimum number of pigs to be enrolled in order to find a possible difference of around 10 ml in GFR between intervention and no intervention groups. We settled an alpha level of 0.05 and a power of 90 % giving us an estimation of a of 6 pigs per group. We decided to add a buffer of 2 pigs per group contemplating possible dropouts and in addition incorporate 8 pigs as control animals.

A total of 24 landrace female pigs with a mean weight of 32 kg (27-38) were enrolled and randomised into four groups (Fig. 1). The randomization was blind to the main investigator. The animals were enrolled by pairs, and one day before the surgery the veterinarian staff choose one of the animals to start administrating the drug to avoid possible bias during the surgery. Only after the surgical procedure the main investigator discovered which animal was receiving LCZ696.

Groups:

1) Laparoscopic PN and treatment with Entresto® (LCZ696 49/51 mg/day) for 15 days, starting one day prior to the surgery (n = 8)

2) Laparoscopic PN and treatment with vehicle (n = 8)

3) Sham surgery and treatment with Entresto® 49/51mg/day for 15 days (n = 4)

4) Sham surgery and vehicle treatment (n = 4).

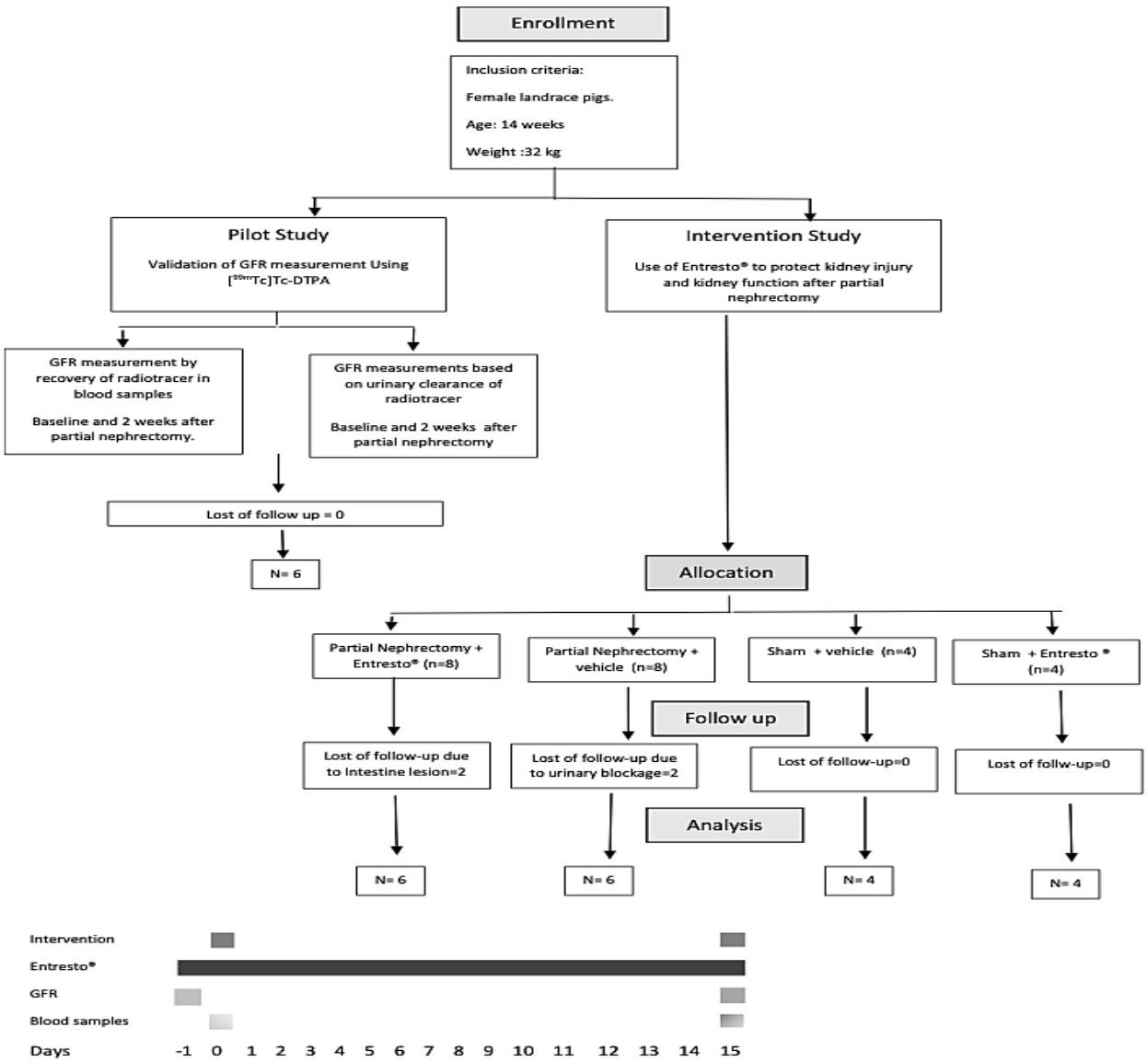

Figure 1 CONSORT diagram

Description of the procedures

Baseline GFR was measured prior to surgery under general anaesthesia, using the protocol mentioned previously. For the laparoscopic surgery groups, the anaesthetic protocol was slightly changed to ensure enough depth of anaesthesia during surgery. The pre-anaesthetic protocol included zoletid 1.5-2.0 mL/10 kg and continuous infusion of propofol 3.3mg/kg/hour and fentanyl 13 µg/kg/hour. Central venous and arterial catheters were placed under ultrasound guidance; afterwards, the pigs were placed in upper right flank position. The skin of the pigs was prepared using chlorhexidine solution, and the surgical field was delimited using sterile single-use cloth. Once the operation area was ready, we proceed with installation of pneumoperitoneum using the Hansson open technique to minimise intrabdominal lesions; a pressure of 8 mmHg was settled as a standard. Two 10 mm ports and one 5 mm port were established to allow camera and instrument entrance, using the same port position as in human procedures[89].

Once the camera was in the abdominal cavity, the first step was to identify the kidney's pedicle (vein and artery), meticulously dissect the components and place a bulldog clamp to close the organ's irrigation; at this precise moment, measurement of the warm ischemia time began[89].

The right upper pole of the kidney was identified and excised. Following the procedure, the specimen was immediately removed, weighed, and divided into two sections. One piece was immersion fixed in 4% paraformaldehyde to allow further histological analysis; the other section was snap frozen in liquid nitrogen. The remaining kidney was sutured, and organ perfusion was stopped for a period of 60 minutes.

The length of the period of warm ischemia was based on the recommended kidney ischemia time in humans (below 30 minutes)[28] and in the results of previous investigations in pigs evaluating the effect of ischemia-reperfusion for periods of around 120 minutes but without the additional insult of partial nephrectomy[87].

The bulldog clamp was then removed, perfusion was assured under visual inspection and haemostasis was controlled. As a last step, the instruments were removed under vision and pneumoperitoneum was evacuated to avoid postoperative pain. The muscular fascia was sutured with Vicryl 2.0 in a separate fashion to avoid postoperative hernias, and the skin was sutured using Vicryl 3.0 in an intracutaneous fashion. Local anaesthesia was administered at the fascia level of the surrounding area of port placements[89].

For the surgical procedures, two pigs were enrolled the same day and the investigator was blinded to which pig received Entresto® or vehicle prior to the laparoscopic PN of the right kidney with 60 minutes of ischemia[89].

After anaesthetic recovery, the pigs were housed in a stall for a period of two weeks. During this period, they had free access to water and were feed following the veterinarian recommendations[89].

Non-steroid anti-inflammatory drugs (NSAIDs) were used only for the first postoperative day, and Amoxicillinum trihydricum 150/mL was used as a single preoperative dose of 1 mL/10 kg intramuscularly. No postoperative treatment was needed in any of the pigs. Administration of oral Entresto® 49/51mg/daily for 15 days, starting the day prior to surgery, was made possible using a small portion of apple given with the medicine. After two weeks, GFR was again measured. On this occasion, 99^{m} TC-DPTA clearance was performed separately for the right and left kidney. For that purpose, the ureters were isolated and clipped distally. Catheters with a size of 5 Fr. were inserted and placed in the renal pelvis, allowing collection of single kidney urine for 4 hours[89].

Afterwards, both kidneys were removed, and sections were frozen or fixed in 4% paraformaldehyde for future analyses. The pigs were then euthanised by using an overdose of 10 mL intravenous sodium pentobarbital 400 mg/dL[89].

Plasma and urine sample collection

For plasma analysis, 10mL blood was drawn at three different time periods: baseline, after 2 hours of surgery and at termination. Blood was collected in two lithium-heparin coated tubes and centrifuged at 3,000 rpm for 10 min to obtain two tubes of 2 mL plasma for each time point. One sample from each time point was stored at -83 °C; the other was analysed for creatinine, Na+, K+, urea and albumin. Urine was sampled at the mentioned time points to measure Na+, K, albumin, total protein, urea and creatinine; and urine was measured continuously during the entire process to obtain data on urine flow[89].

Statistical analysis

Normal distribution was assured by performing the Shapiro-Wilk test and then two-way analysis of variance (ANOVA) with 95% confidence intervals (CIs) and post hoc analysis by Tukey's multiple comparison test. A $p< 0.05$ was considered statistically significant. The differences between and within groups were analysed at baseline and after 15 days. For all the statistical analyses, the software of Prism Graph Pad 9™ was used[89].

Expected findings

We hypothesized to find a difference in GFR, proteinuria and blood levels of natriuretic peptides between the groups with PN + LCZ696 vs PN + placebo.

STUDY 3.

The study was a post hoc study derived from an original study that was conducted from July 2016 to August 2019. In the original protocol a total of 74 participants were enrolled prior to partial or total nephrectomy at the departments of urology of Odense and Zealand University Hospital. The original study was approved by the Scientific Ethics Committee for the Region of Southern Denmark (S-20140044) and is registered at ClinicalTrials.gov, identifier: NCT02646293. After selection fifty-four patients were included in the original trial and assigned to either unilateral TN (n = 35) or PN (n = 19). Kidney function was assessed by both creatinine (Cr) ethylenediaminetetraacetate (EDTA) (Cr^{51}-EDTA) and 99^{m}Techtenium (Tc) diethylenetriaminepentaacetic acid (DPTA) (99^{m}Tc-DPTA) before surgery and 3 and 12 months after nephrectomy. Urine and plasma were collected at several time points: (I) pre-surgery, (II) 24 hours after surgery, (III) 5 days after surgery, (IV) 21 days after surgery, and (V) 3 and (VI) 12 months after surgery. At these time points, levels of albumin and creatinine, NT-proBNP, MR-proANP and aldosterone were determined[43].

The functional distribution of the kidneys was assessed using preoperative renal scintigraphy. Additionally, the patients who received PN had two additional scintigraphies performed at 3 and 12 months after surgery. Estimation of single-kidney GFR was done by assessing the homogeneity of the distribution of kidney function and then dividing total GFR by two[43].

Post hoc study: Volumetric analyses

In the original cohort all the patients from Odense University Hospital diagnosed with RCC were independently controlled by CT scans before nephrectomy and one year after surgery. Thus this allowed us to conduct an observational study with a total of 19 patients of whom 8 underwent PN and 11 TN[43].

For all patients, preoperative CT scans were compared with the one-year postoperative images. The preoperative volume of the kidneys and tumour and the postoperative volume of the contralateral kidneys were assessed by using the image segmentation and three-dimensional (3D) reconstruction open-source software 3D Slicer[43, 92].

The patients who had been donors were excluded from the study as no postoperative CT scans were done as a default practice.

Statistics

All statistical analyses were performed in the GraphPad Prism software version 9. Datasets were analysed using the Shapiro-Wilk test to determine the Gaussian distribution followed by ANOVA with the Sidak *post hoc* analysis. Results were interpreted as significant if $P < 0.05$[43].

Expected findings

We hypothesized to find a persistent elevation of Natriuretic peptides blood levels at different timepoints after partial or total nephrectomy and a possible direct correlation between the magnitude of the kidney postoperative hypertrophy compensation and levels of Natriuretic peptides.

As secondary findings we wanted to document the role and correlation of preoperative GFR levels and magnitude of hypertrophy and the adaptative changes following total nephrectomy based on p-creatine levels, albumin/creatinine ratio and single kidney GFR levels.

RESULTS

STUDY 1

The review provided an overview of the different types of NPs and the different actions at kidney level. The main results of the systematic review were:

Degradation of the Natriuretic peptides.

The binding of NP with NPR-C triggers a mechanism which internalises the receptor-ligand complex, taking the NP out of circulation and facilitating its degradation[10, 72, 73]. In addition to the mechanism of degradation by NPR-C, the NPs are also degraded by NEP and excreted by glomerular filtration in approximately 5 minutes[10, 72]. The mechanism of inactivation of NP's by NPR-C or neprilysin sparked the hypothesis that catabolic degradation inhibition may increase circulating level of peptides and hence exploit their beneficial effect[10] (Figure 2).

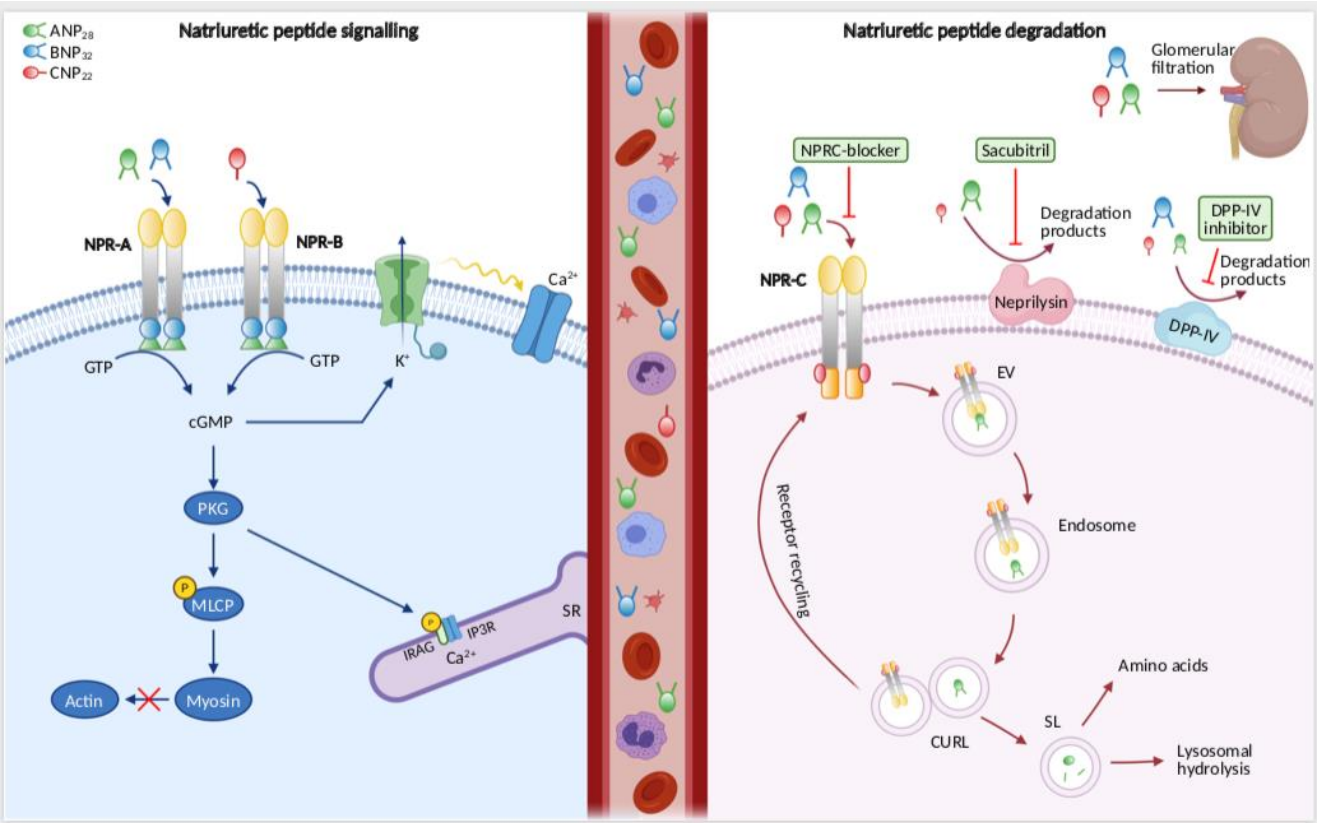

Figure 2 ANP receptors and degradation pathways

Combination of natriuretic peptides and furosemide.

In patients undergoing uncomplicated hearth surgery, furosemide increased renal blood flow and reduced filtration fraction and renal oxygen demand. However, it also reduced GFR. Opposite to this, ANP without the use of furosemide seemed to

increase GFR, keep blood flow stable and could present an increase in filtration fraction and consequently oxygen demand when used at low doses[10, 93].

Studies showed [10, 30, 94, 95] that the combination of the two drugs may have an additive effect, resulting in increased GFR and RBF, decreased renal vascular resistance and unaffected filtration fraction.

A main outcome in patients who received the combination of NPs and furosemide was an increase in creatinine clearance and a reduced risk of dialysis and AKI[10, 30, 94, 95]

Inhibition of neprilysin and its impact on the plasma concentration of natriuretic peptides.

Different approaches have been used to increase the plasma concentration of NPs due to their short life. One simple but difficult way is to administer the peptides under continuous infusion; another innovative path is to focus on the degradation cascade. Since NPR-C inhibition does not correlate with considerable effects observed at the vascular level, several studies have targeted the inhibition of the NEP neprilysin, which drives NP degradation[10].

With a predilection of neprilysin for degradation ANP over BNP, it was shown that ANP can increase several folds during NEP inhibition[10, 96-101]. A major challenge is that neprilysin, beyond NPs, has several substances as substrates, such as endothelin, bradykinin, vasopressin, and ANG II. Therefore, their degradation could carry serious side effects. One well-described side effect is angioedema, which occurs after bradykinin degradation[10, 102].

To decrease the percentage of patients suffering from side effects after use of NEP inhibitors, new drugs were released to the market featuring a combination of NEP inhibition + ANG II inhibition, the so-called ARNIs, of which LCZ696 (Entresto®, Novartis Pharmaceuticals, Basel, Switzerland) has been commercially available since 2015.

In preclinical studies, use of ARNIs increased survival on rats with end-stage renal disease compared with valsartan alone[10, 103] (Table 1). In addition, the combination of neprilysin inhibitors and enalapril or valsartan lowered proteinuria, glomerulosclerosis and tubulointerstitial fibrosis, increasing the production of nitric oxide compared with enalapril alone[10, 103, 104].

ARNI drugs and their effect in kidney warm-ischemia reperfusion models

In pre-clinical kidney ischemia-reperfusion models, the groups not receiving ARNI drugs had higher levels of urea, creatinine and kidney injury markers than animals receiving ARNI drugs after reestablishment of circulation[105]. In addition, ARNI

treatment also had more anti-inflammatory and anti-fibrotic effects than angiotensin blockers only[10, 106].Thus, in Dahl-salt-sensitive rats receiving Entresto®, the decline in GFR was mitigated and proteinuria mildly attenuated[10, 107] (Table 1).

AUTOR	YEAR/TYPE OF STUDY	MODEL	DRUG TESTED	OUTCOMES
Benigni	2004/Randomized/20 rats	5/6Nephrectomy	Oral Vasopeptidase inhibitor vs Enalapril alone	>GFR andrenal blood flow
Chujo	2010/Randomized/60 rats	45min left ischemia/reperfusion+ right nephrectomy	IV Atrial NatriureticPeptide	>Outler medullary flow, decrease of plasma creatinine
Jin	2014/Randomized/24rats	Unilateral Nephrectomy	IV C-type Natriuretic Peptide	Augmented expression of epidermal growth factor
Cao.	2015 Randomized/ 36 rats	Ischemia/reperfusion	Iv Lyophilized recombinant human Brain Natriuretic Peptide	>GFR and renal blood flow
Moriyama	2016 Randomized 18 rats	30min bilateral Ischemia/reperfusion	IV Atrial NatriureticPeptide	Reduction of NGAL marker level
Ushijima	2017 Randomized/ 23rats	Right nephrectomy+2/3left nephrectomy	Oral LCZ696(Entresto) vs ANG TII Blockers	Delay In progression to ESKD
Chen	2017 Randomized/ 30 rats	Hypovolemic Shock	IV C-type Natriuretic Peptide	>GFR andrenal blood flow
Jing	2017/Randomized/32 rats	5/8Nephrectomy	Oral LCZ696(Entresto) vs ANG TII Blockers	Lower levels of cellular hypoxia
Kolsrud	2020/Randomized/20pigs	Cardiopulmonar By-pass	IV infusion of Atrial Natriuretic Peptide	>GFR without affection of renal oxygen delivery
Suematsu	2018 Randomized/ 34rats	5/6Nephrectomy	Oral LCZ696(Entresto)	Delay In progression to ESKD

Table 1 pre-clinical studies testing the effects at kidney level of NPs by infusion or Inhibition of degradation.

ARNI class drugs and their effects on kidney function and tissue protection in clinical studies.

To date (August 2023), seven randomised clinical studies have been conducted in patients with CKD and hearth failure. The conclusions of these studies on the effect of the use of ARNI are not completely aligned[102, 108-112]. The PARAMOUNT[112] and the PARAGON HF [110] studies found that ARNI prevented a change in eGFR at 12 months and reduced the percentage of patients who died due to ESKD[10, 110, 112]. Also, the IMPRESS and OVERTURE studies reported beneficial effects after use of omapatrilat, but both studies had less statistical power than the PARAGON HF and the PARAMOUNT study. They described a lower rate of renal impairment. However, the definition of renal impairment was not

published in IMPRESS and no *P* value was available in OVERTURE[102, 111]. In addition, lower rates of urea and creatinine were found in patients treated with omapatrilat[10, 111].

The PARADIGM study was also able to demonstrate a slower decline in eGFR in patients receiving Entresto® than in patients only receiving enalapril[10, 113].

Contrary to the positive results in the IMPRESS, PARAMOUNT, PARAGON, PARADIGM and OVERTURE studies, the trials EVALUATE[108] and UK-HARP-III[109] were not able to detect differences in changes in eGFR or in the levels of plasma urea or creatinine after follow-up[10, 108, 109] (Table 2).

STUDY	TYPE OF STUDY	MODEL	DRUG TESTED	OUTCOMES
PARAMOUNT	Randomized	Patients with heart failure and CKD	LCZ696 vs Valsartan	< changes in eGFR and mortality due to ESKD
PARAGON	Randomized	Patients with heart failure and CKD	LCZ696 vs Valsartan	< changes in eGFR and mortality due to ESKD
IMPRESS	Randomized	Patients with heart failure and CKD	Omapatrilat vs Enalapril	< renal impairment, blood urea and creatinine
OVERTURE	Randomized	Patients with heart failure and CKD	Omapatrilat vs Enalapril	< renal impairment, blood urea and creatinine
PARADIGM	Randomized	Patients with heart failure and CKD	LCZ696 VS Enalapril	Slower decline in eGFR
EVALUATE	Randomized	Patients with heart failure and CKD	LCZ696 vs Valsartan	similar eGFR and p-creatinine
UK-HARP III	Randomized	Patients with heart failure and CKD	LCZ696 vs Irbesartan	similar eGFR and p-creatinine

Table 2Randomized Clinical Trials testing ARNI drugs

STUDY 2

The first part of the study consisted of a pilot study were GFR was successfully estimated by plasma decay and from bladder urine collected at study entry. Plasma decay curves of GFR were successfully determined and used to estimate GFR. The average values of measured GFR were 65±11 mL/min (Fig 3). When GFR was measured by plasma clearance of 99^{m} TC-DPTA through quantitative urine collection, the result was a mean of 54 ± 12 mL/min (85% of plasma decay results P>0.05) when urine was collected from the bladder [89](Fig.3).When urine was collected from each kidney the sum of single kidney GFRs (25 mL/min from the right side and 31 mL/min from the left side, SE 2.38) matched the value obtained by bladder urine collection (56 ± 14 mL/min) and was not significantly different from plasma decay measurements (P = 0.72) in any of the groups[89](Figure 3). After completion of the pilot study, we continued with the randomized preclinical trial to determine the effect that LCZ696 could have in kidney function after partial nephrectomy and extended ischemia time .

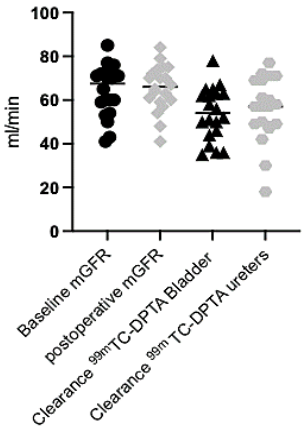

Figure 3 Diagram shows data from the initial pilot series with measured GFR using 99mTc-DTPA by the plasma decay approach and by urinary clearance at baseline and 15 days after PN. The values obtained by the sum of single-kidney clearances were not statistically different from the values obtained by the plasma decay method.

Baseline parameters in pigs subjected to sham or partial nephrectomy.

The pigs included in the intervention trial had a mean entry weight of 32 kg (27-38); MAP, O_2 saturation and hearth rate at baseline were 94 ±mmHg, 78 bpm and 99%, respectively. No differences between groups at baseline or after 15 days were noted (Table 3). Grams and percentage of tissue removed during PN were on average the same in the two groups (Entresto® or vehicle, P = 0.85, corresponding to a mean of 21±4 gr and 25%, respectively[89](Table 3).At termination, 2 weeks after PN, the mean weight of right and left kidney was different for the groups with PN, independently of the treatment (P = 0.03 for the Entresto® group and 0.005 for vehicle group)[89].

The treatment was well tolerated, and no side effects were reported after administration of Entresto ® at a dose of 49/51 mg/daily. The final body weight of the pigs was not different between groups after 15 days [89] (Table 3).

Characteristics	PN + Entresto	PN + vehicle	Control + Entresto	Control + vehicle	*P*-value
Initial weight (kg)	31 ± 3.2	32 ± 3	35 ± 2.5	32 ± 4	.5
Terminal weight (kg)	35 ± 4	36 ± 1.5	39 ± 4	37 ± 3.5	.9
Weight of	21 ± 4	21 ± 2			.85

Effect of Entresto® on baseline measured GFR and plasma creatinine in the four experimental groups.

No differences in baseline GFR measurements were observed between the four groups. Pooled, measured GFR at baseline from all pigs was 65 ±4 mL/min (n = 24). In accordance with this, baseline plasma creatinine concentration was the same in the four groups of this study [89](Fig 4).

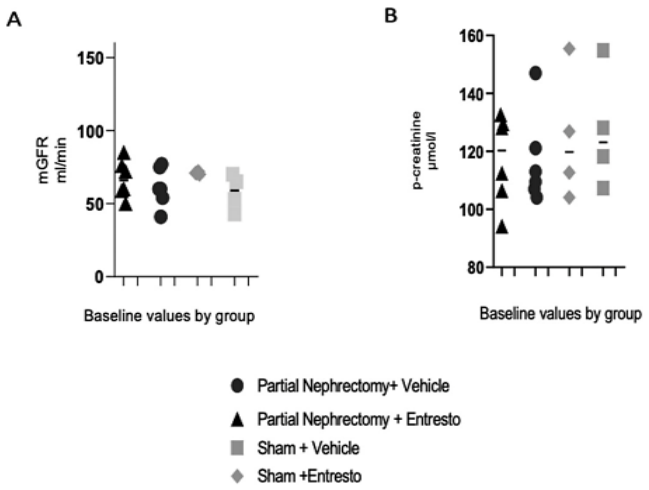

Figure 4 Baseline values of measured GFR (mGFR) (A) and plasma creatinine concentration (B) in the four experimental groups. No statistical diferences were found before interventions.

Effect of Entresto® on measured GFR in pigs with and without partial nephrectomy and warm ischemia

Treatment with Entresto® at a dose of 49/51 mg daily versus placebo for 15 days improved total 99m TC-DPTA clearance 14 days after right-kidney PN (Fig. 5). A mean difference of 23 mL/min (8.370-36.63 P = 0.0007) was found between the group of pigs undergoing laparoscopic PN (Fig. 5)[89]. At termination, left and right-kidney clearances were measured separately. In the group of PN + vehicle treatment, the right injured kidney exhibited lower GFR than kidneys in the sham surgery group treated with Entresto®(Fig. 5). The total difference in GFR by clearance of 99^{m} TC-DPTA was attributed predominantly to improved clearance by the non-ischemic contralateral left kidney (Fig. 5). The difference in clearance between left intact kidneys in the laparoscopic groups was significant; mean 16.7 mL/min (1.53-31.8) (P = 0.02) between the pigs receiving Entresto® daily vs those receiving surgery + vehicle. Furthermore, the same mean difference was observed when comparing left kidneys of pigs that underwent sham surgery and vehicle treatment (Fig.5)[89]. The largest single kidney GFR difference was measured in the surgery group with Entresto®. The mean difference of clearance of the radiotracer between the right and left kidney in the group with PN + Entresto® treatment for 15 days consisted in a 21 mL/min larger radiotracer clearance in the left intact kidney (7-29 mL/min, P = 0.01; Fig. 5); the effect was not present in the group with laparoscopic surgery + vehicle[89].

With the intention to obtain an estimated value of baseline GFR clearance of 99^{m} TC-DPTA from the right and left kidney, we divided the initial clearance by two. This allowed us to analyse single-kidney GFR at baseline vs 14 days after surgery. We found that the adaptive response of the left kidney in the group with PN and treatment with Entresto® was notorious. The increase in clearance of 99^{m} TC-DPTA from the left kidney was not only different from that of the injured right kidney (P = 0.0001); indeed, it also differed from the baseline values of the right kidney (P = 0.008) and left kidney itself (P = 0.0084; Fig. 6)[89].The adaptative changes were not present in the group with PN+ vehicle treatment, and as expected; nor in control group + Entresto® or the control group + vehicle (Fig. 6)[89].

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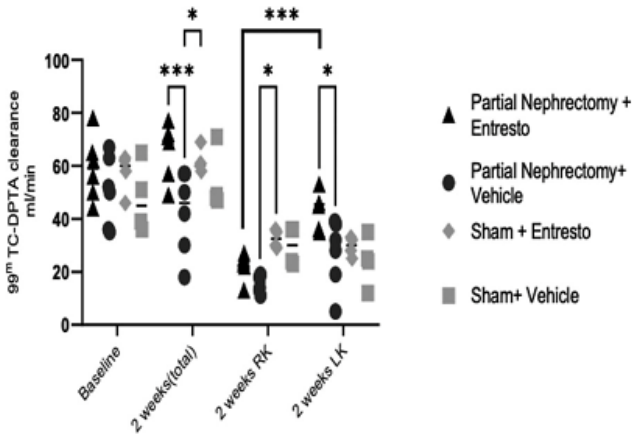

*Figure 5 Measured GFR by urinary clearance of 99mTc-DTPA at baseline and after 15 days. At termination after 2 weeks, urine was collected from the right (RK) and left (LK) kidney by ureter catheter. A statistically significant difference was observed 2 weeks after PN between the groups receiving Entresto or vehicle. Significant difference at ***P = .0007 and *P = .02.*

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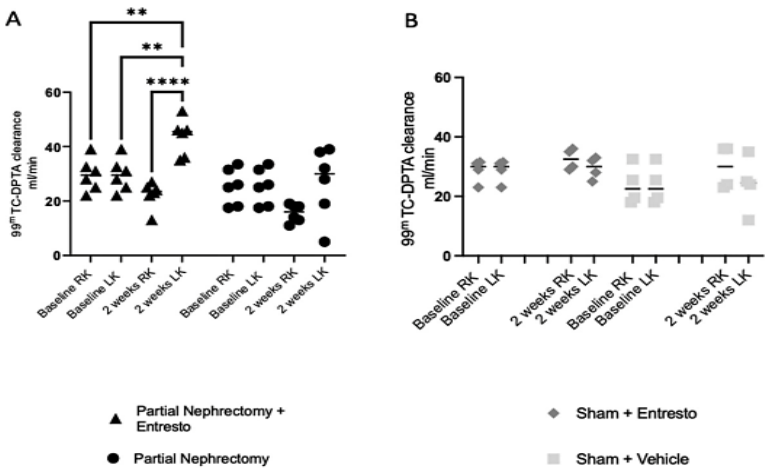

*Figure 6 Comparison of single-kidney GFR at baseline (estimated from total GFR) and after 2 weeks (measured for each kidney). (A) Groups with PN. (B) Groups with sham nephrectomy. Two weeks after PN, the adaptative response in the left kidney was significant in the group receiving Entresto. Significant difference at ****P ≤ .0001 and **P = .036. LK, left kidney; RK, right kidney.*

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Effect of Entresto® on filtration barrier injury in pigs with and without partial nephrectomy and warm ischemia

No differences in total protein/creatinine ratio in urine from the injured kidney were observed between baseline values and values obtained after 14 days of laparoscopic PN and 60 min of warm ischemia in the group receiving treatment with Entresto® (Fig. 7A); nor was any difference observed from baseline to 14 days after surgery in left kidneys in the PN+Entresto® group. Opposite, a significant increase was seen in urine total protein/creatinine ratio 14 days after surgery in the right kidney in the group of PN +vehicle treatment (P<0.05; Fig.7A)[89]. For total protein, no difference between left and right kidneys was observed at 15 days in any of the groups. Using albumin as an injury marker, we discovered an increase in the albumin/creatinine ratio in urine from the right injured kidney 14 days after surgery compared with baseline (P = 0.01; Fig.7B), but also in urine from the left kidney without ischemia in the group undergoing PN + vehicle (P = 0.005)[89]. This increase in albuminuria levels was not seen in the group of PN + Entresto®. It is important to note that the treatment with Entresto® did not change proteinuria or albuminuria levels in healthy (control) specimens (Fig.7 A and B)[89].

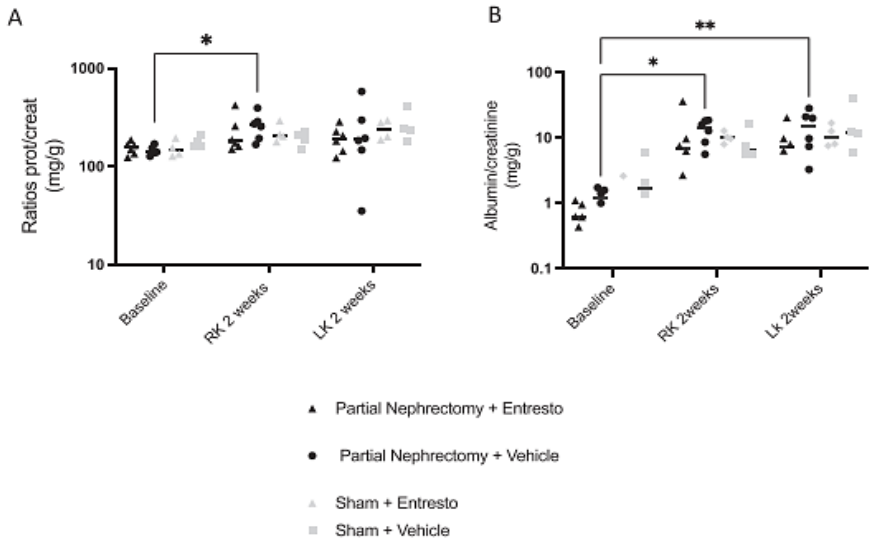

*Figure 7(A) Total protein and (B) albumin: creatinine ratios in urine collected at baseline from bladder and after 2 weeks from the left kidney (LK) and the right kidney (RK). A significant increase was observed in the total urinary protein and albumin: creatinine ratios from the RK 2weeks after PN in the group receiving vehicle. Also, the left intact kidney showed an increase in albumin: creatinine ratio (P = .005). Significant difference at **P = .005 and *P = .01.*

Effect of Entresto® on mid-regional proANP (MR-proANP) in pigs with and without partial nephrectomy and warm ischemia

Prior to the surgical aggression and at its termination, the plasma concentration of MR-proANP did not differ between the four experimental groups. Thus, Entresto® and sham surgery did not alter plasma ANP values. By contrast, 2 h after laparoscopic right-side PN with prolonged warm ischemia, MR-proANP increased significantly in plasma only in the Entresto® group compared with baseline values(P = 0.01; Fig.8)[89]. In both groups subjected to surgery and ischemia, plasma MR-proANP decreased close to baseline values after 15 days and was significantly lower than at to 2 h after surgery (P = 0.003; Fig.8)[89].

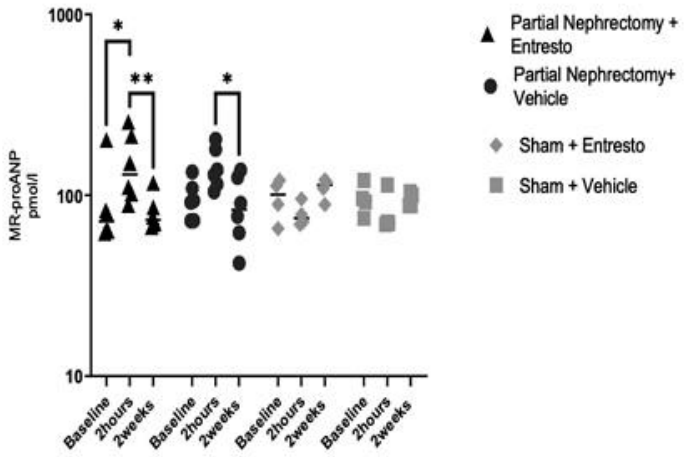

*Figure 8 The plasma concentration of MR-proANP in the experimental groups before and 2 h after surgery and at study completion after 2 weeks. Significant difference at **P = .003 and *P = .01.*

Effect of Entresto® on fractional electrolyte handling in pigs with and without partial nephrectomy and warm ischemia

Fractional excretion of Na at baseline was 0.5-1% (Fig.9A) and there was no difference between the four groups. We found no difference in changes in fractional Na+ excretion at termination compared with baseline, or between kidneys between the sham groups receiving Entresto® or vehicle. A different scenario was seen at termination in the groups exposed to PN and warm ischemia (14 days after surgery). The fractional Na^+ excretion increased above baseline in the injured kidneys from the surgery groups irrespective of treatment with Entresto®. In addition, only in the Entresto® group we did observe an increase in fractional Na^+ excretion also in the non-injured left kidney (Fig.9A)[89]. The mean value of fractional excretion of K^+ at baseline was 10-15%, and no differences were observed between pigs treated with Entresto® or vehicle. As for Na^+, no differences were observed between fractional K^+ excretion from left and right kidneys in sham groups after 15 days (Fig.9B).

Fractional K^+ excretion increased in the injured right kidney in the group of pigs treated with Entresto®, but this effect was not found in the placebo-treated nephrectomy group[89].

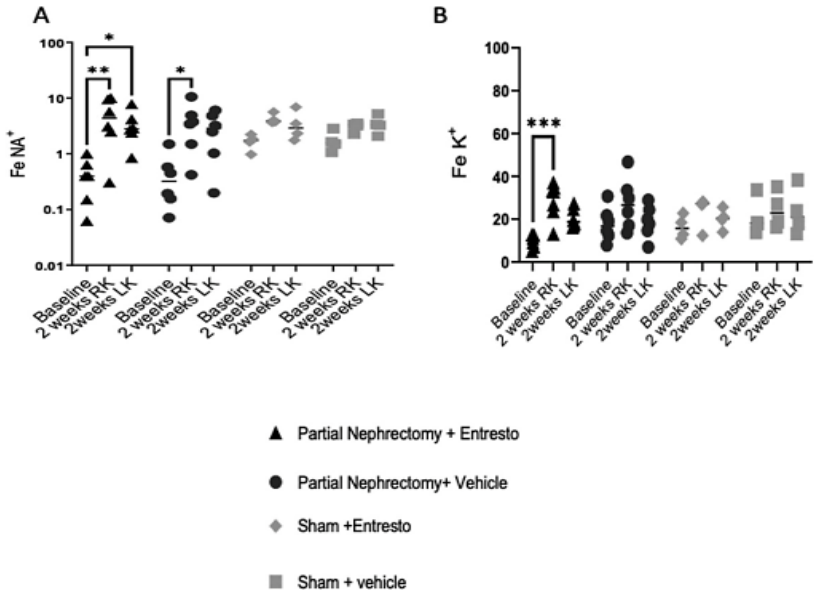

*Figure 9 Fractional excretion of (A) Na+ and (B) K+ at baseline and after 2 weeks from the left kidney (LK) and the right kidney (RK) in the four experimental groups. Treatment with Entresto increased fractional excretion of Na+ both from the RK and the LK 2 weeks after PN. Significant difference at **P = .001 and *P = .04.*

Kidney injury molecule 1 (KIM-1) and kidney morphology images

No major pathological changes were observed; nor were any tubular casts or areas with tubular necrosis observed. At the glomerular level, we found that glomeruli were not sclerotic, vessels appeared with normal wall thickness and the interstitial compartment was not expanded. In only two kidneys we did observe signs of focal inflammation with infiltration of inflammatory cells in defined cortical areas[89].

Measurement of KIM-1 in tissue samples of right PN and TN after 15 days of treatment with Entresto® or vehicle did not show difference within or between groups[89].

STUDY 3

Preoperative values

The TN and PN groups had similar baseline characteristics. Patients in the PN cohort had a mean age of 58 years (SD 9) and a mean BMI of 27.7kg/m^2 (SD 5.2). Those in the TN group had a mean age of 62 years (SD 8) and a BMI of 29 kg/m^2 (SD 7.2)[43].

The functional volume of the sick kidneys (containing the tumour) was on average 176.2 cm^3 (SD 40.05) in the RN group and 132.2 cm^3 (SD 38.8) in the PN group; i.e. no statistical difference was seen between the groups (P = 0.1)[43].

The same tendency was observed when analysing the contralateral (healthy kidneys) in both groups, showing mean volumes of 169 cm^3 (SD 35,05) in the RN group and 158 cm^3 in the PN (SD 59.2) (P = 0-9; Table 4)[43] .

According to the American and European Urological Association guidelines, the size and location of the tumour should determine the choice between RN and PN[8, 9]. For that reason, and as expected, the volume of tumours of patients who underwent PN and TN differed statistically significantly (P = 0.001). The mean volume was 4.34 cm^3 (SD 2.3) in tumours in the PN group, and tumours in this group were predominantly small and exophytic. Opposite to this, the mean volume of tumours in the TN cohort was 34.4 cm^3 (SD 9.4) (P<0.001)[43].

	Total Nephrectomy	Partial Nephrectomy	P=(between groups)
Age (years)	62 +/- 8	58 +/-9	0.4
BMI	29+/-7,2	27,7+/-5,2	0.68
Pre op. Albumin/creatinine ratio (mg/g)	32+/-45	26+/-30	0.92
Pre op. Creatinine(µmol/l)	76+/19	82+/-15	0.99
Pre op mGFR (ml/min)	98+/-15	84+/-8	0.1
Kidney volume cm^3			
Tumoral side	176,2 +/-40	132,2+/-38,8	0.1
Healthy side	169+/-35	158+/-59	0.9
Tumor volume cm^3	34,4+/-9,4	4,34+/-2,3	<0,001
Values expressed as mean and SD			

Table 4General characteristics of the cohort

Volume changes on the contralateral kidney following partial and total nephrectomy.

The mean volume increase in the group of RN was 27 +/- 32% when compared with baseline volumes (Fig.10A)[43]. Similar increases were not seen in the PN group where the corresponding average volume increase was 4% (Fig 10B). The total kidney volume (sum of all healthy tissue) in patients in the RN group was mean 291±66 cm^3 in the preoperative period and mean 191±48 cm^3 after removal of an entire kidney (P = 0.0037). No statistically significant post-surgery changes were observed in patients undergoing PN (pre-surgery total volume 346±72 cm^3 vs. 325±93 cm^3 after surgery) (P = 0.79; Fig 10C and D)[43].

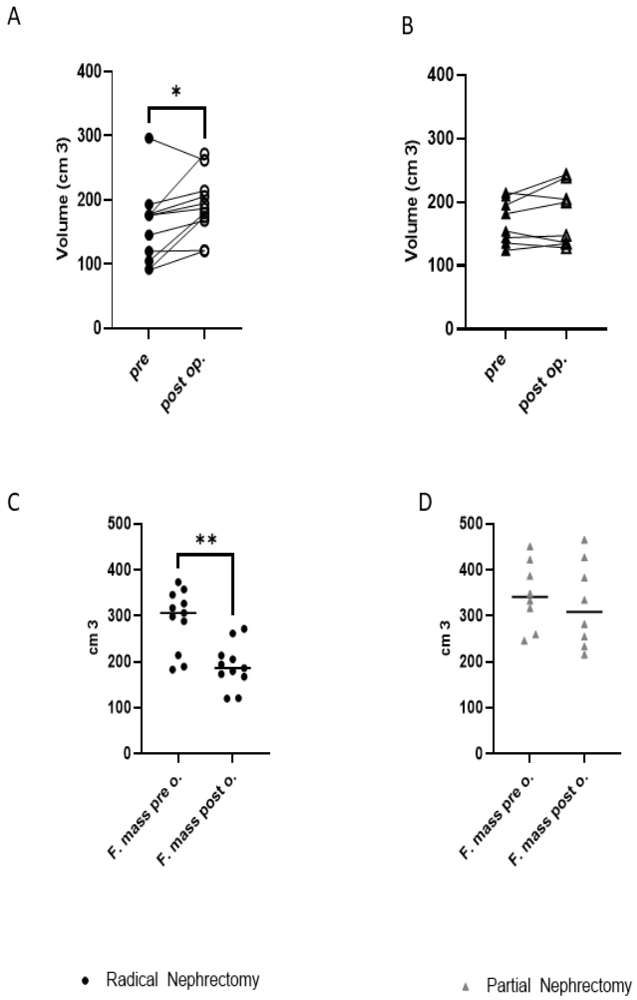

*Figure 10 A Increase in volume after radical nephrectomy in the contralateral kidney. With only one exception, all patients experienced a significant increase in the remnant kidney (N = 11, *: p=0.02 by paired T test).*

*B In the partial nephrectomy group (B) there was no significant change in volume of the contralateral kidney(N = 9). The figure shows the total kidney volume (sum of both kidneys) before and after patients were subjected to RN. After 12 months total volume was significantly reduced. **: p=0.003, (N =11).*

*C The figure shows the total kidney volume (sum of both kidneys) before and after patients were subjected to RN. After 12 months total volume was significantly reduced. **: p=0.003, (N =11).*

D The total kidney volume remained stable in patients undergoing partial nephrectomy (PN) after 12 months. Total volume was not different from total volume before radical nephrectomy in C (N= 8).

Impact of nephrectomy on the filtration barrier and glomerular filtration rate

The values of p-creatinine and the albumin/creatinine ratio remained stable after surgery in both groups (Fig 11A and B)[43].

The mean preoperative value of GFR in the RN group was 98.4±5 mL/min. After removal of an entire kidney, a decrease to a mean of 64 ±15 mL/min one year after surgery (P<0.001) was observed. Based on the preoperative renography, a significant increase was seen in the GFR of the remnant kidney after TN in the present cohort. The mean preoperative single kidney had

a mean GFR of 49±7.5 mL/min, and the mean postoperative single kidney GFR was 64±15 mL/min (P = 0.012). In the PN group, no difference between pre- and postoperative GFR was observed, and the mean reported GFR values for this group were 84±8 mL/min before surgery and 75±14 mL/min after one year of nephron-sparing surgery (P = 0.37)[43].

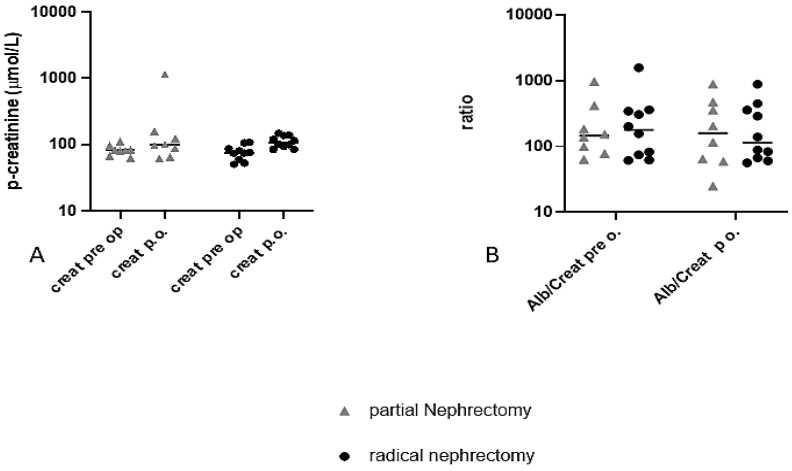

Figure 11 A After one year of radical or partial nephrectomy , there was no significant changes in p-creatinine(N= 19 , P = 0.30)

B The urinary albumin/creatinine ratio remained stable after one year of radical or partial nephrectomy (N =19,P=0.97).

Relation between kidney hypertrophy and changes in the plasma hormones NT-proBNP, MR-proANP and aldosterone after nephrectomy

NT-proBNP values showed a transient increase 24 hours after TN. The average preoperative NT-proBNP value was 88.6±9 ng/L which rose to a mean value of 476±393 ng/L 24 hours after surgery (P = 0.01; Fig 12A). For MR-proANP, the peak of the increase also occurred after 24 hr of RN (P = 0.001); but opposite NT-proBNP, all postoperative values (5, 21 days after surgery and 3 and 12 months after surgery) remained elevated after RN (P = 0.01; 0.038; 0.035 and 0.049, respectively; Fig 12B)[43].

We found a positive correlation between the values of Δ increase of NT-proBNP and volume hypertrophy after one day of TN (r = 0.42; Fig 12C)

For MR-proANP, the positive correlation with volume (Δ increase) was present at all the five postoperative timepoints with the strongest correlation after 12 months of TN (r = 0.78; P = 0.02; Fig 12D)[43]

In the group of RN, the correlation between volume Δ increase and the AUC for NT-proBNP and MR-proANP was also positive (0.25 and r = 0.5, respectively, Fig 12E and F)[43]. For aldosterone, the correlation with volume increase was negative at each postoperative timepoint (mean r = − 0.41, P = 0.1)[43].

The dynamic of NT-proBNP and MR-proANP following TN was not seen in the group of PN, and none of the timepoints were statistically different from one another[43].

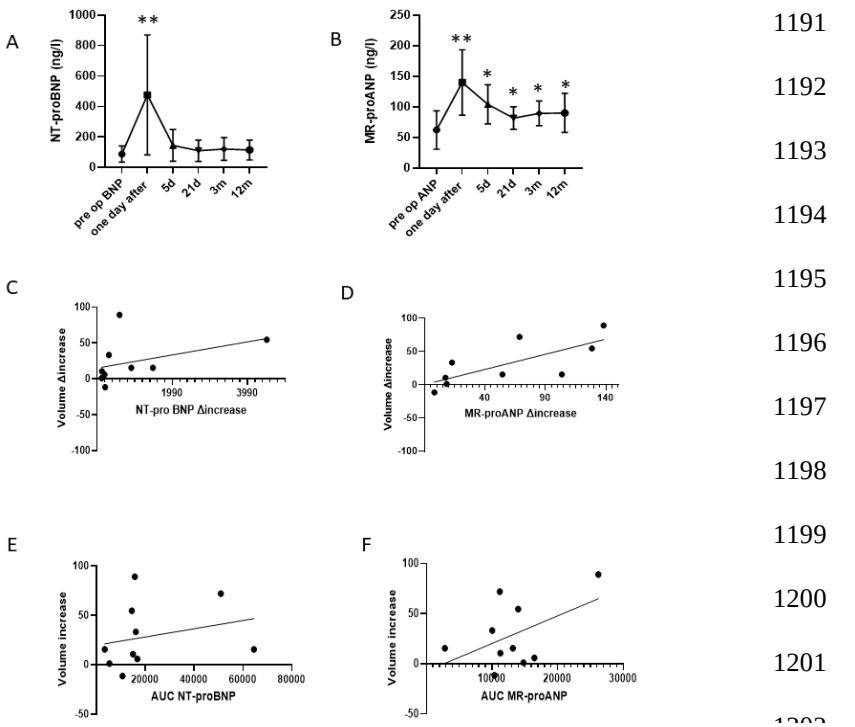

*Figure 12 A The curve shows plasma concentration changes with time of NT-proBNP (mean ±SD in plasma) from patients subjected to radical unilateral nephrectomy (N = 11 . **: P =0.0094 by two-way ANOVA)*

*B The figure shows plasma concentration changes with time of MR-proANP (mean ± XX) in the cohort of patients subjected to radical unilateral nephrectomy, (N = 11). **: P =0.0011(1st 317 day p.o) , * P = 0.01 (5 days p.o) * P = 0.03 (21 days p.o) *P = 0.035 (3 months p.o) *P = 0.04 12 months p.o) by two-way ANOVA.*

C and D: The diagrams show the relation between the increase in kidney volume postoperatively at 12 months after nephrectomy and plasma concentration of NT-proBNP and MR-proANP at 12 months.

E and F The diagrams show the relation between kidney volume increase (delta) 12 months after nephrectomy and area under the curve (AUC) for the time course of plasma concentration changes of NT-proBNP and MR-proANP.

Correlation between postoperative compensatory kidney hypertrophy and preoperative and delta GFR.

A positive direct relation was observed between the volume increase and the preoperative values of GFR (Δ increase value) (r = 0.40). A strong positive correlation was also observed between single-kidney preoperative GFR values and GFR values after TN (r = 0.81, P = 0.008; Fig 13). By contrast, no positive relation was observed between increase in kidney volume and increase in GFR in the TN group (r = - 0.14; P = 0.7)[43].

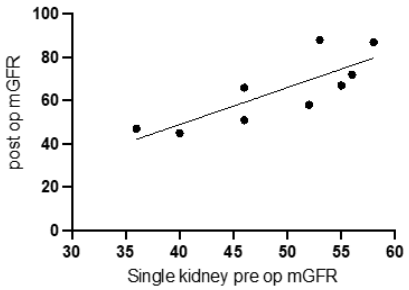

Figure 13 The figure shows the relation between single kidney preoperative measured GFR (by 99m TC-DPTA and 51 Cr 325 EDTA clearance) and postoperative GFR in the patients subjected to radical nephrectomy (N=11) .

DISCUSSION

STUDY ONE

The rate diagnosis of AKI in patients admitted to the intensive care unit, after major surgeries or in direct interventions in the kidneys such as partial nephrectomies or renal transplantation could be around 15 -35%, being the underlying cause a disbalance between O_2 delivery and consumption at kidney level. The clinical consequence will be a higher need of dialysis and the possible development to chronic kidney disease also increasing the total healthcare cost[29, 30]. This scenario could explain the current need to find interventions that can decrease the occurrence of AKI and maintain the GFR without increasing the oxygen consumption in the postoperative phase. As reviewed in this article we enlightened that the natriuretic peptide pathway could be a valid and novel option based on its mechanism of action and the presence of natriuretic peptide receptors at afferent and efferent vessels at glomerular level in the kidney[67].

The activation of the NPRA at kidney level could benefit the vasodilatation at the arcuate and afferent arterioles and at the same time the contraction of the efferent arterioles and in this way contribute to maintain or increase the GFR[30].

The mechanism of action of NPs could explain why GFR is not affected negatively by continuous infusion of natriuretic peptides although a significant systemic blood-pressure-lowering effect was observed [10, 114, 115]. The advantages of the treatment with natriuretic peptides at the kidney level lose power due its short half-life of only 5 minutes which would demand continuous infusion. The atrial natriuretic peptide and the brain natriuretic peptide are degraded by the internalization at the Natriuretic peptide receptor C which is avid at the kidney level or by direct degradation of the neprilysin enzyme which has preference for ANP at an order of magnitude higher than for BNP[72, 73]. This problem prompted us and other researchers to think in alternative ways to increase the circulating level of natriuretic peptides and thus prolong the beneficial effects at kidney level. The combination of neprilysin inhibition plus angiotensin II blockers commercialized as Entresto® represents a valuable solution.

In experimental randomized trials Entresto® was able to prevent a decrease in blood flow at the kidney medullary level during and after major cardiac surgeries; increased 100% the blood flow at the vasa recta and 50% in the arcuate vessels[29, 116]. This notion may also explain why patients within the Entresto® group maintained GFR levels and avoided elevated plasma creatinine (above 2.5 mg/dl) in some trials[10, 114]. The meta-analysis by Dodey et al. of four studies using ARNI drugs also highlighted a renal benefit in patients with heart failure treated with these drugs[117]. Several studies found near optimal dose between 50 and 200 ng/kg/min and detrimental effects when the administration was above 200 ng/kg/min[94, 118].

Another important factor to take into consideration is the concomitant effect of inhibition the angiotensin II AT1 receptors that generates an increase in RBF and pressure related changes in GFR[7].

In favour to the natriuretic peptide treatment some studies reviewed in this article remarked the fact that the patients undergoing major cardiac surgery who received concomitant treatment of NP and furosemide required 75% lower doses of the diuretic than controls during the post operative hospitalization, the length of stay was shorter and the increases in plasma creatinine where significantly smaller in favour to the individuals receiving NP (21 vs 75%)[119].

A well-known side effect of Entresto® is an increased albuminuria level. In the study by Jensen et al., it was shown that NPR-A is not associated with mesangial cells but with podocytes in human kidney[67]. The albuminuria increase could be linked to conductivity changes at this level; however, the increase seems to be transient, and levels normalise after treatment discontinuation[10, 113, 120]. It is also seen that in DOCA-salt hypertensive mice, podocyte NPR-A has a significant and marked protective action that may counterbalance effects of glomerular hypertension. Intriguingly, through elevated ANP/BNP, Entresto® may therefore exert direct podocyte protection in CKD [10, 121]. Moreover, NPs could have an effect, although indirect, at the kidney medullary level which is extremely susceptible to ischemia[29]. The explanation could lie in the effect of the relaxation that Entresto® produces in the efferent arterioles and vasa recta at the juxtamedullary level[10, 78-80].

Conclusion

The literature review showed that some of the properties of the NPs, such as a decrease in the level of plasma creatinine, an improvement of postoperative GFR, and an anti-inflammatory function after warm ischemia-reperfusion syndrome, could be interpreted as the beginning of a new era of use for these drugs to avoid kidney function disease after surgical and non-surgical scenarios. The reviewed articles suggest that neprilysin inhibitors + ANG II blockers could have a determinant role in reducing the number of patients who progress to ESKD. Larger experimental and/or clinical trials with a focus on kidney function and outcomes are needed to make solid conclusions in this regard[10].

STUDY TWO

Until this study was conducted, only little information was available on the use of 99^{m} TC-DPTA as a radiotracer to measure single-kidney GFR in pigs. Most of the previous studies in this field described other methods and radiotracers for measuring total GFR in pigs or another big animal like dogs [122-125]. The only previous model for measuring single-kidney GFR deployed MRI contrast enhancement and was tested only in dogs, and it was technically difficult to conduct[123]. We hence faced the challenge of creating a model for single-kidney determination of GFR; in this respect, 99^{m} TC-DPTA proved to be feasible and the results of the pilot study confirmed the reproducibility of the method and allowed us to continue with the preclinical trial[89].

One of the goals of the study was to create an experimental situation close to the human setting of a partial nephrectomy using an animal model that could represent the kidney anatomy and function in the best possible way. Thus, the decision to choose which was the appropriate duration of ischemia time for the kidneys was challenging. First of all we considered the urological society recommendations to keep the ischemia time bellow 30 minutes[8, 9], furthermore we took the previous publications of Aarhus university group who developed a model of kidney ischemia injury using pigs as the animal model[87] and determined that to achieve a certain degree of kidney damage we should maintain the time of blood deprivation to the right kidney in around 60 minutes. In addition, the set up was created with the tough that the removal of around 1/3 of the kidney would have a detrimental effect.

We demonstrate that removal of around 1/3 of the right kidney plus deprivation of blood supply for 60 minutes have deleterious consequences for GFR and was able to generate damage at the filtration barrier by the finding of increasing levels of albuminuria. At light microscopy level we were not able to document glomerular damage in any of the groups.

A fact to remark was that the treatment with Entresto® enhanced the compensatory response of the contralateral kidney sufficiently to maintain acceptable GFR values and avoid an increase in p-creatinine[89]. Treatment with Entresto® for a total of 15 days also had protective effect on the filtration barrier, avoiding an increase of proteinuria or albuminuria in both kidneys, avoiding the increase of albuminuria that the hyperfiltration may cause in the non-damaged kidney and thus maintained stables levels of urinary albumin/creatinine ratio[89].

Activation of the NP receptor NPR-A increases cGMP in target cells and promotes kidney sodium and water excretion[126]. In preclinical models, activation of the NP receptor NPR-A has also been shown to increase RBF, especially in the outer medulla, which is extremely sensitive to changes in oxygen supply[10, 127-130]. This effect was seen also in preclinical

experiments of high-risk surgery (cardiothoracic by-pass), where continuous NP infusion prevented changes in oxygen supply at the medullary level of the kidney[29, 89].

Treatment with Entresto® has previously been shown to protect the kidney in ischemia-reperfusion scenarios through increasing of circulating levels of ANP[105, 107]. The reports by Ushijima et al. concluded that Entresto® had an anti-proteinuric effect in 5/6 nephrectomised rats compared with treatment with valsartan alone which is in accordance with the results of our study[89, 103]. One side effect of the compensatory increase in GFR of the contralateral kidney could be albuminuria caused by the hyperfiltration effect, but this was also mitigated by the treatment with Entresto® [89].

In some clinical trials, the use of Entresto® or continuous NP infusion increased creatinine clearance and urine flow, but, most importantly, data showed that ANP infusion reduced sixfold the risk of dialysis[94, 95, 131]. Furthermore, ANP infusion increased dialysis-free survival in patients admitted to intensive care unit or patients undergoing major surgery[95]. These finding are in accordance with data from the present study, but our data suggest that particularly the healthy remaining tissue responds to the intervention[89].

Another important fact is that Entresto® plays a role in the protection of the filtration barrier, not only protecting the injured kidney but also the contralateral kidney, avoiding the deleterious effects that the ischemia-reperfusion syndrome and hyperfiltration could cause. Contrary to the group in which PN and vehicle was used as treatment, a deleterious effect at the filtration barrier with an increase in proteinuria and albuminuria values was observed, not only in the right kidney but also in the healthy (contralateral) kidney[89].

The present study did not allow as to identify a maintained increase in ANP plasma concentrations during treatment with the drug as we expected. One possible explanation to this could be the fact that we were not able to measure active ANP in pigs but only a stable translation product.

None of the animals exposed to the treatment with Entresto ® suffered side effects after administration of the drug and variances as mean arterial pressure and weight remained stable[89].

The use of pigs as animal model, demonstrated to be functionally and anatomically closer than rodents to the human anatomy, and allowed us to translate techniques already used in humans (GFR measurement and partial nephrectomy) in the exact same

way as in clinical settings. Moreover the pig kidneys demonstrated to be highly resistant to deleterious conditions as large partial nephrectomy and prolonged deprivation of the blood supply[89].

Conclusions

The method to estimate GFR by plasma decay or clearance of 99^{m} TC-DPTA in pigs used in this study could be a useful technique for future investigations with focus on kidney function and using pigs as animal model. A valid perspective would be to elucidate if future investigations could arise to determinations of GFR using only late blood and urine samples, avoiding a time-consuming process as it was in this study.

After completion of this trial, we can enunciate that the treatment with the ARNI-class drug Entresto® for a period of 15 days in pigs subjected to partial nephrectomy and warm ischemia protected the glomerular filtration barrier and improved the functional adaptation of the non-damaged kidney.

The treatment with Entresto could potentially represent a novel preventive pharmacologic therapy to protect kidney function in different surgical scenarios, and not only on those with direct implication to the kidney.

A weak point of the study was the small sample size, and therefore we conclude that larger studies would help to interpretate in a better way the results that we obtained in this initial study.

Human trials testing the drug will help to arise firm conclusions with respect to the clinical use of the drug.

STUDY THREE

Compensatory changes following PN or RN are well known and have been reported extensively in previous studies. However, the mechanism driving this process remain poorly investigated[44, 46-50].

In our study, we showed a significant volume increase in the remaining kidney tissue specially in the cohort of patients undergoing radical nephrectomy. The changes in the remnant kidney were related directly to tissue mass removal, pre-surgical GFR and post-surgery increase in plasma MR-proANP[43]. It should be noted that the sudden increase in ANP after kidney removal and the maintenance levels above baseline after one year of surgery could be interpreted as such the natriuretic peptides could play a significant role in post-surgery kidney structural hypertrophy. This, however, is purely associative and no causal relation can be concluded which would require intervention.

Additionally, confirming previous studies, we found that the preoperative GFR was positively correlated with postoperative volume increase and a compensatory GFR increase[43, 44, 46-50].In experimental trials it was demonstrated that a humoral factor could be implicated in the post operatory GFR adaptation after radical nephrectomy as the injection of plasma of recently nephrectomised rats into healthy specimens triggered a sudden increase in GFR[57].

In the study, we found a direct relation between MR-proANP and hypertrophy which could contribute causally to the volume increase after RN. Such a relation will await more direct testing and the present observational-associative relation does not demonstrate causality since another factors that might contribute to post operatory kidney hypertrophy were not measured in this observational study [43].

Using patients from the present cohort, the original trial by Azawi et al. demonstrated a persistent increase in MR-proANP after 12 months of kidney removal, supporting the probable implication of NPs in the postoperative kidney adaptation [57].

The NPs could also act as protective agents at the filtration barrier of the kidney, maintaining a stable albumin/creatinine ratio. Experimental trials demonstrated that the treatment with Entresto® for a period could avoid increases in albuminuria and protected the contralateral kidney after ischemia reperfusion scenarios [10, 43].

Plasma aldosterone did not present postoperative changes, as demonstrated in previous investigations[57]. The fact that a negative correlation between p-aldosterone and the postoperative volume gain exists is compatible with reactive suppression of aldosterone and argues against aldosterone-driven kidney hypertrophy[43].

The mechanism of compensatory kidney hypertrophy could lead to a volume increase in the contralateral kidney of up to 39% in patients having undergone RN[44, 46-50]. Patients who experienced no contralateral kidney compensatory hypertrophy were at higher risk of developing CKD[46]. A similar observation was done in different series of healthy, living kidney donors. Those who experienced no early compensatory volume and GFR changes within a month had a tenfold higher risk of developing cardiovascular disease and CKD than the general population[54-56], suggesting that the process of post-surgery hypertrophy and GFR adaptation is a strong biomarker for a positive outcome.

The urinary albumin-creatinine ratio was negatively correlated with those live donors who experienced CKH[132]. In our study, the compensatory response was not associated with increases in the albumin creatinine ratio as observed previously[43, 54-56].

The cohort of patients with PN experienced lesser changes. This may be explained by the limited removal of tissue since tissue removal stimulates hypertrophy only temporarily. One year after PN, no significant change in GFR, kidney volume and albumin/creatinine ratio was observed anymore[43].

Conclusion

This observational study could represent the first step towards the discovery of strong associations between postoperative kidney hypertrophy and humoral factors that could lead to this process. The strengths of the original study were the prospective design with a long follow up of one year after surgery representing enough time to stable hypertrophy levels. The consecutive measurements at fixed timepoints provided a much better-integrated response measure for the correlation analyses.

The mean weak points of this study are the small sample size included for the analysis and the observational nature. The present observational study did not include living kidney donor patients, because they were not CT scanned routinely after surgery.

Longer randomized prospective clinical trials measuring natriuretic peptide level at fixed timepoints after surgery may help to elucidate the possible correlation between these compounds and the magnitude of postoperative kidney hypertrophy.

OVERALL CONCLUSIONS

Previous investigations have shown that the use of natriuretic peptides or ARNI drugs could represent a new therapy to improve or maintain the renal function. The combination of effects of natriuretic peptides and concomitant blockage of ANG II AT1 receptors showed to be effective to decrease plasma creatinine levels and maintain GFR without increasing the filtration fraction. Moreover, neprilysin inhibitors + ANG II blockers could have a determinant role in reducing the number of patients who progress to ESKD[10].

The use of ARNI drugs , even though only for a short period of time, was able to demonstrate the protections of the filtration barrier and facilitated an improvement of the GFR by fast adaptation of the non-injured kidney after partial nephrectomy and prolonged warm ischemia time using an animal model closer to human anatomy and function than rodents[89].

Plasma levels elevations of endogenous atrial and brain natriuretic peptide after total nephrectomy could represent the first leap towards the discovery of strong associations between postoperative kidney hypertrophy and humoral factors that could be of vital importance to drive this process[43].

Human trials testing the drug will help to arise firm conclusions with respect to possible new clinical uses of the drug.

PERSPECTIVES

The natriuretic pathway and treatment with ARNI class drugs stills waits for larger randomized trials with focus on kidney function. Valuable information about GFR measurement methods, drug usage and proper time of blood deprivation to achieve kidney damage could be useful for future investigators. We should empathize that the aim of this study was not to elucidate the role that this drug could play in kidney cancer patients, the aim was to elucidate the role that the treatment could play in protection of renal function after different kind of surgeries and clinical scenarios. One of the obstacles for the use of natriuretic peptides could be that for achieving valuable outcomes, in terms of renal protection, the peptides may be administrated under continuous infusion due the short medium life of the compounds. This represents a challenging scenario for the implementation of home-based treatment, and therefore new ways of prolonging the action of the peptide could signify a valid approach. In that direction we can say that the combination of angiotensin II blockers plus neprilysin inhibitors (ARNI drugs) could play a determinant role.

Probably is time to start thinking that the spectrum of the treatment with natriuretic peptides or ARNI drugs is beyond the cardiovascular system, since it seems that they could play an important role in kidney protection and postoperative kidney hypertrophy. Moreover, the treatment with ARNI drugs could potentially represent a novel preventive pharmacologic therapy to protect kidney function in different surgical scenarios, and not only on those with direct implication to the kidney.

After the development of a reliable method to measure GFR in pigs using a radiotracer, an additional point of valuable interest for future investigations would probably be the estimation of GFR by plasma decay or clearance of 99^{m} TC-DPTA using only late blood and urine samples. This will definitively represent a time saving method.

We strongly believe that the association between the use of natriuretic peptides or ARNI drugs and positive kidney outcomes expressed in the three manuscripts that serve as main work for the realization of this PhD. could represent a starting point for larger investigations that have the aim to elucidate the role of these drugs at kidney level and probably extend the current clinical use of the compounds.

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SUMMARY

The prevalence of renal cancer is increasing, which may, among others, be due to a rise in the number of radiological investigations performed for other reasons, e.g., to determine the origin of abdominal pain or weight loss. Recent years have therefore seen an increase in the number of surgical interventions involving the kidneys. This trend and the rapid aging of the general population command careful considerations of possible treatments and strategies to protect the renal function during and after kidney surgery. Searching for a feasible therapeutic option that avoids or at least minimizes post-surgery loss of kidney function, we conducted studies to evaluate the role of natriuretic peptides and blockage of their degradation as this approach has shown promising results in the cardiovascular area.

The group of NPs counts three vasoactive peptides with a molecular weight below 10 kDa. that are structurally interrelated; they are atrial NP (ANP), B-type NP (BNP), and C-type NP (CNP). NPs are synthetised, stored and secreted at myocardiocyte level.

Aims:

a) To perform a review to determine the potential benefits of the use of NPs and angiotensin-receptor-neprilysin inhibitor (ARNI) drugs to protect kidney function.
b) To determine the feasibility of single kidney glomerular filtration rate (GFR) measure using 99m TC diethylenetriaminepentaacetic acid (DPTA) as radiotracer in a pilot study, using pigs as an animal model.
c) To perform a randomised pre-clinical study investigating the action of LCZ696 at the kidney level after laparoscopic partial nephrectomy with prolonged ischemia time.
d) To elucidate the role of NPs in the mechanism of kidney hypertrophy after kidney injury

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