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The Severe Cardiorenal Syndrome

Het Ernstig Cardiorenaal Syndroom
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op 28 februari 2011 des middags te 4.15 uur

door

Lennart Guido Bongartz
geboren op 5 september 1980
te Duisburg, Duitsland

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In the time of your life, live — so that in good time there shall be no ugliness or death for yourself or for any life your life touches.
Seek goodness everywhere, and when it is found, bring it out of its hiding-place and let it be free and unashamed.
Place in matter and in flesh the least of the values, for these are things that hold death and must pass away.
Discover in all things that which shines and is beyond corruption.
Encourage virtue in whatever heart it may have been driven into secrecy and sorrow by the shame and terror of the world.
Ignore the obvious, for it is unworthy of the clear eye and the kindly heart.

Be the inferior of no man, nor of any man be the superior.
Remember that every man is a variation of yourself. No man's guilt is not yours, nor is any man's innocence a thing apart.
Despise evil and ungodliness, but not men of ungodliness or evil.

These, understand.

Have no shame in being kindly and gentle, but if the time comes in the time of your life to kill, kill and have no regret.
In the time of your life, live — so that in that wondrous time you shall not add to the misery and sorrow of the world, but shall smile to the infinite delight and mystery of it.

William Saroyan (1939)

Voor mijn ouders, mijn broertje, Mayke, en niet te vergeten alle ratten die onvrijwillig hun leven ter beschikking hebben gesteld voor deze studies

Contents

ACE	angiotensin converting enzyme
AT	angiotensin
CI	cardiac index
CO	cardiac output
CRC	Cardiorenal Connection
CRS	Cardiorenal Syndrome
dP/dt-max	maximal rate of pressure increase
dP/dt-min	maximal rate of pressure decrease
Ea	arterial elastance
EDP	end-diastolic pressure
EDV	end-diastolic volume
Ees	end-systolic elastance
EF	ejection fraction
E'max	maximal (end-systolic) elastance
ESP	end-systolic pressure
ESRD	end-stage renal disease
ESV	end-systolic volume
FF	filtration fraction
GFR	glomerular filtration rate
GS	glomerulosclerosis
CKD	chronic kidney disease
L-NNA	N_{ω}-nitro-L-arginine
LV	left ventricle
LVH	left ventricular hypertrophy
LV-FAC	left ventricular fractional area change
L-VNIO	L-N5-(1-Imino-3-butenyl)-ornithine
LVSD	left ventricular systolic dysfunction
MAP	mean arterial pressure
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-kappa B
NO	nitric oxide
NOS	nitric oxide synthase
PAMP	preload-adjusted maximal power
PDTC	pyrrolidine-di-thiocarbamate
PRSW	preload-recruitable stroke work
RAS	renin-angiotensin system
RBF	renal blood flow
ROS	reactive oxygen species
SBP	systolic blood pressure
SCRS	Severe Cardiorenal Syndrome
SNS	sympathetic nervous system
SOD	superoxide dismutase
SV	stroke volume
SVRI	systemic vascular resistance index
SW	stroke work
τ, *tau*	time constant of early diastolic relaxation
Tempol	4-hydroxy-2,2,6,6-tetrametyl piperidine-1-oxyl

Chapter 1

Introduction

1.1 Historical observations of cardiorenal disease.

In recent years, the relationship between the heart and the kidneys in disease has received increasing attention from the clinical and scientific medical community. This was initiated by epidemiological observations in the late 1990's of increasing patient numbers with concurrent heart and kidney problems, and the association with a significantly higher mortality ratio. This has led to intense discussions about the value of the recognition of cardiorenal disease and the existence of a specific "cardiorenal syndrome".

The idea of specific interaction between heart and kidneys is not new. There are numerous examples and anecdotes that show that people in the past from various societies considered the heart and the kidneys to have a special relationship.

Heart and kidneys in ancient times

The Egyptian "Book of the Dead" (1600-1240 B.C.), which served as a reference work to assist the deceased in the afterlife, is one of the first known texts that mentions the heart and kidneys in parallel:

> "*Homage to thee, O my heart! Homage to you, O my kidneys*!".[1]

The heart and the kidneys were the only organs left inside the body during the process of mummification. The heart was weighed against the feather of truth by the jackal-headed Anubis (Figure 1), but the exact role of the kidneys for the passage into afterlife is uncertain. Blood vessels are well preserved in mummies, and there is evidence that cardiovascular disease affecting both the heart and the kidneys were not uncommon.[2]

Eknoyan[3] researched the Bible and found that:

> "[T]he kidneys are mentioned five times in the Bible as the organs examined by God to pass judgment on a person. They are mentioned either before or after but always in parallel with the heart, as for example, "*I, the*

Lord, search the heart, I try the reins, even to give every man according to his ways, and according to the fruit of his doings" (Jer. 17:10), and, "*Examine me, O Lord, and prove me; try my reins and my heart*" (Psalms 26:2)."

In Hebrew lore the kidneys owned the status as the organs which give the heart advice and counsel, and which symbolize the innermost sources of thought and desire, those hardly accessible to man but tested by God.[4]

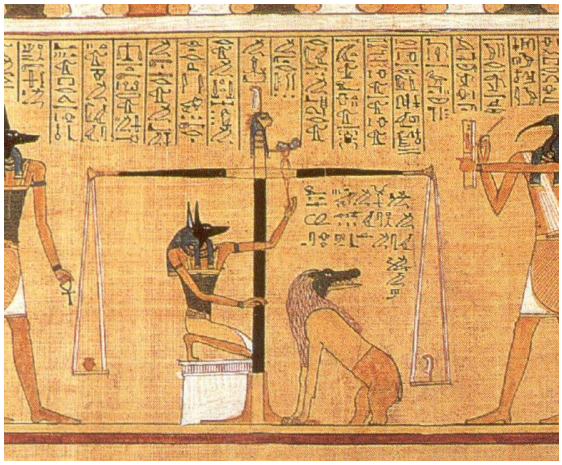


Figure 1. The weighing of the heart against the feather of thruth. This papyrus was found in the tomb of the scribe Hunefer in Thebes. It dates from the 19th Dynasty, about 1285 BC. It can be seen in the British Museum.

Heart and kidneys in Traditional Chinese Medicine

No less lyrical, albeit more clinical descriptions are found in China, where the heart and the kidneys are described in various medical texts. In Traditional Chinese Medicine (TCM), the kidney represents water and is considered a 'yin' organ whereas the heart represents fire and is a 'yang' organ.[5] In TCM, the kidney not only regulates the urinary system, but also controls the reproductive, endocrine and nervous system. It stores Jing, which is considered a vital life force responsible for development and reproduction. The heart rules the blood vessels and blood supply to the organs, but also stores the "spirit", reflected in a person's mental, cognitive and intellectual abilities.

Dr. Shen Jin'ao writes in his book “Dr. Shen's Compendium of Honoring Life (*Shen Shi Zunsheng Shu*)” from 1773:

> “The heart resides in the vessels. It rules the kidney network, not via a controlling position in the restraining circle of relationship between the organ networks [where the kidney actually restrains the heart], but simply because it is the general master of all organ networks. Before the heart fire can harmoniously blend with the kidney water, however, the kidney water must be sufficient. Otherwise the heart fire will flare out of control, and all kinds of heart and kidney ailments will arise.”

In the 5 Elements network of Chinese medicine (Figure 2) a disorder called “heart and kidney failing to link” (*xin shen bu jiao*) is presented, resulting in a variety of symptoms ranging from restlessness and palpitations to dizziness, and dark, scanty urination or nocturia.[6] If both kidneys and heart are weakened, there may be palpitations, shortness of breath, dizziness, darken complexion, purple lips and nails, sensitivity to low temperatures, urinary difficulty, edema that is more apparent in the lower limbs, and a bulky tongue. If the kidneys and heart are in disharmony, there may be palpitations, dream-disturbed sleep, forgetfulness, dizziness, thirst, red cheeks, night sweats, lumbar and knee soreness, nocturnal emission, and a red tongue.

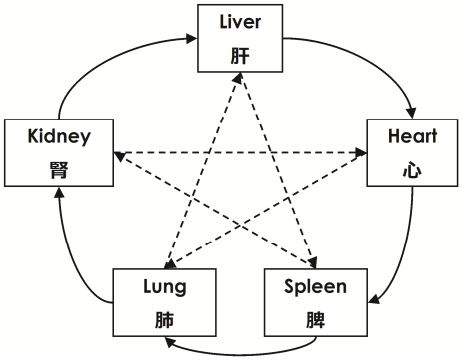


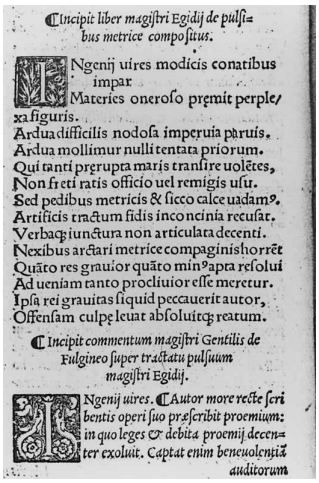
Figure 2. The Five Elements theory of TCM and the relationships between the organs, with generation (solid arrows) and restriction cycles (dashed arrows).

Another piece of traditional Chinese Medicine text gives a pretty accurate description of the symptoms of cardiorenal failure:

> "When the kidney fails to evaporate fluid which then floods and ascends to depress the function of heart 'yang' there may be clinical manifestations such as oedema, chills and cold limbs, accompanied by palpitations, shortness of breath and stuffiness in the chest, indicating retained water affecting the heart."[7]

Cardiorenal disease in the European Middle Ages

In Western society, during the Middle Ages, heart disease per sé was not very well described in medical doctrines, although the heart was considered the source of the *spiritus vitalis*. Medieval doctors viewed the outward appearance and excretions of the whole body or body parts as a reflection of one's state of health, and as such the symptoms of congestive heart failure were approached as separate clinical entities.[8] The examination of urine was however a widely used diagnostic tool. As one of the first Western "cardio-nephrologists", Gentile da Foligno (Gentilis de Fulgineo; 1272? – 1348) considered heart disease as one of the major inflictions modulating the color and output of urine in his commentary on *De pulsibus* (About Pulses) composed by Aegidius Corboliensis (Figure 3).[9]


Incipit liber magistri Egidij de pulsi=
bus metrice compositus.

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xa figuris.
Arduadifficilis nodosa imperuia paruis.
Ardua mollimur nulli tentata priorum.
Qui tanti prerupta maris transire uolētes,
Non freti ratis officio uel remigis usu.
Sed pedibus metricis & sicco calce uadam⁹.
Artificis tractum fidis inconcinia recusat.
Verbaq; iunctura non articulata decenti.
Nexibus arctari metrice compaginis horrēt
Quāto res grauior quāto min⁹apta resolui
Ad ueniam tanto procliuior esse meretur.
Ipsa rei grauitas siquid peccauerit autor,
Offensam culpę leuat absoluitq; reatum.

Incipit commentum magistri Gentilis de
Fulgineo super tractatu pulsuum
magistri Egidij.

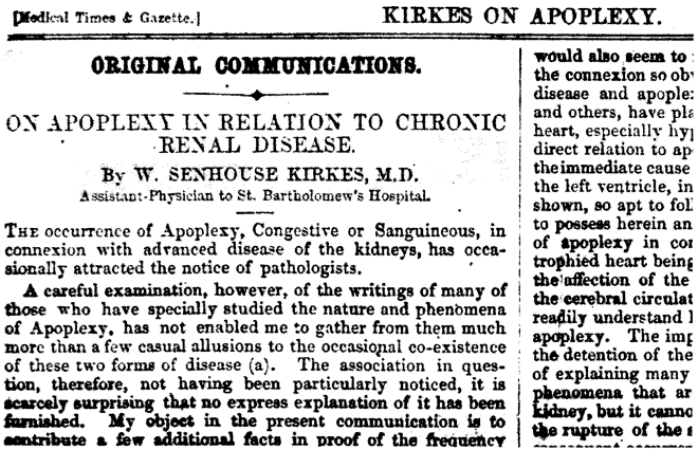
Ingenij uires. Autor more recte scri
bentis operi suo præscribit proemium:
in quo leges & debita proemij decen=
ter exoluit. Captat enim benevolentiæ
auditorum

Figure 3. First page of *De pulsibus.* Town Library, Foligno. Reproduced from ref. 9.

Heart-kidney interactions in the late 19th and early 20th century

During the Industrial Revolution the medical sciences expanded and scientific methods became more and more reliant on experiments and observation. Richard Bright (1789–1858) and Ludwig Traube (1818–1876) both documented that cardiac hypertrophy was a common anomaly resulting from chronic renal disease.[10, 11] Traube refers in his writings to William Senhouse Kirkes (1822 – 1864) who reviewed 14 autopsy cases of with apoplexy and diseased kidneys, of which only one did not have an enlarged heart (Figure 4).[12] He concludes that:

> "... I believe that the affection of the kidneys is the primary disease... [it] has among its most frequent and permanent accompaniments an hypertrophied condition of the left ventricle ... of the various explanations of this pathological fact the most probable perhaps is that which regards the blood as so far altered from its normal constitution ... as to move with less facility through the systemic capillaries, and thus to require increased pressure, and consequently increased growth of the left ventricle, to effect its transmission."



[Medical Times & Gazette.] KIRKES ON APOPLEXY.

ORIGINAL COMMUNICATIONS.

ON APOPLEXY IN RELATION TO CHRONIC RENAL DISEASE.

By W. SENHOUSE KIRKES, M.D.
Assistant-Physician to St. Bartholomew's Hospital.

THE occurrence of Apoplexy, Congestive or Sanguineous, in connexion with advanced disease of the kidneys, has occasionally attracted the notice of pathologists.

A careful examination, however, of the writings of many of those who have specially studied the nature and phenomena of Apoplexy, has not enabled me to gather from them much more than a few casual allusions to the occasional co-existence of these two forms of disease (a). The association in question, therefore, not having been particularly noticed, it is scarcely surprising that no express explanation of it has been furnished. My object in the present communication is to contribute a few additional facts in proof of the frequency

would also seem to
the connexion so ob
disease and apople
and others, have pla
heart, especially hy
direct relation to ap
the immediate cause
the left ventricle, in
shown, so apt to fol
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the cerebral circulat
readily understand
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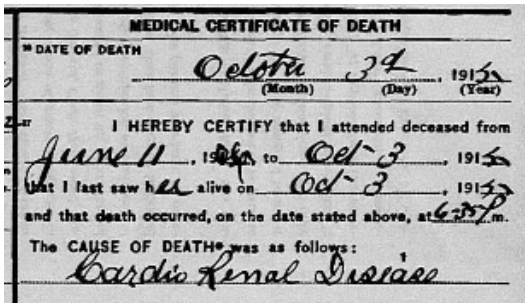
of explaining many
phenomena that ar
kidney, but it canno
the rupture of the

Figure 4. Beginning of Kirkes' publication in the Medical Times & Gazette, 1855.

Alfred Stengel[13] proposed a definition of “cardio-renal disease” (Figure 5) when he wrote in 1914:

> “The clinician encounters many cases, mainly in persons of middle age or older, in which evidences of cardiac weakness and other circulatory disturbances, such as high pressure, are associated with signs of failure of renal function or urinary indications of renal disease. When this combination of symptoms is of such character that the observer cannot readily assign to either the cardiovascular system or to the kidneys the preponderance of responsibility, the term "cardio-renal disease" is often employed. The term, therefore, comprises cases of combined cardiovascular and renal disease without such manifest predominance of either as to justify a prompt determination of the one element as primary and important and the other as secondary and unimportant.”

The observations on the cardiac consequences of chronic kidney disease were later expanded, and Gouley[14] was coined the term “uremic myocardiopathy” in 1940 and in 1944 Raab[15] proposed that cardiotoxic substances accumulate in uremia. Rössle,[16] and Langendorf and Pirani[17] later showed that interstitial widening and fibrosis were common in hearts of patients dying from uremia.


MEDICAL CERTIFICATE OF DEATH

DATE OF DEATH Octobr 3d 1915
(Month) (Day) (Year)

I HEREBY CERTIFY that I attended deceased from June 11, 1915, to Oct 3, 1915
that I last saw her alive on Oct 3, 1915
and that death occurred, on the date stated above, at 6:35 p.m.

The CAUSE OF DEATH was as follows:
Cardio Renal Disease

Figure 5. Example from a 1915 Death Certificate from Massachusetts. From Rudy’s List of Archaic Medical Terms at http://www.antiquusmorbus.com/English/HeartStroke.htm

The Cardiorenal Syndrome in modern times

The advent of the Cimino-shunt and the development of hemodialysis (HD) as the mainstay treatment for end-stage renal disease (ESRD) resulted in further increasing interest in the structural and functional cardiac status of HD patients.[18-21] The full extent of the problem of cardiovascular disease in chronic kidney disease (CKD) and ESRD patients was then charted in the 1990's, showing that a large proportion of patients starting dialysis already suffers from cardiac abnormalities and dysfunction and that survival of these patients after a myocardial infarction (MI) was dismal.[22-24] In 2003, a statement from several councils from the American Heart Association (AHA) was published in Hypertension and Circulation underscoring the problem of increased cardiovascular risk in CKD, and the lack of knowledge on pathophysiology.[25] This was followed by two seminal papers published in the New England Journal of Medicine showing the exponentially increased risk for adverse outcome with decreasing kidney function, in "normal" patients but even more so after they had experienced a myocardial infarction.[26, 27] At the same time, the scientific and clinical community became increasingly aware of the effect of decreased kidney function or kidney damage on the prognosis of patients with heart failure.[28-30] Interestingly, in a study on the predictive value of 10 different biomarkers in over 3000 patients from the Framingham Heart Study, levels of brain natriuretic peptide (BNP) and urinary albumin-to-creatinine ratio most strongly predicted major cardiovascular events.[31] One patient study even suggested that the decline of renal function is accelerated after an acute MI.[32] These epidemiological associations resulted in a strong clinical suspicion that the combination of heart and kidney disease is associated with accelerated disease progression and adverse outcome.

1.2 The Severe Cardiorenal Syndrome and the Cardiorenal Connection

The epidemiological data, the AHA statement, and our own clinical observations of cardiorenal failure in patients led us to propose the “Severe Cardiorenal Syndrome” (SCRS) as a separate disease entity with the “Cardiorenal Connection” (CRC) as the putative pathophysiological model.[33] We defined the SCRS as a condition in which combined cardiac and renal dysfunction amplifies progression of failure of the individual organ, leading to grossly increased cardiovascular morbidity and mortality. The CRC works in conjunction with the hemodynamic control model of heart-kidney interactions as stipulated by the late professor Guyton (Figure 6).

The “cardiorenal connectors” that we put forward were:

- the balance between nitric oxide (NO) and reactive oxygen species (ROS),
- the sympathetic nervous system (SNS)
- the renin-angiotensin system (RAS), and
- inflammation.

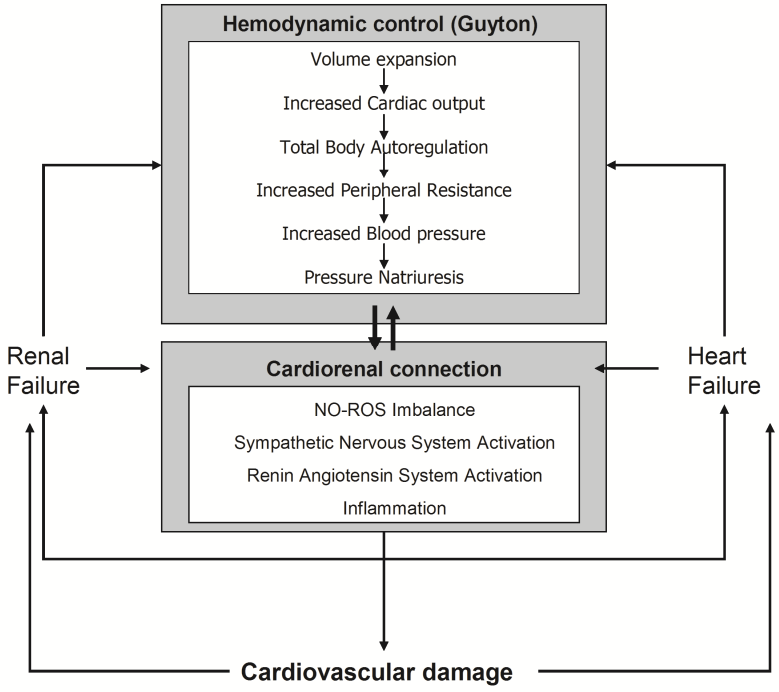


Figure 6. The cardiorenal connection works extensive to Guyton’s model to drive accelerated cardiovascular damage in combined renal and heart failure.

We envisioned that both heart and renal failure lead to derangement of the Guytonian model of hemodynamic control, but also results in activation/disturbance of the connectors of the CRC. The connectors have a modulating effect on hemodynamic control but can also induce cardiovascular damage, thereby mediating further functional deterioration.[33] We proposed that activation of the CRC leads to a vicious cycle in which all the connectors become disturbed, synergize and further activate each other. This ultimately results in worsening of both cardiac and renal damage and failure.

Summary of the Cardiorenal Connection

A shift in the balance between NO and ROS towards ROS is a central event in many cardiovascular diseases.[34] In the SCRS, the balance between NO and the ROS is skewed towards the latter by increased production of ROS, a low anti-oxidant status, and lower availability of NO.[35] In the cardiorenal connection, this imbalance may influence sympathetic nervous activity,[36] release of renin and angiotensin,[37] and promote inflammation by oxidative modification of substances.[38]

Sympathetic nervous activity is also increased in both renal and heart failure. By affecting the other cardiorenal connectors it can play a significant role in the SCRS. It stimulates renin release from the kidneys,[39] generates ROS which induces vascular wall growth,[40] and induces inflammation.[41]

The RAS is activated in both renal and heart failure [42, 43] and angiotensin II (Ang II) affects the other cardiorenal connectors in different ways. It activates the SNS in both heart and kidney failure,[44, 45] it generates ROS via nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase,[46] and activates pro-inflammatory gene expression via nuclear factor-κB.[47]

Persistent inflammation has been found in both renal and heart failure. By altering the functioning of the RAS,[48] and promoting ROS[49] and noradrenaline formation,[50] inflammation can contribute to the positive feedback loops in the cardiorenal connection. The Severe Cardiorenal Syndrome is thus not a syndrome in which cardiac and renal failure simply co-exist side-by-side. Cardiac and renal failure are intimately linked by the cardiorenal connectors, because failure of either organ can excite the cardiorenal connectors, but the connectors themselves also affect the

structure and function of both organs. Logically, the cardiorenal connectors become more pronounced in combined failure.[51]

Previous research on the cardiorenal interactions

Thus the question was raised whether kidney disease and heart disease simply co-exist or that they indeed worsen each others progression. Clinical studies can not provide the answer to this question because they are observational, lack histological end-points, and are confounded by selection bias, inconsistent definition of end-points, and medication use. Therefore, further exploration of the mechanisms of cardiorenal interactions must rely on animal studies, in which timing and severity of the disease are controlled, progression of disease can be followed, and histological end-points are assessed.

Much of what we know today on the structural cardiac consequences of chronic kidney disease results from the extensive research in rats with CKD by the group of Kerstin Amann and Eberhard Ritz in the late 80's and early 90's.[52] Despite numerous cardiac changes, in the rat CKD model of subtotal (5/6th) nephrectomy (SNX) cardiac systolic function is generally maintained.[53, 54] Conversely, after MI by ligation of the left coronary artery in rats, renal histological damage or proteinuria is absent although glomerular filtration rate (GFR) may be decreased.[55, 56] Thus, it appears that both organs need to be affected to cause acceleration of damage and failure typical for the CRS. Only two animal studies investigated the effect of 'dual damage' to heart and kidneys, with MI following shortly after a renal insult in rats, with conflicting results.[55, 57] Different models of nephrectomy exist in mice, but these are not as robust as those in rats, with variable changes in renal function and cardiac abnormalities. The renal hemodynamic response to HF induced by pacing in dogs has also been investigated, but whether there is histological damage is unknown. Furthermore, there is no proven model of CKD in dogs.

Taken together, there is still a paucity of models that investigate the interaction between kidney and heart failure in a chronic set-up with integrated physiological and histological assessment. From the available data, the SNX model of CKD and coronary ligation (CL) model of HF appear the most robust to investigate the SCRS, and we therefore chose these approaches to develop animal models of the SCRS.

Focus on nitric oxide availability: effector and modulator?

In this thesis, we also put special focus on NO availability. Firstly, reduced NO availability is considered a hallmark of CKD.[35, 58] Secondly, reduced NO availability is a pathogenic factor in many cardiovascular diseases, like hypertension and diabetes, which are also the two most common causes of CKD.[34] NO can function as an effector of the CRC by way of its vasodilatory action. It also modulates GFR and tubulo-glomerular feedback (TGF).[59] Reduced NO availability will result in tissue damage by oxidative stress. Furthermore, many effects of the other CRCs may be mediated by changes in the redox-balance and NO availability.[60, 61] Apart from its role in endothelial dysfunction, NO availability also modulates cardiac contractility, as NO synthase (NOS) inhibition reduces cardiac output, and causes cardiac damage in high doses.[62, 63] In SNX, cardiac systolic function generally remains preserved, while in patients left ventricular dysfunction (LVSD) develops during the course of CKD progression.[64] The CKD model in the rat of SNX essentially creates nephron number reduction in an otherwise healthy animal. Thus, the combination of SNX and NO reduction may more accurately mimic the clinical situation of CKD.

1.3 Aims and outline of the thesis

The aim of this thesis was to develop animal models of the Severe Cardiorenal Syndrome with combined longitudinal kidney disease and heart disease, and to test the effect of different late (or "rescue") interventions on cardiorenal structure and function in an integrative physiological fashion. All studies in this thesis were performed in rats.

***General hypothesis*:**

> Chronic kidney disease and cardiac disease interact and worsen each other's structural and functional derangement, which is mediated by the Cardiorenal Connectors.

PART I provides the theoretical background, summarized in the above paragraphs, for the current thesis. As described above, we put forward the Cardiorenal Connection as the pathophysiological model driving the (Severe) Cardiorenal Syndrome (SCRS), and evidence for the interactions between the cardiorenal connectors are reviewed in **Chapter 2** of this thesis.

Study question:

> Based on current knowledge about cardiorenal interactions in health and disease, can we develop a putative pathophysiological model to explain the enhanced morbidity and mortality observed in patients with combined kidney and heart failure?

In **PART II**, we assessed cardiorenal structural and functional progression in a model of combined CKD and MI in the rat. **Chapter 3** describes the development of a rat model for the SCRS, and shows that bidirectional organ damage and dysfunction in combined CKD and MI occurs. In **Chapter 4**, we studied whether pharmacological correction of the Cardiorenal Connectors could abrogate functional decline and improve structural damage to heart and kidneys. We also set out to determine if there is hierarchy present in the Cardiorenal Connectors by assessing the effects of different treatment combinations.

Hepcidin is the main regulator of body iron and is normally produced by the liver. It is also expressed in the heart with inflammation or hypoxia, and may play an important role in cardiomyocyte iron homeostasis. We measured cardiac and hepatic gene expression of hepcidin in rats with CKD, HF and SCRS to show that it reacts to local and systemic stimuli in cardiorenal disease, and studied the effect of different interventions. (**Chapter 5**).

Study questions:

1. Can we develop a model for the SCRS in the rat based on combined CKD and MI, that allows longitudinal follow-up of cardiorenal function, combined with structural assessment?
    a) Does long-term pre-existent CKD adversely affect cardiac structure and function after MI, and conversely does ensuing HF worsen renal structural and functional derangement?
2. Do the Cardiorenal Connectors play a role in the progression of the SCRS, and is there a hierarchy between them?
    a) Does blockade of all Cardiorenal Connectors fully abrogate progression of the SCRS?
    b) Does correction of inflammation and the disturbed NO/ROS balance or of the RAS alone have similar effects?
3. Is hepcidin expression in the heart in CKD, HF and their combination different from systemic (liver) hepcidin expression, and does it react to anti-inflammatory and antioxidant therapy?

In **PART III** the interaction between CKD and NO availability on cardiac function is elaborated. **Chapter 6** describes the effect of transient low dose NO synthase inhibition during development of CKD on long-term progression of cardiorenal variables. Because this was associated with persistent LVSD as well as low NO availability long after cessation of NOS inhibition, we hypothesized that there was a causal relationship between the LVSD and low NO production, and studied the effect of NO supplementation with the tolerance-free NO donor molsidomine (**Chapter 7**). The functional role of the neuronal NOS isoform for the support of

cardiac function and the β-adrenergic response in this model was investigated in **Chapter 8**.

Study questions:

1. Does depletion of NO availability during CKD development induce cardiac dysfunction and amplify progression of cardiorenal failure?
    a) Is this due to an interaction between CKD and NO availability or due to NO depletion per sé?
    b) Are there permanent structural and/or functional effects?
2. Does systemic NO supplementation with an NO donor restore cardiac dysfunction and improve cardiorenal parameters?
3. What is the functional role of the neuronal NOS isoform for *in vivo* cardiac function in this model of the SCRS?

In **PART IV** previous research in rats and other animals on CKD and HF are summarized in **Chapter 9**. Here we review cardiac changes in different models of CKD, and renal changes in different models of heart failure (HF).This summary also identifies the lack of adequate chronic animal models that replicate the SCRS, and highlights the advantages and disadvantages of our models in this respect.

Study question:

Are there animal models available that replicate cardiorenal failure in the SCRS in a controlled and longitudinal fashion, and that show bi-directional cardiorenal interaction?

In **Chapter 10**, the results of the preceding chapters are discussed and a perspective is provided.

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Part I

Chapter 2

The Cardiorenal Connection: "Guyton revisited"

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The incidence of cardiac failure and chronic renal failure is increasing, and it has now become clear that the co-existence of the two problems has an extremely bad prognosis. We propose the severe cardiorenal syndrome (SCRS), a pathophysiological condition in which combined cardiac and renal dysfunction amplifies progression of failure of the individual organ, so that cardiovascular morbidity and mortality in this patient group is at least an order of magnitude higher than in the general population. Guyton has provided an excellent framework describing the physiological relationships between cardiac output, extracellular fluid volume control and blood pressure. While this model is also sufficient to understand systemic hemodynamics in combined cardiac and renal failure, not all aspects of the observed accelerated atherosclerosis, structural myocardial changes and further decline of renal function can be explained. Since increased activity of the renin-angiotensin system, oxidative stress, inflammation, and increased activity of the sympathetic nervous system seem to be cornerstones of the pathophysiology in combined chronic renal disease and heart failure; we have explored the potential interactions between these cardiorenal connectors. As such, the cardiorenal connection is an interactive network, with positive feedback loops, which in our view forms the basis for the SCRS.

2.1 Introduction

Cardiovascular disease is a profound problem in chronic renal failure (CRF), with 43.6% of all deaths in end stage renal disease (ESRD)-patients due to cardiac causes.[1] Death from cardiac causes is 10-20 times more common in patients with CRF than in matched segments of the general population.[2] In ESRD, the prevalence of left ventricular hypertrophy (LVH) and coronary artery disease approximate 75% and 40%, respectively.[3] About half of ESRD-patients will suffer from myocardial infarction (MI) within two years after initiating dialysis therapy, and mortality in these patients is high.[4] Even a slightly decreased kidney function correlates with a substantial increase in cardiovascular disease risk and higher mortality, independent from known other risk factors.[5-10] A recent statement from the AHA[11] determined that both proteinuria and a decline in glomerular filtration rate (GFR) are independent risk factors for the development of cardiovascular disease, and pointed out our lack of knowledge on pathophysiology of this syndrome. Impaired renal function is also associated with adverse outcomes after acute coronary syndromes,[12] percutaneous coronary intervention,[13] coronary artery bypass surgery,[14] or fibrinolytic therapy.[15] The incidence of heart failure as cause of death was inversely related to GFR.[16, 17]

Another major concern is the incipient epidemic of CRF.[18] Not only the prevalence of ESRD increases, but also the number of patients with moderate renal dysfunction.[19] An epidemic of heart failure is also in progress, due to increasing age and better survival after MI.[20] The risk for developing CRF in heart failure has not clearly been defined, but renal dysfunction is often observed in heart failure patients,[21] and is associated with adverse prognosis.[22] The frequency of the combination of heart failure and CRF will thus increase and inescapably come with high morbidity and mortality. The mechanisms that cause decline of kidney function and its repercussions are, however, still poorly understood. In this review, we would like to explore potential pathophysiological interactions that lead to strong interaction between cardiovascular and renal disease.

2.2 The Severe Cardiorenal Syndrome

The strong connection between renal and cardiovascular disease has revived interest in the complex interactions between heart and kidneys. The late Arthur Guyton has extensively described normal physiological interactions between the control of extracellular fluid volume by the kidney and the systemic circulation by the heart (Figure 1).

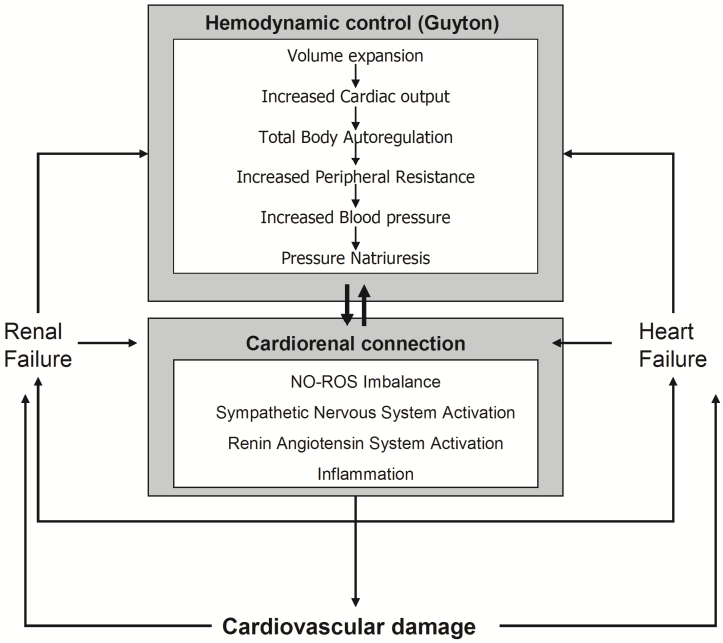

Figure 1. Pathophysiological basis of the severe cardiorenal syndrome.
The model of Guyton explains heart–kidney interaction with respect to extracellular fluid volume, cardiac output, and mean arterial pressure. When one of these organs fails, a vicious circle develops in which the renin–angiotensin system, the NO–ROS balance, the sympathetic nervous system, and inflammation interact and synergize, here called the cardiorenal connection.

The framework of reasoning about the control of extracellular fluid volume (ECFV) and systemic hemodynamics, the concept of total body autoregulation, as well as the renal control mechanisms for sodium excretion with their ”infinite gain”,[23] are of invaluable importance. A recent monologue on volume control in hemodialysis treatment has applied the Guytonian rules to explain and treat cardiovascular

disease.[24] Nevertheless, pathophysiological mechanisms underlying this reciprocal relationship between the heart and kidneys are still enigmatic. We propose the *severe cardiorenal syndrome (SCRS)*, a pathophysiological condition in which combined cardiac and renal dysfunction amplifies progression of failure of the individual organ to lead to astounding morbidity and mortality in this patient group.[25] SCRS is a syndrome with accelerated and extensive cardiovascular disease that has distinct properties not occurring in conditions that affect either organ alone.

In the heart, the consequence of the SCRS is in part due to the described accelerated atherosclerosis in the form of coronary artery stenosis.[26-28] Similarly, LVH is an almost invariable finding, in both clinical and experimental uremia, in the absence of significant hemodynamic stimuli.[29, 30] Rather, the interplay between renal failure and cardiovascular disease reflects an inappropriate remodeling process. The SCRS also involves myocardial micro-angiopathy, manifested in the intramyocardial arterioles by wall thickening and reduced lumen diameter as a consequence of hypertrophy of smooth muscle cells.[31] Clinically, the narrowed lumen diameter may interfere with the already reduced coronary perfusion reserve. The intramyocardial capillaries of uremic rats exhibit decreased capillary density,[32] which increases the oxygen diffusion distance and may further impair the ability of the myocardium to withstand episodes of hypoxia.

Pulse wave velocity (PWV) is a reflection of the elastic properties of 'windkessel' arteries and a high PWV has been recognized as a prognostic factor for cardiovascular events. Uremia affects PWV by functional (angiotensin, volume expansion),[33] and structural (vascular calcification) derangements. The aggressiveness of the calcification process is almost exclusively observed in severe CRF and ESRD,[34] and is not only present in the large arteries but also in coronary plaques of CRF patients.[35-37]

Finally, heart failure can lead to excessive and inappropriate activation of the renin-angiotensin system (RAS),[38] which has been implicated in many ways in the progression of renal disease.[39] Thus, combined renal and cardiac disease invokes a number of forces that are specific for this combination and synergistically aggravate renal and cardiac disease.

2.3 Components of The Cardiorenal Connection Contributing to the SCRS

Central in Guyton's model are the kidney, as regulator of ECFV, and the RAS with its corresponding extensions (aldosterone, endothelin) and its antagonists (natriuretic peptides, nitric oxide (NO)). The model is sufficient to explain the changes in ECFV, blood pressure and cardiac output in combined heart and renal failure. However, can we also explain the accelerated atherosclerosis, cardiac remodeling and hypertrophy, and progression of renal disease observed in the SCRS (Figure 1)?

In this respect, we have recently proposed an extension to the Guytonian model of volume and blood pressure control called the *Cardiorenal Connection (CRC).*[25] Over the past decades, actions have been described by the regulators central in Guyton's model which do not directly control hemodynamics, but affect other aspects of cardiac and renal function. In dissecting the pathophysiological events in the SCRS, we try to couple actions of the regulators of Guyton's model to their extended actions on structure and function of heart and kidney. We propose the renin-angiotensin system (RAS), the balance between NO and reactive oxygen species (ROS), inflammation, and the sympathetic nervous system (SNS) as actual connectors in the CRC (Figure 1). We envision that derangement of one connector of the CRC leads to a vicious circle in which the other connectors become disturbed as well and synergize, ultimately resulting in cardiac and renal functional derangement and structural damage. Accordingly, renal failure and heart failure would lead to the SCRS via common pathophysiological mechanisms: the CRC. The following sections describe evidence on the pathophysiological mechanisms and interactions between connectors of the CRC.

The renin-angiotensin system

Activation of the RAS by low renal perfusion pressure or blood flow serves as a defence against under-perfusion of vital organs, such as in hemorrhage. In heart failure, this response can take a devastating down-hill course: volume retention due to the hemodynamic and reabsorptive actions of angiotensin II (Ang II) develops,[40] with further congestive heart failure as a consequence. Unfortunately, inappropriate activation of the RAS is also one of the characteristics of renal

failure.[41] Besides the (dys)regulation of ECFV and vasoconstriction, one of the most deleterious actions of the RAS in the CRC is activation of NADPH-oxidase by Ang II, resulting in formation of reactive oxygen species (ROS).[42] This has been documented in endothelial cells, vascular smooth muscle cells,[43] renal tubular cells,[44] and cardiomyocytes.[45] Interesting observations in this context are raised NADPH-oxidase activity in hearts of patients with end-stage heart failure,[46] and increased NADPH-oxidase mediated ROS-release in glomeruli of Dahl salt-sensitive rats with heart failure, which could be attenuated by angiotensin-converting enzyme (ACE) inhibition.[47] Moreover, ACE inhibition has been shown to increase NO bioavailability in patients with coronary artery disease, possibly related to reduced vascular oxidative stress or increased extracellular superoxide dismutase (SOD) activity.[48]

Ang II, potentially acting via changes in the cellular redox state, is implicated in vascular inflammation via the nuclear factor kappa B (NF-κB) pathway, which induces production of chemotactic and adhesion molecules.[49, 50]

The RAS interacts with the SNS by complex mechanisms.[51] It has been found that the stimulus for the sympathetic hyperactivity observed in renal failure arises from the failing kidneys,[52] and that increased sympathetic outflow in CRF could be controlled with ACE-inhibition.[53,54] Blocking Ang II signalling reduced SNS hyperactivity after MI in rats, attenuating ensuing development of heart failure.[55] Interactions of the RAS with the other cardiorenal connectors are shown in Figure 2.

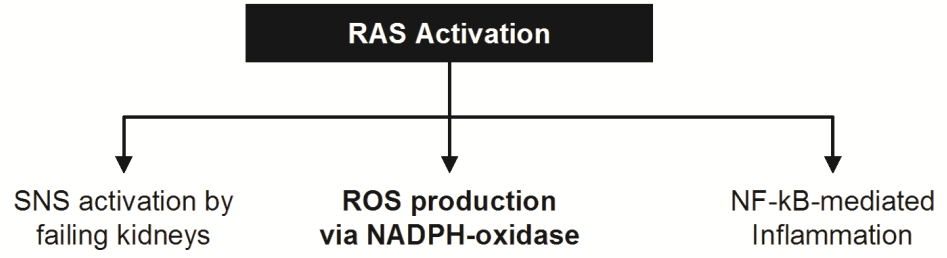

Figure 2. The renin-angiotension system (RAS) affects the other cardiorenal connectors by inducing sympathetic nervous system (SNS) activation in kidney failure, generation of reactive oxygen species (ROS) by nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase , and nuclear factor kappa B (NF-kB) mediated pro-inflammatory gene expression.

The balance between NO and ROS

Nitric oxide is important in renal control of ECFV and blood pressure by causing vasodilation, natriuresis and desensitization of tubuloglomerular feedback.[56] There are now many indications that superoxide has the opposite effect on ECFV control and can contribute to high blood pressure.[57-60] In the SCRS, the balance between NO and the ROS is skewed towards the latter by increased production of ROS, a low antioxidant status, and lower availability of NO. Increased levels of different oxidative stress markers, like F_2-isoprostane,[61] and antibodies against oxidized LDL,[62] have been found in dialysis patients. A low anti-oxidant status is caused by oxidative inactivation, decreased availability of antioxidant vitamins and removal of water-soluble antioxidants through the dialysis membrane.[63] Oxidative stress is further increased by interplay between the uremic state and inflammatory reactions on the dialysis membrane. A relative NO-deficiency in renal failure is caused by reaction of NO with oxygen radicals, as well as by high concentrations of circulating asymmetric di-methyl arginine (ADMA), an endogenous NOS inhibitor.[64] In heart failure, increased oxidative stress has also been demonstrated,[46] and decreased antioxidant status was found in rat myocardium after MI, which was associated with progression to heart failure.[65] Interestingly, hemodynamic improvement by captopril and prazosin led to enhanced antioxidant status.[66] Kielstein *et al*.[67] also showed a relationship between reduced renal perfusion, impaired NO-mediated endothelial vasodilatation and high concentrations of ADMA in patients with normotensive heart failure, markedly resembling the situation in CRF patients.

Additional potential interactions between the NO-ROS imbalance and other cardiorenal connectors in the SCRS are depicted in Figure 3. Oxidative stress by hydrogen peroxide (H_2O_2) has been shown to increase activity of preganglionic sympathetic neurons *in vivo* and *in vitro* in rats, raising mean arterial pressure and heart rate.[68] Also, renal sympathetic nervous activity in spontaneously hypertensive rats was found to be regulated by vascular superoxide concentrations.[69]

In renal failure, oxidative stress imposes damage on DNA (8-oxo-OH-deoxyguanosine), proteins (carbonyl compounds,[70] advanced oxidation protein products)[71], carbohydrates (advanced glycation end-products)[72] and lipids (oxidized LDL)[62]. These substances have pro-inflammatory effects by attracting and activating leucocytes,[73, 74] but they can also damage endothelial cells.[75]

Oxidative stress is a major initiator of an inflammatory response, resulting in a shift towards production (and activation) of pro-inflammatory cytokines, in particular IL-1, IL-6, and tumor necrosis factor alpha (TNFα).

Although as yet not completely resolved, oxidative damage to the renal tubular or interstitial cells may interfere with feedback systems involved in renin secretion and angiotensin formation in the SCRS. Chronic inhibition of NO synthesis causes upregulation of cardiac ACE and Ang II receptors, possibly mediating inflammatory changes.[76]

Himmelfarb *et al*.[77] have termed oxidative stress the "elephant", or key-point, in uremia. Treatments that decrease superoxide production (such as NADPH-oxidase inhibitors), aid in scavenging ROS, or support the function of NO, are intriguing clues that support our concept. One relatively small trial has reported a positive effect of antioxidant therapy on cardiovascular end points in patients with renal failure,[78] but more evidence is needed.

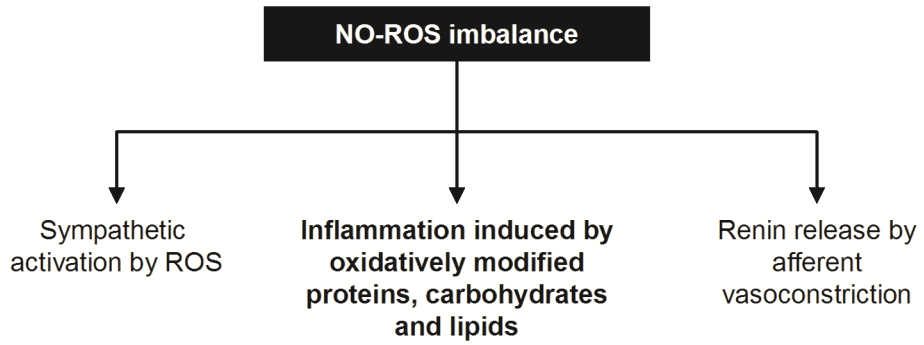

Figure 3. Imbalance between NO and ROS is a central event in cardiovascular diseases. In the cardiorenal connection, this balance may influence sympathetic nervous activity, release of renin and angiotensin, and promote inflammation by oxidative modification of substances.

Inflammation

Together with increased oxidative stress, inflammation has been designated the other common denominator in uremia.[79] The combined occurrence of chronic renal insufficiency and high C-reactive protein (CRP) levels has a more than additive

effect on the incidence of myocardial infarction and death.[80] In CRF, circulating levels of CRP [81] and several pro-inflammatory cytokines such as IL-1b, IL-6 and TNFα, are predictors of atherosclerosis.[82, 83]

It has been suggested that inflammation will aggravate heart failure. In patients with heart failure, elevated levels of TNF-α and IL-6 have been found in both plasma and myocardium, and are related to progression of the disease.[84, 85] Interleukin-18 has also been associated with cardiac dysfunction after MI.[86] The exact role of the activation of inflammatory cells is as yet far from clear; however, in both CRF and heart failure a state of chronic inflammation is present. This low-grade inflammation can cause ROS production by activating leucocytes to release their oxidative contents.[87] In cultured rat vascular smooth muscle cells, IL-6 induced upregulation of the AT_1 receptor and Ang-II mediated production of ROS, providing evidence for a possible link between inflammation and RAS activation.[88] Cytokines may stimulate renin secretion as a component of the systemic stress response, and tubulointerstitial inflammation may have effects on adaptive responses of glomerular hemodynamics to impaired renal function.[89] After myocardial infarction IL-1β is produced,[90, 91] which has been shown to stimulate norepinephrine release from sympathetic neurons.[92] Interactions between inflammatory factors and the other connectors are depicted in figure 4.

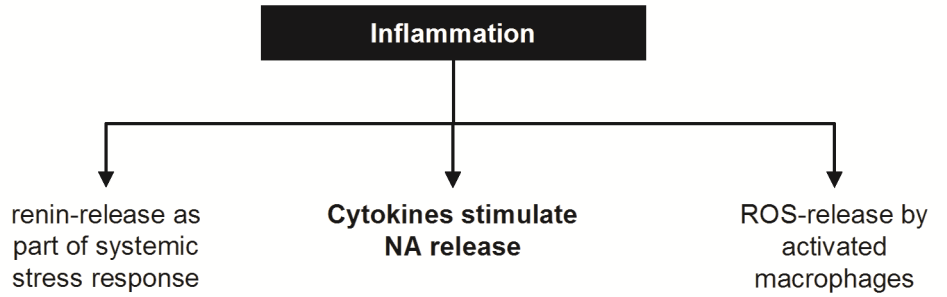

Figure 4. Persistent inflammation has been found in both renal and heart failure. By altering ROS functioning, and promoting ROS and noradrenaline (NA) formation, inflammation contributes to the positive feedback loops in the cardiorenal connection.

Sympathetic nervous system

By stimulating renin release via renal sympathetic neurons, the SNS contributes to long-term regulation of ECFV and blood pressure. Converse[52] was the first to report increased peripheral sympathetic nerve activity in ESRD, which was corrected when the diseased kidneys were removed. The SNS is initially activated in heart failure by the baroreflex to provide inotropic support and preserve cardiac output. However, excessive sympathetic activity can induce cardiomyocyte apoptosis, hypertrophy and focal myocardial necrosis.[93] Cardiac hypertrophy is partly due to direct actions of catecholamines, as several studies have shown that noradrenaline induces hypertrophy of cultured cardiomyocytes.[94, 95] Interestingly, this action involves induction of superoxide.[94, 95] Chronically, sympathetic overactivity causes beta-adrenoceptor insensitivity in both renal failure[96] and heart failure.[97, 98] This can lead to a disturbed baroreceptor reflex, reduction in heart rate variability and increased susceptibility to arrhythmia. Whether the atherosclerotic process is associated with increased sympathetic activation is unclear. However, sympathetic overactivity can affect lipid metabolism, and beta-blockers have been shown to have anti-atherosclerotic properties.[99, 100] There are several indications that the SNS affects the other connectors of the CRC, for instance RAS activation, production of ROS by sympathetic neuroactive substances, and activation of the immune system (Figure 5).

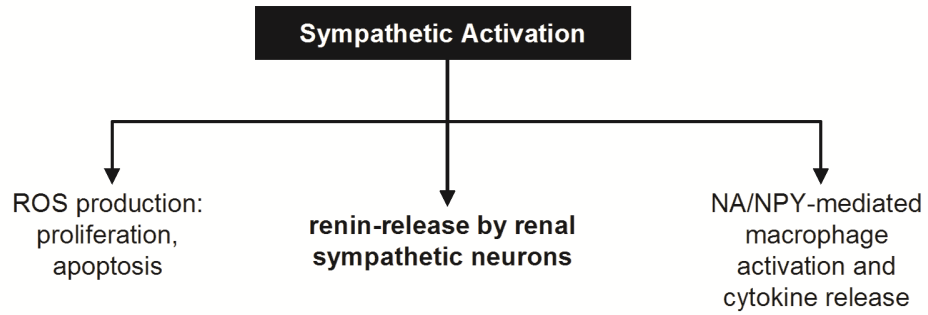

Figure 5. Sympathetic nervous activity is increased in both renal and heart failure. By affecting the other cardiorenal connectors it can play a significant role in the SCRS. It stimulates renin release from the kidneys, generates ROS, and induces inflammation. NPY: neuropeptide Y.

Next to direct sympathetic innervation of the kidneys, renin release can be enhanced because prolonged SNS overactivity has a growth-promoting effect on the wall of intrarenal blood vessels.[101] This effect has recently been found to be mediated by ROS production.[102] In ischemia/reperfusion damage in the kidney, H_2O_2 formation by monoamine oxidase enzymes induced a pro-apoptotic cascade in proximal tubular cells.[103] The SNS may induce inflammation by norepinephrine-mediated cytokine production from liver[104] and heart,[105] and beta-blockade after experimental MI diminished myocardial cytokine gene expression.[106] Neuropeptide Y (NPY) is a neurohormone released by sympathetic activation that is involved in the prolonged vasoconstriction associated with stress. It can act as a vascular growth promoter, leading to neo-intima formation and has been associated with carotid artery atherosclerosis.[107] Thirdly, it affects the immune response by altering cytokine release and immune cell function.[108, 109] High levels of NPY have been demonstrated after MI and in patients with heart failure.[110] Thus, the sympathetic nervous system can modulate the other cardiorenal connectors.

2.4 Conclusion

In this review we extend the solid framework of Guyton for extracellular fluid volume and blood pressure regulation. Epidemiological data point towards reciprocal connections between the heart and kidneys in disease, encompassed in the severe cardiorenal syndrome (SCRS). This connection is, to our opinion, more elaborate than the hemodynamic model of Guyton alone. With the model of the cardiorenal connection (CRC), we hope to unravel the interactions underlying the deleterious consequences of the SCRS by taking into account the extended cardiorenal effects of the RAS, the balance between NO and ROS, inflammation, and the SNS. Oxidative stress and inflammation have been strongly implicated in the SCRS. Although several interventions (e.g. blockade of the RAS and the SNS or physical exercise) have been shown to be effective in reducing oxidative stress and inflammation, at present no effective strategy to directly influence these factors has been devised. Since interactions in the CRC induce positive feedback loops at many points, it is considered likely that multiple interventions are needed to stop the vicious circle. Ideally, when all four factors are taken into account, we will be

able to predict the clinical course of most patients with SCRS. Taken further, if all four factors were to be corrected, we would stand a chance to more effectively control the progression of the SCRS. Because only fragmentary data exist on interactions between the cardiorenal connectors in the setting of the SCRS, experimental and clinical studies are needed to test this model.

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Part II

Chapter 3

Bidirectional organ damage in combined experimental chronic kidney disease and heart failure

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Co-existence of chronic kidney disease (CKD) and heart failure (HF) in humans is associated with worsened outcomes. However, the reciprocal nature of cardiorenal interactions has scarcely been studied in animal models. We hypothesized that pre-existent CKD worsens cardiac outcome after MI, and conversely that ensuing HF worsens progression of CKD.

Male Lewis rats were subjected to subtotal nephrectomy (SNX) or sham operation (CON). In wk 9, we performed coronary ligation (CL) or sham-surgery to realise 4 groups: CON, SNX, CON+CL, and SNX+CL. *In vivo* measurements were performed in wk 8, 11, 13 and 15. In wk 16, cardiac hemodynamics and end-organ damage was assessed.

Blood pressure was significantly lower in SNX+CL vs. SNX. Despite this, glomerulosclerosis was more severe in SNX+CL vs. SNX. Two wks after MI, rats with SNX+CL had more cardiac dilatation compared to CON+CL (end-diastolic volume index: 0.28±0.01 vs. 0.19±0.01 mL/100g BW; $P<0.001$), despite similar infarct size. During follow-up, SNX+CL rats developed a lower ejection fraction, and the only rats that died were SNX+CL (2 out of 9). In SNX+CL, end-diastolic pressure (18±2 mmHg) and *tau* (29±4 msec.), the time-constant of active relaxation, were significantly higher compared to SNX (13±1 mmHg, 20±1 msec.; $P<0.01$) and CON+CL (11±2 mmHg, 17±1 msec.; $P<0.01$).

This study demonstrates the existence of reciprocal organ damage in a new model of combined CKD and HF, which appear to be separate from systemic hemodynamic changes.

3.1 Introduction

Recently a classification of the Cardiorenal Syndromes according to event-order and time-frame has been proposed, based on the long-standing clinical presumption that failure of the heart and kidneys interact.[1-3] We previously defined the "Severe Cardiorenal Syndrome" (SCRS) as a syndrome where pre-existent failure of either kidney or heart will amplify progression of failure of the other organ.[2] Nevertheless, direct evidence for a bidirectional interaction between the two failing organs is scarce. The increased risk for cardiac disease in advancing stages of chronic kidney disease (CKD) is now widely recognized.[4] When CKD is complicated by myocardial infarction (MI) or left ventricular systolic dysfunction, prognosis is dismal.[5, 6] Conversely, in heart failure (HF) decreased kidney function is independently associated with adverse outcome.[7] Exactly how this apparent organ-organ interaction works is unknown.[8] Cardiovascular disease is associated with increased incidence of kidney disease,[9] and after MI the decline of renal function may be accelerated.[10] Several patient studies assessed cardiac remodeling after MI in patients with CKD, but these yielded conflicting results regarding left ventricular (LV) dilatation and systolic function.[11, 12] Underuse of appropriate medical therapy may also play a role in the worse prognosis of CKD patients experiencing an MI.[13]

Results from clinical studies are however observational, lack histological end-points, and are confounded by selection bias, inconsistent definition of end-points, and medication use. Therefore, further exploration of the mechanisms of cardiorenal interactions must rely on animal studies, in which timing and severity of the disease are controlled, progression of disease can be followed, and histological end-points are assessed.

In the rat CKD model of subtotal (5/6th) nephrectomy (SNX) cardiac systolic function is generally maintained.[14, 15] Conversely, after MI in rats, renal histological damage or proteinuria is absent (although GFR may be decreased).[16, 17] Thus, it appears that both organs need to be affected to cause acceleration of damage and failure typical for the SCRS. To try to explain the dismal outcome after MI in CKD, a few animal studies investigated the effect of 'dual damage' to heart and kidneys. Dikow *et al.*[18] showed that rats with short-term uremia have larger myocardial

infarctions after ischemia-reperfusion injury than non-uremic controls, but they did not assess whether this was associated with worsened cardiac remodeling or failure. Two studies from the group of van Dokkum *et al.*[16, 19] assessed the effect of MI shortly after a renal insult in rats. When uninephrectomy (UNX) was followed by MI one week later, progressive proteinuria and glomerular damage developed.[16] Conversely, an MI two weeks after SNX failed to worsen renal damage.[19] These studies may have been hampered by the absence (UNX) or short duration of uremia before MI, and the absence of overt heart failure after MI. Thus, evidence is still lacking as to whether the presence of CKD as such adversely affects cardiac remodeling and cardiac dysfunction after MI, and whether the resultant HF in turn further worsens renal injury and failure

The aim of the present study was to investigate the bi-directional nature of CKD and MI-induced heart failure in a technically feasible longitudinal animal model. We hypothesized that pre-existent CKD worsens cardiac outcome after MI, and conversely that HF following MI aggravates CKD. To study this bidirectional interaction, we used rats with and without SNX, and performed either coronary ligation (CL) or sham operation 8 weeks later.

3.2 Methods & Materials

Overall study setup

The study protocol was approved by the Ethical Committee on Animal Experiments of the University of Utrecht, Utrecht, The Netherlands. Male inbred Lewis rats (Lew/Crl; 180 – 200 grams) were purchased from Charles River, Germany, and housed in a climate-controlled facility with a 12:12-hour light:dark cycle. At t= -1 wk a two-stage subtotal nephrectomy by resection or sham operation was performed as described previously.[20] From t=1 wk onward rats received standard powdered chow supplemented with 6% NaCl until the end of the study. At t= 9 wks, rats from both groups were either subjected to left anterior descending coronary artery ligation (CL) or sham operation. This resulted in four groups: CON (sham-SNX + sham-CL; n=10); SNX (SNX + sham-CL; n=12); CON+CL (sham-SNX + CL; n=9); and SNX+CL (n=9). Rats were followed up to wk 16. *In vivo* measurements were

carried out in a selection of rats from the CON and SNX groups at wk 8 before coronary ligation or sham surgery. After CL, in wk 11, 13 and 15, all rats from each subgroup were measured. CON+CL and SNX+CL rats without visible MI on echocardiography and an ejection fraction (EF) > 40% at wk 11 were excluded from the study. Ejection fraction was calculated from end-diastolic and end-systolic volumes obtained with the area-length calculation, on B-mode cine-loops recorded in the parasternal long axis view.[20] Based on these criteria, approximately 45% of animals qualified for further study. The other rats were excluded because they either had no infarction or a small/medium infarction with EF > 40%. In wk 16, invasive hemodynamic measurements of the LV were performed, rats were euthanised and organs were removed, weighed and processed for histological quantification and determination of mRNA expression.

Surgical procedures

Subtotal nephrectomy was performed by resection as described previously.[20] In short, the right kidney was removed under isoflurane anaesthesia and buprenorfine analgesia, and weighed. One week later, the poles of the left kidney were excised with a total weight approximatelty 2/3^{rds} of the previously removed right kidney. For coronary ligation, rats were anaesthetized with isoflurane and buprenorfine, intubated and ventilated according to the settings advised by Horstick et al.[21] After a left thoracotomy, the left descending coronary artery was ligated with a 5/0 Ethibond suture without externalizing the heart. Lidocaine (10 mg/kg) was administered peri-operatively to reduce the risk of fatal arrhythmias.[22]

In vivo measurements

We performed tail-cuff systolic blood pressure (SBP) registration and collected 24-h urine samples, for determination of creatinine and protein excretion, with the rats in individual metabolic cages while fasting, as described.[20] Echocardiography was performed as described previously[20] (also see Appendix for expanded Methods). We used the echocardiographic images to determine differences in infarct scar size at wk 11, by calculating the ratio of the length of the thin, akinetic, part of the LV divided by the total LV circumference in diastole, measured through the middle of the ventricular wall in the long axis (LAX) and short axis (SAX) views. After

echocardiography, a blood sample (0.5 mL) was collected in EDTA for determination of plasma urea and creatinine.

Hemodynamic measurements

Rats were intubated and ventilated with 2% isoflurane in 40% O_2. The left jugular vein was cannulated for continuous infusion of saline. A Millar micro-pressure catheter (Millar Instruments, Houston, Tx, USA) was inserted into the LV via the right carotid artery. After a 15-min stabilization period, LV pressures were recorded with Chart software (ADInstruments, Spechbach, Germany) at 3 separate intervals of 5-10 seconds with the ventilator turned off. Hemodynamic data were calculated using the Chart Blood Pressure module (ADInstruments, Germany) for the following parameters: maximum LV pressure, end-diastolic pressure, the maximal rate of pressure increase (maximum dP/dt) and pressure decrease (minimum dP/dt), the exponential time constant of active relaxation (*tau)*. Mean arterial pressure was calculated from pressure data registered in the carotid artery, from which systemic vascular resistance (SVRI) was calculated by dividing it by the level of cardiac index (CI) derived from echocardiography.

Organ weights and histology

Focal segmental glomerulosclerosis (FSGS) and tubulo-interstitial damage were scored on periodic acid Schiff (PAS)-stained kidney sections.[23, 24] FSGS was scored on 50 separate glomeruli by quadrants, on a scale of 0 to 4, where 0 means no quadrant affected and 4 means that the whole glomerulus was affected. Cardiomyocyte area was measured on PAS-stained myocardial slices in sections with transversely cut myocardial fibers (in remote myocardium in hearts with MI) by tracing the cellular border on photomicrographs of at least 50 different cardiomyocytes with a computer assisted image analysis system (OptiMas, Houston, TX) in a blinded manner. Infarct scar size was measured on photomicrographs of transverse sections of the heart stained with Sirius Red, by dividing the length of the infarct scar by the circumference of the total LV section, traced in mid-wall using ImageJ software.[25] All measurements were performed by an experienced technician blinded to the group allocation.

Quantitative polymerase chain reaction

Expression of brain natriuretic peptide (BNP; Rn00580641) and connective tissue growth factor (CTGF; Rn00573960) in cardiac apical tissue was assessed by qPCR as described.[26] Cycle time(Ct) values for BNP and CTGF were normalized for mean Ct-values of Calnexin (Canx; Rn00596877) and β-actin (Actb; Rn00667869), which we previously determined to be the two most stable housekeeping genes across all groups.

Statistical Analysis

Data is presented as mean ± SEM. Data were analyzed and graphed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). Two-way analysis of variance (ANOVA) with the Student-Newman-Keuls (SNK) post-hoc test was done per time-point across all groups, and three-way ANOVA with SNK post-hoc test across all time-points and groups. Differences between wk 8 and wk 11 were tested by one-way ANOVA with the Holm-Sidak post-hoc test with data in wk 8 as reference. Data that was not normally distributed was log-transformed or ranked to achieve normality. Statistical significance was reached with p-values below 0.05.

3.3 Results

General characteristics

Rats with SNX had a lower body weight than their non-uremic counterparts, which was not further lowered by subsequent MI (Table 1). Acute post-operative mortality after coronary ligation (within 48h) was similar in SNX rats (28%) and CON rats (22%). Two out of 9 animals (22%) died in the SNX+CL group after CL (one in wk 12 and one in wk 14). There was no long-term mortality in the other groups.

Systolic blood pressure and renal variables

Rats with SNX developed stable hypertension with SBP of about 160 mmHg (Figure 1, A). In both groups with MI, SBP was lower than their respective controls, with a larger difference in SNX+CL vs. SNX. Subtotal nephrectomy caused chronic stable elevations in plasma urea and creatinine from wk 6 onward (data not shown), and creatinine clearance was reduced (Table 1). These renal variables were not

significantly affected by CL in either CON+CL or SNX+CL rats. Proteinuria was not significantly higher in SNX+CL rats vs. SNX alone (Figure 1, B).

Table 1. Biometric parameters at wk 15.

	CON (n=10)	CON+CL (n=9)	SNX (n=10)	SNX+CL (n=7)
Body weight (g)	424 ± 8	426 ± 9	378 ± 11 ***	361 ± 14 $$$
Kidney:				
Creatinine clearance (mL/min/100 g BW)	0.74 ± 0.06	0.67 ± 0.09	0.30 ± 0.03 ***	0.27 ± 0.04 $$$
Heart:				
Heart rate (beats/min)	368 ± 6	349 ± 7	358 ± 7	329 ± 14 $
Cardiac index (mL/min/100 g BW)	30 ± 2	17 ± 1 ***	29 ± 2	14 ± 1 ###

Data as mean ± SEM.
*** *P*<0.001 vs. CON; $ *P*<0.05, $$$ *P*<0.001 vs. CON+CL; ### *P*<0.001 vs. SNX.

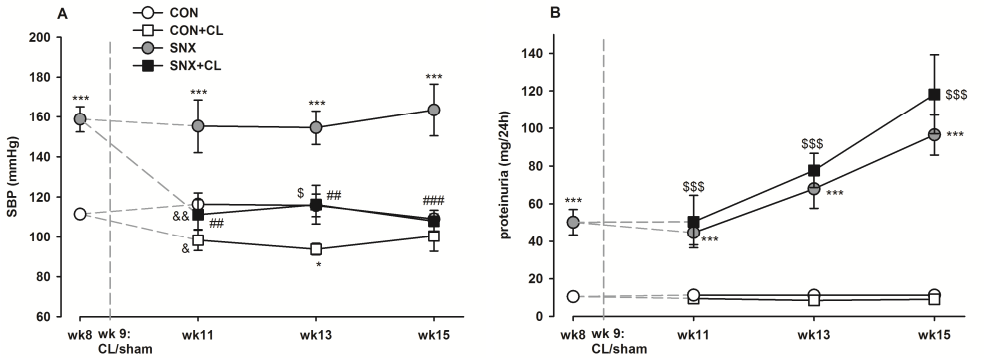

Figure 1. (A) Tail-cuff systolic blood pressure (SBP; mmHg). In SNX+CL vs. SNX rats, the difference in SBP after MI was larger than in CON+CL vs. CON rats. **(B)** Proteinuria in all groups.
Mean ± SEM. If not visible, the error bars fall within the symbol.
* *P*<0.05, ** *P*<0.01, *** *P*<0.001 vs. CON; $ *P*<0.05, $$ *P*<0.01, $$$ *P*<0.001 vs. CON+CL;
# *P*<0.05, ## *P*<0.01, ### *P*<0.001 vs. SNX, & *P*<0.05, && *P*<0.01, &&& *P*<0.001 vs. wk 8 in respective non-CL group.

Focal glomerulosclerosis scores

The left kidney remnant showed marked hypertrophy in all SNX animals, with no effect of CL. Tubulo-interstitial damage was present in both SNX groups, without an effect of MI in either SNX or CON rats (supplementary data in Appendix). In

SNX, predominantly severe focal segmental glomerulosclerosis (FSGS) was seen (Figure 2). A small increase in mild FSGS (1 quadrant affected; score 1) was apparent in animals with CL alone. In SNX+CL, the amount of diseased glomeruli was increased across the spectrum of FSGS but was most apparent in the number of glomeruli in which one or all quadrants (severe FSGS, score 4) were affected. This led to a nearly 50% decrease in the percentage of unaffected glomeruli compared to SNX alone.

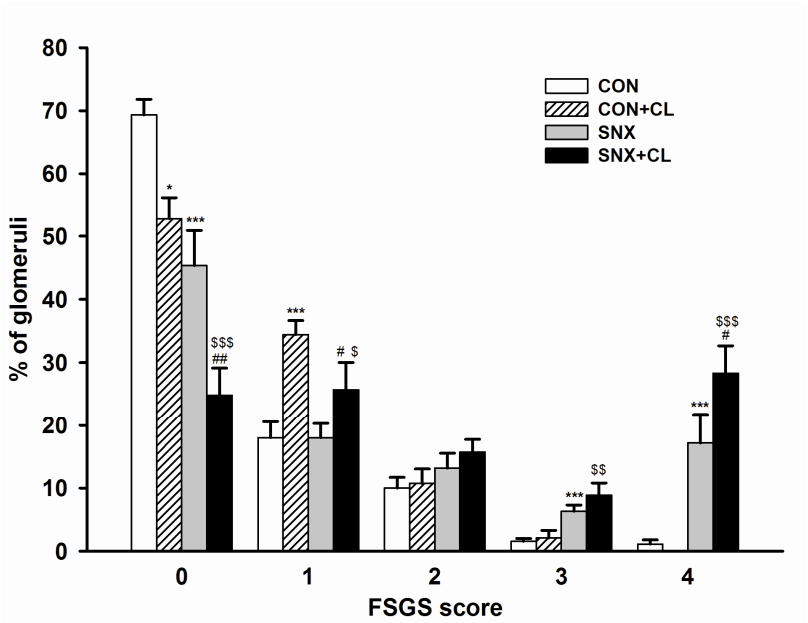

Figure 2. Distribution of focal segmental glomerulosclerosis (FSGS) scores, which indicate the number of quadrants affected. 0 = healthy glomeruli, 1 = 25% damage, 2 = 50% damage, 3 = 75% damage, and 4 = totally sclerotic.
Mean ± SEM, * P<0.05, *** P<0.001 vs. CON; $ P<0.05, $$ P<0.01, $$$ P<0.001 vs. CON+CL; # P<0.05, ## P<0.01.

Cardiac structure and function as assessed by cardiac sonography

Given the differences in body weight (Table 1), cardiac parameters are expressed per 100 g BW, where applicable. Subtotal nephrectomy alone induced progressive end-diastolic dilatation, which was most prominent at wk 15 (Figure 3, panel A). Coronary ligation alone was associated with an acute increase in cardiac volumes that was stable during follow-up. In SNX+CL, cardiac dilatation was significantly

worse ($P < 0.001$) compared to that in CON+CL and SNX at wk 11 (Figure 3, A&B). The difference between SNX+CL and SNX was much greater than that between CON+CL and CON at that time-point, and 2-way ANOVA identified a significant interaction between SNX and CL ($P = 0.01$). Values of end-diastolic and end-systolic volume index remained significantly higher in the SNX+CL group vs. CON+CL during follow-up, although the statistical significance of the interaction disappeared from wk 13 onward.

Rats with SNX alone exhibited a steady decline in EF, and CL rats had significant systolic dysfunction from wk 11 onward (Figure 3, C). In rats with the combined intervention, EF was not significantly different from that in CON+CL at wk 11 but thereafter started to decline and was significantly lower in SNX+CL rats vs. CON+CL rats. Un-indexed stroke volume was significantly lower in SNX+CL vs. CON+CL at wk 15 ($P < 0.05$; Supplementary data in Appendix), but this was not apparent when corrected for BW (SVI; Figure 3, D). Figure 3, panel E, illustrates that, at wk 15, SNX+CL rats have a worse EDVI-SVI relationship, suggesting further decompensation.

Rats with SNX+CL had a slightly lower heart rate, and cardiac index (CI) was borderline decreased at wk 15 compared to rats with CON+CL (Table 1; $P = 0.065$). When tested with 3-way ANOVA and time was factored in, EF and CI were significantly lower in the SNX+CL vs. the CON+CL group during follow-up.

The fractional infarct scar size was similar between CON+CL and SNX+CL at wk 11 measured on echocardiographic images in both the long axis (31.4 ± 2.3% vs. 35.6 ± 1.8%, resp., N.S.) and the short axis view (21.2 ± 1.5% vs. 22.4 ± 1.1%, resp., N.S.).

Invasive cardiac hemodynamics

Table 2 shows LV hemodynamic data acquired in wk 16. Decreases in LV maximum pressure and maximal rate of pressure increase (maximum dP/dt) were observed that were larger in SNX+CL than in CON+CL vs. their respective non-CL groups, similar to the differences observed by tail-cuff SBP. Left ventricular end-diastolic pressure (EDP) was additively raised in CON+CL, SNX, and SNX+CL. Minimum dP/dt, the maximal rate of pressure decline during diastole, was not different between SNX and CON, but reduced in both CL-groups compared to their

respective controls. On the other hand, *tau*, the time-constant of active diastolic relaxation, was prolonged in SNX animals and even further worsened in SNX+CL. Systemic vascular resistance index (SVRI), as an indication of its contribution to afterload, was significantly higher in rats with SNX and SNX+CL compared to their respective non-SNX controls. There was no further increase in SVRI in SNX+CL.

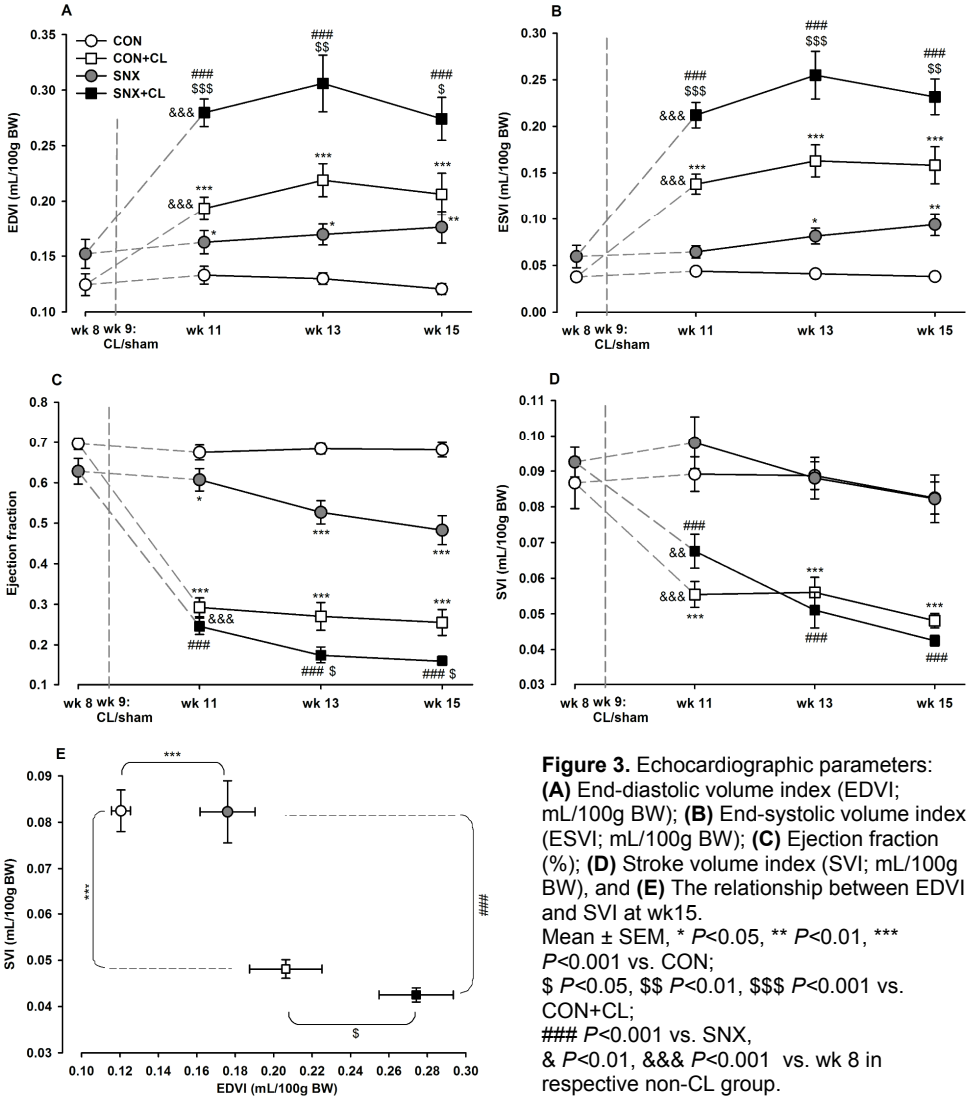

Figure 3. Echocardiographic parameters: **(A)** End-diastolic volume index (EDVI; mL/100g BW); **(B)** End-systolic volume index (ESVI; mL/100g BW); **(C)** Ejection fraction (%); **(D)** Stroke volume index (SVI; mL/100g BW), and **(E)** The relationship between EDVI and SVI at wk15.
Mean ± SEM, * *P*<0.05, ** *P*<0.01, *** *P*<0.001 vs. CON;
$ *P*<0.05, $$ *P*<0.01, $$$ *P*<0.001 vs. CON+CL;
P<0.001 vs. SNX,
& *P*<0.01, &&& *P*<0.001 vs. wk 8 in respective non-CL group.

Table 2. Left ventricular hemodynamic variables, measured in wk 16.

	CON (n=6)	CON+CL (n=5)	SNX (n=6)	SNX+CL (n=5)
Max. LVP (mmHg)	136 ± 5	109 ± 7 *	190 ± 11 ***	141 ± 10 $ ###
EDP (mmHg)	6.2 ± 0.6	10.7 ± 1.2 *	12.5 ± 1.2 **	17.9 ± 1.9 $$ #
Maximum dP/dt (mmHg/s)	7,622 ± 655	5,726 ± 592	11,181 ± 727 **	7,416 ± 1085 ##
Minimum dP/dt (mmHg/s)	-10,013 ± 677	-5,500 ± 546 ***	-9,826 ± 878	-5,605 ± 510 ###
Tau (msec)	14.6 ± 0.8	16.9 ± 0.9	19.6 ± 1.4 *	28.6 ± 4.2 $$ ##
SVRI (mmHg/ml/min/100g BW)	3.65 ± 0.41	4.42 ± 0.46	7.22 ± 0.95 ***	8.09 ± 0.62 $$$

Max. LVP: maximum left ventricular pressure. EDP: end-diastolic pressure; Maximum dP/dt: maximal rate of pressure increase; Minimum dP/dt: maximal rate of pressure decline; *Tau*: exponential time-constant of relaxation; SVRI: systemic vascular resistance index. Data as mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. CON; $ $P<0.05$, $$ $P<0.01$, $$$ $P<0.001$ vs. CON+CL; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs. SNX.

Organ weight and histology

Weight of the LV corrected for BW was slightly increased in CON+CL, and grossly increased in SNX (Figure 4, A). Rats with SNX+CL showed decreased corrected LV weight compared to SNX alone, but this was still significantly higher compared to rats with CL alone. Cardiomyocyte area in the LV, as a measure of cellular hypertrophy, was increased in CL and SNX alone similar to the changes in LV mass, but was not further increased in SNX+CL (Figure 4, B). Assessment of BW-corrected right ventricle RV weight and wet lung weight revealed that both were borderline increased in SNX, and slightly more so in animals with CL only (Figure 4, C&D). Both parameters were additively worsened in SNX+CL. Myocardial infarct size, determined on transverse Sirius Red-stained cardiac slices, was not significantly different between CON+CL (34 ± 4%) and SNX+CL (39 ± 2%).

Cardiac gene expression

Gene expression of brain natriuretic peptide (BNP) and connective tissue growth factor (CTGF) were assessed by qPCR in apical heart tissue. Expression of both proteins showed a similar pattern of stepwise upregulation in CON+CL, SNX, and

SNX+CL (Figure 5), but there was no correlation between individual levels of expression when these 3 groups were combined (R = 0.231; *P* = 0.146).

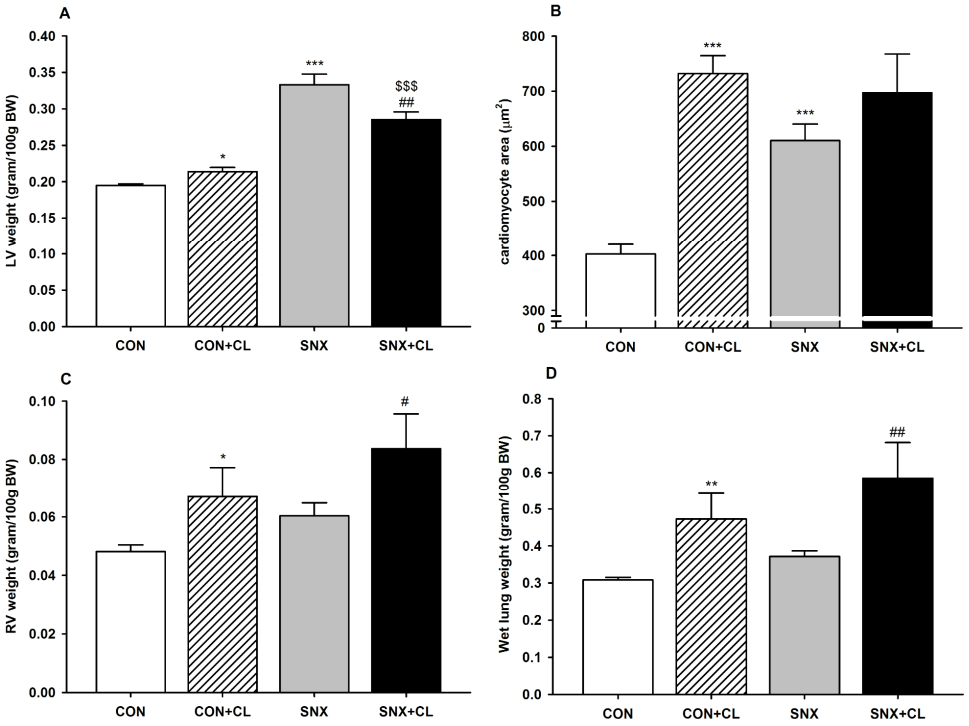

Figure 4. Terminal morphological variables. **(A)** LV weight corrected for BW; **(B)** cardiomyocyte area; **(C)** corrected RV weight, and **(D)** corrected wet lung weight.
Mean ± SEM; * *P*<0.05, ** *P*<0.01, *** *P*<0.001 vs. CON; $$$ *P*<0.001 vs. CON+CL; # *P*<0.05, ## *P*<0.01.

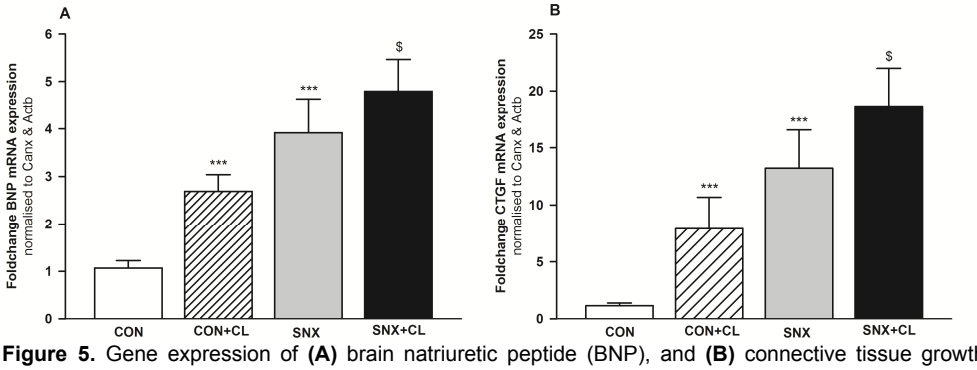

Figure 5. Gene expression of **(A)** brain natriuretic peptide (BNP), and **(B)** connective tissue growth factor (CTGF) in cardiac apical tissue, assessed by qPCR.
Mean ± SEM, *** *P*<0.001 vs. CON; $ *P*<0.05 vs. CON+CL.

3.4 Discussion

Whether the combination of heart and kidney failure can lead to a vicious cycle, both in an acute and in a chronic setting, irrespective of which organ initially fails, is currently the subject of active debate.[1-3] The present study shows bi-directional worsening of organ damage in a rodent model of combined chronic kidney disease induced by SNX, and heart failure induced by MI. The current model replicates the long-term changes seen in patients and provides a feasible, albeit technically challenging, model of the SCRS.

Subtotal nephrectomy induced CKD as evidenced by reduced GFR, progressive proteinuria, and substantial FSGS. When CKD was followed by MI, FSGS was significantly worsened. After SNX, glomerular hypertension and hyperfiltration develops in the remaining nephrons which is associated with progression of glomerulosclerosis.[27] Glomerular hypertension due to efferent vasoconstriction has also been documented in rats with MI and HF.[28] When CKD and MI are combined, glomerular hypertension could be even more pronounced, which could contribute to the worsened FSGS observed in the current study. This occurred while blood pressure was lower, suggesting a dissociation of intraglomerular and systemic hemodynamics and/or direct glomerular damage by neurohormonal factors, such as activation of the renin-angiotensin system (RAS).

Although both the CKD groups exhibited progressive proteinuria over time, this was not worsened by combined CKD and HF. Remarkably, levels of tail-cuff SBP and carotid artery MAP were lower in the SNX+CL group and creatinine clearance (GFR) was unchanged. Proteinuria can worsen separate from changes in BP and GFR, pointing to an intrinsic renal effect.[20, 29] In patients with heart failure, albuminuria was found to be independent of systemic blood pressure as well, but increased when renal blood flow decreased.[30] The fact that the worsened FSGS was not associated with an increase in proteinuria in both the CON+CL and SNX+CL group may be due to type of damage that occurred. In both groups an increase in glomerulosclerosis score 1 was documented which may be too mild to cause protein leakage, as no proteinuria was observed in rats with CON+CL. In the SNX+CL group, a significant increase in FSGS score 4 was also observed. These glomeruli are totally sclerotic, often collapse and hence will not leak protein at all.

The tubular reabsorption of proteins also plays a role in urinary protein loss. However, tubulo-interstitial injury was not increased in SNX+CL rats compared to rats with SNX alone.

Subsequent HF did not further aggravate the decrease in GFR in CKD animals. With a reduction in cardiac output and systemic blood pressure, GFR was expected to decrease.[31] Although we realize well that we have no direct proof for a mechanism, GFR could be maintained due to above-described changes in glomerular pressure. The increased FSGS, as well as maintained GFR in the face of obviously decreased renal perfusion pressure, are compatible with further activation of the RAS in the current SCRS model.

Why patients with CKD have worsened outcome after MI compared to the general population is still unknown. One of the hypotheses is that patients with CKD may develop larger infarct sizes, leading to increased dilatation and worsened failure. However, post-hoc analysis of the VALIANT (VALsartan In Acute myocardial iNfarcTion) study found that infarct segment length assessed by echocardiography was not influenced by decreased GFR.[11] Dikow *et al.*[18] showed that in rats with SNX the size of the non-perfused area at risk, i.e. the vascular territory supplied by the left anterior descending coronary artery, was similar to that in control rats. Also, in a study by Windt *et al.*,[19] CL two weeks after SNX in rats yielded similar infarct sizes as in controls, when assessed ten weeks later. In our study, a larger infarct size as cause for the increased dilatation and worsened function is also unlikely, as infarct size was similar between both CL groups two weeks after infarction. We assessed infarct size by echocardiography, which correlates well with histological determination of infarct size.[32] Furthermore, there was no difference in infarct size at the end of the study.

Two patient studies assessed cardiac dilatation after MI in patients with CKD compared to non-CKD patients. In the above-mentioned study by Verma *et al.*[11] EDV was smaller as GFR declined, but LV mass increased. On the other hand, Naito *et al.*[12] documented increased EDV and ESV, as well as decreased EF 14 days after MI in 30 patients with a GFR < 60 compared to patients with a GFR ≥ 60 mL/min/1.73 m^2. In rats with SCRS, cardiac dilatation was also aggravated after MI. Increased preload or afterload can worsen dilatation after MI by increasing wall

stress.[33] Although cardiac volumes were not significantly larger in SNX vs. CON before CL, LV pressures may well have been higher due to hypertension and LVH, leading to worsened dilatation after MI. However, after MI the drop in SBP was much greater in SNX+CL than in CON+CL. Levels of SBP did not change significantly over time and the terminal carotid artery MAP measurements showed a similar pattern. Furthermore, SVRI in SNX+CL was similar to that in SNX, which suggests that afterload was not a likely causative factor for further dilatation and functional deterioration. Neurohormonal factors could have aggravated the remodeling process as well, by worsening fluid retention and cardiac damage.

Impaired diastolic relaxation has been documented in rats with CKD,[15] and the increased EDP and *tau*, a relatively load-independent measure for active diastolic relaxation,[34] suggest worsened diastolic function in SCRS rats. This could well be associated with more pulmonary congestion, corroborated by the observed increases in RV and lung weight. [35]

Despite a progressive decrease in EF, cardiac output was maintained in rats with CKD alone. In line, the dP/dt-max, as an index of pressure generation, was increased in CKD rats compared to controls, similar to findings of others in *in vivo* models,[19, 36] and in an isolated heart set-up.[37] This suggests that in rats with CKD contractility is maintained or compensated by higher preloads.

Increased dilatation after MI is associated with adverse outcome in patients, although a faster decline in systolic function may not be apparent in the earlier phases.[38] Similarly, systolic function was not lower in SCRS rats at wk11 compared to MI rats. However, during follow-up, EF in SCRS rats fell to significantly lower levels, and the mortality rate was 22% in this group. Therefore, the acutely worsened dilatation appears to be associated with a poorer long-term outcome in the SCRS animals. The larger difference in maximum developed LV pressure and dP/dt-max in SCRS rats also suggest an exacerbated loss of contractile performance compared to rats with CKD alone, especially because SVRI was similar. Furthermore, the higher EDP and EDV (i.e. preload) observed in SCRS rats did not lead to a higher output in these animals, suggesting that these rats have worsened HF and decompensation. This was supported by the increased lung weight and RV hypertrophy. Conversely, LV hypertrophy appeared to be

impaired in SCRS rats. Cardiac load and fibrosis may have been worsened in SCRS rats, as expression of BNP and CTGF was slighty, albeit not significantly, higher vs. CKD rats. In a model of pressure overload, mRNA expression levels of these proteins were correlated.[35] We did not find such an association, which is probably related to the different pathophysiology of our model with regard to pressure overload.

Our studies were performed in Lewis rats subjected to SNX by resection. We chose the Lewis rat because this strain exhibited a lower mortality rate after coronary ligation.[39] Although the Lewis rat is believed to be more resistant to renal mass reduction,[40] the addition of 6% salt to the diet we were able to create a model of stable CKD.[20, 41] Lewis rats are resistant to high (8%) salt diets with respect to blood pressure and development of proteinuria.[42] In our study the high-salt diet also did not appear to have any significant effects on SBP and proteinuria in CON and CON+CL, but it may have slightly worsened the fluid overload and hypertension in rats with CKD.

Taken together, the present study shows that, in a combined model of CKD and MI, reciprocal worsening of organ damage occurs. Although GFR was maintained at similar levels in the animals with the SCRS compared to rats with CKD alone, they displayed more severe FSGS. The fact that this occurred at lower systemic blood pressures and cardiac output than in animals with CKD alone indicates that this may be not mediated by systemic hemodynamic factors, but likely by substantial alterations in intraglomerular hemodynamics. The presence of CKD did not lead to a larger infarct size after MI. However, aggravated cardiac dilatation occurred shortly after MI. Ultimately, this was associated with a lower EF, worsened diastolic dysfunction, further decompensation, and mortality. This makes it a suitable model to further understand mechanisms and therapeutic options in the SCRS. Possibly, enhanced activation of the RAS or the sympathetic nervous system mediated the current observations in the kidney and the heart. The discrepancy between the worsened FSGS and unchanged GFR leads to the question whether CKD patients who suffer from MI and HF develop aggravation of renal injury that goes undetected using standard renal markers.

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Chapter 4

Targeting the cardiorenal connectors in experimental severe cardiorenal syndrome

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Previously we proposed the Cardiorenal Connection (CRC) as the pathophysiological mechanism behind the accelerated cardiorenal damage in the Severe Cardiorenal Syndrome (SCRS). We recently found that rats with chronic kidney disease show aggravated cardiac dilatation after MI, associated with a decrease in systolic function, worsened diastolic dysfunction, further decompensation and death. We hypothesized that simultaneous interventions targeting all the cardiorenal connectors would be optimal to break the vicious cycle and halt progression of the SCRS. We further hypothesized that the combination targeting all connectors would yield superior benefit compared to combinations targeting only some or one connector.

Rats underwent subtotal nephrectomy (SNX) and 8 weeks later coronary ligation (CL) was performed. After 2 wks (wk 11), rats were stratified according to cardiorenal function and divided into subgroups, receiving vehicle (VEH), a 5 medication combination (5MED: losartan, metoprolol, tempol - a superoxide dismutase mimetic, PDTC – an NF-κB inhibitor, and molsidomine – an NO donor), a 3 medication combination (3MED: tempol, PDTC, molsidomine), or losartan alone (1MED). In vivo cardiorenal function was again measured in wk 13 and wk 15. In wk 16, invasive cardiac hemodynamics were assessed, rats were sacrificed, and organs processed for histological analysis.

Compared to SNX+CL+VEH, rats on 5MED had practically no renal structural injury and improved renal function. Renal injury was also reduced by 3MED and 1MED but to a lesser degree. The decline of cardiac ejection fraction and stroke volume seen in rats on VEH was abrogated by all medication combinations. However, with 5MED, heart rate was reduced, which mitigated the effect on cardiac output. Arterial pressure and cardiac pressure development were also reduced by 5MED.

An intervention combination targeting all cardiorenal connectors halted progression of cardiorenal failure in rats with the SCRS, with almost complete reversal of renal injury. This effect was larger than that achieved by correction of only 3 or 1 of the connectors and appeared to be independent of the effects on cardiac function.

4.1 Introduction

The combination of chronic kidney disease (CKD) and cardiac disease was found to be associated with a marked increase in cardiovascular morbidity and mortality.[1, 2] In the "Severe Cardiorenal Syndrome" (SCRS), CKD and heart failure (HF) may induce worsened cardiovascular damage due to the activation of mechanisms that bidirectionally aggravate the structural and functional deterioration of both organs.[3, 4] We recently developed a rat model for the SCRS, in which we documented reciprocal organ damage when CKD was followed by HF induced by myocardial infarction (MI). We proposed the following "Cardiorenal Connectors" (CRC): (1) activation of the renin-angiotensin system (RAS), (2) activation of the sympathetic nervous system (SNS) , (3) a systemic inflammatory state, and (4) an imbalance between NO and ROS favoring oxidative stress and endothelial dysfunction.[4] We reviewed available evidence and showed that these connectors contribute to the progression of both CKD and HF.[4] Furthermore, we proposed that they interact and synergize with each other to cause accelerated CV damage, leading to further organ failure, and ultimately the SCRS.

Following our hypothesis on the CRC and the synergistic nature of the connectors, we hypothesized that simultaneous interventions targeting all the cardiorenal connectors would break the vicious cycle and halt progression of the SCRS. We further hypothesized that the combination targeting all connectors would yield superior benefit compared to combinations targeting only some or just one connector. Within all the interactions of the cardiorenal connectors, we have not yet established a clear hierarchy. One of the leading connectors could be the RAS. [5, 6] It has been extensively studied and has solid associations with the other cardiorenal connectors. However, we argued that blocking only one connector would never adequately control SCRS, because of the potent interactions and positive feedback loops.

To substantiate the claim that blockade of all connectors is essential in controlling SCRS, we aimed to test different combinations of treatments targeting the connectors. Primarily, we assessed combined blockade of all connectors. It is established that the RAS and SNS share an intricate relationship, especially in renal disease.[7-9] Inflammation and NO/ROS imbalance are also inextricably linked

in cardiovascular diseases, forming a vicious cycle.[10] We next tested partial targeting of the cardiorenal connectors.

We induced the SCRS in male Lewis rats by subtotal nephrectomy (SNX), followed by coronary ligation (CL) 8 weeks later to induce HF. We chose to start with the interventions two weeks after MI for several reasons. One is that we were primarily interested in the role of the CRCs in combined CKD and HF, and the progression to end-stage SCRS. Secondly, we did not want to interfere with the acute remodeling process after MI, which takes about two weeks in rats.[11] With this set-up, the medication combinations also function as rescue therapy rather than preventive interventions, more closely resembling clinical practice. In the SCRS, it is often indistinguishable whether kidney failure or heart failure is the culprit,[12] which was an additional reason to start at a stage when both are present.

4.2 Methods

Overall study setup

The study protocol was approved by the Ethical Committee on Animal Experiments of the University of Utrecht, Utrecht, The Netherlands. Male inbred Lewis rats (Lew/Crl; 180 – 200 grams) were purchased from Charles River, Germany, and housed in a climate-controlled facility with a 12:12-hour light:dark cycle. At t= -1 wk a two-stage subtotal nephrectomy (SNX) by resection or sham operation (CON) was performed as described previously.[13] From t=1 wk onward rats received standard powdered chow supplemented with 6% NaCl until the end of the study. At t= 9 wks, rats from the SNX group were subjected to left anterior descending coronary artery ligation (CL). The CON group received sham operation. This resulted in two groups: CON and SNX+CL. Rats were followed up to wk 16. *In vivo* measurements were carried out in a selection of rats from the CON and SNX groups at wk 8 before coronary ligation or sham surgery. After CL, in wk 11, 13 and 15, all rats from each subgroup were measured. Ejection fraction (EF) was calculated from end-diastolic and end-systolic volumes obtained with the area-length calculation, on B-mode cine-loops recorded in the parasternal long axis view.[13] SNX+CL rats without visible MI on echocardiography and an EF > 40% at wk 11 were excluded from the study. Rats from the SNX+CL group that were

eligible for further study were then stratified according to plasma urea and EF, and randomized to four different treatment groups. One group received only normal drinking water (SNX+CL+VEH). To study the hierarchy of the CRC's, we tested different combinations of medications, administered in drinking water (Table 1). Losartan was a generous gift from MSD, the other substances were bought at Sigma-Aldrich (St. Louis, MO, USA).

The first combination was a five-medication intervention targeted at complete correction of all connectors (SNX+CL+5MED). The second combination consisted of three substances (SNX+CL+3MED), targeted at correction of inflammation, oxidative stress and NO deficiency. This was done to separate the hemodynamic effects of losartan and metoprolol from the non-hemodynamic effects of 3MED. Also, inflammation, oxidative stress and NO deficiency are not only considered as effectors in the CRC but also as mediators of the RAS and SNS.

Table 1. The cardiorenal connectors and their respective interventions and doses.

Targeted cardiorenal connector	Intervention	Mechanism	Dosage
RAS	Losartan (LOS)	AngII type 1 receptor antagonist	5 mg/kg/day
SNS	Metoprolol (MET)	β-adrenergic receptor antagonist	50 mg/kg/day
Inflammation	Pyrrolidine dithiocarbamate (PDTC)	Inhibitor of NF-κB activation[14]	30 mg/kg/day
NO/ROS-balance	Molsidomine (MOLS)	tolerance-free NO donor	25 mg/kg/day[15]
	Tempol	SOD mimetic	20 mg/kg/day[16]

AT: angiotensin, NF-κB: nuclear factor –κB; NO: nitric oxide; SOD: superoxide dismutase

Third, we treated the rats with RAS blockade alone (losartan; 1MED), because we found that metoprolol induced significant bradycardia, even at low doses. β-blockade will also inhibit renin release,[17] and we wanted to test whether the effect of RAS blockade was independent of the bradycardia. In pilot experiments we found that the initially administered doses of losartan (200 mg/L)[18] and metoprolol (150 mg/kg/day)[19] induced severe hypotension, edema and death. Hence we

adjusted the doses so that tail-cuff systolic blood pressure (SBP) was reduced to approximately 100 mmHg with the 5MED combination. In wk 16, invasive hemodynamic measurements of the LV were performed, rats were euthanised and organs were removed, weighed and processed for histological quantification.

Surgical procedures

Subtotal nephrectomy was performed by resection as described previously.[13] In short, the right kidney was removed under isoflurane anaesthesia and buprenorfine analgesia, and weighed. One week later, the poles of the left kidney were excised with a total weight approximately 2/3rds of the previously removed right kidney. For coronary ligation, rats were anaesthetized with isoflurane and buprenorfine, intubated and ventilated according to the settings advised by Horstick et al.[20]. After a left thoracotomy, the left descending coronary artery was ligated with a 5/0 Ethibond suture without externalizing the heart. Lidocaine (10 mg/kg) was administered peri-operatively to reduce the risk of fatal arrhythmias.[21]

In vivo measurements

We performed tail-cuff systolic blood pressure (SBP) registration and collected 24-h urine samples, for determination of creatinine and protein excretion, with the rats in individual metabolic cages while fasting, as described.[13] Echocardiography was performed as described previously.[13] After echocardiography, a blood sample (0.5 mL) was collected in EDTA for determination of plasma urea and creatinine.

Hemodynamic measurements

Rats were intubated and then ventilated with 2% isoflurane in 40% O_2. The left jugular vein was cannulated for continuous infusion of saline. A Millar micro-pressure catheter (Millar Instruments, Houston, Tx, USA) was inserted into the LV via the right carotid artery. After a 15-min stabilization period, LV pressures were recorded with Chart software (ADInstruments, Spechbach, Germany) at 3 separate intervals of 5-10 seconds with the ventilator turned off. Hemodynamic data were calculated using the Chart Blood Pressure module (ADInstruments, Germany) for the following parameters: maximum LV pressure, end-diastolic pressure, the maximal rate of pressure increase (maximum dP/dt) and pressure decrease

(minimum dP/dt), the exponential time constant of active relaxation (*tau)*. Mean arterial pressure was calculated from pressure data registered in the carotid artery, from which systemic vascular resistance (SVRI) was calculated by dividing it by the level of cardiac index (CI) derived from echocardiography.

Organ weights and histology

Focal segmental glomerulosclerosis (FSGS) and tubulo-interstitial damage were scored on periodic acid Schiff (PAS)-stained kidney.[22, 23] FSGS was scored on 50 separate glomeruli by quadrants, on a scale of 0 to 4, where 0 means no quadrant affected and 4 means that the whole glomerulus was affected. Tubulo-interstitial damage was scored on a scale of 1-5 in at least 10 different non-overlapping fields per animal. Scored variables were the amount of per-tubular inflammatory infiltrate, interstitial fibrosis, tubular atrophy and tubular dilatation. The cardiomyocyte circumference was measured on PAS-stained myocardial slices in sections with transversely cut myocardial fibers by tracing the cellular border on photomicrographs of at least 50 different cardiomyocytes with a computer assisted image analysis system (OptiMas, Houston, TX). All measurements were performed by an experienced technician blinded to the group allocation.

Statistical Analysis

Data is presented as mean ± SEM. Data were analyzed and graphed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). Differences between CON and SNX+CL+VEH were assessed by Student's t-test. Differences between SNX+CL+VEH and the MED groups were tested by one-way ANOVA with the Holm-Sidak post-hoc test with the VEH group as control. Longitudinal data was tested by two-way RM ANOVA with the Holm-Sidak post-hoc test with the data of the VEH group at wk 11 as the reference group. Data that was not normally distributed was log-transformed or ranked to achieve normality. Statistical significance was reached with p-values below 0.05.

4.3 Results

Tail-cuff SBP

After CL, SBP decreased to levels of controls in the SNX+CL+VEH group (Figure 1). With 5MED, SBP decreased slightly but significantly in wk 13 and 15 compared to the level in this group at wk 11 (both $P<0.05$). In the 1MED group, levels of SBP tended to be higher than those in the VEH group but this did not reach statistical significance.

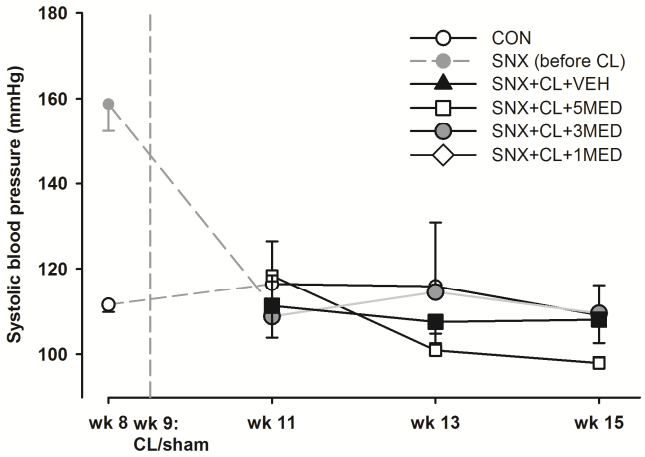

Figure 1. Tail-cuff SBP. Mean ± SEM. Where not visible, error bars fall within the symbol.

Renal function

Plasma urea was consistently high in SNX+CL+VEH vs. CON (Figure 2A). In rats on 3MED, levels plasma urea tended to be higher than in rats on VEH at wk 15 (P=0.054), but they were significantly higher than those in rats on the 5MED combination (P = 0.009). Creatinine clearance (per 100 g BW) showed no major differences between SNX groups, and all were clearly lower than CON (Figure 2B). At wk 15, creatinine clearance was slightly higher in rats on 5MED and 1MED, but this was only significant for 5MED in direct comparison with the VEH group with Student's t-test (P = 0.031).

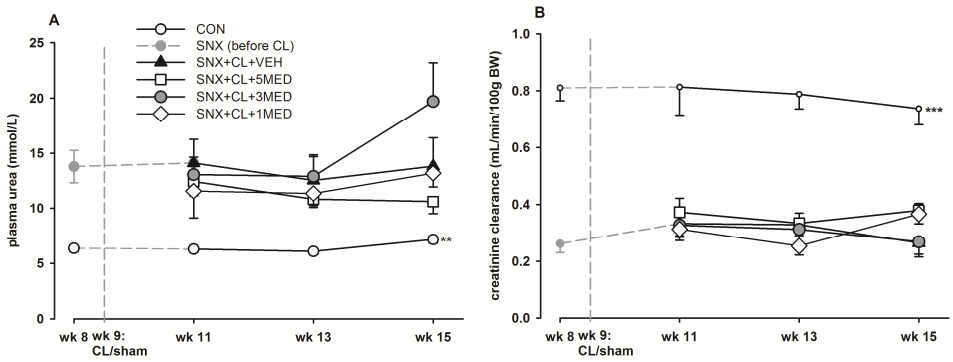

Figure 2. Plasma urea (A) and creatinine clearance (B). Mean ± SEM. ** *P*<0.01 vs. CON, *** *P*<0.001 vs. SNX+CL+VEH.

The medication combinations had minor but differential effects on proteinuria (Figure 3). Urinary protein excretion at wk 15 was lower in 5MED animals than rats on VEH, which was only significant with Student's t-test (*P*<0.05). Similar to plasma urea, rats on 3MED displayed increased proteinuria compared to rats on VEH, but this was not significant. Losartan alone (1MED) did not affect proteinuria.

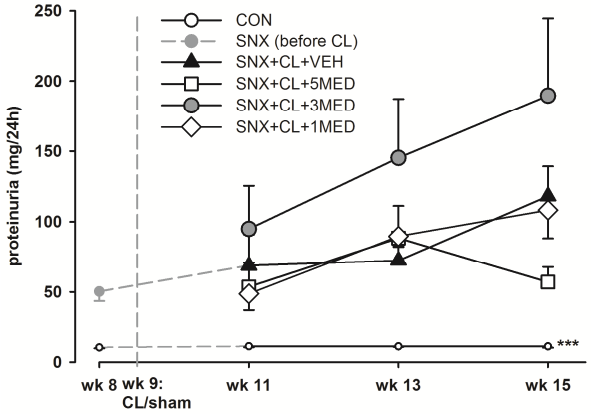

Figure 3. Proteinuria. Mean ± SEM. *** *P*<0.001 vs. CON.

Renal Histological Damage

The weight of left kidney remnant (corrected per 100 g BW) was significantly higher in all three SNX+CL-groups when compared to the corrected weight of the left kidney of CON rats, but there was no effect of any treatment combination (data not shown). A differential, but significant effect of the medication combinations was observed on the amount and distribution of glomerulosclerosis (Figure 4). Compared to CON, the number of normal unaffected glomeruli was significantly lower in all SNX+CL-groups, with no significant differences between these 4 groups, although the groups on medication had slightly more unaffected glomeruli. The number of partially sclerotic glomeruli (25-50% damage) was higher in the SNX+CL-groups vs. CON.

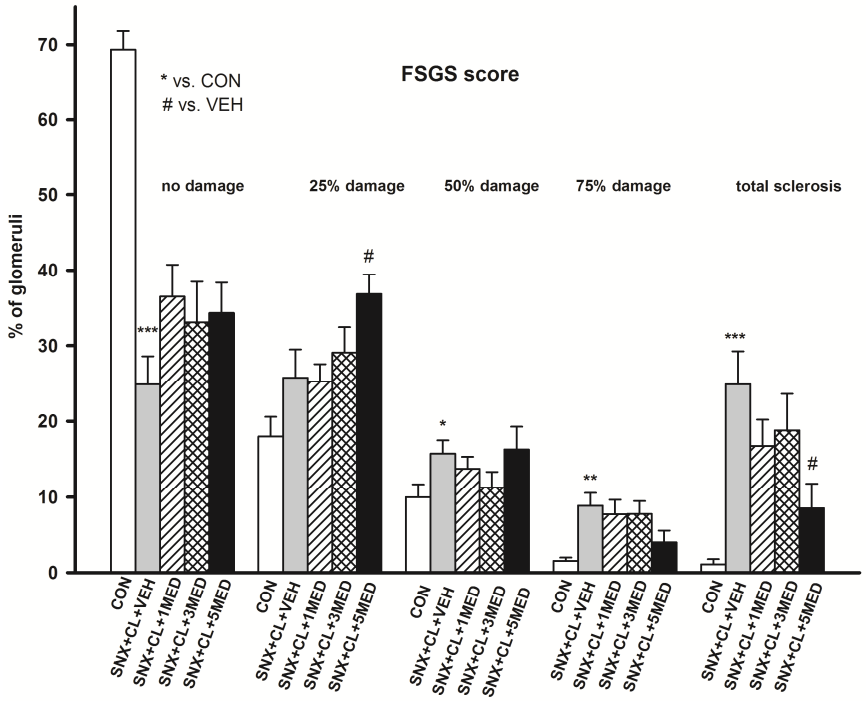

Figure 4. Focal segmental glomerulosclerosis (FSGS) scores. The bars represent the percentage of glomeruli in each group that are affected by increasing severity of damage. Mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. CON; # $P<0.05$ vs. SNX+CL+VEH.

Within these groups, rats on 5-MED displayed significantly more glomeruli with mild (25%) damage than rats on VEH (P<0.05). For the amount of severe FGS the pattern was opposite, with the 5MED group showing significantly less totally sclerotic glomeruli than the VEH group (P<0.05), and a borderline decrease in the percentage of glomeruli with 75% damage. Both the 1MED and 3MED group showed only minor decreases in the number of damaged glomeruli. Tubulo-interstitial damage scores are shown in Figure 5. Both 1MED and 3MED showed a mitigating effect on tubulo-interstitial damage, primarily on the degree of interstitial fibrosis. Importantly, the severity of inflammatory infiltration, fibrosis and dilatation were reduced to levels similar to those in control rats by 5MED.

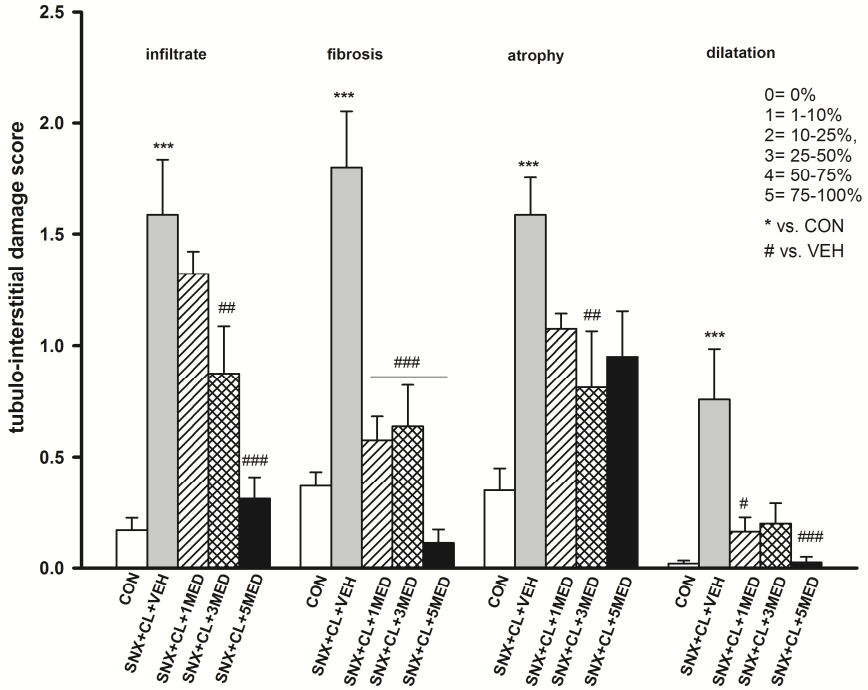

Figure 5. Tubulo-interstitial damage scores. Bars represent the amount of damage per field. Mean ± SEM. * P<0.05, ** P<0.01, *** P<0.001 vs. CON; # P<0.05, ## P<0.01, ### P<0.001 vs. SNX+CL+VEH.

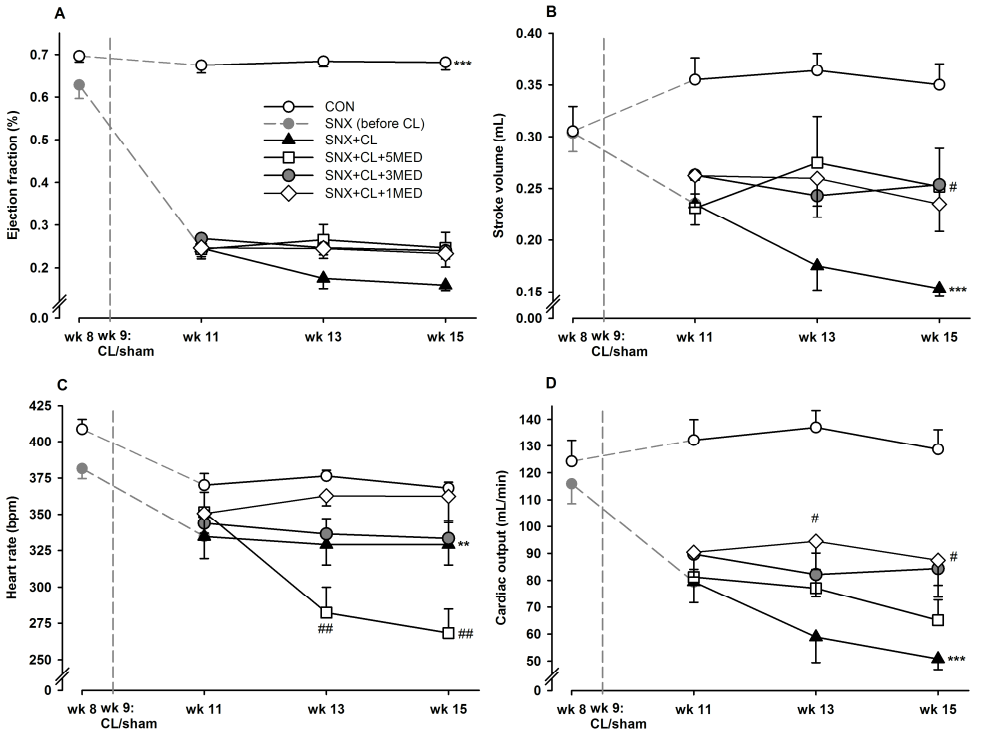

Figure 6. Echocardiographic variables. (A) Ejection fraction, (B) Stroke volume, (C) Heart rate, (D) Cardiac output. Mean ± SEM. ** *P*<0.01, *** *P*<0.001 vs. CON; # *P*<0.05, ## *P*<0.01 vs. SNX+CL+VEH.

Echocardiographic structure and function

B-mode echocardiography was performed with a 15-MHz linear array probe at 2 week intervals. Although EDV and ESV were significantly larger in all four SNX+CL groups compared to CON, there were no significant treatment effects (See supplementary figure in Appendix). Despite only minor differences in volumes, calculated systolic parameters were significantly better in SNX+CL rats receiving treatment. In SNX+CL rats, EF was very low two weeks after MI, and similar in all four groups before start of treatment (Figure 6A). During follow-up, EF decreased further in untreated rats (*P*<0.001 vs. wk11) while it was maintained in all treatment groups.

Stroke volume showed a largely similar pattern, with a steady decline in VEH-treated rats down to 0.153 ± 0.008 mL, and maintained stroke volume in rats on all different medication combinations (Figure 6B). When heart rate (HR) was analyzed,

we observed a steady and strongly significant decline in HR in the 5MED group (Figure 6C). The drop in HR was not observed when non-CKD rats with a MI were treated with 5MED, and HR even slightly increased in rats on losartan alone. This led to a differential effect on CO (Figure 6D). While SNX+CL+VEH rats showed a steady deterioration in CO over time (P<0.01 vs. wk 11), the decline in CO was abrogated by both the 3MED and 1MED intervention (P<0.05).

Invasive hemodynamics

Mean arterial pressure (MAP) and maximum LV pressure (P-max) were both significantly lower in the 5MED group compared to the VEH-group (Table 2). In the 3MED and 1MED groups, both these variables were also lower than in the VEH-treated groups. Differences did not reach statistical significance versus 5MED or VEH. End-diastolic pressure tended tp be lower in the 1MED and 5MED groups compared to VEH, but this did not reach statistical significance. Compared to untreated SNX+CL-rats, the maximal rate of pressure increase (dP/dt-max) and pressure decrease (dP/dt-min) were both significantly lower in the 5MED group. Although tau, the time-constant of early relaxation was significantly prolonged in SNX+CL+VEH rats vs. controls, none of the treatments had any effect on this variable. The arterial elastance (Ea) and systemic vascular resistance index (SVRI) were however both significantly reduced by all treatments, with no significant difference among the treatment combinations.

Mortality during treatment

In the SNX+CL+VEH group 3 out of the initial 10 animals died during follow-up from wk 11 onward. In the 5MED group (n = 8) no mortality was observed, while 2 animals died in both the 3MED (n = 9) and 1MED (n = 8) group. Mortality was either spontaneous or rats were sacrificed due to severe morbidity (cachexia, dyspnea, edema).

Cardiac histological damage

The SNX+CL+VEH group had a higher LV weight index compared to controls, as well a higher RV weight index and corrected wet lung weight (Table 3). LV hypertrophy and cardiomyocyte area tended to be less in rats that had been

treated with the 5-MED combination when compared to VEH rats (P = 0.055 and P = 0.051, respectively).

Table 2. Invasive cardiac hemodynamics.

	CON	***SNX+CL +VEH***	***SNX+CL +1MED***	***SNX+CL +3MED***	***SNX+CL +5MED***
	n = 8	n = 7	n = 6	n = 7	n = 7
MAP (mmHg)	103 ± 8	116 ± 9	92 ± 8	99 ± 9	74 ± 6 ###
***P*-max** (mmHg)	130 ± 5	141 ± 8	118 ± 9	119 ± 9	90 ± 5 ###
EDP (mmHg)	6.7 ± 0.7	17.9 ± 1.3 ***	10.1 ± 2.1	16.6 ± 5.1	12.1 ± 2.4
dP/dt-max (mmHg/s)	7112 ± 534	7416 ± 917	5451 ± 566	6326 ± 844	3809 ± 380 ##
dP/dt-min (mmHg/s)	-9081 ± 792	-5164 ± 361 ***	-4981 ± 690	-4148 ± 574	-3182 ± 361 ##
tau (msec)	14.1 ± 0.66	27.1 ± 3.2 ***	23.0 ± 2.2	25.5 ± 3.0	24.4 ± 1.2
Ea (mmHg/mL)	422 ± 34	895 ± 42 ***	530 ± 60 ###	477 ± 42 ###	417 ± 53 ###
SVRI (mmHg/mL* min^{-1} per 100g BW)	3.65 ± 0.41	8.09 ± 0.52 ***	4.92 ± 1.05 ###	4.15 ± 0.46 ###	4.48 ± 0.57 ##

MAP: mean arterial pressure; P-max: maximum left ventricular pressure; EDP: end-diastolic pressure; dP/dt-max: rate of pressure increase; dP/dt-min: rate of pressure decrease; tau: time constant of diastolic relaxation; Ea: arterial elastance; SVRI: systemic vascular resistance index.
Mean ± SEM. *** P<0.001 vs. CON, ## P<0.01, ### P<0.001 vs. SNX+CL+VEH.

Table 3. Organ weights corrected for BW and cardiomyocyte area.

	CON	***SNX+CL +VEH***	***SNX+CL +1MED***	***SNX+CL +3MED***	***SNX+CL +5MED***
	n=9	n=7	n=6	n=7	n=8
LVWI (g/100g)	0.19 ± 0.00	0.29 ± 0.01 ***	0.29 ± 0.02	0.26 ± 0.02	0.25 ± 0.01
RVWI (g/100g)	0.048 ± 0.002	0.084 ± 0.012 **	0.077 ± 0.016	0.086 ± 0.012	0.074 ± 0.010
wet lung WI (g/100g)	0.31 ± 0.01	0.60 ± 0.08 **	0.48 ± 0.08	0.67 ± 0.12	0.43 ± 0.03
cmc area (μm^2)	429 ± 13	697 ± 71 ***	632 ± 32	633 ± 31	592 ± 21

LVWI: left ventricular weight index; RVWI: right ventricular weight index; cmc: cardiomyocyte.
Mean ± SEM. ** P<0.01, *** P<0.001 vs. CON.

4.4 Discussion

The central premise of our theorem was that in the development of the SCRS, the cardiorenal connectors interact and synergize, and form positive feedback loops creating a vicious cycle of accelerated cardiovascular damage and dysfunction.[4]

We hypothesized that correction of all cardiorenal connectors is needed to halt progression of cardiorenal damage and failure.[4] Indeed, targeting all the cardiorenal connectors with 5MED significantly reduced glomerular injury and almost completely reversed tubulo-interstitial damage. It also slightly improved renal function, and abrogated the decline in cardiac ejection fraction. The fact that this occurred in the presence of established long-term cardiorenal failure make these results all the more striking. Although complete CRC blockade reduced HR, which mitigated the effects on CO, the beneficial effects on renal structure and function were greater than correction of inflammation and NO/ROS imbalance alone and RAS blockade monotherapy. We also questioned whether there was a hierarchy among the cardiorenal connectors. Inflammation and disturbed NO/ROS balance are tightly linked pathways in the pathogenesis of cardiovascular diseases,[10, 24] and are generally considered mediators of RAS and SNS induced damage. However, our results point to an independent effect of these cardiorenal connectors on the progression of the SCRS.

Effects of 5MED: complete blockade of the cardiorenal connnectors

The RAS contributes to progression of renal injury and RAS blockade protects the kidney.[5, 6] Furthermore, blockade of the SNS, by dorsal rhizotomy or by β-blockade, was associated with decreased renal injury in rats with SNX.[25, 26] Clinically, reduction of blood pressure with β-blockers appears to slow progression of kidney disease.[27] The combination of quinapril and dorsal rhizotomy was found to exert even better protection against SNX-induced renal damage.[28] Both RAS blockade and SNS blockade in non-hypotensive dosage reduced cardiac histological injury in rats with CKD, but whether this had an effect on cardiac function has not been described.[19, 29, 30] Correction of inflammation, oxidative stress and NO availability in models of CKD also showed promising results.[15, 16, 31, 32] However, these medications have not yet been examined as a combination in established CKD and/or HF.

Plasma urea and proteinuria were slightly lower, and creatinine clearance was slightly improved by 5MED, while the 1MED or 3MED groups showed no effect or even a worsening of these variables. Although these two groups exhibited slightly less renal injury than VEH-treated SNX+CL rats, severe glomerulosclerosis and tubulo-interstitial (TI) damage were substantially more reduced by 5MED.

While the number of severely and totally sclerotic glomeruli was reduced by 5MED, the number of mildly sclerotic glomeruli (25% damage) was increased compared to VEH-treated rats. This shift in the distribution of the FSGS score suggests that progression of glomerular injury is abrogated or even reversed by 5MED. Effects on TI damage were even more pronounced, with 5MED inducing a very significant reduction in the amount of injury compared to VEH rats, and in fact practically eliminating TI damage. The fact that these therapeutic interventions were started 11 weeks after SNX, with established CKD, makes these results even more striking. It should be noted that studying dose-dependency of these beneficial effects was not an objective of this study, and that the dosage of RAS blockade with SNS blockade in 5MED or without SNS blockade in 1MED were only mildly hypotensive. Furthermore, our primary objective was to study whether the effects of 5MED were dependent on combined inhibition of these factors.

All treatments resulted in a similar maintenance of EF and SV, compared to a significant decline over time in the VEH-treated group. However, HR was significantly lower in the 5MED group, which was most likely due to a negative chronotropic effect of the β-blockade. This led to a less prominent effect on CO assessed by echocardiography. Tail-cuff SBP, MAP and LV maximum pressure were lower in the 5MED group than in the VEH group or the other treatment groups, suggesting a mildly anti-hypertensive effect of 5MED. On the other hand, Ea and SVRI were similarly reduced in all treatment groups and dP/dt-max was only significantly reduced by 5MED. This suggests that a certain amount of negative inotropy also played a role in the mitigating effect of 5MED on cardiac output, separate from an effect on systemic resistance and afterload. Previous studies in rats with MI treated with β_1-adrenoceptor antagonists showed similar effects on EF and CO, but also a positive effect on myocardial energy metabolism and coronary perfusion.[33, 34] Interestingly, in the above-described experimental MI studies β-blockade did not affect arterial pressure.[26, 33, 34]

Effects of 3MED: targeting inflammation and NO/ROS

One previous investigation showed that inhibition of NF-κB activation with PDTC given as preventive therapy in the rat SNX model reduced blood pressure, proteinuria and renal injury.[32] Treatment with tempol 5 weeks after SNX also reduced blood pressure, and improved NO availability.[35] Furthermore, we and others documented beneficial effects of the NO donor molsidomine in rats with early or established CKD.[15, 31] In the rat model of MI, NF-κB is activated and induces a pro-inflammatory cascade.[24] Inhibition of NF-κB improved post-MI remodeling and capillary density but did not affect ejection fraction,[36, 37] while tempol reduced MI size and prevented the decline of systolic function and the dilation in rats with MI.[38, 39] Positive effects of molsidomine in cardiac dysfunction and heart failure have been described by others and by us, in experimental as well as the clinical setting.[15, 40-43]

In our study, we also documented beneficial effects of the combination of these three medications in the SCRS. The 3MED combination did not significantly affect glomerular injury. However, in the tubulo-interstitial compartment, 3MED significantly reduced the severity of damage. These changes were not associated with an amelioration of renal function. In the heart, 3MED abrogated the decline of EF and SV, and, because HR was not affected, CO was also preserved. Systolic and mean arterial blood pressure were not significantly affected.

Effects of 1MED: RAS blockade monotherapy

Treatment with losartan alone did not affect renal function, and resulted in similar reductions in renal injury as 3MED. Also, effects on cardiac function were similar. Inhibition of angiotensin action is considered standard therapy for patients after MI. Whether there is an active local RAS inside the heart is still a matter of debate, but several studies suggest that components of the RAS are upregulated after MI, and contribute to remodeling by activating the inflammatory response and induction of oxidative stress (reviewed in [44]). After experimental MI in rats, activation of the RAS contributes to an increase of intraglomerular pressure in the face of reduced renal blood flow, thus maintaining glomerular filtration rate.[45, 46] Several studies found that in the first weeks after experimental MI the systemic RAS was not activated,[47, 48] which suggests that in this initial period local RAS components play a major role.

It is likely that in this model of the SCRS the RAS was already activated by the pre-existent CKD and that this was worsened by subsequent MI. The dose of losartan employed in our study may not have been high enough to fully antagonize the RAS activation in this model, which may explain the lack of effect on blood pressure of 1MED. Higher doses of losartan may have provided better reno-protection. However, as mentioned above, only low, mildly hypotensive doses of losartan and metoprolol were tolerated in this model of the CRC. Despite their lack of effect on blood pressure 1MED and 3MED still had beneficial effects, which appeared to be additive when combined in 5MED.

4.5 Conclusion

The results from the present study suggest that complete CRC blockade (5MED) in the SCRS elicited beneficial effects on cardiorenal structure and function, even when given in established combined CKD and HF. Renal injury was practically eliminated and the decline in cardiac systolic function was abrogated, despite a decrease in heart rate. Although the reduction in glomerular injury may have been secondary to the decline in blood pressure and hence intra-glomerular pressure, the marked diminution of tubulo-interstitial damage is likely blood pressure independent.

With regard to the hierarchy of the cardiorenal connectors, our results suggest that inflammation and an imbalance between NO and ROS contribute separately to progression of cardiorenal failure. The triad of inflammation, oxidative stress and NO deficiency are generally considered to mediate structural cardiovascular effects of the RAS and the SNS, and therapeutic correction of this triad may provide additional treatment benefit for patients with the SCRS without significantly affecting hemodynamics.

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Chapter 5

Organ-specific gene expression of hepcidin in experimental cardiorenal failure

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Hepcidin is mainly produced in the liver and decreases cellular iron export by degrading ferroportin 1 (Fpn1). It responds to serum iron levels, is upregulated in inflammation, and downregulated with hypoxia and anemia. Hepcidin is also expressed and functional in cardiomyocytes and increases in hypoxia and inflammation, but also with cardiac injury. Because iron is an essential component for oxidative energy metabolism, but can also contribute to oxidative stress, perturbations in cardiac iron homeostasis may play a role in myocardial dysfunction and injury.

We hypothesized that hepcidin is upregulated not only after myocardial infarction (MI) but also in chronic kidney disease (CKD) and the combination, and that the degree of upregulation relates to the amount of cardiac injury. We assessed mRNA expression of hepcidin by qPCR in heart and liver tissue from control (CON) rats, rats with CKD, rats with MI, and rats with cardiorenal failure (CKD+MI). We assessed the correlation between hepcidin expression and markers of hypoxia and damage. Because cardiac hepcidin expression was highest when CKD and MI are combined, we also tested whether cardiac hepcidin expression in this model was affected by anti-oxidant and anti-inflammatory treatment.

Rats with CKD had reduced GFR. Rats with MI had a strong decrease in ejection fraction, which was lowest in those with cardiorenal failure. Cardiac hepcidin expression was upregulated in rats with MI, but also in rats with CKD and the combination, while liver expression of hepcidin was downregulated in CKD+MI. Hepcidin expression was not strongly correlated with levels of hematocrit. Furthermore, the cardiac expression of hepcidin was tightly correlated with that of BNP (Figure 6B, $R = 0.734$, $P < 0.001$). Treatment with a nitric oxide (NO) donor alone or in combination with anti-oxidant and anti-inflammatory medication was associated with a further increase in cardiac hepcidin expression.

Thus, cardiac hepcidin was upregulated in states of cardiac stress, whether locally induced or secondary to CKD. It correlated with markers of cardiac injury, but not with hematocrit. The precise mechanisms of iron regulation and the role of hepcidin need to be elucidated in further research.

5.1 Introduction

Iron-dependent modulation of energy metabolism is of special importance in tissues with high metabolic demand, such as the myocardium. Iron is an essential trace element that can donate electrons in its ferrous form-Fe(II)-and accept electrons in its ferric form-Fe(III). This capacity makes it a useful component of cytochromes and oxygen-binding molecules, such as haemoglobin and myoglobin.

Several key enzymes involved in the citric acid (Krebs) cycle have iron-responsive elements (IREs), and iron supplementation increases NAD(P)H production, mitochondrial oxygen consumption and ATP formation. Weanling rats raised on an iron-deficient diet developed left ventricular dilatation, with abnormal mitochondrial and sarcomere structure.[1] Sustained iron deficiency induced cardiac fibrosis and lung congestion with hypoxia-inducible factor (HIF)-1α upregulation.[2] On the other hand, iron overload may promote the generation of free radicals, induce toxicity and is associated with development of cardiomyopathy.[3]

In this respect, from a clinical point of view, the results of the FAIR-HF trial are of major importance.[4] In this trial, patients with heart failure (HF) and iron deficiency experienced significant improvements in quality-of-life and exercise capacity when treated with iron supplementation by ferric carboxymaltose. Interestingly both patients with and without anemia at baseline benefited from the treatment, suggesting a hematocrit-independent effect.

Hepcidin is the main regulatory protein of body iron metabolism. It is mainly produced in the liver and binds to ferroportin, resulting in internalization and degradation of the complex.[5] Hepcidin thus inhibits cellular iron efflux from enterocytes, macrophages and hepatocytes. The hepcidin antimicrobial peptide (HAMP) gene codes for a 84 amino-acid pre-prohepcidin protein, which is subsequently cleaved into the bio-active 25 amino acid hepcidin molecule. Because hepcidin expression is primarily controlled at the transcriptional level,[6] HAMP mRNA expression appears to be a good indication of the amount of hepcidin protein produced. Hepatic gene expression of HAMP is regulated by several mechanisms related to iron metabolism, inflammation and hypoxia. Hyperferremia and inflammation increase hepcidin synthesis[7]. Conversely, iron deficiency downregulates HAMP expression that appears to be dependent on HIF

signaling.[8] Indeed, systemic hypoxia and anemia can down-regulate hepatic hepcidin expression.[9]

Tight regulation of cardiomyocyte iron content requires powerful intracellular iron controllers such as hepcidin. Both hepatic and cardiac hepcidin are upregulated by systemic inflammation. However in systemic hypoxia, hepatic hepcidin expression is reduced, while cardiac hepcidin is upregulated.[10] Recently, hepcidin expression was also found to be upregulated in rat hearts with myocardial infarction (MI) and myocarditis, as well as in human hearts with myocarditis.[11] The expression of hepcidin in the infarcted and inflamed myocardium was correlated with the expression of BNP and IL-6, suggesting a link between iron regulation and cardiomyocyte stretch and inflammation, respectively. Furthermore, increased expression of hepcidin was found by immunofluorescence in the hearts of rats with chronic kidney disease (CKD), which was associated with levels of iron deficiency and anemia.[12] In cardiomyocytes *in vitro* hepcidin reduces ferroportin-1 content and iron release,[13]which suggests that hepcidin is biologically active in the heart.

We hypothesized that cardiac hepcidin is upregulated in response to damage, both in models that induce damage to the myocardium directly (i.e. coronary ligation) as well as in models that indirectly damage the myocardium (i.e. renal failure). Furthermore we hypothesized that the degree of hepcidin upregulation relates to the severity of cardiac injury. We assessed gene expression of hepcidin in rat heart and liver tissue to study whether regulation of hepcidin expression is organ-specific, and assessed the correlation between hepcidin expression and markers of hypoxia and damage. Finally we tested whether cardiac hepcidin expression was affected by NO supplementation, and anti-oxidant and anti-inflammatory treatment.

5.2 Methods

The study protocol was approved by the Ethical Committee on Animal Experiments of the University of Utrecht, Utrecht, The Netherlands. Male inbred Lewis rats (Lew/Crl) were purchased from Charles River, Germany, and housed in a climate-controlled facility with a 12:12-hour light:dark cycle. At t= -1 wk a two-stage subtotal

nephrectomy by resection or sham operation was performed as described previously.[14] From t=1 wk onward rats received standard powdered chow supplemented with 6% NaCl until the end of the study. At t= 9 wks, rats from both groups were either subjected to left anterior descending coronary artery ligation (CL) or sham operation. This resulted in four groups: CON (sham-SNX + sham-CL; n=10); SNX (SNX + sham-CL; n=12); CON+CL (sham-SNX + CL; n=9); and SNX+CL (n=9). Rats were followed up to wk 16. *In vivo* measurements were carried out in a selection of rats from the CON and SNX groups at wk 8 before CL or sham surgery. After CL, all rats from each subgroup were measured in wk 11, 13 and 15. CON+CL and SNX+CL rats without visible MI on echocardiography and an ejection fraction (EF) ≥ 40% at wk 11 were excluded from the study. In addition, SNX+CL rats (with EF <40%) were either treated with the tolerance-free NO donor molsidomine (MOLS: 25 mg/kg/d, N=4) or a 3-medication combination (3MED: tempol - a superoxide dismutase (SOD) mimetic, 20 mg/kg/d, Pyrrolidinedithiocarbamate (PDTC) – a nuclear factor-kappa B (NF-kB) inhibitor, 30 mg/kg/d, and molsidomine, 25 mg/kg/d). All drugs were administered in the drinking water. EF was calculated from end-diastolic and end-systolic volumes obtained by echocardiography using the area-length calculation, on B-mode cine-loops recorded in the parasternal long axis view.[14]

In wk 16, rats were subjected to invasive cardiac hemodynamic assessment under isoflurane anaesthesia, euthanised and organs were removed, weighed and processed for histological quantification and determination of mRNA expression. Expression of hepcidin (HAMP; Rn00221783), brain natriuretic peptide (BNP; Rn00580641), connective tissue growth factor (CTGF; Rn00573960) and erythropoietin receptor (EPOR; Rn00566533) in cardiac apical tissue, and of hepcidin, C/EBP-α (Rn00560963), bone morphogenetic protein-6 (BMP-6; Rn00432095) and monocyte chemoattractive protein-1 (MCP-1; Rn00580555) in liver tissue was assessed by qPCR as described previously.[15]

Cycle time(Ct) values for all genes were normalized for mean Ct-values of Calnexin (Canx; Rn00596877) and β-actin (Actb; Rn00667869), which we previously determined to be the two most stable housekeeping genes across all groups in both organs. Statistical analysis was performed by 2-way ANOVA for changes over

time, and 1-way ANOVA for the difference between wk 8 and wk 11 and for treatment effects, where indicated.

5.3 Results

Functional data

Changes in cardiac ejection fraction (EF) over time, as shown in a previous study (Chapter 3), are presented in Figure 1. While rats with SNX alone showed a gradual decline in EF, it was strongly reduced 2 weeks after CL in both CON+CL and SNX+CL rats. However, SNX+CL rats showed an even further decline of EF down to 16 ± 1% at end of follow-up compared to 26 ± 3% in CON+CL rats. A similar pattern was observed for cardiac index (CO per 100 g BW; Table 1). Hematocrit was significantly lower in SNX rats vs. controls, with no further effect of CL (Table 1). Creatinine clearance was lower in SNX rats compared to CON rats, but was not affected by CL in either group.

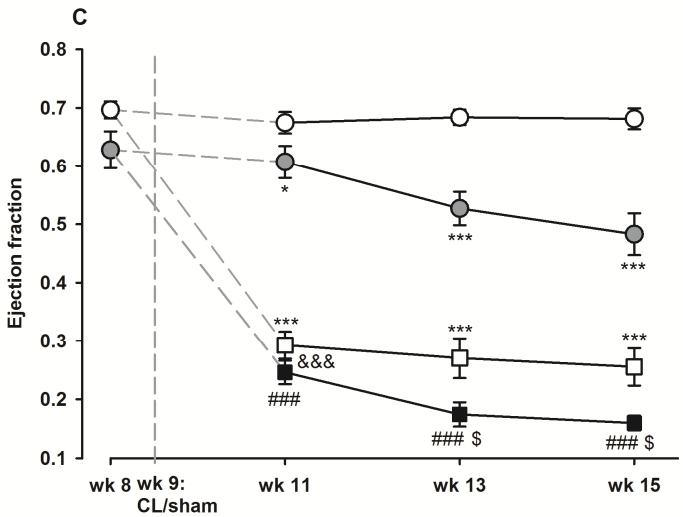

Figure 1. Ejection fraction values in controls rats (CON; open circles), rats with subtotal nephrectomy and sham coronary ligation (SNX; gray circles), control rats with coronary ligation (CON+CL; open squares), and rats with SNX+CL. Mean ± SEM. If not visible, the error bars fall within the symbol.
* *P*<0.05, ** *P*<0.01, *** *P*<0.001 vs. CON; $ *P*<0.05 vs. CON+CL; ### *P*<0.001 vs. SNX; &&& *P*<0.001 vs. wk 8 in respective non-CL group.

Table 1. Biometric parameters at wk 15.

	CON	CON+CL	SNX	SNX+CL
	(n=10)	(n=9)	(n=10)	(n=7)
Body weight (g)	424 ± 8	426 ± 9	378 ± 11 ***	361 ± 14 $$$
Hematocrit (ml/ml)	0.47 ± 0.01	0.46 ± 0.01	0.42 ± 0.01 **	0.43 ± 0.02
Kidney:				
Creatinine clearance (mL/min/100 g BW)	0.74 ± 0.06	0.67 ± 0.09	0.30 ± 0.03 ***	0.27 ± 0.04 $$$
Heart:				
Cardiac index (mL/min/100 g BW)	30 ± 2	17 ± 1 ***	29 ± 2	14 ± 1 ###

Mean ± SEM. *** *P*<0.001 vs. CON; $ *P*<0.05, $$$ *P*<0.001 vs. CON+CL; ### *P*<0.001 vs. SNX.

Iron content in the heart

We stained iron in cardiac tissue with Prussian Blue. In cardiac tissue of rats with MI, iron deposition was seen in the infarct zone and to some extent in the peri-infarct zone (Figure 2). In the remote myocardium of MI rats practically no iron was observed, similar to hearts of non-infarcted CKD or CON rats. The staining confirmed the well-known accumulation of iron in MI. Differences in staining were not quantified.

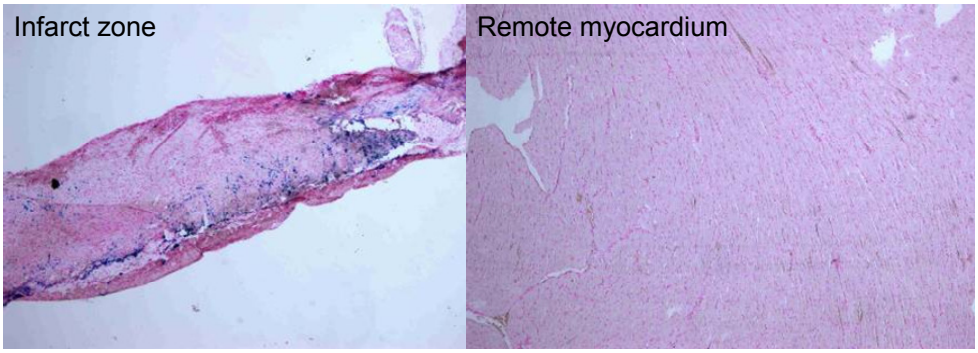

Figure 2. Prussian Blue staining of iron in the myocardial infarct zone and remote myocardium.

Cardiac gene expression in relation to damage

Gene expression of brain natriuretic peptide (BNP) and connective tissue growth factor (CTGF) showed a stepwise upregulation in CON+CL, SNX, and SNX+CL

(Figure 3), but there was no correlation between individual levels of expression when these 3 groups were combined (R=0.231; *P* = 0.146).

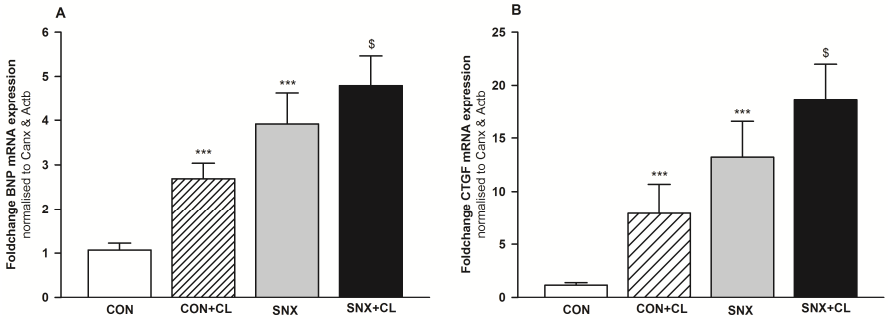

Figure 3. Gene expression of (A) brain natriuretic peptide (BNP) and connective tissue growth factor (CTGF) in cardiac apical tissue, assessed by qPCR.
Mean ± SEM, *** *P*<0.001 vs. CON; $ *P*<0.05 vs. CON+CL.

Hepcidin expression in heart & liver

Cardiac expression of hepcidin was increased in both the SNX and CON+CL group, and slightly more so in SNX+CL (Figure 4). In contrast, liver expression of hepcidin was unaffected by SNX and CL alone, while it was significantly decreased in the SNX+CL group.

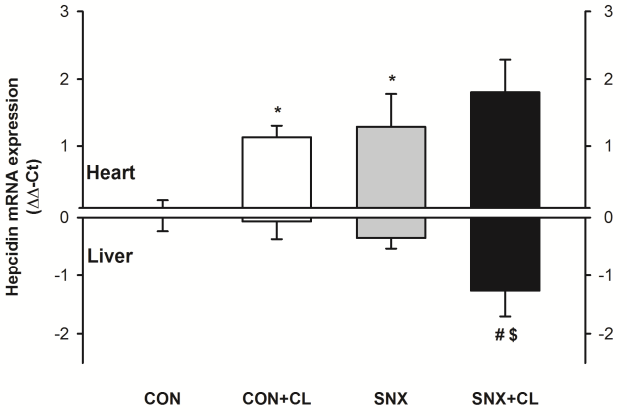

Figure 4. Hepcidin mRNA expression in heart and liver. ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group. Mean ± SEM, * *P*<0.05 vs. CON; $ *P*<0.05 vs. CON+CL; # *P*<0.05 vs. SNX.

Correlation of Hepcidin expression with Ht.

Cardiac hepcidin expression ($\Delta\Delta C_t$ values) correlated with values of hematocrit (R = 0.459, *P* = 0.032; Figure 5), while liver expression of hepcidin did not (R = 0.252; *P* = 0.271). The observed correlation in the heart was due to a slightly more negative association between Ht and hepcidin expression in the SNX rats, irrespective of whether CL was performed or not.

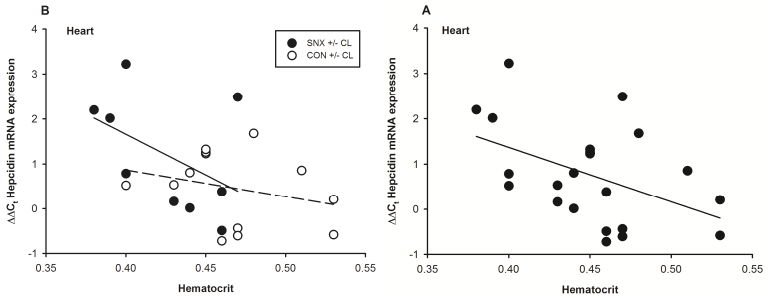

Figure 5. The relationship between hematocrit and cardiac hepcidin expression in all groups (panel A), or divided according to the presence or absence of CKD (SNX and CON respectively, panel B). ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group.

Hepcidin expression in the heart correlated with BNP and CTGF expression

Cardiac expression of hepcidin correlated significantly with cardiac expression of CTGF (R =0.431, *P* = 0.022; Figure 6A). Furthermore, the cardiac expression of hepcidin was tightly correlated with that of BNP (Figure 6B, R = 0.734, *P*< 0.001)

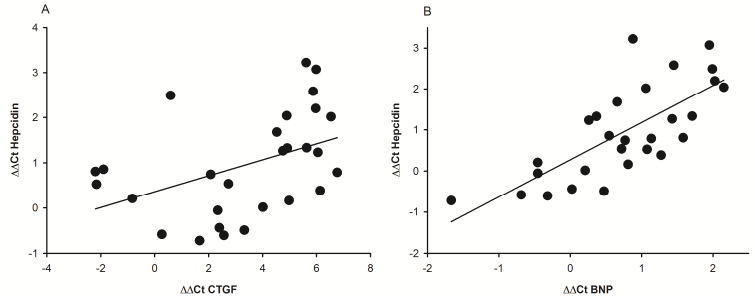

Figure 6. The correlation between cardiac expression of CTFG and hepcidin (A), and between BNP and hepcidin (B). ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group.

EPO-receptor expression in heart was decreased with CL

Cardiac expression of the EPO receptor was variable across the groups. Although it tended to be increased in SNX rats vs. CON rats (*P* = 0.066), it was decreased in rats with CON+CL and SNX+CL (Figure 7). Cardiac expression of EPO mRNA was undetectable in all groups.

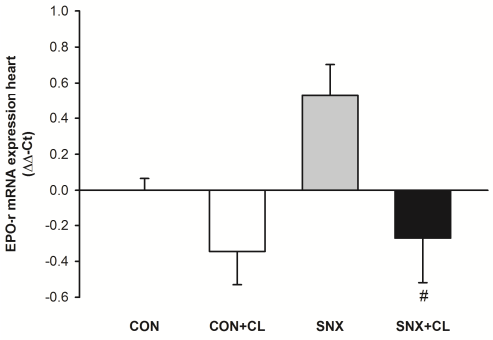

Figure 7. Cardiac expression of EPO receptor. ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group.
Mean ± SEM. # *P*<0.05 vs. SNX.

Correlation of hepatic hepcidin expression with C/EBP-α

Hepatic hepcidin expression correlated with expression of the hypoxia-apoptosis marker CCAAT/enhancer-binding protein α (C/EBP-α; R = 0.682, *P*< 0.001; Figure 8), but not with expression of BMP-6 (R = 0.285, *P* = 0.149) or MCP-1 (R = 0.122, *P* = 0.546). Expression of C/EBP-α was decreased progressively across groups, and was nearly significant in SNX vs. CON (*P* = 0.06; Figure 8B).

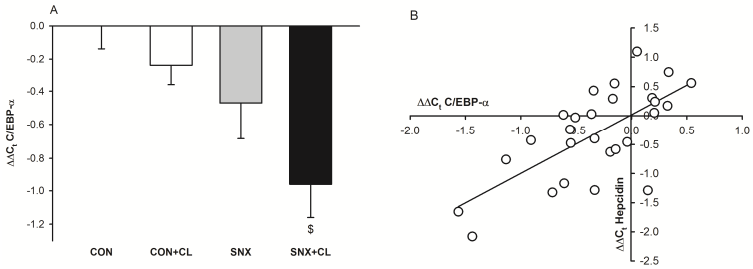

Figure 8. Correlation between gene expression of C/EBP-α and hepcidin (A), and expression of C/EBP-α across groups (B). ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group. $ *P*<0.05 vs. CON+CL.

Effect of medication on hepcidin expression

Subsequently, we assessed cardiac hepcidin expression in hearts from SNX+CL rats treated with the NO donor molsidomine (MOLS), and with a combination of MOLS, a NF-κB inhibitor (PDTC) and a SOD mimetic (tempol). Contrary to our expectations, these interventions further increased cardiac hepcidin expression and tended to normalize hepatic hepcidin expression (Figure 9).

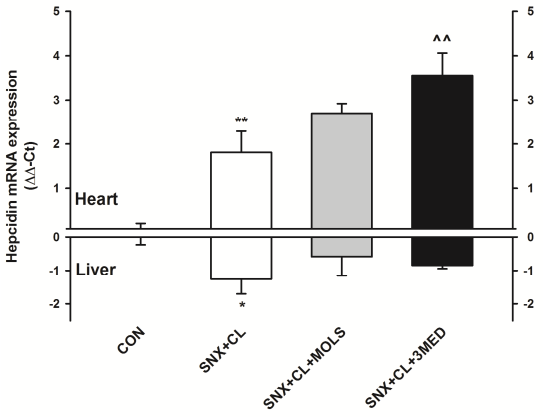

Figure 9. Effect of NO supplementation with molsidomine (MOLS), and of anti-inflammatory and anti-oxidant 3MED therapy on hepcidin in heart and liver. Mean ± SEM, * P<0.05, ** P<0.01 vs. CON;
^^ P<0.01 vs. SNX+CL.

5.4 Discussion

The main findings of this study were:

1. Hepcidin gene expression in rat hearts was increased in local (MI), and remote (CKD) cardiac injury. In the combined injury model, cardiac hepcidin expression was markedly increased whereas, in contrast, hepatic hepcidin gene expression was decreased
2. Cardiac hepcidin gene expression correlated strongly with gene expression of damage markers CTGF and BNP, and hepatic hepcidin gene expression correlated to expression of the hypoxia/apoptosis marker C/EBP-α.

3. With NO supplementation, anti-oxidant and anti-inflammatory medication in rats with cardiorenal failure, cardiac expression of hepcidin was further increased.

The increased cardiac expression of hepcidin with local or remote injury is in agreement with findings by others.[10-12] As hepcidin in cardiomyocytes reduces ferroportin content and iron efflux[13] and sequesters iron inside the cells one could speculate that hepcidin expression plays a protective role in myocardial injury. The maintenance of intra-cellular iron levels could contribute to improved myocardial energy metabolism in times of cardiac stress. It may also reduce the amount of extra-cellular iron that participates in generation of oxidative stress. On the other hand, the increased intra-cellular iron levels may enhance oxidative stress inside the cell. Contrary to our expectations, liver hepcidin was decreased in rats with cardiorenal failure. Cardiorenal failure is likely associated with inflammation and oxidative stress, which would have resulted in hepcidin upregulation. Alcoholic and viral liver cell damage has been found to reduce hepcidin expression, mediated by reactive oxygen species and C/EBP-α.[16-18] Indeed, C/EBP-α expression in the liver was also decreased in rats with cardiorenal failure, and there was a positive correlation between the gene expression of C/EBP-α and that of hepcidin in the liver. Therapeutic interventions aimed at supplementing NO and reducing oxidative stress and inflammation had little affect on liver hepcidin gene expression, an, unexpectedly, tended to increase cardiac hepcidin gene expression. We previously found that the 3MED combination improved cardiac function. This may have resulted in a greater metabolic activity, and an increase in cardiomyocyte iron demand. Alternatively, alleviating oxidative stress may reduce iron consumption in the Fenton reaction, and thus necessitate further intracellular sequestration of iron by hepcidin. Finally, it is possible that NO, ROS and/or NF-kB have direct effects on cardiomyocyte HAMP expression.

This study was performed as an additional analysis to studies performed earlier to elucidate bidirectional organ damage in combined CKD and HF, and to assess the effect of different interventions. The results of this study suggest that hepcidin reacts to cardiac injury and may play a role in pathogenesis, although at this point the mechanism still need to be elucidated.

5.5 Perspectives

Our results suggest a role for iron and iron regulation by hepcidin as a biologically active mechanism in cardiorenal failure. In the future, animal and cell experiments should be developed that study the mechanisms of systemic and local hepcidin production and action in different tissues. Effects of different stress conditions (uremia, hypoxia, heart failure) and interventions (hepcidin antagonism, iron supplementation or iron chelation) on cellular iron distribution should be investigated and coupled to structural and functional outcome.

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Part III

Chapter 6

Transient nitric oxide reduction induces permanent cardiac systolic dysfunction and worsens kidney damage in rats with chronic kidney disease

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Left ventricular systolic dysfunction (LVSD) in patients with chronic kidney disease (CKD) is associated with poorer prognosis. As patients with CKD often exhibit progressively decreased nitric oxide (NO) availability, and inhibition of NO production can reduce cardiac output, we hypothesized that loss of NO availability in CKD contributes to pathogenesis of LVSD.

Subtotally nephrectomised (SNX) rats were treated with a low dose of the NO synthase inhibitor Nω-nitro-L-arginine (L-NNA; 20 mg/L water; SNX+L-NNA) and compared to relevant control groups. To study permanent changes separate from hemodynamic effects, L-NNA was stopped after wk 8 and rats were followed up to wk 15, until blood pressure was similar in SNX+L-NNA and SNX. To study effects of NO depletion alone, a control group with high dose L-NNA (L-NNA-High: 100 mg/L) was included.

Mild systolic dysfunction developed at wk 13 after SNX. In SNX+L-NNA, systolic function decreased by almost 50% already from wk 4 onward, together with markedly reduced whole body NO production, and high mortality. In L-NNA-High, LVSD was not as severe as in SNX+L-NNA and renal function was not affected. Both LVSD and NO depletion were reversible in L-NNA-High after stopping L-NNA, but were persistently low in SNX+L-NNA. Proteinuria increased compared to rats with SNX, and glomerulosclerosis and cardiac fibrosis were worsened.

We conclude that SNX+L-NNA induced accelerated and permanent LVSD that was functionally and structurally different from CKD or NO depletion alone. Availability of NO appears to play a pivotal role in maintaining cardiac function in CKD.

6.1 Introduction

Progression of chronic kidney disease (CKD) coincides with increased incidence of cardiovascular disease,[1] and left ventricular (LV) systolic dysfunction is one of the most important risk factors for development of heart failure (HF) and mortality.[2-4] We, and others, designated the co-existence of kidney and heart dysfunction as the (Severe) Cardiorenal Syndrome.[5-7] As patients are far more likely to die of cardiovascular disease during the progression of CKD than to reach end-stage renal disease and dialysis,[1] it is important to identify pathogenic factors that drive this increased risk. We proposed the Cardiorenal Connection (CRC) as the putative pathophysiological mechanism,[6] and further postulated that the balance between nitric oxide (NO) and reactive oxygen species (ROS) is a key modulator of the other cardiorenal connectors.[8]

Subtotal (5/6th) nephrectomy (SNX) in rats is one of the most widely used models to study the cardiac sequelae of chronic kidney disease (CKD). However, measures of *in vivo* cardiac systolic function appear to be unchanged during study periods up to 8 weeks.[9, 10] In humans, the most common causes of CKD are hypertension, diabetes and/or aging which are associated with reduced NO availability. Indeed, CKD is accompanied by a long-standing and progressive decrease in nitric oxide (NO) availability.[11, 12] Furthermore, inhibition of NO synthase (NOS) causes hypertension and cardiovascular damage, and can functionally decrease systolic function and cardiac output in rats, dogs and humans.[13-17] We therefore aimed to explore the interaction between experimental CKD and chronically diminished NO availability on development of systolic dysfunction. We hypothesized that depletion of NO availability during progression of experimental CKD would cause cardiac systolic dysfunction and accelerate the development of cardiorenal failure. We studied this by treating SNX rats on a high-salt diet with low dose NOS inhibition up to 8 weeks after surgery (SNX+L-NNA), and compared *in vivo* heart and kidney function and whole body NO production with that in relevant control groups, also on high salt. To distinguish combined effects of CKD and L-NNA on cardiorenal variables from those of systemic NO depletion per se, we also included a control group of normal rats treated with high dose L-NNA designed to achieve similar levels of hypertension and NO depletion as in SNX+L-NNA. Furthermore, while hemodynamic effects may reverse after

discontinuation of chronic NOS inhibition, the resultant underlying cardiovascular damage can negatively affect renal function in the long term.[18, 19] Therefore, we studied whether NO inhibition had caused persistent changes and studied cardiorenal functional variables not only during L-NNA treatment, but also after discontinuation of treatment, followed by evaluation of end-organ damage upon termination**.**

6.2 Methods

The study protocol was approved by the Ethical Committee on Animal Experiments of the University of Utrecht, Utrecht, The Netherlands, and conformed to Dutch law on Laboratory Animal Experiments. Male inbred Lewis rats (Lew/Crl), 180 – 200 g, were purchased from Charles River, Germany, and housed in a climate-controlled facility with a 12:12-hour light:dark cycle.

Study set-up and experimental groups.

Rats were divided into five groups with similar initial body weight: **CON**: sham-operated; **SNX**: subtotal nephrectomy; **L-NNA-Low:** sham-operated + 20 mg/L L-NNA (Nω-nitro-L-arginine; Sigma-Aldrich Chemie, Steinheim, Germany) in drinking water; **L-NNA-High:** sham-operated + 100 mg/L L-NNA in drinking water; **SNX+L-NNA:** subtotal nephrectomy + 20 mg/L L-NNA in drinking water (Figure 1)**.** The doses for L-NNA were determined in pilot experiments. The dose of 20 mg/L was chosen because it caused an increase in tail cuff systolic blood pressure (SBP; see below) up to 200 mmHg during L-NNA-treatment. The dose for L-NNA-High was chosen to achieve similar levels of hypertension and NO depletion as SNX+L-NNA. Animals in the L-NNA-Low, -High and SNX+L-NNA groups were pre-treated with L-NNA during two wks before surgery. CON and SNX rats received normal water. All groups were fed standard pellet rodent chow (CRM-E; Special Diet Services Ltd., Witham, Essex, UK). Baseline (BL) measurements of *in vivo* cardiac and renal function were performed to document dose-dependent effects of L-NNA before surgery. Then, starting in wk –1, we performed a two-stage SNX or sham-procedure. In short, the right kidney was first removed and one week later, the

poles of the left kidney were cut off, equaling approx. 2/3rds of the weight of the previously removed kidney. In sham-operated rats, the kidneys were only decapsulated. After one week of recovery, rats again received L-NNA or normal water according to group, and all rats were fed standard powdered chow (CRM-FG; Special Diet Services Ltd., Witham, Essex, UK) supplemented with 6% NaCl, to induce acceleration of development of CKD. Although Szabo *et al.* [20] found that Lewis rats developed only mild CKD after SNX (by ablation/infarction; A/I), others have shown that A/I SNX in Lewis rats caused chronic stable kidney disease up to 15 weeks with progressive proteinuria from 4 weeks onward, and a two- to three-fold increase in serum creatinine levels.[21] In pilot-studies, we determined that SNX by surgical resection combined with 6% NaCl feed induced stable chronic kidney disease after 6 – 8 wks, comparable to that described by Vercauteren *et al.*[21] We performed i*n vivo* measurements of renal and cardiac function in wk 4 and wk 8 after SNX. All L-NNA-treatments were then stopped at the end of wk 8 in respective groups. After a three-week washout period, cardiac and renal function was re-evaluated every two weeks up to wk 15 (Figure 1), until tail-cuff systolic blood pressure (see below) in SNX+L-NNA had decreased to the level of SNX alone.

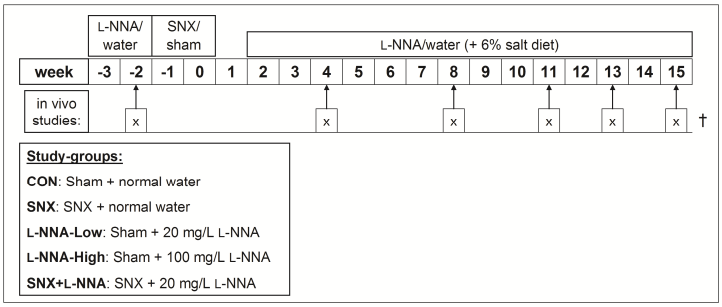

Figure 1. Study design: rats in the two L-NNA groups and in the SNX+L-NNA group were pretreated with L-NNA 2 wks before SNX or sham surgery at wk 0. L-NNA-treatment was continued from wk 1 to wk 8. After wk 8, L-NNA was stopped and rats were followed up to wk 15. *In vivo* measurements were performed after pre-treatment with L-NNA in respective groups before surgery (BL: baseline), the L-NNA phase (wk 4 and 8), and during the recovery phase (wk 11, 13, and 15) After wk 15, animals were sacrificed and end-organ damage was assessed.

Systolic Blood Pressure and 24-hour Urine Samples

Systolic blood pressure (SBP) was measured by the tail-cuff method as described previously.[22] Directly after each SBP measurement rats were placed in metabolism

cages without food for 24 h, but with free access to water with 2% glucose, supplemented with L-NNA as appropriate, for determination of urinary protein, measured with Coomassie blue. Urine was collected on 1 mL of antibiotic/antimycotic solution (Sigma, St. Louis, MO; A5955) and stored at –80°C. Urinary excretion of stable NO metabolites NO_2 + NO_3 (NO_x) were determined by fluorometric quantification of nitrite content.[23] The rats were fasted during the 24h collection period to minimize the effect of dietary protein intake. Thus, analysis of the 24-h NOx excretion can provide a solid estimate of whole body NO production during 24 hours.[24] Creatinine clearance was calculated by the standard formula. Urinary sodium content was determined by flame photometry.

Echocardiography

Trans-thoracic echocardiography was performed with a digital ultrasound machine (Philips Sonos 5500, Eindhoven, NL) and a 15-MHz linear array transducer (Hewlett Packard Company, Palo Alto, USA). Animals were anesthetized with isoflurane and placed in a supine position on a warming pad and a three-lead ECG system was connected to the paws. Anesthesia was adjusted to the lowest possible level to maintain physiological heart rates. Two-dimensional B-mode cine-loops with continuous ECG-registration were recorded in the parasternal long axis (LAX) and the mid-papillary short axis (SAX) views. Typical study duration was 15 min. We coded the acquisitions, and results were decoded after analysis. The recordings were analyzed off-line using the software present on the system, and the variables were measured in at least three heartbeats at end-diastole and corresponding end-systole. The investigator performing the analyses was blinded to treatment group.

Details on calculations:

Long axis: LV area (LVA) was measured by tracing the endocardial border; LV length (LVL) was measured from the apical trace border to the middle of the trace border on the LV outflow tract. LV volume was calculated with the ellipsoid area–length method: $V\ (ml) = [8*(LVA)^2]/(3\pi*LVL)$ at end-diastole (end-diastolic volume; EDV) and end-systole (end-systolic volume; ESV).[25] Calculated variables: Stroke volume (SV, mL) = EDV – ESV; Ejection fraction (EF) = (EDV-ESV)/ESV.

Short axis: the endocardial border was traced at end-diastole (LVEDa) and end-systole (LVESa) at the mid-papillary level. Fractional area change of the LV (LVFAC) was calculated with the formula: LVFAC (%) = [(LVEDa-LVESa)/LVEDa] x 100%.

Plasma parameters

After echocardiography, a blood sample (500 µL) was collected from the tail vein. Plasma was separated and levels of urea (blood urea nitrogen) and creatinine were measured. Urea was determined by DiaSys Urea CT FS (DiaSys Diagnostic Systems, Holzheim, Germany) and creatinine was enzymatically determined with Creatinine F L-Type R1 and R2 (Wako Chemicals, Neuss, Germany).

Organ Weights and Histology

After exsanguination under anesthesia, we harvested the organs and weighed them. Kidneys were cut transversely, fixed in formalin, and embedded in paraffin. Glomerulosclerosis and tubulo-interstitial damage were scored on PAS-stained kidney sections in a blinded manner as described previously.[26, 27]

After fixation in formalin, the heart was cut in three transverse sections and embedded in paraffin. Cardiomyocyte circumference was measured on PAS-stained myocardial slices in sections with transversely cut myocardial fibers by tracing the cellular border on photomicrographs of at least 50 different cardiomyocytes with a computer assisted image analysis system (OptiMas, Houston, TX) in a blinded manner.

Digital photomicrographs of transverse sections of the heart stained with Sirius Red were taken to measure collagen content of the heart. The extent of cardiac fibrotic patches was scored in a blinded manner on joined digital images acquired at 20x magnification using ImageJ software.[28] Images were converted to an RGB stack and collagen area fraction was measured on the green channel, where the red-stained areas appear black. The percentage of the collagen area was calculated by dividing the Sirius Red stained area by the total LV tissue area.
Perivascular collagen was analyzed by dividing the area of collagen around the vessel by total vessel area using ImageJ software on digital images acquired at 200x magnification, visualized with circular polarized light.

Data Analysis

Data are shown as mean ± SEM. Data were analyzed and graphed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). One-way analysis of variance (ANOVA) with the Student-Newman-Keuls (SNK) post-hoc test was done per time-point across all groups, and one-way repeated measures ANOVA with SNK post-hoc test across all time-points within each group. Data that was not normally distributed was log-transformed or ranked to achieve normality. Statistical significance was reached with p-values below 0.05.

6.3 Results

Systolic blood pressure.

Low dose L-NNA in rats without SNX caused mild hypertension. Mild hypertension was also present in SNX rats and treatment with low dose L-NNA in SNX+L-NNA induced an increase in SBP up to levels of 200 mmHg (Figure 2). Thus, the effect of low-dose L-NNA and SNX on blood pressure appeared to be additive. In sham-operated rats, treatment with high dose L-NNA (L-NNA-High), led to a rise in blood pressure to similar levels as observed in SNX+L-NNA rats during (low dose) L-NNA-treatment. After cessation of L-NNA treatment, SBP quickly normalized to pretreatment levels in L-NNA-Low and -High. In contrast, in SNX+L-NNA, SBP tapered off slowly to the level of SNX.

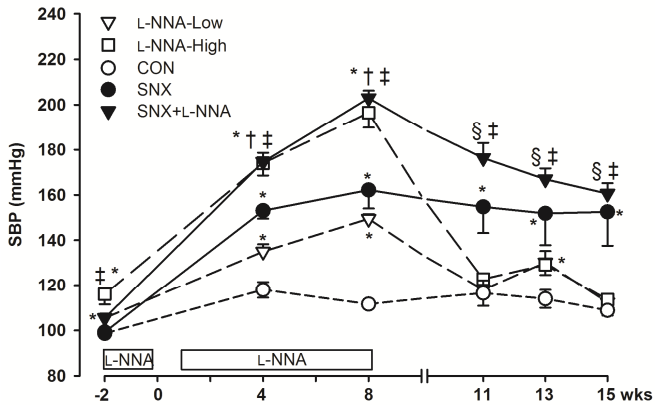

Figure 2. Tail-cuff systolic blood pressure (SBP; mmHg) in all groups. Mean ± SEM,
* *P*<0.05 vs. CON;
† *P*<0.05 vs. SNX;
‡ *P*<0.05 vs. L-NNA-Low.
§ *P*<0.05 vs. L-NNA-High. For clarity, different levels of significance are not indicated.

Table 1. *Kidney functional data at 8 weeks and 15 weeks.*

	8 weeks					*15 weeks*				
	CON	**SNX**	**LNNA-Low**	**LNNA-High**	**SNX+LNNA**	**CON**	**SNX**	**LNNA-Low**	**LNNA-High**	**SNX+LNNA**
	(n=10)	(n=8)	(n=7)	(n=9)	(n=19)	(n=10)	(n=8)	(n=7)	(n=9)	(n=14)
Plasma urea (mmol/L)	6.3 ± 0.4	12.4 ± 0.6 *	7.7 ± 0.8	7.2± 0.4	18 ± 1.1 †‡§	8.9 ± 0.6	14 ± 1.1 *	7.6 ± 0.3	6.5 ± 0.8	16 ± 0.9 ‡§
Creatinine clearance (ml/min/100 g BW)	0.85 ± 0.09	0.25 ± 0.03 *	0.77 ± 0.07	1.00 ± 0.07	0.27 ± 0.02 ‡§	0.77 ± 0.06	0.21 ± 0.04 *	0.89 ± 0.08	0.90 ± 0.06	0.28 ± 0.03 ‡§
Urinary NOx (µmol/24h/100 g BW)	1.23 ± 0.14	0.89 ± 0.13	0.89 ± 0.10	0.50 ± 0.11 *‡	0.48 ± 0.04 †‡	1.03 ± 0.12	1.19 ± 0.28	0.99 ± 0.05	1.19 ± 0.11	0.63 ± 0.05 †‡§
Natriuresis (µmol/24h/100 g BW)	736 ± 77	782 ± 68	627 ± 36	617 ± 57	851 ± 83	638 ± 57	637 ± 55	592 ± 92	724 ± 117	463 ± 54

BW: body weight; NOx: nitric oxide metabolites.
Mean ± SEM. * $P<0.05$ vs. CON; † $P<0.05$ vs. SNX; ‡ $P<0.05$ vs. L-NNA-Low; § $P<0.05$ vs. L-NNA-High.
For clarity, different levels of significance are not indicated.

Urinary NO metabolites

Urinary NO metabolite (NOx) excretion, as a measure for whole-body NO production, was mildly (P = 0.06) reduced in both SNX and L-NNA-Low vs. CON at 8 wks, but markedly depressed in L-NNA-High and SNX+L-NNA to similar low levels (Table 1). At wk 15, 7 wks after stopping L-NNA, levels of urinary NOx in L-NNA-Low and L-NNA-High, as well as in SNX, had increased to levels similar to CON. This is in contrast to what we observed in SNX+L-NNA where NO production remained significantly depressed compared to all other groups.

Echocardiography

Figure 3 shows the temporal changes of systolic function, expressed as left ventricular fractional area change (LV-FAC), in all groups. At the baseline measurement, after pre-treatment in relevant groups before surgery (Figure 1), there was a clear dose-dependent effect of L-NNA on systolic function. In SNX, LV-FAC was similar to CON up to wk 8. Cardiac function was already severely impaired in SNX+L-NNA at wk 4, with LV-FAC dropping by almost 50% from baseline levels. In this group, we found an increase in end-systolic volume (ESV) at wk 8 vs. SNX, L-NNA-Low and L-NNA-High (Figure 4). This resulted in a decreased ejection fraction (EF) and stroke volume (SV), despite the fact that end-diastolic volume (EDV) was also slightly larger in SNX+L-NNA vs. SNX. Systolic dysfunction was also seen in L-NNA-High, but this was not as pronounced as in SNX+L-NNA and almost completely restored after stopping NOS inhibition. Surprisingly, LVSD did not recover in SNX+L-NNA after cessation of L-NNA.

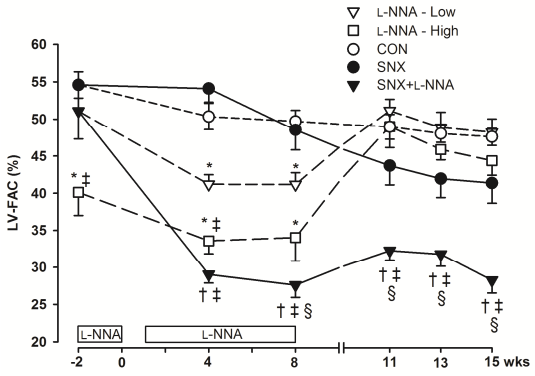

Figure 3. Cardiac systolic function expressed as LV fractional area change (LV-FAC,%) by echocardio-graphy in the mid-papillary short-axis view.
Mean ± SEM, * P<0.05 vs. CON;
† P<0.05 vs. SNX;
‡ P<0.05 vs. L-NNA-Low.
§ P<0.05 vs. L-NNA-High. For clarity, different levels of significance are not indicated.

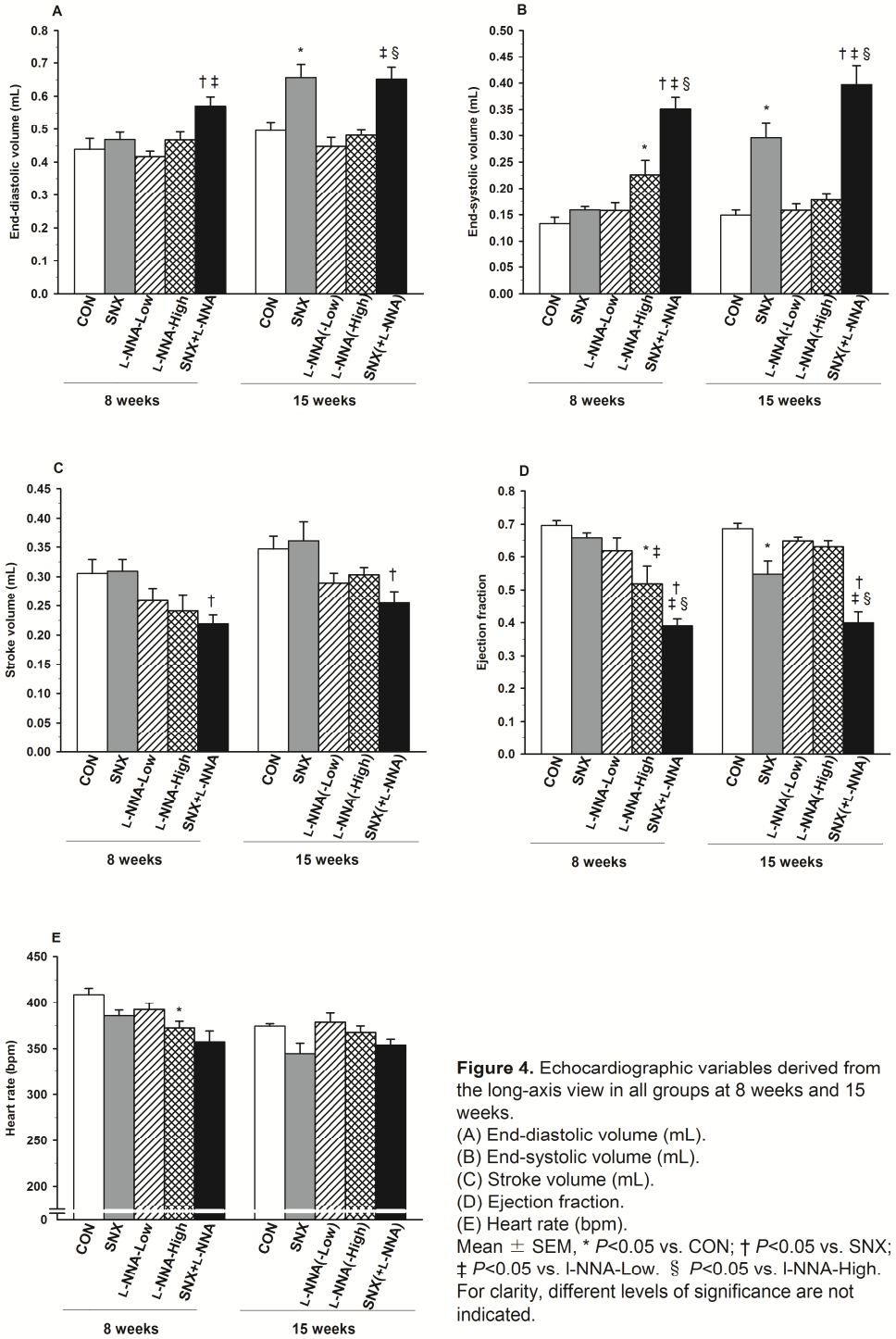

Figure 4. Echocardiographic variables derived from the long-axis view in all groups at 8 weeks and 15 weeks.
(A) End-diastolic volume (mL).
(B) End-systolic volume (mL).
(C) Stroke volume (mL).
(D) Ejection fraction.
(E) Heart rate (bpm).
Mean ± SEM, * P<0.05 vs. CON; † P<0.05 vs. SNX; ‡ P<0.05 vs. l-NNA-Low. § P<0.05 vs. l-NNA-High. For clarity, different levels of significance are not indicated.

At wk 15, EF and LV-FAC were both mildly depressed in SNX vs. CON, but only EF was significantly lower at wk 15 in SNX vs. CON (Figure 4). On the other hand, stroke volume was similar because of significant LV dilatation. Levels of LV-FAC of both CON and SNX were significantly lower after wk 8 compared to their baseline levels. Systolic function was still markedly low in SNX+L-NNA at the end of the study with an increase in ESV and SV. Even when levels of LV-FAC of SNX+L-NNA rats after wk 8 were corrected for the decline in SNX rats compared to CON, it was still significantly worse than SNX alone. Heart rate (HR; Figure 4E) was significantly lower in SNX rats compared to controls at wk 15. However, because SV was slightly larger, calculated cardiac output was not different (125 ± 14 mL/min vs. 130 ± 8 mL/min in SNX vs. CON resp.). Heart rate was reduced by high dose L-NNA at wk 8, but not at wk 15. There were no significant differences in HR between SNX and SNX+L-NNA.

Renal variables

Treatment with L-NNA-Low and L-NNA-High did not affect renal function throughout the whole study period. Natriuresis levels were not significantly different between groups at wk 8 and wk 15. It should be noted that these values were obtained under fasting conditions. Subtotal nephrectomy induced CKD and resulted in raised plasma urea, plasma creatinine, and progressive proteinuria (Figure 5, Table 1, and supplementary figures in Appendix). Calculated creatinine clearance (CrCl) in SNX was one-third of CON at wk 8 (P<0.001) and declined further at wk 15 (P<0.001). At wk 4, there were no significant differences between SNX and SNX+L-NNA in either plasma urea (12 ± 1 mmol/L vs. 13 ± 1 mmol/L, resp.) or plasma creatinine (66 ± 6 µmol/L vs. 76 ± 5 µmol/L, resp.). Plasma urea was significantly higher in SNX+L-NNA compared to SNX at wk 8 (Table 1; P<0.001 vs. SNX) and wk 13 (see supplementary figure). At 15 wks, no significant differences were apparent in plasma urea, creatinine or CrCl between SNX and SNX+L-NNA. On the other hand, proteinuria approximately doubled in SNX+L-NNA compared to SNX at wk 8 (Figure 5) and remained higher until the end of the study, despite the fact that SBP decreased to similar levels. From wk 8 onward, levels of proteinuria in both SNX and SNX+L-NNA were significantly higher than those at BL or wk 4. At the end of the study, levels in SNX were significantly higher than those at wk 8. In

SNX+L-NNA, proteinuria was borderline increased at wk 13 and 15 compared to wk 8 and 11 (*P*=0.08).

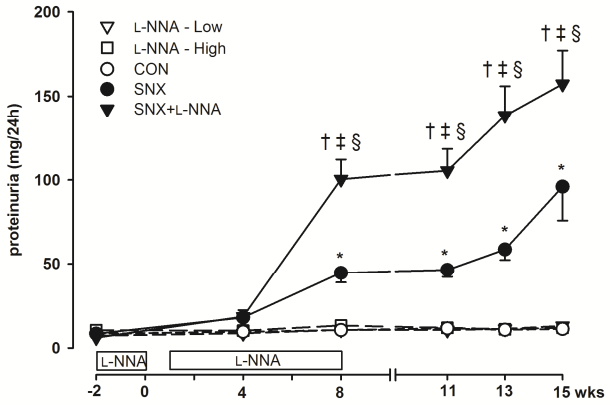

Figure 5. Proteinuria (mg/24h). Mean ± SEM,
* *P*<0.05 vs. CON;
† *P*<0.05 vs. SNX;
‡ *P*<0.05 vs. L-NNA-Low.
§ *P*<0.05 vs. L-NNA-High.
For clarity, different levels of significance are not indicated.

Mortality

Mortality in SNX+L-NNA was 30% (9 out of the initial 30 animals) by wk 8. After stopping L-NNA, mortality continued to increase to 40% (12/30) by wk 11, and 53% (16/30) by wk 15. Half of the mortality was spontaneous, and cause of death was undetermined. All other rats were euthanized because of severe morbidity. This consisted mainly of cachexia and dyspnea related to end-stage cardiorenal failure with visibly enlarged atria and thoracic edema. Others exhibited neurological deficits due to brain or spinal hemorrhage, a well-known side-effect of long-term NOS inhibition.[29] No morbidity or mortality occurred in any of the other groups.

Terminal biometric data

At the end of the study, both hematocrit and body weight were slightly but significantly lower in all rats with CKD compared to the other groups (Table 2). The left kidney remnant was hypertrophied to a larger degree in SNX than in SNX+L-NNA (Table 2). Terminal LV weight was also higher in all CKD rats compared to the other groups. Both right ventricle weight and wet lung weight were increased in SNX vs. CON, and wet lung weight was slightly worsened by SNX+L-NNA (borderline significant).

Table 2. Terminal biometric data.

	CON	SNX	L-NNA-Low	L-NNA-High	SNX+L-NNA
	(n=10)	(n=8)	(n=7)	(n=9)	(n=14)
Ht (g/g)	0.49 ± 0.01	0.43 ± 0.01 *	0.46 ± 0.00	0.48 ± 0.01	0.44 ± 0.01 ‡§
Body weight (g)	426 ± 8	371 ± 17 *	433 ± 9	425 ± 7	362 ± 7 ‡§
LK-WI (g/kg)	3.34 ± 0.09	4.15 ± 0.12 *	3.59 ± 0.08	3.62 ± 0.06	3.80 ± 0.09 †
LV-WI (g/kg)	1.93 ± 0.02	3.47 ± 0.21 *	1.89 ± 0.02	2.07 ± 0.05	3.30 ± 0.06 ‡§
RV-WI (g/kg)	0.49 ± 0.03	0.67 ± 0.06 *	0.44 ± 0.03	0.48 ± 0.03	0.60 ± 0.05
Wet lung WI (g/kg)	3.09 ± 0.09	3.91 ± 0.28 *	3.09 ± 0.04	3.28 ± 0.07	5.31 ± 0.55 ‡§

Ht: hematocrit; WI: weight index (g/kg body weight;) LK: left kidney; LV: left ventricle; RV: right ventricle.
Mean ± SEM. * *P*<0.05 vs. CON; † *P*<0.05 vs. SNX; ‡ *P*<0.05 vs. L-NNA-Low; § *P*<0.05 vs. L-NNA-High.
For clarity, different levels of significance are not indicated.

Table 3. Cardiorenal histological damage.

	CON	SNX	L-NNA-Low	L-NNA-High	SNX+L-NNA
	(n=7)	(n=8)	(n=7)	(n=9)	(n=8)
Kidney:					
Normal glomeruli (score 0, %)	72 ± 2	54 ± 5 *	60 ± 2	59 ± 6	42 ± 4
Partial GS (score 1-2, %)	26 ± 1	29 ± 4	33 ± 2	33 ± 4	24 ± 3
Severe GS (score 3-4, %)	3 ± 1	17 ± 3 *	7 ± 2	9 ± 3	33 ± 6 † ‡ §
TI injury score (a.u)	0.63 ± 0.11	7.14 ± 0.68 *	0.64 ± 0.09	0.68 ± 0.11	6.46 ± 0.62 ‡ §
Heart:					
cmc area (μm^2)	390 ± 22	542 ± 23 *	441 ± 18	591 ± 24 * ‡	596 ± 28 ‡
Collagen area (%)	0.28 ± 0.10	2.67 ± 0.42 *	0.34 ± 0.06	1.80 ± 0.51 * ‡	4.25 ± 0.43 † ‡ §
Perivascular collagen (%)	26 ± 2	34 ± 2 *	30 ± 1	40 ± 1 * ‡	38 ± 2 ‡

GS: glomerulosclerosis, numbers indicate percentage of glomeruli affected; score 1-2: 25 – 50% damage; score 3-4: 75% damage – total sclerosis; TI: tubulo-interstitial; cmc: cardiomyocyte.
Mean ± SEM. * *P*<0.05 vs. CON; † *P*<0.05 vs. SNX; ‡ *P*<0.05 vs. L-NNA-Low; § *P*<0.05 vs. L-NNA-High.
For clarity, different levels of significance are not indicated.

Histology

Histological damage is summarized in Table 3. SNX induced glomerulosclerosis and tubulo-interstitial injury. The amount of severely sclerotic glomeruli was two-fold increased by SNX+L-NNA. Parallel to the increase in LV mass, cardiomyocyte area was higher in SNX compared to controls (P<0.001), but this was not further aggravated in SNX+L-NNA. Cardiomyocyte hypertrophy was also observed in the L-NNA-High group (P<0.001 vs. CON) despite the absence of increased LV weight (Table 2). SNX induced patchy fibrosis in the heart with increased collagen area fraction, which was worsened in SNX+L-NNA. High dose NOS inhibition alone (L-NNA-High) also caused a moderate degree of fibrosis. Increased perivascular collagen was apparent in SNX, SNX+L-NNA and L-NNA-High, but not in the L-NNA-Low group.

6.4 Discussion

The main finding of this study is that minimal NOS inhibition in the context of renal failure (SNX+L-NNA) was sufficient to cause marked and persistent LV systolic dysfunction and worsened kidney damage. This occurred in association with a more profound and sustained depletion of whole body NO production than that observed with SNX or low dose L-NNA alone. Proteinuria and severe glomerulosclerosis, as well as cardiac fibrosis were more severe in SNX+L-NNA rats when compared to rats with SNX alone. Cardiorenal failure was also worse in SNX+L-NNA than in sham-operated rats treated with a high dose of L-NNA, despite similar levels of hypertension and NO depletion. The combination of SNX and low dose L-NNA induced combined cardiorenal failure that was functionally and structurally different from that induced by either CKD or NO depletion alone (as in L-NNA-High).

Cardiac changes.

Our study suggests an important role for NO availability for maintenance of cardiac systolic function in CKD. Measures of *in vivo* systolic function generally remain preserved or even increase in rats with CKD with study periods up to 8 weeks.[9, 10, 30] Reddy *et al.*[10] argued that extending the period of CKD after SNX might induce a

more progressive decline in cardiac function. We found only a slight decrease in systolic function in prolonged CKD up to 15 weeks, and stroke volume was not different from controls.

Addition of low-dose NOS inhibition during development of CKD in rats was sufficient to cause a striking decrease in LV systolic function. The cardiac dysfunction in SNX+L-NNA was due to an increase in ESV (Figure 4). The difference in ESV at wk 8 was much larger in SNX+L-NNA vs. SNX than in L-NNA-High vs. CON, while SBP was comparable between SNX+L-NNA and L-NNA-High (203 ± 3 mmHg vs. 197 ± 7, resp.). Furthermore, LVSD was worse in SNX+L-NNA compared to L-NNA-High. Levels of SBP were also similar in SNX+L-NNA and SNX at the end of the study, while cardiac systolic function was significantly more impaired in the former. It has been shown before that NOS inhibitors reduce cardiac output to a larger degree than equal pressor doses of vasoconstrictors.[16] In acute NOS inhibition in dogs,[16] as well as chronic NOS inhibition in rats [31] a decrease in cardiac output was observed without an effect on coronary blood flow. This suggests that negative inotropic effects of NOS inhibition could also play a role. However, total blood pressure load in SNX+L-NNA over the course of the whole study was likely higher than in rats with SNX alone, which might have played a role in the development of more cardiac fibrosis.

End-diastolic volume was slightly larger in SNX+L-NNA at wk 8, which could be secondary to increased volume overload or compensation for loss of systolic ejection. Systolic function can also be affected by changes in preload, but both EDV and hematocrit were not different in SNX+L-NNA vs. SNX at the end of the study. On the other hand, the increased cardiac fibrosis in SNX+L-NNA may have hampered diastolic filling, leading to lower stroke volumes. Constitutive NOS isoforms have diverse autocrine and/or paracrine effects on cardiomyocyte function (reviewed in [32]). For example, neuronal NOS-derived NO appears to modulate excitation-contraction coupling of the cardiomyocyte by regulating calcium fluxes. Isolated cardiomyocytes from uremic rats showed reduced sarcoplasmic reticulum calcium ATPase-2a (SERCA2a) activity and disturbed calcium cycling,[33] and inhibition of NO production in SNX+L-NNA might have worsened these effects. Finally, the absence of meaningful differences in heart rate between groups suggests that cardiac output followed changes in stroke volume. The level of

anesthesia during echocardiography was specifically adjusted to the lowest possible level to avoid reductions in heart rate and blood pressure. The small, non-significant reduction in HR at wk 8 in SNX+L-NNA compared to SNX alone means that cardiac function in the former group was even worse than estimated by stroke volume and ejection fraction. More importantly, no compensatory increase in HR, which might have confounded the observed LVSD, was seen in any of the treated groups. The fact that we used a high-salt diet to accelerate progression of CKD in SNX rats might have affected the observed cardiac changes by altering fluid volume status or cardiac damage. However, all groups received the high-salt diet and natriuresis was not significantly different between groups.

Thus, a combination of functional and structural effects of the combined CKD and NO depletion likely caused the persistent systolic dysfunction. Our observations indicate that the experimental CKD made the heart more susceptible to the cardiodepressive effects of NO depletion. A CKD-specific effect on cardiac dysfunction is supported by clinical observations that LVSD and LV hypertrophy (LVH) can reverse in a significant number of patients after renal transplantation, but the underlying mechanisms are incompletely understood (reviewed by Zolty *et al.*[34]).

Inhibition of NO production alone in L-NNA-Low and L-NNA-High caused dose-dependent hypertension and systolic dysfunction that reversed to control levels after stopping L-NNA, which is in accordance with previous findings.[13, 18, 19, 31] Some mild permanent effects on cardiac remodeling and function were appa9rent in L-NNA-High at wk 15, which are probably linked to the cardiomyocyte hypertrophy and mild fibrosis present in these hearts. Cardiomyocyte size was, however, similarly increased in the hypertrophied SNX and SNX+L-NNA hearts as in the non-hypertrophic L-NNA-High hearts. The different hypertrophic and fibrotic responses in SNX, SNX+L-NNA, and L-NNA-High are in agreement with previous observations that the LVH and fibrosis observed in CKD appear to be partly independent of blood pressure load.[35-37]

Changes in NO production.

Both SNX alone and L-NNA-Low alone exhibited a mild reduction in urinary NOx excretion compared to controls, while in SNX+L-NNA it was markedly decreased.

This suggests that the separate interventions reduce whole body NO production via alternate pathways, and have additive effects when combined. This also suggests that in SNX alone there was reserve capacity of NO production, which was blocked by addition of an exogenous NO synthase inhibitor. Rats with L-NNA-High also had a significantly lower NOx excretion at 8 wks compared to untreated controls, which was not statistically different from SNX+L-NNA. However, cardiac dysfunction was significantly worse in SNX+L-NNA. Thus, a similar reduction in NO availability has a stronger impact on LV systolic function in combination with CKD than either of these alone. The additive effects of low dose NOS inhibition in the CKD rats were most likely not due to accumulation of L-NNA, because L-NNA is not excreted through the kidneys.[38] Furthermore, it is highly likely that all L-NNA was fully eliminated during the 3-week wash-out period between wk 8 and wk 11 in all groups. Although salt intake can influence effects of NOS inhibition,[39] the fact that there was no renal dysfunction and only minimal damage in L-NNA-Low and L-NNA-High excludes a major interaction. Urinary NOx excretion was not reduced in SNX animals at wk 15 compared to controls. This could either be due to increased NO production needed to maintain natriuresis,[40] or to iNOS-derived NO related to injury. However, the latter is unlikely considering that TI injury was not significantly increased in SNX+L-NNA vs. SNX. The high-salt diet may have slightly worsened hypertension in SNX, which can increase NO production due to higher shear-stress in resistance vessels.

Urinary NOx excretion was still significantly reduced at wk 15 in SNX+L-NNA. The combination of CKD and temporary NOS inhibition apparently induced changes that persistently reduce net NO production. These might include decreased NOS enzyme density or activity, L-arginine deficiency, or increased levels of endogenous inhibitors like asymmetric dimethylarginine (ADMA).[41]

Renal changes.

Previously, Fujihara and co-workers [42] treated SNX rats with low do0se N(G)-nitro-L-arginine (L-NAME), a similar NOS inhibitor, up to 3 weeks after SNX. They found increased hypertension and intraglomerular pressure, associated with more severe renal failure and kidney damage. Kang *et al.* [43] administered L-NAME over a 4 week period, starting 4 weeks after SNX surgery, and found similar effects. Thus,

NOS inhibition during both the initial and later stage of CKD development appears to worsen renal endpoints. In our study, renal failure was only mildly aggravated during L-NNA-treatment, as evidenced by significantly higher plasma urea and numerically higher plasma creatinine in SNX+L-NNA compared to SNX at wk 8. However, these differences were no longer appreciable at wk 15. Furthermore, creatinine clearance was not lower in SNX+L-NNA at wk 8, and even numerically higher at wk 15 compared to SNX alone. In contrast, we observed consistently higher levels of proteinuria from wk 8 onward in SNX+L-NNA compared to SNX alone. Inhibition of NOS can worsen proteinuria in rats with diabetic nephropathy with no effect on creatinine clearance.[44] Thus, different disease states that are generally associated with a decrease in NO availability make the kidneys more sensitive to (further) reductions in NO, and the permanent reduction in NO availability seen in SNX+L-NNA may be the driving factor for the increased protein leakage, despite the fact that SBP had returned to levels of SNX at wk 15.

Kidney function was not affected by either L-NNA-Low or L-NNA–High alone in sham-operated rats, and the high dose group displayed only a minor degree of glomerulosclerosis. A similar high dose of L-NNA has been shown to induce significant hypertension, proteinuria, and mortality in Wistar Kyoto rats.[45] This suggests that the inbred Lewis rat, like the Wistar Furth rat,[46] is more resistant to NO depletion. The fact that renal function in Lewis rats was not compromised in L-NNA-High allows more accurate separation of the effects of reduced vs. intact kidney function, combined with systemic NO depletion. The Wistar Furth rat was also more resistant to development of CKD after SNX, but this was greatly accelerated by subsequent treatment with low dose NOS inhibition, concurrent with a high mortality rate (28%).[47] This increased mortality may well have been due to cardiac events.

We used a high-salt diet in our experiments. This was done to accelerate progression of CKD after SNX, because Lewis rats appear to be more resistant to SNX than other strains.[20] Nevertheless, with this combination we could produce a model of chronic stable CKD similar to the model of Vercauteren *et al.*.[21] All groups received the high-salt diet, and natriuresis was not significantly different between groups at wk 8 and wk 15 (Table 1), suggesting that all groups were in sodium balance. The absence of significant renal changes in the L-NNA-Low and –High

groups suggests that the interaction between the high-salt diet and NOS inhibition on renal function was minimal, but we cannot rule out that results may have been different in rats on normal salt intake.

At 4 wks post-SNX, the levels of plasma urea, plasma creatinine, and proteinuria were all similar in SNX+L-NNA as compared to SNX, but systolic function was already severely compromised in SNX+L-NNA. Thus, the initial development of systolic dysfunction in SNX-L-NNA could not be attributed to more severe renal damage or dysfunction. Nevertheless, the persistence of LVSD at later stages might well relate to the worsened kidney damage that developed later on, as evidenced by increased proteinuria and more severe glomerulosclerosis in SNX+L-NNA.

6.5 Conclusions

Transient low dose NOS inhibition during development of experimental CKD induced severe and permanent cardiac systolic dysfunction and persistent NO depletion in association with high mortality. The combination of SNX and temporary low dose L-NNA caused end-organ dysfunction and structural damage in heart and kidneys that was worse than that induced either by long-term CKD, or by temporary high dose NOS inhibition only, despite an equal degree of hypertension and NO depletion. Our results underscore the importance of adequate NO availability for maintenance of cardiorenal function in the face of reduced kidney function. Furthermore, the cardiorenal failure observed in the model of SNX+L-NNA might serve as a more accurate representation of advanced CKD in patients, associated with decreased NO availability and cardiac systolic dysfunction.

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Chapter 7

The nitric oxide donor molsidomine rescues cardiac function in rats with chronic kidney disease and cardiac dysfunction

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We recently developed a rat model of cardiorenal failure that is characterized by severe left ventricular systolic dysfunction (LVSD) and low nitric oxide (NO) production which persist after temporary low dose NO synthase inhibition. We hypothesized that LVSD was due to continued low NO availability, and might be reversed by supplementing NO.

Rats underwent subtotal nephrectomy and were treated with low dose NO synthase inhibition with Nω-nitro-l-arginine up to wk 8. After three weeks of wash-out, rats were treated orally with either the long-acting, tolerance-free NO donor molsidomine (MOLS) or vehicle (VEH). Cardiac and renal function was measured in wk 11, 13, and 15. In wk 16, LV hemodynamics and pressure-volume relationships (PVR) were measured invasively and rats were sacrificed to quantify histological damage.

At wk 15, blood pressure was mildly reduced and creatinine clearance was increased by MOLS (both $P<0.05$). Molsidomine improved ejection fraction (53 ± 3 vs. 37 ± 2 % in VEH; $P<0.001$) and stroke volume (324 ± 33 vs. 255 ± 15 µL in VEH; $P<0.05$). Rats with MOLS had lower end-diastolic pressures (8.5 ± 1.1 mmHg) than VEH rats (16.3 ± 3.5 mmHg; $P<0.05$), and reduced *tau* (the time-constant of relaxation; 21.9 ± 1.8 vs. 30.9 ± 3.3 ms, respectively; $P<0.05$). The LV end-systolic PVR was shifted to the left in MOLS compared to VEH.

Summarized, in a model of cardiorenal failure with low NO availability, supplementing NO significantly improves cardiac systolic and diastolic function, without a major effect on afterload.

7.1 Introduction

Chronic kidney disease (CKD) is associated with a strongly increased risk for cardiovascular disease, including left ventricular (LV) hypertrophy (LVH), systolic dysfunction (LVSD) and heart failure.[1] Prognosis of patients with CKD is poorer when LVSD is present, and conversely outcome in patients with heart failure is worse when renal dysfunction is present.[2-4] This co-existence of cardiac and renal failure was designated as the (Severe) Cardiorenal Syndrome.[5, 6] We recently developed a rat model of combined CKD and LVSD, based on the CKD model of subtotal (5/6th) nephrectomy (SNX). The LVSD was induced in SNX rats with a low-dose of NO synthase (NOS) inhibition given up to 8 weeks after SNX, and ejection fraction (EF) was almost half of that in rats with SNX alone.[7] This was associated with strongly decreased NO production and worsened proteinuria. When NOS inhibition was stopped, systemic NO availability remained suppressed, and this was accompanied by persistent LVSD.

Reduced NO availability has a negative impact on cardiac function, especially in failing hearts, but it is also a common feature in CKD.[8, 9] Our previous study suggests that the cardiac dysfunction observed in our model is related to NO availability. However, the effect of changes in NO availability on cardiac function in CKD has not yet been fully established. We thus hypothesized that persistence of LVSD in our model is due to low NO availability and that supplementing NO *in vivo* with an NO donor could reverse cardiac dysfunction and ameliorate cardiorenal failure. Molsidomine (MOLS) is an NO-releasing pro-drug,[10, 11] previously shown to have beneficial effects in SNX and cholesterol-fed rats,[12, 13] but also in patients with heart failure.[14] We studied *in vivo* cardiorenal effects of MOLS given as a rescue therapy during the last 4 weeks of the protocol.

7.2 Methods

The study protocol was approved by the Utrecht University Committee on Animal Experiments, and conformed to Dutch Law on Laboratory Animal Experiments. Male inbred Lewis rats (Lew/Crl), 180 – 200 g, were purchased from Charles River, Germany, and housed in a climate-controlled facility with a 12:12-hour light:dark cycle.

Study set-up and subtotal nephrectomy

Rats were divided into two groups with similar initial body weight: CON: sham-operated; SNX+L-NNA: subtotal nephrectomy + 20 mg/L L-NNA (Nω-nitro- L-arginine; Sigma-Aldrich, St. Louis, MO, USA) in drinking water. Animals were pre-treated with L-NNA or normal (acidified) drinking water for two weeks, and fed standard pellet rodent chow (CRM-E; Special Diet Services Ltd., Witham, Essex, UK). Then, at wk –1, a two-stage subtotal (5/6th) nephrectomy (SNX) by resection or sham-procedure was performed.[7] After one week of recovery, rats were re-started on L-NNA or normal water, and all groups were fed standard powdered chow (CRM-FG; Special Diet Services Ltd.) supplemented with 6% (w/w) NaCl. This was done to accelerate progression of CKD, and results in stable chronic kidney disease comparable to the model of Vercauteren *et al*. [15] in the same rat strain. Normal Lewis rats are resistant to high (8%) salt diets with respect to blood pressure and development of proteinuria.[16]

In vivo measurements of renal and cardiac function (see below) were performed in wk 8 in a selection of animals, after which L-NNA treatment was stopped. After a three-week washout period, cardiac and renal function were re-evaluated at 11 weeks in all rats. Rats in the SNX+L-NNA group were stratified according to plasma urea and echocardiographic left ventricular fractional area change (LV-FAC; [LV end-diastolic area – LV end-systolic area]/LV end-diastolic area x 100%) and divided into two groups: treated with normal water (VEH), or treated with 120 mg/L molsidomine (MOLS; N-(Ethoxycarbonyl)-3-(4-morpholino)sydnone-imine, Sigma-Aldrich). Molsidomine is metabolized in the liver to linsidomine (SIN-1), which releases NO without further enzymatic activation. Although SIN-1 can generate peroxynitrite in vitro, at the *in vivo* levels of oxygen concentration it functions as a donor of NO.[11, 17] Because molsidomine is sensitive to light,[18] the medication was supplied in dark-brown bottles. In vivo measurements were then again performed 2 and 4 wks after start of treatment in all groups and in wk 16, terminal measurements were performed, and organs were harvested. Data from the treatment phase were censored for deaths occurring in that period.

Systolic blood pressure and 24-h urine sampling

Systolic blood pressure (SBP) was measured by the tail-cuff method.[19] After SBP measurement, a 24-h urine sample was collected in individual metabolic cages on 1 ml of antibiotic/antimycotic solution (Sigma-Aldrich) to prevent degradation of NO metabolites. These measurements were performed in week 8, 11, 13 and 15. In metabolism cages rats received no chow but had access to water with 2% glucose, supplemented with L-NNA or MOLS if indicated. After 24-h, urinary volume was measured and samples were stored at – 80ºC. Urine samples were analyzed for total protein, creatinine, nitric oxide metabolites, as described.[7] Creatinine clearance was calculated by the standard formula.

Echocardiography and blood samples

Transthoracic echocardiography was performed as previously.[7] In short, animals were anesthetized with isoflurane and placed in a supine position on a warming pad. A three-lead ECG system was connected to the paws, the chest shaved and excess hair removed with depilatory cream. Two-dimensional B-mode cine-loops with continuous ECG-registration were recorded in the parasternal long axis (LAX) and the mid-papillary short axis (SAX) views, while isoflurane anaesthesia was adjusted to the lowest possible level (1.75-1.85%) to minimize effects on heart rate and blood pressure. Typical study duration was 15 min. Acquisitions were coded and results were decoded after analysis. The recordings were analyzed off-line using the software present on the system, and the variables were measured in at least three heartbeats at end-diastole and corresponding end-systole, as described.[7] LV volume was calculated with the prolate ellipsoid area–length method,[20] and LV mass by the formula described by Litwin *et al.*.[21] After echocardiography, a blood sample (500 µL) was collected from the tail vein, and analysed for urea (blood urea nitrogen) and creatinine.[7]

Hemodynamic studies

Terminal LV hemodynamic measurements were performed via the closed-chest approach in week 16. After induction with isoflurane, the rats were placed on heating pads, intubated and mechanically ventilated with 2% isoflurane at a rate of 65/min with 40% oxygen. Buprenorfine (0.015 mg/kg i.m.) was injected for

analgesia. The left internal jugular vein was isolated and cannulated with a PE-50 catheter for infusion of saline at a rate of 8-10 ml/kg BW/min. LV hemodynamics were assessed with 2-Fr. pressure micromanometer (Millar Instruments; Houston, Tx, USA) and recorded with Chart software (ADInstruments; Spechbach, Germany). After a 15-min stabilization period, LV pressures were recorded during a short period with the ventilator switched to continuous positive airway pressure.

Hemodynamic variables were calculated using the Blood Pressure module. Data from at least three separate intervals each consisting of at least five cardiac cycles were averaged and the following parameters were calculated: heart rate (HR; bpm), end-diastolic pressure (EDP; mmHg), end-systolic pressure (ESP; mmHg), maximum change of pressure with time (dP/dt-max; mmHg/sec), minimum first time-derivative of pressure (dP/dt-min; mmHg/sec), and regression of dP/dt versus pressure (Tau; msec). Carotid artery pressure was measured, and mean arterial pressure (MAP) was calculated.

Cardiac pressure-volume analysis

Induction and basic surgical preparation was performed as above. The upper abdomen was opened by a small incision in the linea alba to provide access to the inferior vena cava (IVC) between the liver and diaphragm for occlusion studies (see below). A catheter was inserted in the bladder via a supra-pubic approach. The right common carotid artery was isolated and a 2-Fr. pressure conductance catheter (SPR-838, Millar Instruments) was inserted. The conductance catheter was advanced retrograde into the left ventricle. The pressure-volume signals were registered continuously with Chart-software (ADInstruments) at a sample rate of 1000/s. Adequate placement of the catheter was verified by the PV-loop signals. After a stabilization period of at least 15 minutes, baseline (BL) hemodynamic studies were performed as described above. These were followed by IVC occlusion (IVCO) studies during a short period of apnea and continuous positive airway pressure ventilation.

Measurements were recorded on Chart-software and analyzed off-line with the PVAN software (PVAN v3.6, Millar Instruments, Houston, Tx). Volume calibration of the conductance values was done by plotting the mean conductance catheter values for maximal and minimal volume of the baseline loops preceding

the IVCO-experiment against the end-diastolic and end-systolic volumes determined by echocardiography (see above.) This yielded an equation with a value for slope and y-intercept that were entered in the PVAN-program. The following parameters calculated by the PVAN-program were used to assess changes in contractility and elastance: End-systolic elastance (Ees; mmHg/µL) calculated from the linear the end-systolic PV relationship (ESPVR) and the linear volume intercept (V_0; µL); the maximal elastance calculated from quadratic curve fit of the ESPVR (E'max; mmHg/µL) and its volume intercept (V_0-quad; µL); the slope of the relationship between EDV and dP/dt-max (dP/dt-EDV; mmHg/s/µL) and its volume intercept (dP/dt-EDV intercept; µL), and the slope of the end-diastolic pressure volume relationship (EDPVR; mmHg/µL).

End-organ damage and histology

After exsanguination through the abdominal aorta under anaesthesia, organs were harvested and weighed. Glomerulosclerosis and tubulo-interstitial damage was scored on PAS-stained kidney sections.[7] Cardiomyocyte circumference was measured on PAS-stained myocardial slices in sections with transversely cut myocardial fibres and was traced on the cellular border on photomicrographs of at least 50 different cardiomyocytes with a computer assisted image analysis system (OptiMas, Houston, TX). Digital photomicrographs of transverse sections of the heart stained with Sirius Red were taken to measure collagen content of the heart using the ImageJ software.[22] The percentage of the collagen area was calculated by dividing the Sirius Red stained area by the total LV tissue area.[7]

Quantitative polymerase chain reaction

Expression of atrial natriuretic peptide (ANP; Rn00561661), neuronal NOS (nNOS; Rn00583793), inducible NOS (iNOS; Rn00561646) and endothelial NOS (eNOS; Rn02132634) in cardiac apical tissue was assessed by qPCR as described.[23] Cycle time(Ct) values were normalized to mean Ct-values of Calnexin (Canx; Rn00596877) and β-actin (Actb; Rn00667869), which we previously determined to be the two most stable housekeeping genes across all groups using the geNorm-program (http://medgen.ugent.be/~jvdesomp/genorm/). Statistical analysis was performed on ΔΔCt values, and results were graphed as fold-change ($2^{\Delta\Delta Ct}$).

Western Blot for 3-nitrotyrosine

Frozen heart (LV), left kidney and liver tissue samples from 4 rats per group were homogenized in a lysis-buffer (20mM Tris pH 7.4, 10% Glycerol, 0.1% SDS, 1% Triton-X100, 1mM EDTA, 1mM EGTA, 50mM NaF, 2mM Na3VO4, 0.150M NaCl, 0.5% sodium deoxycholate, 5% Protease inhibitor cocktail 1ml). Protein content was measured with a DC Protein Assay (500-0113, 500-0114, 500-0115; BioRad, USA). Then, 10 µg of protein was loaded for LV and liver, and 25 µg of protein for LK on a 8.5% tris-glycine gel and blotted on nitrocellulose membranes. The blots were stained with antibodies to 3-nitrotyrosine (3-NT; Santa Cruz, USA, SC-55256) and β-actin (Sigma, USA, A5441). Bands were visualized using chemiluminescence blotting substrate (POD, Roche) and membranes were stripped with Re-Blot Plus Strong (Millipore, USA, 2504).

Statistics

Data are shown as mean ± SEM. Some of the data from 5 CON rats and 12 SNX+L-NNA+VEH rats have been described in the previous study.[7] Data were analyzed and graphed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). One-way analysis of variance (ANOVA) with the Holm-Sidak post-hoc test was done per time-point across all groups, with the SNX(+ L-NNA)+VEH group as the reference group. For longitudinally assessed variables, a two-way repeated measures (RM) ANOVA was also performed. Survival analysis was done by Kaplan Meier Log-Rank test with Holm-Sidak post-hoc testing for multiple comparisons.Data that was not normally distributed was log-transformed or ranked to achieve normality. Statistical significance was reached with p-values below 0.05.

7.3 Results

Survival and clinical state

During the treatment period, no further mortality was observed in the MOLS-group, while 5 animals died in the VEH-group (Figure 1). Overall differences in survival curves were statistically significant by Kaplan Meier Log-Rank testing (P = 0.045) but with the post-hoc Holm-Sidak test for multiple comparisons there were no

significant differences between individual groups. Rats receiving MOLS also exhibited less morbidity, generally fared better, and were more active.

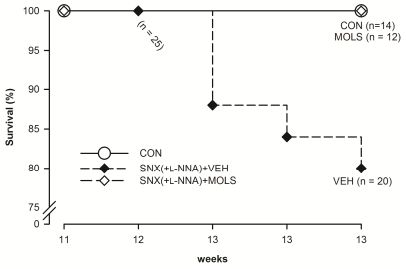

Figure 1. Survival curves from start of treatment at wk 11 up to wk 15. Group size, prior to within-group mortality, is also indicated.

Tail-cuff systolic blood pressure

As described before,[7] rats with SNX+L-NNA became hypertensive with a systolic blood pressure (SBP) of 202 ± 3 mmHg by week 8 (Figure 2). After withdrawing L-NNA, SBP dropped gradually in VEH rats, which was significant over time. Treatment with MOLS mildly reduced blood pressure compared to VEH-rats which was only significant at wk 15 (151 ± 5 vs. 165 ± 4 mmHg, $P < 0.05$).

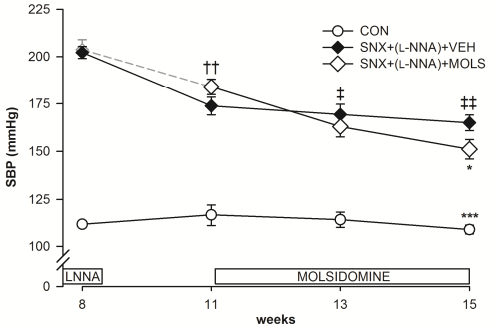

Figure 2. Tail-cuff systolic blood pressure (SBP, mmHg). Values at wk 8 for rats assigned to MOLS three weeks later are depicted as gray diamonds and dashed line. Mean ± SEM. * $P<0.05$; *** $P<0.001$ vs. SNX(+L-NNA)+VEH; †† $P<0.01$ vs. wk 8; ‡ $P<0.05$ vs. wk 11; ‡‡ $P<0.01$ vs. wk 11.

Renal function and urinary NOx excretion

At time of stratification, levels of plasma urea were not significantly different between rats allocated to VEH (13.3 ± 1.1 mmol/L) and MOLS (14.2 ± 1.5 mmol/L).

Plasma urea was not affected by MOLS therapy (Supplementary data in Appendix). Calculated creatinine clearance (CrCl) was also similar at wk 11 (VEH: 0.31 ± 0.02 vs. MOLS: 0.29 ± 0.04 mL/min/100 g). At wk 13, CrCl was still comparable between these groups (data not shown). At wk 15, CrCl was higher in the MOLS group (0.42 ± 0.04 mL/min/100 g) vs. the VEH group (0.30 ± 0.03, P = 0.014; Figure 3, A). Treatment with MOLS did not influence levels of proteinuria (Supplementary data). Urinary excretion of stable NO metabolites over 24h, obtained under fasting conditions, was greatly enhanced by MOLS, confirming enhanced production of NO (Figure 3, B).

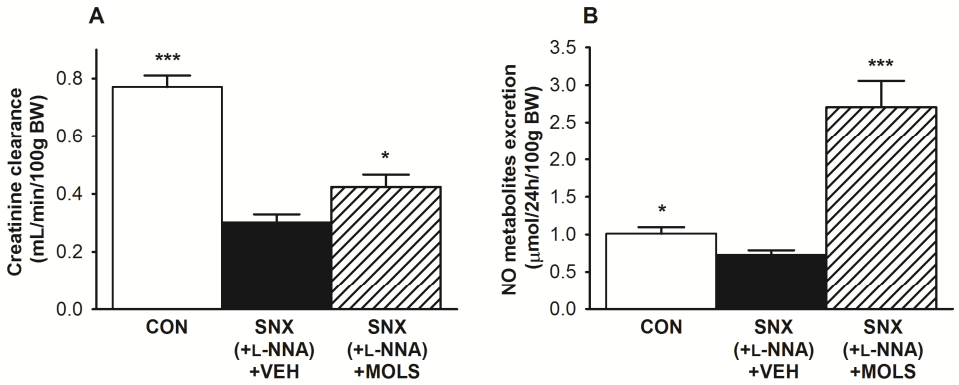

Figure 3. Creatinine clearance (A) and NO metabolite excretion (B) at wk 15. Mean ± SEM. * P<0.05; *** P<0.001 vs. SNX(+L-NNA)+VEH.

Echocardiographic structure and function

Cardiac volumes and systolic function, measured on B-mode cine-loops obtained with 15-MHz cardiac ultrasound, are presented in Figure 4 and Table 1. Three weeks after stopping L-NNA (wk 11), rats were stratified and allocated to either the MOLS-group or the VEH-group. Accordingly, at the start of treatment, levels of left ventricular fractional area change (LV-FAC) were similar in both groups (31.6 ± 0.9% in VEH vs. 31.2 ± 2.1% in MOLS; Figure 4). MOLS significantly improved LV-FAC to 41 ± 2 % during the 4-wk treatment period, while there were no significant changes over time in untreated rats and controls by 2-way RM ANOVA.

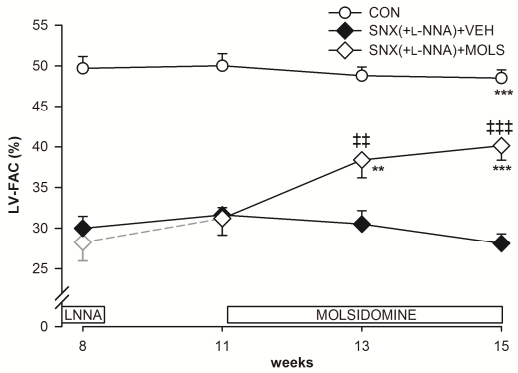

Figure 4. Echocardiographic left ventricular fractional area change (LV-FAC, %) measured in the parasternal short axis view. Values at wk 8 for rats assigned to MOLS three weeks later are depicted as gray diamonds and dashed line.
Mean ± SEM.
** *P*<0.01; *** *P*<0.001 vs. SNX(+L-NNA)+VEH; ‡‡ *P*<0.01 vs. wk 11; ‡‡‡ *P*<0.001 vs. wk 11.

Table 1. Echocardiographic measurements before treatment (week 11) and after 4 weeks of treatment (week 15).

	wk 11 (pre-treatment)			wk 15		
	SNX (+L-NNA) (+VEH)	**SNX (+L-NNA) (before MOLS)**	**CON**	**SNX (+L-NNA) +VEH**	**SNX (+L-NNA) +MOLS**	**CON**
	n=25	n=12	n=10	n=20	n=12	n=14
BW (g)	348 ± 7	364 ± 11	398 ± 9 **	370 ± 5	353 ± 13	423 ± 7 ***
EDV (µL)	677 ± 20	623± 25	515 ± 33 ***	698 ± 33	596 ± 33 *	515 ± 22 ***
ESV (µL)	414 ± 21	356 ± 22	165 ± 16 ***	442 ± 29	272 ± 19 ***	162 ± 10 ***
EF (%)	39.3 ± 2.0	42.8 ± 2.5	68.0 ± 1.8 ***	37.3 ± 2.3	53.3 ± 3.4 ***	68.6 ± 1.4 ***
SV (µL)	263 ± 14	267 ± 20	349 ± 22 **	255 ± 15	324 ± 33 *	354 ± 17 **
HR (bpm)	349 ± 6	356 ± 7	371 ± 6	352 ± 9	332 ± 9	375 ± 5
CO (mL/min)	92 ± 5	94 ± 6	129 ± 8 ***	89 ± 5	109 ± 12	132 ± 6 ***
CI (mL/min/100g BW)	26.4 ± 1.4	26.2 ± 1.9	32.5 ± 1.9 *	24.1 ± 1.3	30.7 ± 3.1 *	31.3 ± 1.5 **
LVM (mg)	1146 ± 33	1126 ± 56	781 ± 20 ***	1236 ± 40	1092 ± 67 *	840 ± 23 ***

BW: body weight; EDV: end-diastolic volume; ESV: end-systoplic volume; EF: ejection fraction; SV: stroke volume; HR: heart rate; CO: cardiac output; CI: cardiac index; LVM: LV mass, calculated by echocardiography.
Mean ± SEM. * *P*<0.05; ** *P*<0.01; *** *P*<0.001 vs. SNX(+L-NNA)+VEH.

There were no significant differences in other cardiac variables at wk 11 before start of treatment (Table 1). By wk 15, end-diastolic volume (EDV) was lower in MOLS rats compared to VEH rats, and end-systolic volume (ESV) even more so (Table 1). This resulted in a higher ejection fraction (EF) and stroke volume (SV).

Heart rate was slightly lower in rats on MOLS (N.S.), resulting in a trend towards higher cardiac output (*P*=0.07) compared to VEH rats. Cardiac index (CI; cardiac output corrected for BW) was significantly higher in the MOLS group (*P* = 0.02). Calculated LV mass was lower in SNX(+L-NNA) rats on MOLS vs. VEH at wk 15.

LV hemodynamics

To further elucidate the mechanisms responsible for the cardiac changes observed with MOLS therapy, we assessed invasive LV hemodynamics, including pressure-volume relationships (PVR), in a subset of rats (Table 2). Rats with SNX(+L-NNA)+VEH showed increased end-systolic and end-diastolic pressures (ESP, EDP), but only EDP was significantly reduced by MOLS therapy. The maximum rate of pressure increase (dP/dt-max) was not different in rats on MOLS compared to VEH, although the maximum rate of pressure decrease (dP/dt-min) tended to be higher in MOLS rats (*P* = 0.07). The time-constant of diastolic relaxation, *tau* (τ, Glantz formula), was more than doubled in rats in the SNX(+L-NNA)+VEH group compared to CON, and MOLS significantly attenuated this (*P*<0.05). Arterial elastance (Ea) was calculated as the ratio of LV end-systolic pressure and stroke volume. In SNX(+L-NNA)+VEH, Ea was significantly higher than in CON, and this was reduced in MOLS-treated rats. The systemic vascular resistance index (SVRI) was calculated by dividing mean arterial pressure (MAP) by cardiac index. Similar to the tail-cuff SBP and ESP, MAP was significantly higher in SNX(+L-NNA)+VEH rats vs. CON, and was slightly lower in MOLS rats (*P* = 0.09). Consequently, SVRI was strongly increased in VEH vs. CON, and significantly ameliorated by MOLS.

We also recorded pressure-volume (PV)-loops during inferior vena cava occlusion. Although the slope of the end-systolic PV relationship (ESPVR) was not significantly altered by MOLS, V_0 (the extrapolated volume at which pressure is 0 mmHg) was significantly lower in MOLS vs. VEH (*P* = 0.02). This was also observed for the ESPVR when a quadratic curve fit was applied to correct for contractility-dependent curvilinearity of the ESPVR.[24] The relationship between EDV and dP/dt-max showed a similar pattern. There were no differences in the slope of the end-diastolic PV relationship (EDPVR).

Table 2. Left ventricular hemodynamic variables and pressure-volume relationships at week 16.

	SNX(+L-NNA) +VEH	SNX(+L-NNA) +MOLS	CON
Baseline LV hemodynamics	*n=10*	*n=9*	*n=7*
End-systolic pressure (mmHg)	170 ± 9	159 ± 8	130 ± 5 **
End-diastolic pressure (mmHg)	16.3 ± 3.5	8.5 ± 1.1 *	7.2 ± 0.6 *
dP/dt-max (mmHg/s)	9,112 ± 635	9,415 ± 676	7,622 ± 565
dP/dt-min (mmHg/s)	-6,592 ± 370	-8,201 ± 687	-9,081 ± 791 **
tau (msec)	30.9 ± 3.3	21.9 ± 1.8 *	15.0 ± 1.3 ***
Arterial elastance (mmHg/μL)	0.61 ± 0.05	0.48 ± 0.04 *	0.40 ± 0.04 **
Mean arterial pressure (mmHg)	151 ± 9	129 ± 9	103 ± 8 ***
SVRI (mmHg/mL/min/100g)	5.60 ± 0.34	3.78 ± 0.23 ***	3.31 ± 0.28 ***
Pressure-volume relationships	*n=8*	*n=8*	*n=7*
Ees (mmHg/μL)	0.54 ± 0.08	0.43 ± 0.08	0.30 ± 0.02
V_0 (μL)	91 ± 45	-149 ± 104 *	-265 ± 43 **
E'max (mmHg/μL)	2.03 ± 0.50	1.66 ± 0.46	1.30 ± 0.30
V_0-quad (μL)	225 ± 43	47 ± 75 *	-20 ± 34 **
dP/dt-EDV (mmHg/s/μL)	19 ± 3	12 ± 1	12 ± 2
dP/dt-EDV intercept (μL)	185 ± 37	-191 ± 109 **	-207 ± 122 **
EDPVR slope (mmHg/μL)	0.017 ± 0.003	0.021 ± 0.003	0.013 ± 0.002

Please see text for explanation of abbreviations. Mean ± SEM.
* *P*<0.05; ** *P*<0.01; *** *P*<0.001 vs. vs. SNX(+L-NNA)+VEH.

Figure 5 depicts the changes in the PV-loops based on the average volume and pressure data for the three groups. This clearly demonstrates a left-ward shift of the PV-loop and ESPVR in the MOLS-group with an increase in SV despite a minor decrease in afterload.

Organ weights, histological damage and cardiac gene expression

Rats on MOLS had a significantly lower LV mass than VEH rats (Table 3) and the left kidney remnant mass was also numerically lower (N.S.), but the significance disappeared when organ weights were corrected for body weight.

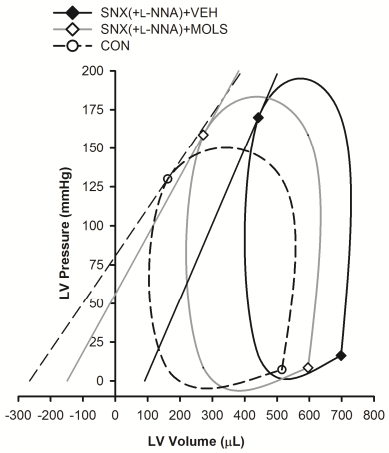

Figure 5. Schematic PV loops based on average end-diastolic and end-systolic pressures and volumes for the three conditions, and corresponding ESPVRs. Treatment with MOLS induced an increase in SV and a leftward shift of the PV-loop without significantly altering Ees.

However, we also measured tibia length in 6 rats from the MOLS group and 5 rats from the VEH group, and found these to be similar (4.25 ± 0.04 vs. 4.25 ± 0.02 cm, respectively, N.S.). Right ventricular weight and wet lung weight were not affected by MOLS therapy (Supplementary data). Cardiomyocyte area was slightly larger in MOLS vs. VEH, but this was also not significant (Table 3). Furthermore, the collagen area fraction in the heart, assessed on Sirius Red stained transverse sections, was not affected by MOLS. In the kidney, MOLS decreased the amount of tubulo-interstitial injury (P = 0.045), but did not influence glomerulosclerosis.

Table 3. Cardiorenal histological damage at week 16.

	SNX(+L-NNA) +VEH	SNX(+L-NNA) +MOLS	CON
Organ weights:	*n=20*	*n=12*	*n=12*
LV mass (g)	1.23 ± 0.02	1.12 ± 0.03 **	0.83 ± 0.02 ***
LV mass /100g BW (g/g)	0.33 ± 0.01	0.33 ± 0.01	0.19 ± 0.00 ***
LK weight (g)	1.49 ± 0.05	1.31 ± 0.08	1.47 ± 0.05
LK weight/100g BW (g/g)	0.40 ± 0.01	0.38 ± 0.02	0.34 ± 0.01 **
Heart:	*n = 11*	*n = 8*	*n = 9*
Cardiomyocyte area (μm^2)	600 ± 31	662 ± 33	396 ± 18 ***
Collagen Area Fraction (%)	4.04 ± 0.38	5.75 ± 1.02	0.24 ± 0.09 ***
Kidney:	*n = 10*	*n = 8*	*n = 7*
Normal glomeruli (score 0)	40 ± 3	48 ± 7	70 ± 2 ***
Partial glomerulosclerosis (score 1-2)	32 ± 4	23 ± 3	27 ± 2
Severe glomerulosclerosis (score 3-4)	28 ± 5	29 ± 8	3 ± 1 **
Tubulo-interstitial Injury Score	6.37 ± 0.63	4.96 ± 0.52 *	0.81 ± 0.15 ***

LV: left ventricle; LK: left kidney (remnant); BW: body weight. Mean ± SEM.
* $P<0.05$; ** $P<0.01$; *** $P<0.001$ vs. SNX(+L-NNA)+VEH.

Cardiac expression of atrial natriuretic peptide (ANP) and the NOS isoforms was determined by qPCR in apical tissue. SNX(+L-NNA)+VEH rats (n=12) showed grossly increased expression of ANP vs. CON (n=9): fold-change: 15 ± 1 vs. 1.1 ± 0.2 ($P< 0.001$), and MOLS (n=8) decreased ANP expression (11 ± 1, $P< 0.05$). Fold change expression of NOS isoforms is shown in Figure 6. Compared to CON, expression of neuronal NOS was significantly higher in SNX(+L-NNA)+VEH rats. Expression of iNOS was also numerically higher (N.S.) vs. CON, but significantly reduced in the MOLS group. Expression of eNOS was not different across groups.

3-Nitrotyrosine levels in heart, kidney and liver

In heart, kidney or liver tissue the amount of 3-nitrotyrosine (3-NT) was not significantly different between groups (Figure 7).

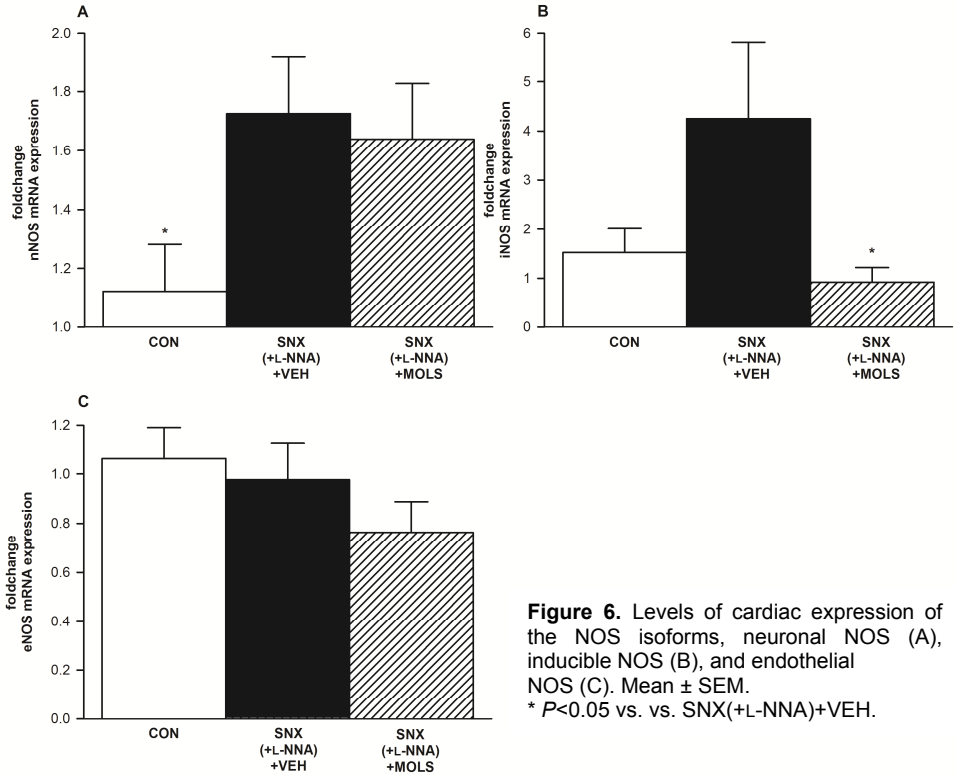

Figure 6. Levels of cardiac expression of the NOS isoforms, neuronal NOS (A), inducible NOS (B), and endothelial NOS (C). Mean ± SEM.
* *P*<0.05 vs. vs. SNX(+L-NNA)+VEH.

7.4 Discussion

The results confirmed our hypothesis that persistent LVSD in SNX(+L-NNA)+VEH rats is linked to the low systemic NO availability. Nitric oxide appears to be a critical modulator of cardiac function in the setting of reduced kidney function. In this rat model of combined CKD and cardiac dysfunction, supplementing NO with molsidomine (MOLS) significantly improved cardiac systolic function, reduced LV mass, improved creatinine clearance and reduced tubulo-interstitial injury, and abrogated further mortality. The positive effects were sustained over a 4-wk treatment period.

Effect of MOLS on renal function and structure

Although it is well known that NOS inhibition can induce and worsen renal injury, data on effects of NO supplementation on existing renal injury are scarce. Benigni

et al.[12] reported that MOLS, starting 3 weeks after SNX, significantly reduced SBP with only minor effects on serum creatinine and proteinuria. In rats with dietary hypercholesterolemia, MOLS prevented proteinuria and both glomerular and tubulo-interstitial injury.[13] In our study, plasma urea and proteinuria were unaltered by MOLS therapy. This may have been related to the time-point at which treatment was started. Nevertheless, CrCl increased at the end of the 4 wk treatment period.

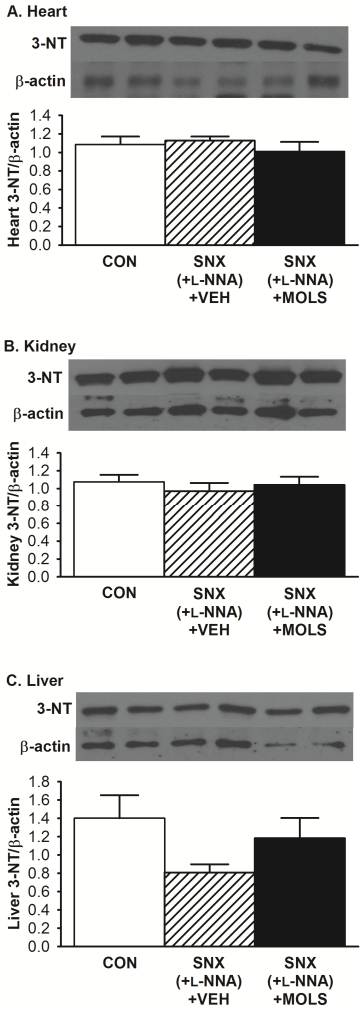

Figure 7. Nitrotyrosine (3-NT) content in heart (A), kidney (B) and liver (C) tissue samples (n=4 per group). Mean ± SEM. Levels of 3-NT were not significantly different across groups in any of the organs.

This could be due to pre-glomerular vasodilatation, an increase in cardiac output and hence renal perfusion, or a combination of these. Because blood pressure was reduced this mechanism will not have been dampened by renal autoregulation (i.e. afferent arteriolar constriction), in so far as this was still operating in the remnant kidney.[25] However, MOLS may also have reduced venous congestion, thus decreasing renal "afterload".[26] Finally, the reduction in tubulo-interstitial injury in MOLS rats may have reduced hydrostatic pressure in Bowman's capsule. It is well known that tubulo-interstitial injury is a strong determinant of glomerular filtration.[27] Because the increase in CrCl was only apparent at wk 15, it is unlikely that improved renal function preceded the observed improvement in cardiac function.

Effects of MOLS on cardiac function and hemodynamics.

The combination of echocardiography and invasive hemodynamic assessment allows us to draw several conclusions regarding the cardiac effects of MOLS in this model of combined CKD and cardiac dysfunction. Both cardiac systolic and diastolic function were significantly ameliorated by MOLS therapy and appear to be almost normalised towards control level. However, afterload was only mildly affected, which can explain why EF was still somewhat lower, and ESP and ESV still somewhat higher than in controls.

Molsidomine was found to have strong anti-hypertensive effects in rats with SNX,[12] and when administered together with NOS inhibition,[28] but not in normal or spontaneously hypertensive rats.[13, 29] This suggests that MOLS mainly affects blood pressure in disease states where NO production is lowered. In our model, the resistance vessels may have been less responsive to exogenous NO as tail-cuff SBP and carotid artery MAP were only mildly lowered by MOLS. Thus, the changes in calculated Ea and SVRI appeared to be mainly mediated by the improved systolic function, which was able to overcome the increased afterload.

Molsidomine, like other NO donors, can function as a venodilator and thereby decreases cardiac preload and wall stress.[30, 31] Indeed, the reduced EDV and EDP in the MOLS group suggest lower end-diastolic wall stress. The decrease in preload has no negative consequences as output is almost normalised at significantly lower filling pressures, and the decrease in EDV is likely secondary to the increase in SV. Analysis of PV relationships showed that MOLS induced a

leftward shift of the linear ESPVR without a change in slope (Ees). Similar changes were seen in dP/dt-EDV relationship. This is indicative of increased contractility, as similar end-systolic pressures could be generated at a lower volume (Figure 5). Because in the rat the ESPVR shows contractility-dependent curvilinearity,[24] we also determined E'max and V_0 derived from a quadratic curve-fit. This yielded similar results to the linear ESPVR, with a significant left-shift of the ESPVR in MOLS rats.

The reduction in *tau*, a relatively load-independent measure of relaxation,[32] however indicates that active diastolic relaxation (lusitropy) was also more efficient in the MOLS group. Many of the same mechanisms that influence lusitropy also affect contractility, by improving excitation-contraction coupling (ECC). Molsidomine-derived NO may have enhanced ECC by increasing Ca^{2+} reuptake by the sarcoplasmic reticulum or by altering S-nitrosylation of Ca^{2+} channels.[9]

Furthermore, in isolated rat hearts, 4 weeks after SNX, the group of Raine *et al*. found reduced responsiveness to increasing Ca^{2+} concentrations,[33] and abnormal Ca^{2+} cycling together with reduced contraction and relaxation velocities in single cardiomyocytes.[34] Thus, reduced sensitivity of cardiac myofilaments to Ca^{2+} may also play a role in the cardiac dysfunction observed in our severe SNX model, and this may have been restored by NO supplementation. A positive effect of MOLS on coronary perfusion, either directly or secondary to decreased wall stress, may also have been present.[35]

Wilson *et al.*[36] recently investigated the effect of concomitant hydralazine and isosorbide dinitrate (H-ISDN) on cardiac function in aldosterone-infused mice. Untreated mice exhibited diastolic dysfunction but no decrease in EF, and H-ISDN improved the (Doppler–derived) diastolic indices. Positive effects of NO donors have also been described on cardiac diastolic function in humans with normal hearts,[37] and with pressure-overload hypertrophy.[38] Furthermore, a beneficial effect on cardiac function, remodeling and mortality in patients with heart failure was documented for long-term treatment with H-ISDN.[39, 40] With respect to MOLS, several studies have shown a beneficial hemodynamic profile of this compound compared to organic nitrates in patients with angina pectoris, ischemic heart

disease, and heart failure, even when tolerance to organic nitrates was present.[14,41,42]

In patients with heart failure, infusion of MOLS for 24h induced an increase in cardiac output that was not seen with similar infusion of organic nitrates.[14] To our knowledge, we are the first to study the role of reduced NO availability and NO supplementation on *in vivo* cardiac function in CKD in a model of cardiorenal failure. Molsidomine, or other tolerance-free nitrates, may be a future therapeutic option for patients with cardiorenal disease.

Effects of MOLS on cardiac histology and mRNA expression.

Longitudinal assessment of calculated LV mass from cardiac sonography showed that in VEH-rats LV hypertrophy progressed while this appeared to be halted in MOLS-rats. These differences were confirmed by crude terminal organ weights. Although these differences disappeared when correcting for body weight, we found that tibia length was similar in the VEH and MOLS group, indicating that growth was not affected and the differences in body weight were likely related to changes in body composition.

Cardiomyocyte-restricted overexpression of endothelial (e)NOS was found to attenuate LVH and cardiomyocyte hypertrophy in mice with pressure overload,[43] and treatment of spontaneously hypertensive rats with MOLS significantly reduced heart weight.[29] In a model of aortic constriction in rats the NO donor LA419 reduced cardiomyocyte hypertrophy but also lessened cardiac patchy and perivascular fibrosis.[44] Conversely, H-ISDN treatment did not prevent development of cardiac fibrosis in aldosterone-infused mice compared to untreated aldosterone-infused mice.[36] In our study, the extent of cardiac collagen deposition was also not different in hearts of MOLS-treated rats vs. VEH. Both the unchanged fibrosis and EDPVR suggest that passive diastolic properties of the heart were not significantly affected by MOLS. The slightly lower cardiac expression of ANP in MOLS is in line with the reduced loading conditions observed with echocardiography and hemodynamic assessment.

Expression in cardiac tissue of constitutive NOS isoforms (e- and nNOS) was not decreased in either the VEH or MOLS group compared to controls, suggesting that at least reduced cardiac expression did not play a significant role in

the persistent LVSD. Expression of nNOS was even significantly higher than CON in the SNX(+L-NNA)+VEH, which was also documented in rats with heart failure.[45] On the other hand, MOLS reduced iNOS expression, which is associated with worsened damage and cardiac function.[9]

Nitrotyrosine protein assay

We measured 3-NT protein levels in different tissues (heart, liver, kidney) to assess the amount of nitrosative stress due to peroxynitrite formation occurring in each study group and found these to be similar across groups in each organ, even in the liver where SIN-1, the active metabolite of MOLS, is generated. Peroxynitrite has been implicated in the pathophysiology of cardiac dysfunction [46] and may have been increased in our model. Although SIN-1 can generate peroxynitrite in vitro, it appears to function solely as a NO-donor at the *in vivo* oxygen concentrations.[11, 17] We also found no increase in 3-NT formation while levels of NO metabolites excretion (and hence production) were greatly increased by MOLS (Figure 3).

7.5 Conclusions

Supplementation of NO with the tolerance-free NO donor molsidomine improved cardiac systolic and diastolic function and improved survival in a rat model of CKD, LVSD and low NO availability. It also ameliorated LVH, reduced tubulo-interstitial injury in the kidney and improved creatinine clearance. Our study suggests that in CKD cardiac function is strongly modulated by NO availability through actions on active diastolic relaxation and systolic ejection, with minor effects on afterload. Although a reduction of NO availability is widely regarded as one of the hallmarks of CKD,[8] the effect of direct supplementation has not yet been investigated in detail. The absence of significant effects on systemic arterial pressure and the resistance to tolerance make MOLS an attractive adjuvant therapeutic option, for example in hemodialysis patients in whom abnormalities of cardiac function are often seen and associated with adverse outcome.[2] Supplementation of NO with MOLS might thus help support cardiac function in patients with advanced CKD and LVSD or heart failure.

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Chapter 8

Neuronal nitric oxide synthase-derived NO supports lusitropy in rats with the Cardiorenal Syndrome

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We have recently described a model of the Cardio-Renal Syndrome (CRS) in rats characterized by chronic kidney disease, cardiac dysfunction, and low NO availability. The neuronal isoform of NO synthase (nNOS), an important regulator of cardiac function, particularly of active diastolic relaxation (lusitropy), can be upregulated in cardiac disease and can modulate the β-adrenergic response. Compared to controls, CRS rats showed increased cardiac gene expression of nNOS, but not eNOS. Hence, we studied the role of nNOS for cardiac function in our model of the CRS (5/6th nephrectomy and temporary low dose non-specific NOS inhibition).

Cardiac function was assessed *in vivo* in control and CRS rats with a conductance catheter. Neuronal NOS activity was blocked by acute infusion of L-VNIO (l-N5-(1-Imino-3-butenyl)-ornithine), a highly specific irreversible inhibitor of nNOS, and dobutamine was infused for β-adrenergic stimulation. Left ventricular hemodynamics and pressure-volume relations (PVR) were studied during administration of dobutamine, L-VNIO, and their combination.

Rats with CRS had reduced cardiac power, prolonged diastolic relaxation at baseline and showed a significant right-shift of the end-systolic PVR. During nNOS blockade with L-VNIO, active diastolic relaxation (lusitropy) of CRS rats was significantly worsened. Rats with CRS showed reduced cardiac β-adrenergic responsiveness as compared to controls, as evidenced by a smaller increase in pressure and power generation. Inhibition of nNOS in CRS rats did not affect the β-adrenergic response of systolic and diastolic variables.

Neuronal NOS-derived NO supports lusitropy in rats with CRS, but does not modulate the decreased β-adrenergic responsiveness.

8.1 Introduction

Cardiac dysfunction in patients with chronic kidney disease (CKD) is associated with adverse outcome,[1, 2] and is one of the recognized Cardio-Renal Syndromes (CRS).[3, 4] We recently showed that transient inhibition of nitric oxide (NO) production with low dose non-specific NO synthase (NOS) inhibition induced a strong reduction in systemic NO availability and permanent left ventricular systolic dysfunction (LVSD) in rats with CKD. Both the cardiac dysfunction and low NO production persisted long after cessation of the NOS inhibitor, compared to only minor and reversible effects in control rats.[5] We later showed that supplementing systemic NO with the tolerance-free NO donor molsidomine rescued cardiac function by improving both diastolic and systolic function, with a minor effect on afterload.[6] These findings led us to hypothesize that cardiac NO production is impaired in our model of the Severe Cardiorenal Syndrome (SCRS) of combined CKD and temporary NOS inhibition, presumably due to persistent down-regulation of NOS. Surprisingly, we found that cardiac nNOS gene expression was upregulated, whereas eNOS gene expression was unaffected.[6]

In the regulation of myocardial function, a distinct role has been described for neuronal NOS (nNOS). Neuronal NOS appears to be the most important intracellular NOS isoform and is associated with the ryanodine receptor calcium (Ca^{2+}) release channel (RyR) and sarcoplasmic reticulum Ca^{2+} ATP-ase (SERCA), where it modulates Ca^{2+} cycling and excitation-contraction coupling.[7] In various pathological conditions induction of nNOS expression appears to exert cardioprotective effects.[8-11] In rats with heart failure (HF), where nNOS was upregulated, blockade of the enzyme did not significantly affect *in vivo* cardiac function.[8] The response to dobutamine was not altered by pharmacological nNOS blockade in normal rat hearts both *ex vivo* and *in vivo*, but it significantly improved the β-adrenergic sensitivity of failing hearts. Both in HF,[12] and CKD[13] the response of cardiac function to β-adrenergic stimulation is depressed. This apparent dampening of the β-adrenergic response by upregulated nNOS was proposed as an adaptation to protect the heart from long-term adverse effects of sympathetic stimulation. Another study in mice with pressure overload showed that conditional overexpression of nNOS preserved *in vivo* ejection fraction and *in vitro* cardiomyocyte Ca^{2+} cycling.[11]

We therefore investigated whether the nNOS upregulation in our CRS model serves as a beneficial or a negative adaptation, by assessing the functional role of nNOS in the acute regulation of cardiac function and the β-adrenergic response. Because nNOS cannot be stimulated pharmacologically, we blocked the enzyme with l-N5-(1-Imino-3-butenyl)-ornithine (vinyl L-NIO or L-VNIO), which has a high specificity (> 100 times) for nNOS compared to eNOS.[14]

8.2 Methods

The study protocol was approved by the Utrecht University Committee on Animal Experiments, and conformed to Dutch Law on Laboratory Animal Experiments and the US National Institutes of Health guidelines. Male inbred Lewis rats (Lew/Crl), 180–200 g, were purchased from Charles River, Germany, and housed in a climate-controlled facility with a 12:12-hour light:dark cycle.

Study set-up, subtotal nephrectomy, and in vivo measurements

Rats were divided into two groups with similar initial body weights: control rats: sham-operated; Cardio-Renal Syndrome (CRS): subtotal nephrectomy + 20 mg/L L-NNA (Nω-nitro-L-arginine); Sigma-Aldrich, St. Louis, MO, USA) in drinking water. Animals were pre-treated with L-NNA or normal (acidified) drinking water for two weeks, and fed standard pellet rodent chow (CRM-E; Special Diet Services Ltd., Witham, Essex, UK). Then, at wk 1, a two-stage subtotal (5/6th) nephrectomy (SNX) by resection or a sham-procedure was performed.[5] After one week of recovery, rats were re-started on L-NNA or normal water, and all rats were fed standard powdered chow (CRM-FG; Special Diet Services Ltd.) supplemented with 6% (w/w) NaCl to accelerate progression of CKD. This method results in stable CKD in Lewis rats.[15] Normal Lewis rats are resistant to high (8%) salt diets with respect to blood pressure and development of proteinuria.[16] Treatment with L-NNA was stopped after wk 8, and rats were followed up until wk 15, and *in vivo* measurements were performed, as recently described.[5] In short, systolic blood pressure (SBP) was measured with the tail-cuff method, and a 24-h urine sample was collected. LV volumes were determined on echocardiographic cine-loops acquired in the parasternal long-axis view and calculated with the prolate ellipsoid

area–length method.[17] After echocardiography, a small blood sample was drawn to determine plasma creatinine.

Hemodynamic and pressure-volume relationship studies

Terminal LV hemodynamic and PV loop measurements were performed via the closed-chest approach in wk 16. After induction with isoflurane, the rats were intubated, placed on a thermo-controlled operating table, and mechanically ventilated with 2% isoflurane at a rate of 65/min with 40% oxygen. Buprenorfine (0.015 mg/kg i.m.) was injected for analgesia. The left internal jugular vein was isolated and cannulated with a PE-50 catheter for infusion of saline at a rate of 8 ml/kg BW/min. The upper abdomen was opened by a small incision in the linea alba to provide access to the inferior vena cava (IVC) between the liver and diaphragm for occlusion studies (see below). A catheter was inserted in the bladder via a supra-pubic approach. The right common carotid artery was isolated and a 2-Fr. pressure-conductance catheter (SPR-838, Millar Instruments, Houston, Texas, USA) was inserted. The conductance catheter was advanced retrograde into the left ventricle. The pressure-volume signals were registered continuously with Chart-software (ADInstruments, Spechbach, Germany) at a sample rate of 1 kHz. Adequate placement of the catheter was verified by the PV loop signals. After a stabilization period of at least 15 min, baseline steady-state and short, temporary IVC occlusion (IVCO) hemodynamic studies were performed with the ventilator temporarily switched to continuous positive airway pressure. Steady state and IVCO interventions were repeated at least three times, with recovery periods in between. After baseline measurements, dobutamine (dobu) was infused at a rate of 5 µg/kg/min. When a stable increase of LV pressure (LVP), LV volume, and dP/dt-max (see below) was reached, steady state and IVCO studies were performed as above. Subsequently, the dobutamine infusion was stopped and hemodynamic variables were allowed to return to baseline values. Then, L-VNIO (Alexis Biochemicals (Axxora), Zandhoven, Belgium) was infused for 30 min up to a total of 0.12 µmol.[18] To correct for any reversible inhibition of eNOS by L-VNIO [Cupples W.A., personal communication], this was followed by a stabilisation period of 30 min after infusion of L-VNIO. Subsequently, steady state and IVCO studies were performed as above. Then, dobutamine was again infused at a rate of

5 µg/kg/min as described above, followed by at least three steady state and IVCO studies. The infusion rate of the saline was adjusted during L-VNIO and dobutamine infusion during the experiment. Finally, the dobutamine was switched off and after a wash-out period rats were euthanized by exsanguination via the abdominal aorta. Organs were harvested and weighed.

Hemodynamic measurements were analyzed off-line with the PVAN software (PVAN v3.6, Millar Instruments, Houston, Tx). Volume calibration of the conductance values was done by plotting the mean conductance catheter values for maximal and minimal volume of the baseline steady state loops against the end-diastolic and end-systolic volumes determined by echocardiography (see above.) This yielded an equation with a value for slope and y-intercept that were entered in the PVAN-program. At least three separate data samples, each consisting of at least five cardiac cycles, were averaged and the following variables were calculated to assess changes in steady state hemodynamics : heart rate (HR; bpm), end-systolic pressure (ESP; mmHg), end-diastolic pressure (EDP; mmHg), arterial elastance (Ea; mmHg/µL), maximum and minimal rate of LV pressure change (dP/dt-max and dP/dt-min; mmHg/sec), and regression of dP/dt versus pressure during isovolumic relaxation (*tau*; Glantz-method; msec); stroke work (SW; mmHg.µL); maximal power (mWatts); preload-adjusted maximal power (PAMP; $mW/\mu L^2$), and PAMP corrected for heart rate (PAMP x RR; $mW.sec/\mu L^2$).
The data from the IVCO studies were used to calculate the following variables: End-systolic elastance (Ees; mmHg/µL) calculated from the linear end-systolic PV relationship (ESPVR), and the ESPVR volume intercept (V_0; µL); the maximal elastance calculated from quadratic curve fit of the ESPVR (E'max; mmHg/µL) and the corresponding volume intercept (V_0-quad; µL); the preload-recruitable stroke work relationship (PRSW; SW vs. EDV, mmHg), and its volume intercept ($PRSW_0$, µL).

Statistics

Data are shown as mean ± SEM. Data were analyzed and graphed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). Student's t-test and one-way ANOVA was used for group comparisons. For hemodynamic studies, a two-way repeated measures (RM) ANOVA was performed. Data that were not normally

distributed were log-transformed or ranked to achieve normality. Statistical significance was reached with p-values below 0.05.

8.3 Results

General characteristics

Rats with CRS had a lower body weight, higher systolic blood pressure and a lower creatinine clearance than control rats (Table 1). CRS rats also showed marked LV hypertrophy, and increased lung weight.

Table 1. General characteristics and organ weights of control rats and rats with cardiorenal syndrome (CRS)

	controls	**CRS**
	n=6	*n=7*
Body weight (g)	429 ± 7	380 ± 8 ***
Tail-cuff SBP (mmHg)	106 ± 3	168 ± 10 ***
Creatinine clearance (mL/min/100 g BW)	0.74 ± 0.06	0.34 ± 0.05 ***
LV weight / 100 g BW (g/g)	0.192 (0.185 – 0.204)	0.319(0.277 – 0.411) ***
RV weight / 100 g BW (g/g)	0.046 (0.040 – 0.069)	0.052 (0.041 – 0.090)
Lung weight / 100 g BW (g/g)	0.308 (0.278 – 0.348)	0.369 (0.328 – 0.649) **

SBP, systolic blood pressure; BW, body weight; LV, left ventricle; RV, right ventricle.
Data as mean ± SEM or median (range). ** P< 0.01, *** P< 0.001 vs. controls.

Hemodynamic profile at baseline

Compared to control rats, CRS rats had a lower HR but evidence of a higher afterload at baseline (Table 2). The rates of pressure increase (dP/dt-max) and decrease (dP/dt-min) were numerically lower (Figures 1A and 1B, both N.S.). The amount of maximal power generation was also comparable, but it was markedly lower in CRS rats when corrected for the difference in preload (PAMP) alone or with the difference in HR (PAMP x RR) (Table 2 and Figure 1C). The time constant of relaxation, *tau*, was increased in CRS rats (P = 0.012; Figure 1D).

At baseline, the end-systolic elastance (Ees) of the CRS rats was higher than that in control rats (P = 0.029) together with a higher volume intercept ($V_{0;}$ P = 0.001), indicative of a steeper ESPVR that was shifted to the right in CRS rats.

Because the ESPVR of the rat heart shows contractility-dependent curvilinearity, [19] we also calculated E'max, which is the quadratic curve-fit of the ESPVR. This was also slightly but not significantly higher in CRS rats. As with the linear ESPVR, the volume intercept was higher in CRS rats (P = 0.001), indicating a significant right shift of the ESPVR. Similarly, as a measure of LV performance, the volume intercept of the preload-recruitable stroke work (PRSW) relationship was higher (P = 0.014). The slope of the EDPVR, a measure of passive diastolic properties, was not significantly different between groups.

Hemodynamic effects of nNOS blockade

Heart rate decreased in both groups after L-VNIO infusion, albeit to a greater degree in CRS rats (Table 2). In controls, L-VNIO did not significantly affect measures of afterload (ESP, Ea) but induced a minor increase in EDP and a slight decrease in PAMP (both P< 0.05). In the CRS group, however, afterload was reduced. Furthermore, dP/dt-max was lower compared to baseline (Figure 1A). Although SW was decreased by L-VNIO (P = 0.013) and maximal power generation tended to be lower, PAMP and PAMP corrected for HR were not significantly affected (Table 2 & Figure 1C).

Effects of L-VNIO on lusitropy in CRS rats were more pronounced, with a strong decrease in negative dP/dt (P< 0.001; Figure 1B) and prolongation of the time constant of relaxation, *tau* (P = 0.012; Figure 1D). Figure 2 depicts the changes in steady-state PV-loops graphed from mean pressure and volume values. In CRS rats, L-VNIO caused a decrease in end-diastolic filling and end-systolic pressure, leading to a decrease in SW (the area of the PV-loop).

With nNOS inhibition, both the linear and quadratic ESPVR of control rats did not change significantly, although the volume intercepts were slightly higher (Table 3). The PRSW became less steep, resulting in a lower volume intercept. In CRS rats, nNOS inhibition caused a slight decrease in end-systolic elastance with a slightly flatter ESPVR (lower volume intercept). The PRSW relation was reduced in CRS

rats (P = 0.001), with a reduction in the slope without a change in the volume intercept.

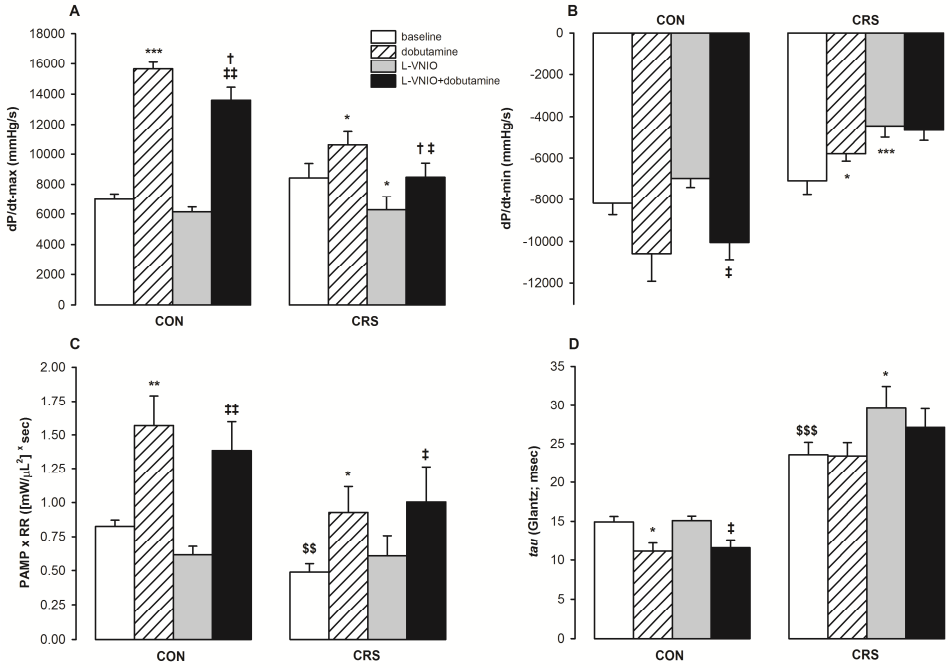

Figure 1. The *in vivo* hemodynamic response of control rats (CON) and rats with Cardio-Renal Syndrome (CRS) in response to sequential infusions of dobutamine and/or L-VNIO. Because L-VNIO irreversibly inhibits nNOS, a first dobutamine infusion was performed after which a wash-out period was observed. Then, L-VNIO was infused, measurements performed and a second dobutamine infusion was started to assess effects of the combination.
(A) Maximal rate of pressure change (dP/dt-max; mmHg/s), (B) Maximal rate of pressure decline (dP/dt-min; mmHg/s); (C) preload-adjusted maximal power corrected for heart rate (PAMP x RR; mW*sec/μL^2); (D) Time-constant of relaxation (*tau*, Glantz; msec).
Mean ± SEM. Symbols: $$ P< 0.01, $$$ P< 0.001 vs. CON at baseline;* P< 0.05, *** P< 0.001 vs. baseline; † P< 0.05 vs. dobu; ‡ P< 0.05, ‡‡ P< 0.01 vs. L-VNIO.

Table 2. Left ventricle hemodynamics

	controls (n=6)				*CRS (n=7)*			
	baseline	***dobu***	***L-VNIO***	***L-VNIO +dobu***	***baseline***	***dobu***	***L-VNIO***	***L-VNIO +dobu***
Heart rate *(bpm)*	372 ± 7	391 ± 5 **	343 ± 8 ***	364 ± 6 ††† ‡‡	328 ± 14 $	340 ± 11	273 ± 13 ***	291 ± 12 ††† ‡
End-systolic pressure *(mmHg)*	115 ± 4	113 ± 8	104 ± 5	118 ± 9	151 ± 10 $$	126 ± 9	122 ± 11 *	117 ± 6
End-diastolic pressure *(mmHg)*	5.3 ± 0.5	6.0 ± 0.5	6.5 ± 0.5 *	7.2 ± 0.6 †	9.8 ± 2.6	9.7 ± 2.6	11.1 ± 2.1	10.1 ± 1.9
Arterial elastance *(mmHg/µL)*	0.35 ± 0.02	0.27 ± 0.03	0.33 ± 0.07	0.26 ± 0.06	0.55 ± 0.05 $$	0.37 ± 0.05 **	0.45 ± 0.05 *	0.32 ± 0.04 ‡‡
Stroke work *(mmHg x µL) x 10 ^{3}*	33.9 ± 1.6	51.1 ± 4.4 **	33.4 ±5.6	56.9 ± 7.6 ‡‡‡	33.5 ± 2.9	37.3 ± 4.0	24.9 ± 1.5 *	36.4 ±3.2 ‡‡
Max. power *(mWatts)*	127 ± 11	237 ± 30 **	119 ± 25	247 ± 46 ‡‡	136 ± 15	201 ± 24 **	95 ± 9	166 ± 21 ‡‡
Preload-adjusted max. power *(mW/µL ^{2})*	5.1 ± 0.3	10.2 ± 1.4 **	3.5 ± 0.4 *	8.4 ±1.3 ‡‡	2.7 ± 0.4 $$$	5.2 ± 1.0 *	2.6 ± 0.5	4.7 ± 1.0 ‡‡
End-diastolic volume *(µL)*	529 ± 16	524 ± 39	584 ± 36	583 ± 51	756 ±47 $$	689 ± 39	667 ± 52	671 ± 47
Stroke volume *(µL)*	334 ± 16	443 ± 39 *	359 ± 43	504 ±53 ‡	282 ± 19	378 ± 46 *	278 ± 18	395 ± 51 ‡
Ejection fraction *(%)*	63 ± 2	84 ± 2 ***	61 ± 5	86 ± 3 ‡‡‡	38 ± 3 $$$	55 ± 5 **	43 ±5	59 ± 7 ‡‡

Hemodynamic responses of the LV were assessed invasively under sequential infusions of dobu (dobutamine), the selective nNOS inhibitor L-VNIO (l-N5-(1-Imino-3-butenyl)-ornithine) and their combination. Because L-VNIO irreversibly inhibits nNOS, a first dobutamine infusion was performed after which a wash-out period was observed. Then, L-VNIO was infused, measurements performed and a second dobutamine infusion was started to assess effects of the combination.

Mean ± SEM; Symbols: $ P< 0.05, $$ P< 0.01, $$$ P< 0.001 vs. control rats at baseline; * P< 0.05, ** P< 0.01, *** P< 0.001 vs. baseline; † P< 0.05, ††† P< 0.001 vs. dobu; ‡ P< 0.05, ‡‡ P< 0.01, ‡‡‡ P< 0.001 vs. L-VNIO.

Table 3. Cardiac hemodynamics and pressure-volume relationships during preload reduction.

	controls (n=6)				CRS (n=7)			
	baseline	**dobu**	**L-VNIO**	**L-VNIO +dobu**	**baseline**	**dobu**	**L-VNIO**	**L-VNIO +dobu**
Ees (mmHg/µL)	0.25 ± 0.02	-	0.25 ± 0.04	-	0.54 ± 0.11 $$	0.71 ± 0.10	0.38 ± 0.08	0.67 ± 0.14 ‡
V_0 (µL)	-436 ± 114	-	-254 ± 92	-	87 ± 53 $$	2 ± 46	-36 ± 44 *	-79 ± 83
E'max (quadratic)	1.12 ± 0.11	-	1.08 ± 0.10	-	1.85 ± 0.51	2.48 ± 0.23 *	1.12 ± 0.18	2.71 ± 0.64 ‡‡
V_0 – quadratic (µL)	-12 ± 6	-	66 ± 35	-	218 ± 49 $$	124 ± 25	163 ± 41	62 ± 54
PRSW	107 ± 23	-	69 ± 16	-	122 ± 11 $	177 ± 11 *	85 ± 13 **	142 ± 15 †† ‡‡
$PRSW_0$	172 ± 49	-	-109 ± 111 *	-	379 ± 49 $	283 ± 37	322 ± 35	244 ± 46
linear EDPVR slope	0.016 ± 0.004	-	0.027 ± 0.006	-	0.015 ± 0.003	0.023 ± 0.002 *	0.017 ± 0.002	0.023 ± 0.004 ‡

Pressure-volume relationships were assessed invasively under sequential infusions of dobu (dobutamine), the selective nNOS inhibitor L-VNIO (l-N5-(1-Imino-3-butenyl)-ornithine) and their combination. Because L-VNIO irreversibly inhibits nNOS, a first dobutamine infusion was performed after which a wash-out period was observed. Then, L-VNIO was infused, measurements performed and a second dobutamine infusion was started to assess effects of the combination.

Mean ± SEM; Symbols: $ P< 0.05,$$ P< 0.01 vs. control rats at baseline, * P< 0.05, ** P< 0.01 vs. baseline; †† P< 0.01 vs. dobu; ‡ P< 0.05, ‡‡ P< 0.01 vs. L-VNIO.

The β-adrenergic response and effects of nNOS blockade in control rats

Beta-adrenergic stimulation with dobutamine strongly increased variables of contractility in control rats (Table 2 & Figure 1). This response was not greatly affected by concurrent nNOS blockade, although dP/dt-max was slightly lower and EDP was somewhat higher than during the first dobutamine infusion. Measures of diastole also improved during β-adrenergic stimulation in controls, without an effect of concurrent nNOS blockade. Assessment of PV relations during preload reduction by dobutamine stimulation was difficult in control rats. The increased contractility caused end-systolic pressure to sharply increase during preload reduction, most likely due to impingement of the catheter. This artefact distorts the PV loops making calculation of load-independent parameters unreliable. In CRS rats, this impingement did not occur and PV relationships during preload reduction could be analysed during dobutamine infusion.

The β-adrenergic response and effects of nNOS blockade in CRS rats

In CRS rats, dobutamine infusion also increased heart rate, and a slight non-significant drop in ESP. Although dP/dt-max increased during dobutamine infusion, this was only by ~25% from baseline values (Figure 1A). When dobutamine was infused after L-VNIO, a similar small increase was seen. Although stroke work was not affected during the first dobutamine infusion, it increased after nNOS blockade (P = 0.006; Table 2). The β-adrenergic responses of maximal power, PAMP and PAMPxRR were largely similar with and without nNOS blockade (Table 2 & Figure 1C). Diastolic variables showed an inverse response in CRS rats compared to control rats. During the first dobutamine infusion, dP/dt-min became less negative and *tau* did not improve (Figure 1B and 1D, resp.). When nNOS was blocked, these parameters tended to improve (both P = 0.06).

Similar to the blunted response of dP/dt-max, the ESPVR only increased minimally in CRS rats with β-adrenergic stimulation under baseline conditions, with a borderline increase in Ees (P = 0.06). The slope of the EDPVR increased with dobutamine. Under nNOS inhibition, the β-adrenergic response was largely similar to that seen under baseline conditions. Both ESPVR's became steeper and were shifted to the left, and the PRSW increased.

8.4 Discussion

The main finding of this study is that blockade of nNOS with L-VNIO worsens diastolic relaxation (lusitropy) in rats with the cardiorenal syndrome (CRS). Furthermore, we found that the β-adrenergic response to dobutamine was blunted in CRS rats, which was not significantly affected by acute nNOS blockade.

Effects of in vivo nNOS blockade in control rats

Data from experimental studies suggest that under basal conditions nNOS inhibits Ca^{2+} influx and contractility and promotes relaxation, but results from gene deletion and over-expression studies have not been entirely consistent.[7, 11, 20, 21] In the present study we document cardiac hemodynamic responses to nNOS blockade with the highly specific nNOS inhibitor L-VNIO in rats. The effects of nNOS blockade with L-VNIO on *in vivo* heart function were only minor in control rats. Despite a significant drop in HR, only PAMP was significantly lower, and EDP was slightly raised. Also, assessment of the ESPVR's did not show a significant effect of L-VNIO on contractility in healthy rats. These results are different to what has been reported previously, where NOS blockade with S-methyl-l-thiocitrulline (SMTC) in control rats increased measures of contractility, prolonged tau, but also increased afterload.[8] In this regard, L-VNIO is more selective for nNOS vs. eNOS than SMTC. SMTC has been found to have a pressor effect *in vivo*,[14, 22] and a certain amount of eNOS inhibition can not be excluded. Moreover, in healthy rats an increase in afterload will most likely be matched by a reflex increase in contractility and end-systolic elastance. In our study we did not see an increase in ESP and Ea with L-VNIO in control rats; hence blockade of eNOS with a subsequent rise in systemic resistance was very unlikely. Indeed, no pressor effects of systemic L-VNIO infusion have been reported *in vivo*. Previous studies found no change in mean arterial pressure,[18, 23] or even describe a hypotensive effect at higher doses.[24, 25] Thus, L-VNIO appears to be highly selective for nNOS vs. eNOS and cardiac hemodynamic changes in response to L-VNIO were not confounded by eNOS inhibition.

In vivo nNOS blockade in CRS rats: effects on diastolic relaxation

In CRS rats, nNOS inhibition appeared to slightly reduce afterload, as suggested by the decreases in ESP and Ea. Furthermore reductions in dP/dt-max, SW and maximal power suggest a decrease in cardiac contractility and efficiency. This was corroborated by the data derived from PV relationships during preload reduction (Table 3). Most notably, diastolic variables of pressure decay were worsened by nNOS blockade indicating that nNOS-derived NO supports lusitropy in rats with CRS. As depicted in Figure 2, EDV decreases in CRS rats after L-VNIO. This decreased filling occurs at a slightly higher EDP, which together with the prolonged *tau* and less negative dP/dt-min, strongly suggests decreased lusitropy. Thus, the reductions in systolic variables (dP/dt-max and SW) are at least partly due to less efficient filling.

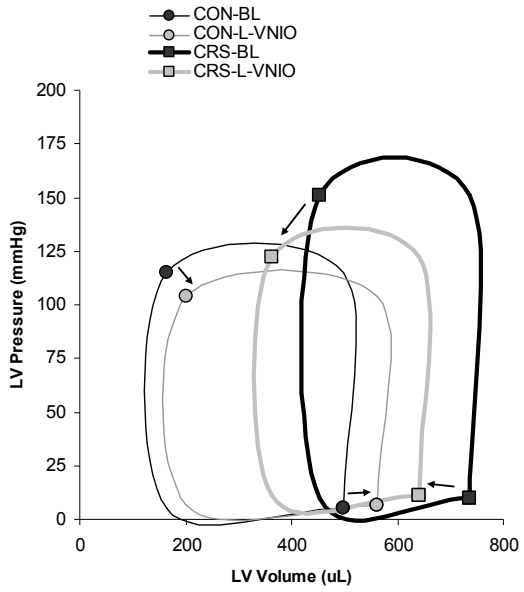

Figure 2. Steady state PV-loops graphed from group averages in control (CON, dashed line, circles) and Cardio-Renal Syndrome rats (CRS , thick line, diamonds) before (BL) and after L-VNIO infusion (open vs. closed symbols). Arrows indicate the responses of preload and afterload to L-VNIO.

The precise role of nNOS on lusitropy in healthy and diseased hearts has been difficult to ascertain. The time constant of relaxation *tau* was significantly prolonged in $nNOS^{-/-}$ mice compared to WT mice,[9] whereas Burger *et al*. [26] documented similar dP/dt-min values in $nNOS^{-/-}$ vs. WT mice. In healthy rats nNOS blockade with SMTC *in vivo* prolonged *tau*.[8] After MI, $nNOS^{-/-}$ mice had a similarly depressed diastolic function as WT mice,[9] and in rats with MI-induced heart failure SMTC did not have a significant effect on cardiac hemodynamics, although both dP/dt-min and *tau* were slightly worsened.[8] We now show that myocardial nNOS plays a role in relaxation of the hypertrophic and dilated left ventricle of rats with the CRS *in vivo*. The slope of the EDPVR was not changed, suggesting that the passive properties of the LV were not affected.

In our previous studies, short-term low-dose NOS inhibition induced marked LVSD that was permanent after stopping NOS inhibition, together with low NO availability.[5] Systemic supplementation of NO with the tolerance-free NO-donor molsidomine significantly improved diastolic and systolic function in CRS rats.[6] Thus, reduced NO availability appears to be detrimental for cardiac function in the setting of CKD. However, expression of cardiac eNOS was unchanged in CRS rats compared to control rats, and expression of nNOS was found to be increased. In various pathological situations nNOS is upregulated and appears to exert protective effects. Conditional over-expression of cardiomyocyte nNOS in mice reduced cardiac dilatation and preserved ejection fraction after transverse aortic constriction, inducing concentric remodeling instead of the dilatory phenotype observed with this model in WT mice.[11] After MI, remodeling and outcome was worse in $nNOS^{-/-}$ mice compared to WT mice, although measures of contractility were increased both at baseline and 8 weeks after MI.[9] Our results suggest that nNOS primarily has a protective role for LV diastolic function in CRS. Diastolic dysfunction is a common finding in CKD in both animal models and patients,[27-29] it can worsen fluid overload and progress to diastolic HF. It may occur early in the disease, and in one study CKD-associated mortality was found to be higher in patients with diastolic HF than with systolic HF.[30] Our studies underscore the importance of adequate NO availability for diastolic function,[31] especially in the setting of reduced kidney function and LVSD.

Beta-adrenergic responses in control and CRS rats, and the effect of L-VNIO

In control rats, the dobutamine infusion induced a strong increase in dP/dt-max (> 200%) and other contractile parameters. Studies on how nNOS modulates the β-adrenergic response in healthy hearts are inconclusive, which may be due to variations in methodology. The response to dobutamine in control rats was not altered by SMTC administration,[8] which is however less selective for nNOS than L-VNIO. With acute nNOS inhibition with L-VNIO murine cardiomyocytes showed an enhanced inotropic response to isoproterenol, but only at low concentrations.[20] On the other hand, the lusitropic response to dobutamine *in vivo* was blunted in $nNOS^{-/-}$ mice.[9, 32] In our study, the β-adrenergic responses of control rat hearts were not strongly influenced by L-VNIO infusion, and the improvement of diastolic variables was similar with and without concurrent nNOS blockade.

Reduced β-adrenergic responsiveness in patients has been documented in HF [12], CKD and end-stage renal disease,[13] and was found to be secondary to adrenoceptor desensitisation.[33] Our results of blunted β-adrenergic response in CRS rats are in line with these findings. Increased production of nNOS-derived NO was found to reduce the β-adrenergic responsiveness in failing rat hearts after MI, as nNOS inhibition with SMTC resulted in significantly higher values for parameters of contractility compared to dobutamine alone,[8] although eNOS inhibition by SMTC may have also played a role. The upregulation of nNOS was suggested to serve as a protective inhibitory adaptation, sacrificing the “acute” inotropic effect for protection against damage induced by long-term sympathetic stimulation.[8]

In rats with the CRS, nNOS blockade did not significantly affect the blunted β-adrenergic response to dobutamine, as parameters of contractility were not higher with than without nNOS blockade. This may be due to a difference in cardiac phenotype, i.e. “uremic” cardiomyopathy vs. ischemic HF. Exogenous NO was found to decrease the response to isoprenaline in ventricular strips from control rats, but this was not the case in heart tissue from rats subjected to either SNX or aortic banding.[34] In a rat model of pressure overload, nNOS upregulation also occurred,[10] but whether this upregulation had consequences for baseline cardiac function and the β-adrenergic response was not elucidated. Thus, in models of hypertrophy and pressure overload, modulation of the β-adrenergic response by nNOS may be different from that in ischemic heart failure. Furthermore, the

absence of an inhibitory effect of nNOS on sympathetic stimulation may be specific for the CRS. As we proposed, sympathetic activation can be one of the "cardiorenal connectors" inducing accelerated cardiovascular damage and dysfunction in the CRS.[3]

8.5 Conclusions

In rats with the cardiorenal syndrome, cardiac neuronal NOS gene expression was upregulated and the nNOS-derived NO was found to support lusitropy *in vivo*. The β-adrenergic response was blunted in these rats, but this effect was not mediated by the nNOS pathway. Our results underscore the role of NO in diastolic function, and the importance of adequate NO availability to maintain baseline cardiac function. The absence of an interaction between nNOS and the β-adrenergic response suggests that the pathophysiology of cardiac dysfunction in this model of the CRS is different from that in MI-induced HF, and that nNOS-derived NO does not influence the adrenergic signalling in combined CKD and cardiac dysfunction. Precise cellular targets for nNOS-generated NO that support cardiac function remain to be elucidated.

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Part IV

Chapter 9

Animal models of the Severe Cardiorenal Syndrome: where do we stand?

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The combination of chronic kidney disease (CKD) and heart failure (HF) is associated with adverse prognosis. We previously defined this as the "Severe Cardiorenal Syndrome" (SCRS) and proposed the Cardiorenal Connection as the putative pathophysiological mechanism. However, in the past years insight in the pathogenesis of the SCRS remains minimal. Although several clinical studies hint at a specific bidirectional interaction between HF and CKD, animal models are needed in which the cardiorenal interaction can be studied in detail. In this paper, we summarize and review available evidence on cardiorenal interactions from animal models of CKD and HF.

A plethora of cardiac changes have been described on the structural, cellular and biochemical level in models of CKD in the rat and mouse. However, despite these changes, parameters of cardiac remodeling are equivocal, and cardiac systolic function generally remains preserved. In models of heart failure, renal changes have been described in rats and dogs, but these are mostly limited to functional changes such as decreases in GFR and sodium excretion. Renal damage has not been documented. Only a few studies have looked at the effect of myocardial infarction (MI) on pre-existent renal injury, but these were hampered by the short duration of renal injury before MI, and the apparent lack of HF.

Because of this lack of 'dual failure' models, we have recently developed two rat models of combined long term CKD and HF. Renal dysfunction was induced by subtotal nephrectomy (SNX). Cardiac dysfunction was induced either functionally by NO depletion, or structurally by MI. In both models, we found evidence of worsened cardiorenal damage and dysfunction. We also propose several characteristics of animal models of the SCRS that are required to more accurately reflect the bidirectional nature of this syndrome.

9.1 Introduction

The current epidemics of CKD and cardiac diseases are major health care problems. Patients with CKD suffer greatly from cardiac disease. The risk rises steeply as the glomerular filtration rate (GFR) progressively falls, and patients have a far greater chance of dying than reaching the stage where renal-replacement therapy is mandatory.[1] Conversely, cardiac disease, most often of ischemic origin, will ultimately lead to heart failure (HF). Renal dysfunction in HF is an independent risk factor for adverse outcome that has received increasing attention in the past decades.[2] Outcome after MI is independently determined by the degree of GFR loss,[3] and is especially dismal in patients on dialysis.[4]

During the course of CKD in patients cardiac output (CO) *in vivo* is generally maintained or even increased by an increase in stroke volume, which is probably secondary to volume retention and anemia (reviewed in [5]). However, as CKD progresses the incidence of LV systolic dysfunction increases, and this is an important predictor for HF and adverse outcome.[6-8] Conversely, outcome after a cardiac event (e.g. MI or a revascularization procedure) or in HF is independently determined by the level of kidney dysfunction.[3, 9-16]

This apparent interaction between kidney and heart disease has been termed the “Cardiorenal Syndrome”. The exact definition of the Cardiorenal Syndrome is still a highly debated topic in the scientific literature, and multiple review articles have been published in recent years to try to structure current knowledge. However, most review papers are based on epidemiological data and most acknowledge that the pathophysiology of the Cardiorenal Syndrome is still poorly understood.[17]

We proposed that in both kidney and heart dysfunction, similar pathophysiological pathways are activated independently of cardiorenal mechanisms governing blood volume and pressure control (Guyton model).[18] These pathways include (disproportionate) activation of the renin-angiotensin system (RAS) and the sympathetic nervous system (SNS), a systemic inflammatory state, and an imbalance between reactive oxygen species (ROS) and nitric oxide (NO) leading to oxidative stress. These connectors interact and synergize in response to both renal and cardiac injury and have the capacity to induce accelerated cardiovascular damage and cause further structural and

functional deterioration. We defined the “Severe Cardiorenal Syndrome” (SCRS) as a disease state where both CKD and HF are present, and the (pre-existent) failure of one organ accelerates progression of structural damage and failure of the other organ. Thus, in the SCRS, cardiorenal interactions work in a bi-directional fashion via the Cardiorenal Connectors. This is one step further in the disease process from what is commonly termed cardiorenal disease or cardiorenal syndrome, where the effect of either kidney or HF on its “normal” or “healthy” counterpart is described. Regarding this approach, several researchers have questioned whether the sequence of events warrants distinction between “cardiorenal” and “renocardiac” disease.[19, 20] In our view, the bi-directional nature of the Cardiorenal Connection obviates this distinction as well as further sub-classification of the cardiorenal syndromes.[21] Several patient studies allude to a certain degree of reciprocity between kidney and HF. One autopsy study found tubulo-interstitial injury and glomerulosclerosis in 15 out of 16 patients who died of end-stage HF.[22] Sub-analysis of two trials from Groningen in the Netherlands showed increased deterioration of renal function after a first MI, particularly in patients who already had impairment of renal function.[23, 24] The decline was abrogated by ACE inhibition.[23]

Worsening renal function acutely after MI was also associated with a worse prognosis, compared to patients who had stable renal function after MI.[2, 25, 26] However, the observation that HF and CKD interact is based upon data from observational or retrospective clinical studies, which cannot be used to draw definitive conclusions about pathophysiological processes. Interactions between organ systems are most effectively studied in animal models, which allow for standardized initiation of a disease process, long-term follow up and assessment of end-organ damage. The aims of this review are (1) to summarize key findings of animal models that studied cardiac consequences of CKD, and renal consequences of HF. Practically no adequate animal models address effects of the combination of CKD and HF, the SCRS. This review also addresses characteristics of models for the SCRS that are required to study the bidirectional nature of heart-kidney interactions.

9.2 Cardiac effects of CKD

Subtotal nephrectomy in the rat

The most widely used animal model for human CKD is that of 5/6th or subtotal nephrectomy (SNX) in the rat. The model is based on renal mass reduction by one-sided nephrectomy and either ligation of two of the three renal artery branches [SNX(lig)] or resection of the poles [SNX(res)] of the contralateral kidney (ablation model), usually 1 week later. The differences between these models have been extensively investigated by Griffin and Bidani.[27] The infarction model is accompanied by higher renin release, a more acute and greater rise in blood pressure and more initial glomerular injury.[28] In both models, a state of chronic stable kidney disease (also called uremia) develops over the course of 4 to 8 weeks.

Cellular derangements

Several studies identified that calcium homeostasis of the individual cardiomyocytes from uremic hearts was impaired. When isolated cardiomyocytes were incubated with serum from hemodialysis patients, they showed increased fractional shortening but impaired recovery of calcium concentration and relaxation.[29] A later study from the same group described impaired fractional shortening and increased systolic and diastolic calcium concentrations in isolated cardiomyocytes from uremic rats, associated with reduced SERCA2a activity.[30] Conversely, cardiomyocytes from uremic rats did not behave significantly different from cardiomyocytes from control rats under baseline conditions. However, at rising Ca^{2+} concentrations uremic cells exhibited decreased contraction amplitude, lower contraction and relaxation velocities, and were slower to return to diastolic Ca^{2+} levels.[31, 32] Cardiomyocytes from SNX rats exhibited rapid inactivation of the L-type Ca^{2+} current and shortening of action potential duration,[33, 34] which may have implications for cardiac rhythmogenesis. Also, the cardiac response to β-adrenergic stimulation was found to be decreased in CKD, both in patients and in cardiac muscle strips from uremic rats.[35] Further research pointed out desensitization of the β-adrenoceptor due to increased G-protein receptor kinase activity as the most likely cause, which appeared to be specific for the left ventricle.[36]

Ex vivo cardiac changes

Already in 1993, Raine *et al.*[37] demonstrated that isolated hearts from 4-wk uremic rats exhibited a 32% reduction in phosphocreatine and phosphocreatine/ATP ratio, suggesting reduced myocardial energy supply. These hearts were also more sensitive to low-flow ischemia, showing increased ATP breakdown. Uremic hearts had on average a 21% lower CO and high calcium perfusate failed to increase CO, in contrast to control hearts. A later study from the same group did not document impaired *ex vivo* cardiac function after 6 weeks of uremia.[38] The reason for this may be that the degree of uremia was worse in the earlier study. Nevertheless, Hatori *et al.*[39] also did not find a decreased baseline cardiac performance in hearts isolated from rats after 8 weeks of CKD, even though variables of renal failure (urea, creatinine, hematocrit), and perfusate calcium content were comparable to those in the study by Raine *et al*..[37] Thus, *ex vivo* cardiac systolic function is not uniformly decreased in CKD rats, and may depend on experimental conditions rather than the degree and duration of renal failure.

Cardiac histological damage

The extensive work of Amann, Ritz, and co-workers has brought considerable insight into the cardiac consequences of CKD (Table 1). They documented the nearly ubiquitous LVH in rats with SNX,[40] as well as in patients during the early stages of CKD.[41] This LVH was due to an increase in cardiomyocyte diameter and volume, despite loss of cardiomyocytes.[42] Reduction of blood pressure alone did not affect development of LVH suggesting that the process of LVH is independent of afterload.[40] Several other studies have corroborated this finding.[43, 44] Amann and Ritz also documented increased wall thickness of intra-myocardial arteries,[45] and a reduction of capillary length density (the total length of capillaries within a unit volume of myocardium) compared to normal rats.[46] These measures were also largely independent of blood pressure.[47] Interstitial fibrosis and/or expansion of the extracellular matrix in the heart is also a widely reported feature of CKD,[39, 43, 48] and is believed to contribute to myocardial stiffening and diastolic dysfunction (see below). These cardiac structural changes were documented in several other studies as well, including our own.[49]

Table 1. Cardiac histological changes in rat SNX models.

Strain	CKD-model	Time	Cardiac histological changes	Changes in renal variables	Refs.
SD	SNX (res)	2-3 wks	LVH Cardiomyocyte hypertrophy Fibrosis	mild hypertension increased serum creatinine & BUN	40 43
SD	SNX (res)	8 wks	LVH Fibrosis Wall thickening of intramyocardial arterioles & SMC hypertrophy Reduced capillary length density Cardiomyocyte apoptosis	No hypertension increased BUN	47 50 42
SD	SNX (lig)	4 wks	LVH Cardiomyocyte hypertrophy Fibrosis	Hypertension increased serum creatinine	51 52 53
SD	SNX (res)*	5 wks	LVH Cardiomyocyte hypertrophy Fibrosis	increased serum creatinine	54
Lewis	SNX (res)	16 wks	LVH Cardiomyocyte hypertrophy Fibrosis	sustained increase in plasma creatinine & BUN reduced CrCl progressive proteinuria	49

SD: Sprague Dawley, SNX(res): subtotal nephrectomy by resection, SNX(lig): subtotal nephrectomy by ligation, LVH: left ventricular hypertrophy, SMC: smooth muscle cell, BUN: blood urea nitrogen, CrCl: creatinine clearance. * one-stage procedure

Echocardiographic changes

Several investigations report on *in vivo* cardiac function after a varying length of uremia in rats (Table 2). For example, Takahashi *et al.*[55] examined cardiac function by M-mode echo-cardiography 2 weeks after SNX(lig) in SD rats. The end-diastolic diameter was increased in SNX rats compared to controls, and fractional shortening (FS) was stable at 55% because of slightly increased end-systolic diameters. Another study reported similar changes in dimensions and increased FS 4 wks after SNX (lig).[51] These findings were expanded by the same group with conductance catheter measurements, showing increased ejection fraction (EF), but reduced stroke volume (SV). [52, 53] Michea and co-workers[54] found larger diastolic diameters and slightly decreased systolic diameters in SD rats 5 weeks after SNX

by resection, which would translate to an increased FS of ~51% compared to ~45% in control rats. Similarly, Rabkin *et al*.[56] documented an increased FS of 50 ± 9% in SNX vs. 41 ± 7 % in controls (NS?) and also no major change in ejection fraction (EF; 86 ± 8% vs. 78 ± 8% (NS?) after 16 weeks of CKD by M-mode echocardiography. Different results were obtained by Koleganova *et al.*,[57] who found a slightly decreased FS by M-mode compared to controls (~75% vs. ~81% in controls) after 4 weeks of uremia. The reason why their FS-values are much higher than those reported in other studies might have been due to use of a different anaesthesia, or sampling of the M-mode images at a different level in the heart. In our model of SNX(res) and 6% NaCl in Lewis rats,[49] we also documented cardiac dilatation, calculated with the area-length method.[58] Ejection fraction was decreased but only after 13 wks of uremia, but both SV and CO were unchanged compared to controls. Thus, variable changes in cardiac dilatation are seen in both models of SNX, but measures of systolic function generally appear to be maintained.

Invasive cardiac assessment

Invasive assessment of cardiac hemodynamics and pressure-volume relationships provide more insight into the functional adaptations of the heart in uremia (Table 3). Follow-up of rats with 1¾ nephrectomy up to 26 weeks did not results in differences in cardiac hemodynamic parameters in Wistar-Han (measured with open thorax), despite more than doubling of plasma creatinine levels.[59] Conversely, SNX(res) resulted in a decreased rate of maximal pressure change (dP/dt-max) after 12 wks in Sprague Dawley (SD) rats.

The group of Shapiro has studied *in vivo* cardiac function in the SNX-ligation model in rats using echocardiography and invasive assessment of LV hemodynamics and pressure-volume relationships. They report increases in ejection fraction, and maximal rate of pressure increase of the LV (dP/dT-max) 4 weeks after SNX.[51, 52] They also document increased LV end-diastolic pressure, and a steeper slope of the end-systolic pressure volume relationship (ESPVR). The latter finding may be due to the LV hypertrophy and higher afterload, with end-systolic elastance matching the increase in vascular resistance rather than reflecting increased inherent contractility. On the other hand, the time constant of

diastolic relaxation, *tau*, was prolonged and the end-diastolic PVR (EDPVR) was steeper, which is indicative of diastolic dysfunction and decreased cardiac compliance.

Table 2. Cardiac in vivo remodeling in CKD models

Strain	CKD-model	Time	method	Changes compared to controls	Refs.
SD	SNX (lig)	2 wks	Echo	EDD ↑ ESD ↓ FS ↔	55
SD	SNX (lig)	4 wks	Echo	EDD ↓ ESD ↓ FS ↑	51
SD	SNX (lig)	4 wks	CC	EDV ↓ ESV ↓ EF ↑ SV ↓	52 53
SD	SNX (res)	5wks	Echo	EDD ↑ ESD ↓ FS ↑	54
SD	SNX (res)†	16 wks	Echo	EDD ↓ FS ↑ EF ↔	56
SD	SNX (res)	4 wks	Echo	EF ↓	57
Lewis	SNX (res)	16 wks	Echo	EDV ↑ ESV ↑ EF ↓ SV ↔ CO ↔	49

SD: Sprague Dawley, SNX(res): subtotal nephrectomy by resection, SNX(lig): subtotal nephrectomy by ligation, EDD: end-diastolic dimension, ESD: end-systolic dimension, FS: fractional shortening, EDV: end-diastolic volume, ESV: end-systolic volume, EF: ejection fraction, SV: stroke volume, CO: cardiac output. † reverse procedure.

Table 3. Cardiac invasive hemodynamic assessment in rat SNX models.

Strain	CKD-model	Time	type	Changes compared to controls	Refs.
SD	SNX (res)	12wks	*closed chest*	dP/dt-max ↓	60
Wistar-Han	SNX (res)	26 wks	*open chest*	no change in cardiac hemodynamics	59
SD	SNX (lig)	4wks	*closed chest*	dP/dt-max ↑ LVEDP ↑ τ ↑	51
SD	SNX (lig)	4wks	*closed chest*	τ ↑ steeper EDPVR & ESPVR	52 53

SD: Sprague Dawley, SNX(res): subtotal nephrectomy by resection; SNX(lig): subtotal nephrectomy by ligation. dP/dt-max: maximal rate of pressure increase of the left ventricle. LVEDP: left ventricular end-diastolic pressure, τ: tau, time constant of diastolic relaxation, EDPVR: end-diastolic pressure-volume relationship, ESPVR: end-systolic pressure-volume relationship.

Cardiac changes in mouse models of CKD

Although mice are an attractive species because of the possibility of genetic modifications, achieving stable CKD is much more difficult than in rats. Cardiac histological and functional changes in different murine models of CKD are presented in Table 4 and 5, respectively.

Kennedy *et al*.[61] employed a model of kidney cauterization together with contralateral nephrectomy in male CD1 mice, and followed them up to 8 weeks after surgery. In this model, both mild LVH and cardiomyocyte hypertrophy were seen, as well as increased fibrosis. Systolic blood pressure was significantly higher than controls but variables of renal function were not reported (Table 4). The EF, measured by echocardiography, was slightly increased, the slopes of both the ESPVR and EDPVR were steeper than in control mice, and *tau* was prolonged (Table 5). This is indicative of diastolic dysfunction but augmented systolic function. A similar model of renal injury in 129/SvJ mice resulted in raised BUN and creatinine levels, but not in hypertension.[44] The hearts of these mice also showed hypertrophy and fibrosis, but invasively determined cardiac hemodynamic function was unchanged vs. controls.

In a SNX resection model in C57BL/6 mice, hypertrophy and fibrosis of the heart was also found, as well as increased diastolic diameter and decreased FS by echocardiography.[62] Hemodynamically, dP/dT-max and dP/dT-min were reduced

12 wks after SNX. However, levels of creatinine and urea were lower at this time-point compared to 4 weeks after SNX. Conversely, even at 36 weeks after 5/6th nephrectomy Bro *et al*.[63] did not find any changes in echocardiographic parameters of diastolic and systolic function in ApoE knock-out mice on a C57BL/6 background. Although heart weight corrected for body weight was increased, there was no evidence of increased cardiac fibrosis, and systolic blood pressure was similar to sham-operated mice. Subtotally nephrectomized (resection) BalB/c mice showed a slightly decreased EDD on echocardiography with decreased EF.[64]
Although they displayed consistently raised blood urea nitrogen levels and increased cardiac fibrosis, these mice had no proteinuria and no glomerulosclerosis. Furthermore, cardiomyocyte size and corrected heart weight were similar to non-nephrectomized mice. Thus, mice appear to be more resistant to subtotal nephrectomy than rats. Secondly, strain differences exist with regard to both renal and cardiac changes after renal injury.

In conclusion, several subtotal nephrectomy models have been employed in rats and mice to study the effects of uremia on cardiac structure. In itself the uremia does not appear to cause significant systolic dysfunction or HF for a prolonged period after initiation of CKD, despite the occurrence of multiple morphological, vascular, and biochemical changes. Left ventricular hypertrophy (LVH) is almost invariably present, and measures of diastolic dysfunction are generally impaired, while results on LV dilatation vary. Diastolic dysfunction is a more common finding in these models and likely relates to increased interstitial fibrosis and disturbed calcium cycling in cardiomyocytes.

9.3 Renal Dysfunction in Models of Heart Failure

Rat models of heart failure

One of the most commonly used models of HF is that of MI by coronary ligation (CL) in the rat and several studies have looked into the renal changes occurring in this model (Table 6). Despite the reduced renal perfusion, whole body GFR or creatinine clearance (CrCl) is not always significantly affected.[65, 66] Studies on single nephron (SN) level, however, show a reduction in SN-GFR and plasma

flow.[65, 67] This is counteracted by an increased filtration fraction and glomerular pressure, brought on by efferent arteriolar vasoconstriction. This glomerular hypertension upholds single nephron filtration to a certain extent, but it may induce glomerular damage when persistent as has been shown in the remnant kidney.[68] However, in three studies that looked at renal injury after MI, significant glomerular and tubulo-interstitial damage as well as proteinuria were absent.[69-71] The rat model of arteriovenous fistula induces high-output HF, and exhibits much of the same renal characteristics as the low-output model of MI.[67, 72] The end-diastolic pressure, as a measure of decompensation, and the glomerular pressure were both higher in MI-induced HF than in AVF.[67]

Table 4. Cardiac histological changes in mouse models of CKD.

Strain	CKD-model	Time	Cardiac histological changes	Changes in renal variables	Refs.
CD1	SNX (caut)	4-8 wks	mild LVH mild cardiomyocyte hypertrophy fibrosis ↑	sustained hypertension renal function not reported	61
129/SvJ	SNX (caut)	8 wks	LVH cardiomyocyte hypertrophy fibrosis ↑	no hypertension increased BUN and serum creatinine	44
C57BL/6	SNX (res)†	4-12 wks	LVH cardiomyocyte hypertrophy fibrosis ↑ capillary density ↓	serum creatinine and BUN increased, but lower at 12 wks than at 4 wks	62
BALB/C	SNX (res)*	8-24 wks	fibrosis no increase in heart weight and cardiomyocyte size	mild hypertension increased BUN with mild regression no proteinuria no glomerulosclerosis	64
ApoE$^{-/-}$	SNX (res)*	36 wks	LVH, no fibrosis	no hypertension increased BUN and serum creatinine reduced CrCl	63

SNX(res): subtotal nephrectomy by resection, SNX(caut): uninephrectomy and cauterization, LVH: left ventricular hypertrophy, BUN: blood urea nitrogen, CrCl: creatinine clearance. * one-stage procedure; † reverse procedure

Table 5. Cardiac echocardiographic and hemodynamic alterations in mouse CKD models.

Strain	CKD-model	Time	Method	Changes compared to controls	Refs.
C57BL/6	SNX (res)	12 wks	Echo	EDD ↑ FS ↓	62
ApoE$^{-/-}$	SNX (res)†	36 wks	Echo	EDD ↔ ESD ↔ FS ↔	63
CD1	SNX (caut)	4-8 wks	CC	EDV ↓ ESV ↓ EF ↑	61
129/SvJ mice	SNX (caut)	8 wks	Echo	EDD ↔ FS ↔	44
BALB/c	SNX (res)*	8-24 wks	Echo	EDD ↓ ESD ↔ FS ↓	64
C57BL/6	SNX, (res)†	12 wks	closed	dP/dt-max & -min ↓	62
CD1 mice	SNX (caut)	4, 6, 8 wks	closed	τ ↑ steeper ESPVR & EDPVR	61
129/SvJ mice	SNX, caut	8 wks	closed	No changes in dP/dt-max, τ, LVEDP	44

SNX(res): subtotal nephrectomy by resection; SNX(caut): uninephrectomy and cauterization.
*: one-stage procedure; †: reverse procedure. Echo: echocardiography; CC: conductance catheter, closed: closed chest invasive hemodynamic assessment. EDD: end-diastolic dimension, ESD: end-systolic dimension, FS: fractional shortening, EDV: end-diastolic volume, ESV: end-systolic volume, EF: ejection fraction, dP/dt-max: maximal rate of pressure increase of the left ventricle, dP/dt-min: maximal rate of pressure decrease of the left ventricle, LVEDP: left ventricular end-diastolic pressure, τ: tau, time constant of diastolic relaxation, EDPVR: end-diastolic pressure-volume relationship, ESPVR: end-systolic pressure-volume relationship.

Table 6. Renal changes in different HF models.

Species & strain	Model	Time	Renal Changes	Refs.
SD	CL	4 wks	renal resistance ↑ RBF/CO ↓	66
Munich-Wistar	CL	4 wks	whole body GFR n.s.↓; $U_{Na}V$ ↓ single nephron GFR and plasma flow ↓ Pg ↑ ; FF ↑ fractional proximal tubule reabsorption ↑	65
Wistar	CL	12 wks	CrCl ↓; FE_{Na} ↔ no proteinuria, no glomerulosclerosis or interstitial damage	69
Wistar	CL	8 wks	GFR ↓ ; RPF ↓; $U_{Na}V$ ↓ RVR ↑ FF ↑	73
Wistar	CL	16 wks	Cr ↑ no proteinuria, no glomerulosclerosis no increase in LVEDP	70
Wistar	CL	10 wks	CrCl ↔ no proteinuria, no glomerulosclerosis or matrix expansion no increase in EDP	71
SD	CL & AVF	CL: 4wks AVF: 6-8 wks	whole body GFR n.s.↓ RVR and FF n.s.↑ $U_{Na}V$ ↓ single nephron GFR and plasma flow ↓; FF ↑ Pg ↑; higher Pg in CL rats vs. AVF.	67
Wistar	AVF	7 days	GFR ↓ or n.s.↓ RBF ↓ RVR ↑ FE_{Na} ↓	74 75 72

SD: Sprague Dawley, CL: coronary ligation, AVF: arteriovenous fistula, RBF: renal blood flow, CO: cardiac output, GFR: glomerular filtration rate, n.s.: non-significant, $U_{Na}V$: urinary sodium excretion, Pg: glomerular pressure, FF: filtration fraction, CrCl: creatinine clearance, FE_{Na}: fractional sodium excretion, RPF: renal plasma flow, RVR: renal vascular resistance, LVEDP: left ventricular end-diastolic pressure.

Pacing-induced HF in dogs

The group of Burnett Jr. has performed several studies on dogs with HF induced by rapid ventricular pacing (Table 7). In this model, renal blood flow was decreased and renal vascular resistance (RVR) increased.[76, 77] A significant decrease in whole

body GFR and urinary sodium excretion was only seen in severe or advanced HF.[76, 78] An effect of HF on renal histology was not reported.

Table 7. Renal changes in the dog model of pacing-induced HF.

Strain	Model	Time	Renal Changes	Refs.
mongrel	pacing	10 days (180/245 bpm)	GFR n.s.↓ RBF n.s.↓ RVR n.s.↑ $U_{Na}V$ ↓ in severe HF	78
mongrel	pacing	38 days (180 up to 240 bpm)	GFR ↓ RPF ↓ $U_{Na}V$ ↓	76
mongrel	pacing	45 minutes (206 bpm)	GFR ↔ RBF ↓ RVR ↑ FF ↑ FE_{Na} ↓	77

bpm: beats per minute, n.s.: non-significant, GFR: glomerular filtration rate, RBF: renal blood flow, RVR: renal vascular resistance, $U_{Na}V$: urinary sodium excretion, RPF: renal plasma flow, FF: filtration fraction, FE_{Na}: fractional sodium excretion.

9.4 Models with combined cardiac and renal damage

Only a few studies addressed the combination of cardiac and renal failure. In the resection model of SNX, Dikow *et al.*[79] performed a study to determine differences in infarct size between CKD and normal rats. Three weeks after SNX, they ligated the left anterior descending (LAD) coronary artery for 60 min., followed by 90 min. of reperfusion. Hearts from SNX animals showed larger infarct areas than hearts from control animals, both as a fraction of total LV area, and as a ratio to the non-perfused area at risk. The non-perfused area at risk corresponds to the area supplied by the LAD, and this was not significantly different between the groups. Thus, although infarct size after transient ischemia and reperfusion is larger in uremic hearts, after permanent LAD occlusion it may not be different as the amount of myocardium supplied by the LAD was similar. Different interventions correcting blood pressure, sympathetic activity, and salt intake did not alter the findings. The short duration of CKD, as well as the lack of (longitudinal) data on cardiac function

and renal injury limit the possibility to draw conclusions about cardiorenal interactions. Interestingly, animals with SNX developed fatal arrhythmias (ventricular fibrillation) far more often than control animals (38% vs. 10%), which may be explained by the well-described electrophysiological abnormalities.[33]

Studies from the group of van Dokkum *et al*. documented diverse effects of MI on glomerular injury. In their earlier studies, they showed that MI induced a minor, insignificant, degree of focal glomerulosclerosis (FGS) in control rats together with increased creatinine levels, but no proteinuria.[70] However, MI did worsen FGS and proteinuria in rats that were uninephrectomised (UNX) two weeks earlier. However, the UNX in itself did not instigate CKD. In a subsequent study from this group, coronary ligation 2 weeks after SNX(res) failed to induce significantly more FGS assessed 10 weeks later, compared to SNX alone.[71] Proteinuria was also unaffected, but creatinine clearance and renal blood flow were decreased. Despite an increase in LV mass and a decrease in the number of capillaries, cardiac hemodynamic variables did not differ between rats with SNX and rats with SNX+MI. Most likely, the period of CKD before the MI (2 weeks) was too short to induce significant changes that might accelerate subsequent progression of cardiac failure. Infarct size was the same in rats with MI alone and SNX+MI (34 ± 2%), which is more or less in agreement with the size of the non-perfused area at risk found by Dikow *et al.*[79] (median values of 38 ± 13%, range 34 ± 8% to 45 ± 9%). Left ventricular EDP was only mildly raised in rats with MI, and dP/dt-values were minimally affected compared to controls, which suggests that cardiac decompensation and HF was not (yet) present. Together with the short duration of renal injury before MI, this limits the applicability of these models to study the interaction between CKD and HF.

Thus, all in all, no studies are available that have combined CKD of longer duration with adequate cardiac dysfunction in a longitudinal fashion. Consequently, knowledge on how chronic damage and failure of the one organ actually affects damage and failure of the other organ is still lacking, as well as the effect of different interventions in this process.

Hence, we recently developed a rat model of combined CKD and LSVD,[49] based on the hypothesis that nitric oxide (NO) is an important regulator of systemic

hemodynamics and renal function, but also of cardiac contractility.[80] We also postulated that the balance between nitric oxide (NO) and reactive oxygen species (ROS) is a key modulator of the other cardiorenal connectors.[81] Furthermore, NO synthase (NOS) inhibition induces both cardiac and renal damage in high doses,[82-86] and renal injury and function in rats is worsened when low dose NOS inhibition is given to rats with SNX.[87, 88] In control rats, low dose NOS inhibition is usually associated with mild reversible hypertension, but it can also functionally reduce CO in higher doses. The effect of NOS inhibition on cardiac function *in vivo* in CKD was unknown, and we hypothesized that NO depletion would induce LVSD in rats with CKD. Male Lewis rats were subjected to SNX (res) and treated with a low dose of the potent non-selective NO synthase inhibitor Nω-nitro-L-arginine (L-NNA) from week -1 to week 8 (SNX+ L-NNA). Sham-operated rats, untreated SNX rats and sham-operated rats treated with the same dose of L-NNA (20 mg/L) served as controls. The low dose NOS inhibition induced severe LVSD in the SNX rats together with increased blood pressure, while in controls it caused only mild hypertension, no renal function abnormalities, and practically no LVSD. Furthermore, in SNX+ L-NNA rats, urinary NO metabolite (NOx) excretion (as a measure for systemic NO production) was strongly depressed, and there was high mortality in this group. We then stopped NOS inhibition to see whether it had induced any permanent changes in cardiac and renal function, and followed up the rats for 7 weeks, after which histological damage was assessed. In the control+ L-NNA group all hemodynamic changes were reversible and there was no evidence of worsened cardiorenal damage. In the SNX+ L-NNA rats however, proteinuria was worsened as well as severe FGS and cardiac fibrosis compared to rats with SNX alone, and, most remarkably, the LVSD and the low urinary NOx excretion persisted.

To test whether the changes observed in SNX+ L-NNA were due to the interaction between the SNX and the NOS inhibition and not merely dependent on the severely reduced NO production, we also included a group of control rats with high dose L-NNA (100 mg/L) that showed a similar increase in systolic blood pressure and decrease in urinary NOx excretion during NOS inhibition. Despite the high blood pressure, this dose did not induce any change in renal function or proteinuria, and the LVSD was not as severe as in SNX+ L-NNA rats. Furthermore,

after cessation of L-NNA, the hemodynamic changes were almost fully reversible, with no appearance of renal injury. Thus, it appears that both the heart and the kidney are very vulnerable to reduced NO availability during development of CKD, leading to severely depressed cardiac function, and worsened cardiorenal damage.

Next to this functional model of combined CKD and cardiac dysfunction, we also developed a chronic model for the SCRS with structural damage. We performed CL 8 weeks after SNX (res) in male Lewis rats, and found that the pre-existent CKD adversely affected cardiac remodeling after MI, independent of infarct size. Furthermore, diastolic dysfunction and decompensation were worsened. Conversely, the ensuing HF aggravated glomerular injury compared to rats with CKD alone, despite significantly lower blood pressure and CO. Although we found more severely damaged glomeruli in rats with SNX+CL, we could not document a significant decrease in GFR with creatinine clearance. Inulin clearance measurements are probably required in rodents to detect the relatively subtle changes in GFR in the lower ranges, especially when a catabolic state is present.

We thus provided the first evidence that combined CKD and HF can indeed adversely affect each other, and developed a model for the SCRS. The advantages of our approach may include the longer period of CKD, during which adverse cardiac effects of CKD can take place that adversely affect cardiac outcome after MI. Also, the fact that we waited until CKD was established may have played a role. In the early phase after SNX, progressive renal injury develops due to glomerular hypertension and neuro-hormonal activation.[68, 89, 90] This results in a rapid loss of nephrons and a progressive decrease in renal function. At some point, a new balance is established between blood pressure, glomerular pressure and renal filtration. A chronic phase of renal dysfunction with slowly progressive damage develops, resembling the slow progressive nature of CKD in patients. Induction of HF by MI in this phase induces an acute-on-chronic change in hemodynamics and neuro-hormonal activation, and allows assessment of worsening of existing renal damage.

9.5 Requirements of SRCS models

We hereby propose several requirements for animal models to more accurately reflect the SCRS in a chronic set-up. It should consist of either:

(1) CKD with subsequent cardiac insult or dysfunction, or

(2) chronic cardiac dysfunction or HF with additional kidney injury or renal failure.

For combination (1) the CKD (of at least 4 wks duration) should display the following changes:

a) stable/progressive increases in plasma/serum creatinine and/or BUN values,
b) reduced GFR, either by creatinine clearance or (preferably) 'gold standard' inulin clearance,
c) an stable/progressive increase in urinary protein and/or albumin excretion,
d) (persistent) histological changes characteristic of CKD: glomerulosclerosis and tubulo-interstitial damage.

The cardiac insult should typically result in a decrease or worsening of cardiac function (systolic or diastolic), with aggravated cardiac damage or remodeling.

For combination (2) the chronic cardiac dysfunction should exhibit:

a) stable/progressive systolic dysfunction as assessed by EF, SV, and preferably CO/Cardiac Index.
b) cardiac remodeling and damage, including increased fibrosis, LVH, cardiomyocyte hypertrophy, preferably with dilatation.
c) secondary measures of cardiac dysfunction or failure, like increased brain natriuretic peptide levels, increased EDP, pulmonary fluid congestion with increased lung weight.

The renal injury should produce (aggravated) renal damage such as more FGS or tubulo-interstitial damage, together with a decrease in or worsening of renal function variables, preferably with proteinuria. This way, effects of secondary organ damage on progression of primary organ damage and dysfunction and vice versa may be studied and quantified.

9.6 Conclusion

Ample data exist on structural and functional cardiac changes occurring in animal models of CKD and the rat model of SNX is the most widely studied. On a cellular

level, abnormalities in calcium homeostasis have been described. Structurally, LVH is a nearly ubiquitous finding, as is increased fibrosis. Cardiac dilatation is not always documented, but diastolic dysfunction is a frequent functional finding. Conversely, systolic function appears to be only minimally affected, even after longer periods of CKD. In several models of HF, the kidney is also involved to a certain degree. Although measures of whole body GFR may be reduced due to a decrease in renal perfusion, glomerular pressure and filtration fraction is increased. Only few studies examined kidney histology after MI and found no evidence of glomerular or interstitial injury. Animal models of dual failure are scarce and have thus far been unable to accurately show whether a bidirectional interaction exists between CKD and HF. We have recently developed two rat models of combined CKD and HF in a chronic set-up, in which we could document enhanced cardiorenal damage and dysfunction. These models provide evidence for a bidirectional interaction between heart and kidneys, with worsening of renal injury (proteinuria and glomerulosclerosis), aggravation of cardiac remodeling (dilatation, fibrosis), and a decline of both systolic and diastolic dysfunction. Both models can serve as a basis for further research into the pathophysiological mechanism of combined CKD and HF. However, development of SCRS models in other species such as mice, allowing genetic manipulation, and large animals, allowing non-pharmacological treatment, are urgently needed. These new models should combine both HF and CKD in a longitudinal fashion and integrate both structural and functional correlates of cardiorenal function.

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Chapter 10

Discussion & Perspectives

The combination of renal and cardiac disease has received increasing attention in the past years. Although the relationship between the heart and kidneys in health and disease has been considered as special for many centuries, the developing epidemics of chronic kidney disease (CKD) and heart failure (HF) have sparked renewed interest in this intriguing interaction. We proposed the Severe Cardiorenal Syndrome (SCRS) as a state in which combined cardiac and renal dysfunction causes accelerated and progressive cardiovascular damage which amplifies progression of failure of the individual organ.[1, 2] We further postulated the Cardiorenal Connection as the underlying pathophysiological mechanism, which works as an extension to hemodynamic control mechanisms extensively described by the late professor Guyton (Figure 1). The "cardiorenal connectors" (CRC) that we put forward were:

- the balance between nitric oxide (NO) and reactive oxygen species (ROS),
- the sympathetic nervous system (SNS)
- the renin-angiotensin system (RAS), and
- inflammation.

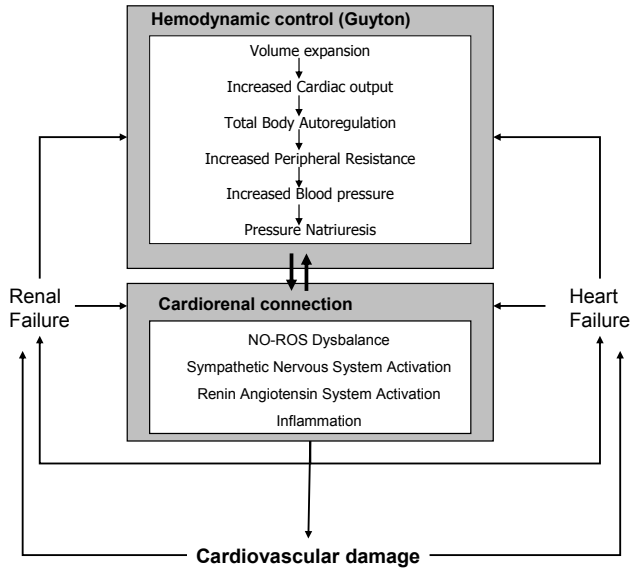

Figure 1. The cardiorenal connection works extensive to Guyton's model to drive accelerated cardiovascular damage in combined renal and heart failure.

In a recent comprehensive review in *Circulation,* Bock and Gottlieb[3] state that: "…each dysfunctional organ has the ability to initiate and perpetuate disease in the other organ through common hemodynamic, neurohormonal, and immunological/biochemical pathways." They also write: "…our understanding of the complex physiological, biochemical, and hormonal derangements that encompass the CRS is woefully deficient…".

Despite general acknowledgement of the adverse prognosis of concurrent cardiac and renal disease, many clinicians and researchers are skeptical about the true existence of a specific heart-kidney interaction that goes beyond known physiological interactions. To accurately investigate the pathophysiology of the Severe Cardiorenal Syndrome, animal studies are the preferred method primarily because they allow standardization of the degree of kidney disease and heart disease, and secondarily because they allow assessment of both physiological dysfunction and morphological damage. Animal models of single organ failure of heart or kidneys do not induce progressive failure in the other organ.[4-7] Thus, it appears that both organs need to be affected to cause acceleration of damage and failure typical for the SCRS. Only two previous animal studies investigated the effect of 'dual damage' to heart and kidneys, with MI following shortly after a renal insult, but these yielded conflicting results.[6, 8] On the other hand, the previously used animal models do not adequately replicate the pathophysiological changes present in patients because they induce organ damage in an otherwise healthy animal.

10.1 Experimental investigations of the Severe Cardiorenal Syndrome.

The main theme of the current thesis was the development of animal models with combined CKD and cardiac dysfunction. We set out to study whether bidirectional organ interaction occurs, and to study the effect of different treatment combinations aimed at the cardiorenal connectors on organ damage and dysfunction in the SCRS in rats.

The Cardiorenal Connection in combined experimental CKD and MI

When a myocardial infarction (MI) was superimposed on long term CKD in the rat, both cardiac and renal injury worsened, with a significant interaction between the

CKD and MI. Hereby we developed a structural model of the SCRS, with reduced cardiac function due to loss of cardiomyocytes. Infarct size (in comparison to rats with MI only) did not play a role, and our results are in agreement with other studies.[8, 9] Despite the worsened glomerulosclerosis and reduced cardiac output (CO), glomerular filtration rate (GFR; assessed by creatinine clearance) was not further reduced. This was unexpected, but may be related to an increase in glomerular pressure and filtration fraction, by post-glomerular constriction. A reduction in GFR due to the more severe renal injury may become evident with longer follow-up, although the creatinine clearance may not be sufficiently accurate to monitor this due to a catabolic state or low tubular flow. In the future, gold standard assessment of GFR with inulin clearance may provide a definitive answer and continuous *in vivo* measurement of GFR with fluorescein-labeled inulin is an attractive approach to assess progressive changes in GFR in experimental SCRS.[10]

We subsequently found that a combination of interventions targeted at all the cardiorenal connectors provided additional reno-protection compared to combinations aimed only at the triad of inflammation, oxidative stress and NO deficiency, or to RAS blockade monotherapy. The combination of interventions targeted at all the cardiorenal connectors appeared to halt progression of glomerular injury and almost completely reversed tubulo-interstitial damage. Preservation of cardiac ejection fraction and stroke volume was similar with all three interventions, but the complete CRC blockade had a negative chronotropic and inotropic effect, most likely because it contained a β-blocker. Cardiac output was thus less well preserved in the complete blockade. Furthermore, blood pressure was lower with complete intervention (which contained low dose losartan and metoprolol) compared to the other interventions, although measures of afterload and vascular resistance were similarly decreased. Despite this, proteinuria and GFR were slightly ameliorated in complete blockade compared to no effect or worsening with the other treatments. Thus, complete correction of the CRC was more effective in abrogating progression of the SCRS, despite the negative chronotropic effect and minor improvement of CO. This suggests dissociation between systemic and intra-renal hemodynamics, with mildly improved renal function despite lower systemic blood pressure. Also direct effects of

correction of the Cardiorenal Connectors on histological damage may have occurred separate from the effects on hemodynamics.

All in all, correction of all the cardiorenal connectors by combination therapy appears to be safe and beneficial for cardiorenal progression in the SCRS, even at the low doses used in our study. Effects of correction of inflammation and NO/ROS imbalance, generally considered mediators of RAS- and SNS-induced cardiovascular damage, was equally effective as low dose losartan monotherapy in preventing the decline in cardiac function. However, when these two interventions were combined, marked effects on renal injury were observed. We found no effect on LVH and cardiomyocyte size, but whether cardiac fibrosis or inflammation was also additively affected by the medication combinations remains to be determined. The dose of losartan was likely too low to provide full RAS blockade, which may explain the lack of effect on blood pressure and renal damage, but in complete CRC blockade only low, mildly hypotensive doses of losartan and metoprolol were tolerated. On a positive note, it might be argued that in the SCRS even a low, non-hypotensive dose of an ARB has a beneficial effect on cardiorenal disease progression, as cardiac systolic function was also stabilized with low dose losartan. Blockade of down-stream effects of the RAS and SNS by interventions that are not hemodynamically active, like correction of inflammation and NO/ROS imbalance, may provide a useful additional treatment strategy and deserve further research.

Recent investigations suggest an important role for iron and hepcidin in the cardiomyocyte. We found that cardiac gene expression of hepcidin is upregulated in rats with MI, CKD and the SCRS and is correlated with markers of cardiac stress. Hepcidin expression in the liver, considered the source of systemic hepcidin, was downregulated and correlated with the hypoxia-apoptosis gene CCAAT/enhancer-binding protein α (C/EBP-α). The upregulation of cardiac hepcidin appeared to independent of decreased NO availability, oxidative stress and inflammation, as different medications targeted at these factors increased cardiac hepcidin expression even further. A greater metabolic activity by improved function, increased extracellular iron not participating in oxidative stress, or a direct effect of these medications on gene expression may have played a role in this upregulation.

Our results suggest that cardiac hepcidin serves a biological function and reacts to different disorders that affect the heart.

The role of nitric oxide in the Severe Cardiorenal Syndrome

We also developed a model of the SCRS based on CKD and depletion of NO availability. The rationale for these investigations was that the pathogenesis of CKD (in the presence of hypertension, diabetes or aging) is associated with low NO availability,[11, 12] while experimental SNX induces nephron number reduction in a healthy animal. In extension to our proposal of the Cardiorenal Connection,[1] we postulated that the balance between NO and ROS is a key modulator of the other cardiorenal connectors.[13] Also, it has been shown that constitutive NO production supports basal cardiac function,[14] For a prolonged period after SNX, cardiac systolic function generally remains preserved, and we hypothesized that a reduction in NO availability would accelerate the development of cardiac dysfunction. Indeed, treatment with an oral NO synthase (NOS) inhibitor (L-NNA), at a very low dose, induced NO depletion and severe cardiac dysfunction. Furthermore, proteinuria, severe glomerulosclerosis and cardiac interstitial fibrosis were worsened compared to rats with CKD without NOS inhibition. Another remarkable finding was that the effects on cardiorenal dysfunction but also on systemic NO production were irreversible after cessation of the NOS inhibitor, during a 7 week follow-up. A five times higher dose of NOS inhibition in control rats, which caused a similar level of hypertension and NO depletion, induced LVSD that was not as severe as in the CRS rats. Furthermore, all effects on blood pressure, cardiac function and NO availability were completely reversible, and had no effect on kidney structure and function. Combining NOS inhibition with SNX also, worsened kidney injury. The more severe hypertension and direct effects of NOS inhibition may have played a role in this.

We conclude that during CKD development the heart is very sensitive to depression of systemic NO availability. Compared to the normal kidney, the damaged kidney is more sensitive to alterations of NO availability as well, possibly because of a loss of autoregulation.[15] Thus, maintaining adequate NO availability appears to be very important for progression of cardiorenal failure during

progression of CKD, and the combination of CKD and NO depletion appears to produce a functional model of the SCRS in which cardiac function is further compromised.

That supplementation of NO is useful as a rescue therapy was shown in a subsequent study, where treatment with the oral tolerance-free NO donor molsidomine (MOLS) significantly improved cardiac diastolic and systolic function, abrogated mortality, and also slightly improved kidney function and injury. The cardiac effect of MOLS appeared to be a combination of reduced cardiac loading and improved contractility and relaxation. Systolic blood pressure was only mildly reduced and GFR was even slightly improved. Thus, MOLS appears to be an attractive and safe therapeutic option for CKD patients suffering from cardiac dysfunction of non-ischemic origin.

The pathophysiology of the continuing low NO production in this model is likely very complex and may include low NOS expression or activity, substrate deficiency, high oxidative stress levels, and increased amounts of endogenous NOS inhibitors.[16] In the hearts of CRS (SNX+L-NNA) rats, we found an unexpected increase in the mRNA expression of the neuronal NOS (nNOS) subtype. This upregulation appeared to be a functional adaptation as selective nNOS inhibition worsened early diastolic relaxation (lusitropy), and reduced cardiac filling. Although others have found that inhibition of upregulated nNOS improved the β-adrenergic response of failing rat hearts after MI,[17] we did not find a modulating effect of nNOS blockade on the decreased cardiac response to dobutamine of CRS rats. The difference in pathogenesis of the cardiac dysfunction (structural vs. functional) and cardiac remodeling (post-MI dilatation vs. concentric hypertrophy and dilatation) may play a role in the observed differences. A dampened β-adrenergic response is found in both CKD and HF, resulting from chronic sympathetic overstimulation.[18, 19] In agreement with our proposal of the CRC and the continuing feedback loops leading to stimulation of the Cardiorenal Connectors, persistent and perhaps progressive SNS activation and cardiac β-adrenoceptor desensitization may have led to the reduced response to dobutamine and the lack of effect of nNOS blockade in our model.

Are there other connectors, not included in the initial model?

Anemia is a common finding in both kidney and heart failure, but correction with supplementary erythropoietin (EPO) does not appear to result in significant benefits for patients. Moreover, anemia is not very pronounced in our model and treatment with rhEPO failed to abolish the cardiac capillary deficiency seen in uremia.[20] Furthermore, EPO generally has deleterious effects in CRF in rats.[21] We have therefore not explored the role of anemia and EPO in further detail.

Aldosterone is an important extension of the RAS that has been implicated in cardiovascular diseases. Treatment with aldosterone-antagonists improves outcome in heart failure patients.[22] It reduces micro-albuminuria in patients and proteinuria and renal injury in animals.[23, 24] Aldosterone synthesis "escape" in patients on RAS-blockade is common and an important problem.[25] By itself aldosterone can cause myocardial fibrosis.[26] In relation to the other cardiorenal connectors, aldosterone induces a pro-inflammatory cascade,[27] increases ROS production,[28] and diminishes NO synthesis.[29] Thus, aldosterone may be an extension of the cardiorenal connector RAS. One major drawback of aldosterone antagonism is hyperkalemia, especially in the presence of chronic renal failure, although Tobian has advanced that mild hyperkalemia may also have beneficial vascular effects.[30]

10.2 Definition and classification of the Cardiorenal Syndrome

Several attempts have been made to define and classify the cardiorenal syndrome. From a clinical perspective, the definition proposed by the NHLBI Working Group on Cardio-Renal Connections which states that the Cardiorenal Syndrome is a state "in which therapy to relieve congestive symptoms of heart failure is limited by further decline in renal function", is adequate albeit very succinct.[31] As Alfred Stengel[32] already proposed in 1914 it is often difficult in advanced disease to dissect whether renal failure or heart failure is the principal disease causing the observed symptoms and signs. However, the pathophysiological mechanisms behind the SCRS remain largely unknown, as also acknowledged by the Working Group, the UK Cardiorenal Forum, and recent reviews on the subject.[3, 33] The Acute Dialysis Quality Initiative (ADQI), under the leadership of Claudio Ronco,

published several consensus papers with a proposed classification of the Cardio-Renal Syndromes.[34-37] They divided cardio-renal interactions on the basis of the primary failing organ (heart vs. kidney) and time-frame (acute vs. chronic). The proposed types were:

- type 1: acute cardiac dysfunction leading to acute kidney injury.
- type 2: chronic cardiac dysfunction causing progressive chronic kidney disease
- type 3: acute kidney dysfunction causing acute cardiac dysfunction
- type 4: chronic kidney disease causing decreased cardiac function, hypertrophy and increased risk for adverse cardiovascular events.

A fifth group was included to encompass cardiorenal disease caused by systemic diseases. This classification was published in multiple journals and has since then pervaded the clinical and scientific literature and generated much debate. The classification by Ronco *et al*.[35] is based on the assumption that the secondary organ is largely unaffected before the primary insult. In the case of the acute CRS (type 1 and 3) this may be probable, but in the chronic setting (types 2 and 4) the distinction between heart and kidney failure as the primary cause of the symptoms may be difficult.[38]

Chronic kidney disease and heart disease share many risk factors, primarily because they are both exponents of systemic cardiovascular disease (CVD). Thus, it seems that both CKD and HF share pathophysiological mechanisms, which are activated in both diseases. Whether cardiac disease or HF is independently associated with increased risk for the development of CKD is less well defined, but the presence of CVD or HF seems to be associated with a higher risk for CKD.[39, 40] In patients with hypertension, the presence of LVH was associated with a higher prevalence of micro- and macro-albuminuria.[41] Furthermore, hypertrophic changes of subcutaneous small resistance vessels in patients with hypertension and LVH in patients with high cardiovascular risk were recently found to predict the decline of renal function.[42, 43]

Secondly, a decrease in GFR and/or proteinuria confer a greatly increased risk for cardiovascular problems, independent of traditional risk factors.[44-48] A recent study showed that when hypertensive patients also had diabetes and/or CKD the severity of LVH and of latent diastolic dysfunction was progressively

increased.[49] When GFR falls below 60 ml/min the risk for cardiovascular events increases exponentially,[44] and when CKD progresses the prevalence of cardiac diseases increases.[50, 51] This is likely one of the main reasons why only a few percent of patients initially diagnosed with CKD eventually reach the stage where renal replacement therapy is advocated.[44, 52]

When cardiac disease is already present, for example MI, risk for a second major adverse cardiac event rises when GFR falls below 75 ml/min.[53] In HF, worsening of renal function is associated with an adverse prognosis.[54] Also, the rate of decline of GFR appears to be accelerated after a first MI, especially in patients with already decreased kidney function.[55, 56] Thus, the presence of cardiac and renal disease appears to be associated with an increased risk for development of failure of the other organ. At later stages, when both diseases are present, the risk for adverse outcome appears to be amplified.

Clinical relevance of the experimental data

Based on our investigations presented in this thesis and the review of current literature on cardiorenal interactions we found the following experimental evidence that describes cardiorenal interactions in the different stages of development towards the SCRS and end-stage disease:

- Myocardial infarction and ensuing HF does not cause any change in renal structure or function, apart from a functional decrease in GFR.
- CKD is almost invariably associated with LVH, fibrosis, and other myocardial abnormalities. Functionally this leads to some degree of diastolic dysfunction, but generally systolic function is maintained for prolonged periods.
- In rats with pre-existent CKD, MI-induced heart failure worsens the severity of glomerular damage. Systolic dysfunction induced by NO depletion in rats with CKD is associated with worsened proteinuria and glomerulosclerosis.
- Pre-existent CKD aggravated cardiac dilatation after MI in rats.

- Rats with CKD and HF have worsened diastolic and systolic dysfunction. Rats with SCRS based on CKD and decreased NO availability show chronic SCRS with high mortality.
- All cardiorenal connectors appear to play a role in the progression of the SCRS.

10.3 Perspectives

The enigmatic pathophysiology of the Severe Cardiorenal Syndrome is a highly active field of research. The continuous debate between clinicians and researchers will hopefully lead to a better understanding of underlying mechanisms. As our knowledge on hemodynamic, neurohormonal and biochemical mediators increases, the development of adequate animal models should continue, to provide a solid basis to explore the relevance of findings in patient studies.

Our research into the SCRS provides evidence of a bidirectional interaction between CKD and HF, and of a beneficial effect of correction of the cardiorenal connectors. Furthermore, we emphasize the role of adequate NO availability for cardiac function in the SCRS.

Both the presence of renal dysfunction in patients with high cardiovascular risk or existing cardiac disease and the presence of cardiac abnormalities in renal patients should be considered as significant prognostic risk factors. Evidence-based therapies to alter this increased risk are still lacking, primarily because cardiorenal patients have been under-represented in clinical trials. Nevertheless, several studies showed that treatment with RAS or SNS blockade may provide benefit for patients with concurrent renal and cardiac disease.[57-60] We showed that combination treatment targeted at all the Cardiorenal Connectors provided significant benefit as a rescue therapy in rats with the SCRS secondary to CKD and MI. Mildly hypotensive doses of RAS and SNS blockade were well tolerated, and correction of inflammation and NO/ROS imbalance appears to be a useful additive therapy. Furthermore, we found that NO supplementation rescues cardiac function and improves GFR in rats with the SCRS based on CKD and NO deficiency.[61] In the future, carefully designed trials should be performed evaluating the effect of interventions targeted at the cardiorenal Connectors in patients with different stages of the Severe Cardiorenal Syndrome.

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Chapter 11

Summary

In this thesis we investigated the interactions between heart and kidney in disease in a longitudinal and integrative fashion. We developed two different rat models of the SCRS and studied the effects of different late interventions on progression of cardiorenal failure and on structural and functional outcome.

Part I

1. *Based on current knowledge about cardiorenal interactions in health and disease, can we develop a putative pathophysiological model to explain the enhanced morbidity and mortality observed in patients with combined kidney and heart failure?*

In **Chapter 2** we proposed the Cardiorenal Connection as a pathophysiological model in which the cardiorenal connectors interact and synergize to cause accelerated cardiovascular damage in the SCRS. The proposed connectors are:

- an imbalance between nitric oxide (NO) and reactive oxygen species (ROS),
- (disproportionate) activation of the sympathetic nervous system (SNS)
- (disproportionate) activation of the renin-angiotensin system (RAS), and
- induction of chronic (low-grade) inflammation.

Part II

1. *Can we develop a model for the SCRS in the rat based on combined CKD and MI that allows longitudinal follow-up of cardiorenal function, combined with structural assessment?*

In **Chapter 3** we described the development of a model of combined CKD and HF, and found evidence of bi-directional organ damage. We performed SNX in Lewis rats, and allowed CKD to develop over a period of 8 weeks. Then, MI was induced by permanent ligation of the LAD coronary artery. Follow-up lasted 6 weeks. Acute mortality after MI was relatively low (~25%) but large myocardial infarctions resulting in an ejection fraction <35% was only achieved in about 40 – 45% of all ligated animals. Therefore, combined SNX+CL with longitudinal follow-up is feasible but labor- and time-intensive.

a. *Does long-term pre-existent CKD adversely affect cardiac structure and function after MI, and conversely does ensuing HF worsen renal structural and functional derangement?*

We found that pre-existent CKD aggravated cardiac dilatation after MI that was associated with worsened cardiac diastolic and systolic function, and mortality. This was not due to an increase in infarct size or afterload in CKD rats compared to control rats. Conversely, the ensuing HF exacerbated glomerular damage in CKD rats, which was not associated with worsening of creatinine clearance or proteinuria.

2. *Do the Cardiorenal Connectors play a role in the progression of the SCRS, and is there a hierarchy between them?*

    a) *Does blockade of all Cardiorenal Connectors fully abrogate progression of the SCRS?*

Blockade of all postulated cardiorenal connectors with low doses of angiotensin receptor blockade and beta-blockade, systemic supplementation of NO, and anti-oxidant and anti-inflammatory therapy (5-MED) had significant beneficial effects on cardiorenal outcome, as described in **Chapter 4**. Therapy was started two weeks after MI when infarct remodeling was assumed to be complete. Complete (5MED) CRC blockade resulted in an abrogation of the decline of cardiac systolic parameters like EF and SV, but because beta-blockade resulted in significant heart rate reduction cardiac output was less well preserved. Blood pressure was lowered by 5MED. In the kidney, 5MED resulted in small improvements in creatinine clearance, plasma urea and proteinuria. Furthermore, there were less severely sclerotic glomeruli and tubulo-interstitial damage was markedly ameliorated.

    b) *Does correction of inflammation and the disturbed NO/ROS balance or of the RAS alone have similar effects?*

The combination of an NO donor with an anti-oxidant and anti-inflammatory compound (3MED) and the RAS blockade alone resulted in similar improvements of cardiac EF and SV as 5MED. The absence of beta-blockade and the associated negative chronotropic effect resulted in a maintained CO in both these groups, and blood pressure was unaffected. In contrast to 5MED,, creatinine clearance and

proteinuria were unaffected or even worsened, and the effects on renal histological damage were less marked.

This suggests that the low dose of RAS and SNS blockade had additional positive effects on renal injury in the SCRS, despite the negative chronotropic effect. Whether the lower blood pressure in the 5MED group is cause or consequence of the improved renal injury cannot be determined. The lower cardiac output did not lead to reduced GFR, which suggests a dissociation between systemic and intrarenal hemodynamics.

3. *Is hepcidin expression in the heart in CKD, HF and the combination different from systemic (liver) hepcidin expression, and does it react to anti-inflammatory and antioxidant therapy?*

Compared to controls, increased cardiac expression of hepcidin was induced by both CKD and HF, as well as in the combination. In contrast, in the liver, hepcidin expression was not affected by both single insults but down-regulated in combined SNX and CL. Cardiac hepcidin expression was significantly correlated to expression of CTGF and BNP, while liver hepcidin correlated with the hypoxia-apoptosis marker CCAAT/enhancer-binding protein α. Therapeutic intervention with 3MED led to increased expression of cardiac hepcidin, while liver expression tended to decrease. Thus, we concluded that cardiac hepcidin expression is induced by local and remote injury. Hepcidin may be an important regulator of cardiac iron distribution, and its precise function in the SCRS remains to be elucidated.

Part III

4. *Does depletion of NO availability during CKD development induce cardiac dysfunction and amplify progression of cardiorenal failure?*
    a) *Is this due to an interaction between CKD and NO availability or due to NO depletion per sé?*
    b) *Are there permanent structural and/or functional effects?*

Low dose NOS inhibition during development of CKD in rats induced worsened proteinuria, severe LVSD and markedly reduced systemic NO production, compared to rats with CKD alone and controls treated with a similar dose of NOS

inhibition (**Chapter 6)**. Furthermore, the increased protein excretion, the LVSD and the low NO production persisted for a long time after cessation of the NOS inhibition, while the mild hemodynamic effects of low dose NOS inhibition observed in controls were fully reversible. In control rats treated with a high dose of NOS inhibition, LVSD was not as severe as in rats with SNX and temporary low dose NOS inhibition, despite similar levels of hypertension and systemic NO depletion. Hemodynamic effects were also reversible in the high-dose group, and no renal abnormalities developed during the study. In rats with CKD and temporary low dose NOS inhibition besides the persisting functional effects that persisted long after stopping NOS inhibition, cardiac fibrosis and severe glomerulosclerosis were also aggravated compared to rats with CKD only. Thus, in CKD significant sensitivity exists to reductions of systemic NO availability with deleterious long term consequences for cardiorenal structure and function, changes which were not observed with temporary severe NOS inhibition or CKD alone.

5. *Does systemic NO supplementation with an NO donor restore cardiac dysfunction and improve cardiorenal parameters?*

Because the findings of the previous study strongly suggested a causal link between NO availability and cardiac function we investigated the effect of systemic NO supplementation with the tolerance-free NO donor molsidomine (MOLS) as a rescue therapy in **Chapter 7**. MOLS significantly improved both diastolic and systolic heart function, with mild effects on cardiac loading conditions and LVH, and no effects on cardiac fibrosis. Creatinine clearance and tubulo-interstitial injury were improved, but no effect was seen on proteinuria. Cardiac expression of iNOS was slightly reduced, but expression of cardiac eNOS and nNOS were not affected. Interestingly, expression of nNOS was increased compared to controls in this model of the SCRS.

6. *What is the functional role of the neuronal NOS isoform for in vivo cardiac function in this model of the SCRS?*

We then investigated in **Chapter 8** the functional role of the nNOS upregulation observed in cardiac tissue of the SCRS rats. We performed invasive *in vivo* measurements of cardiac hemodynamics and pressure-volume relationships in

control rats and rats with the CRS induced by SNX and temporary NOS inhibition. We assessed the effect of selective nNOS blockade in baseline and under beta-adrenergic stimulation and found that nNOS derived NO supports diastolic relaxation in CRS rats, but does not modulate the impaired beta-adrenergic response.

Part IV

Are there animal models available that replicate cardiorenal failure in the SCRS in a controlled and longitudinal fashion, and that show bi-directional cardiorenal interaction?

As reviewed in **Chapter 9**, there is a paucity of animal models that address combined chronic kidney and cardiac dysfunction. The most widely studied model of CKD is that of SNX in the rat, and multiple cardiac structural abnormalities have been described, including LVH, fibrosis and decreased capillary density. Despite the fact that diastolic dysfunction has been reported in this model in several studies, *in vivo* cardiac systolic function is generally maintained, even after longer study periods. In models of heart failure, mostly functional derangements of renal function secondary to decreased renal perfusion have been described but induction of renal damage is absent. The few studies of combined renal and cardiac injury were generally too short of duration, or did not show cardiac dysfunction or heart failure. Thus, we proposed several requirements for future models of the SCRS and reviewed our findings from our models of the SCRS.

In **Chapter 10**, the findings and implications of our animal experiments were discussed. The clinical relevance of our findings were summarized and a perspective for future research was provided.

Samenvatting in het Nederlands

De relatie tussen hart en nieren heeft in de afgelopen decennia steeds meer aandacht gekregen in wetenschappelijk onderzoek en bij de behandeling van patiënten. Grotendeels is dit te verklaren door het toenemende aantal patiënten dat leidt aan gelijktijdig bestaande ziekte van hart en nieren, wat gepaard gaat met een sterk verhoogde kans op verergering van deze ziekte, ziekenhuisopnames en vroegtijdig overlijden. In de afgelopen jaren is een wetenschappelijke discussie ontstaan over de kenmerken van “cardiorenaal falen” en over het bestaan van het zogenaamd “cardiorenaal syndroom”.

Het idee dat hart en nieren een speciale band hebben bestaat echter al eeuwen. In verschillende tijden en culturen worden het hart en de nieren nauw met elkaar in verband gebracht. Zowel in geschriften uit het oude Egypte als in de Bijbel worden hart en nieren samen genoemd. In de traditionele Chinese geneeskunde zijn het hart en de nieren ook met elkaar verbonden en reguleren wederzijds de activiteit. In oude Chinese teksten vinden we uitgebreide en beeldende beschrijvingen terug van patiënten met cardiorenaal falen.

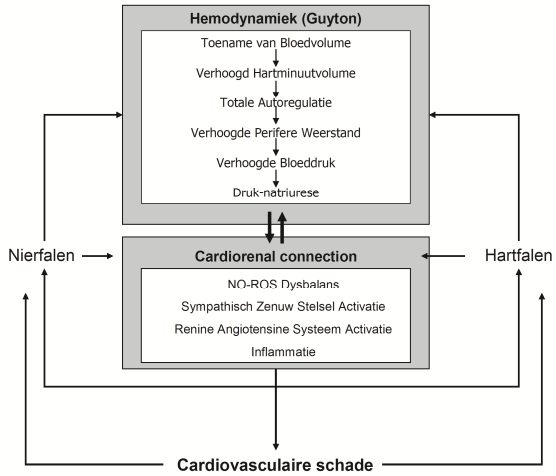
In de Europese Middeleeuwen was Gentilis de Fulgineo (1272? – 1348) een van de eerste artsen die de functie van het hart koppelde aan de kleur en hoeveelheid van de urine. Tijdens de Industriële Revolutie nam de wetenschap een vlucht en werd steeds meer gebaseerd op observaties en experimenten. Door diverse artsen en wetenschappers werd een verdikking van de hartspier (hypertrofie) beschreven bij patiënten met nierfalen bij postmortaal onderzoek. Alfred Stengel poneerde in 1914 een definitie van “cardiorenaal lijden” en de term “uremische cardiomyopathie” (hartspierziekte veroorzaakt door nierfalen) verscheen in de wetenschappelijke literatuur. Na de komst van de hemodialyse werd steeds meer aandacht besteed aan de gevolgen daarvan op het hart, zowel functioneel als structureel. Ook werd beschreven dat dialyse-patiënten die een hart-infarct doormaken een zeer slechte prognose hebben.

Daarnaast werd steeds duidelijker dat nierfalen, of een verslechtering van de nierfunctie, een sterke voorspeller is van een slechte uitkomst bij patiënten met hartfalen. Deze epidemiologische observaties deden vermoeden dat hart- en nierfalen elkaars progressie verergeren en dat de combinatie gepaard gaat met versneld cardiovasculair lijden. Wij noemden dit het “Ernstig Cardiorenaal Syndroom” (ECRS –of Severe Cardiorenal Syndrome; SCRS)

In dit proefschrift onderzochten wij, op een longitudinale en integratieve wijze, de interacties tussen hart en nieren in ziekte. Hiervoor ontwikkelden wij twee modellen van het ECRS in de rat en bestudeerden de effecten van therapeutische interventies op de progressie van cardiorenaal falen en op de structurele en functionele uitkomsten.

Het Ernstig Cardiorenaal Syndroom en de Cardiorenale Connectie

In hoofdstuk 2 poneerden wij de Cardiorenale Connectie (Cardiorenal Connection; CRC; Figuur 1) als model om de ziekteverschijnselen van het ECRS mee te verklaren. De interactie tussen hart en nieren kan voor een groot deel verklaard worden door het bloeddruk-volume model dat uitgebreid beschreven is in de jaren 70 door wijlen professor Guyton. In de decennia daarna zijn een aantal regulatoren van dit model beschreven, die op directe wijze weefselschade kunnen induceren.



Figuur 1. De Cardiorenale Connectie (Cardiorenal Connection). Een toename van het bloedvolume zorgt via een verhoogd hartminuutvolume en bloeddruk voor meer water- en zoutuitscheiding in de nier, waardoor het bloedvolume weer afneemt. Bij zowel hart- als nierfalen kan dit systeem ontregeld raken, met een verhoogde activatie en een verstoring van de regulatoren van dit systeem. Deze ontregeling van regulatoren zorgt daarnaast ook voor toegenomen schade, en kunnen elkaar steeds verder aanzetten. Hierdoor wordt het hart- of het nierfalen steeds erger, maar kan ook verergering van falen van het andere orgaan ontstaan. Uiteindelijk falen beide organen en ontstaat het Ernstig Cardiorenaal Syndroom (ECRS).

Wij stelden dat de actieve factoren van de CRC, de “cardiorenale connectoren”, als gevolg van zowel hartfalen als nierfalen geactiveerd worden. Vervolgens gaan zij met elkaar een interactie aan en stimuleren elkaar verder. Als gevolg daarvan onstaat versnelde schade aan hart en nieren. Hierdoor ontstaat progressie van ziekte en falen in beide organen. Als uiteindelijk beide organen falen is de activatie

van de CRC het sterkst en spreekt men van het ECRS. De voorgestelde connectoren zijn:

- een afname van de hoeveelheid stikstof-oxide (NO) en toename van zuurstofradicalen, waardoor oxidatieve stress ontstaat.
- activatie van het sympathische zenuwstelsel (SZS)
- activatie van het renine-angiotensine systeem (RAS)
- inductie van chronische inflammatie (ontsteking).

In hoofdstuk 2 bespraken wij resultaten uit studies die bewijs leverden dat deze factoren elkaar wederkerig kunnen beïnvloeden en elkaars activiteit kunnen versterken. Gezien de vele interacties tussen de connectoren, stelden wij dat het zeer waarschijnlijk is dat alle connectoren behandeld zouden moeten worden om de vicieuze cirkel te kunnen doorbreken en de progressie van het ECRS te kunnen stoppen. Een duidelijke hiërarchie in het systeem van connectoren is moeilijk aan te geven. Enerzijds zijn het SZS en het RAS in de regulatie van de bloeddruk en het volume nauw met elkaar verbonden, en gaan oxidatieve stress en inflammatie hand in hand. Anderzijds worden oxidatieve stress en inflammatie juist aangezet door het SZS en het RAS. Daarnaast kunnen NO en zuurstofradicalen stimuleringsroutes en processen in de cel moduleren.

Wederkerige orgaanschade in het ECRS

Omdat het idee van een interactie tussen hart en nieren ontstaan is uit epidemiologische observaties wilden wij onderzoeken of er op orgaan-niveau daadwerkelijk een interactie bestaat. Omdat dit enkel goed te onderzoeken is in proefdiermodellen, waarin structurele en functionele uitkomstmaten aan elkaar gekoppeld kunnen worden, ontwikkelden wij een diermodel van gecombineerd chronisch nierfalen (CNF) en hartfalen (HF) in de rat. Onze hypothese was dat pre-existent nierfalen de cardiale structuur en functie na een hartinfarct verslechterd en dat vice versa het hartfalen de renale structuur en functie verder verergerd.

Het chronisch nierfalen werd geïnduceerd in mannelijke Lewis ratten middels subtotale nefrectomie (SNX), waarbij één nier werd verwijderd en van de andere nier de boven- en onderpool werden afgeknipt. Acht weken later werd een hartinfarct aangebracht door de linker kransslagader af te binden en werden de

dieren zes weken lang gevolgd. In dieren met CNF vonden wij na het hartinfarct een verergerde dilatatie (verwijding) van de linkerkamer, wat gepaard ging met verslechtering van de systolische (contractie) en diastolische (relaxatie) functie van het hart, en mortaliteit. De progressie van het hartfalen was niet gerelateerd aan de grootte van het infarct of een hogere bloeddruk na het infarct. Vice versa zagen wij in de ratten met gecombineerd hart- en nierfalen dat de schade aan glomeruli, de functionele eenheden van de nier bestaande uit een kluwen haarvaatjes waar de pre-urine wordt gevormd, was toegenomen ten opzichte van de ratten met enkel chronische nierschade. Deze veranderingen gingen niet gepaard met veranderingen in de creatinine-klaring (als maat voor de glomerulaire filtratie-snelheid) of proteïnurie (eiwit-verlies in de urine).

Correctie van de Cardiorenale Connectoren

Opvolgend aan de hypothese van de Cardiorenale Connectie onderzochten wij of blokkade van alle cardiorenale connectoren de progressie van het ECRS tot staan zou kunnen brengen. Verder bestudeerden wij of er een hiërarchie in de cardiorenale connectoren te ontdekken was en een bepaalde combinatie van interventies meer effectief was dan een andere (hoofdstuk 4). Hiervoor gaven wij ratten met gecombineerd hart- en nierfalen, zoals beschreven in hoofdstuk 3 gedurende de laatste vier weken van de studie medicamenten die aangrijpen op de cardiorenale connectoren. Dit waren een angiotensine receptorantagonist (losartan), een beta-adrenerge blokker (metoprolol), een NO donor (molsidomine), een anti-oxidant (tempol) en een anti-inflammatoir middel (PDTC). Deze combinatietherapie werd twee weken na het aanbrengen van het hartinfarct gestart, na de acute fase van herstel.

De complete combinatie van medicijnen gaf een stabilisatie van de systolische hartfunctie gemeten met echocardiografie, maar vanwege de vertraging van de hartfrequentie door de beta-blokker was de verbetering van de cardiac output (hartminuutvolume) minder uitgesproken. Met betrekking tot de nier gaf de medicatie-combinatie een lichte verbetering van de functie. De schade aan de glomeruli was verminderd , maar vooral aan de nierbuisjes en het tussenliggende weefsel (tubulo-interstitiele schade) was een sterke verbetering te zien. De angiotensine receptorantagonist en de beta-blokker werden in een lage dosering

gegeven, omdat deze anders een te sterke bloeddrukdaling veroorzaakten. Om de grote effecten op de bloeddruk en de hartfrequentie te scheiden van de andere effecten van de cardiorenale connectoren, bestudeerden wij het effect van de combinatie van de NO donor, antioxidant en ontstekingsremmer, en het effect van de lage dosis losartan. Deze combinaties leverden hetzelfde effect op de hartfunctie, zonder de afgenomen hartfrequentie en bloeddruk, maar hadden een minder uitgesproken effect op de nierschade als de totale combinatie. Het lijkt er op dat een totale blokkade van de cardiorenale connectoren een beter effect heeft als 'rescue'-interventie in het ECRS dan mindere combinaties. Dit is grotendeels in lijn met onze eerdere hypothese dat alle connectoren moeten worden behandeld om de vicieuze cirkel van interacties in het ECRS te kunnen doorbreken. Er leek geen duidelijk verschil te zijn tussen enerzijds correctie van oxidatieve stress en inflammatie en anderzijds correctie van het RAS, wat te maken kan hebben met de gebruikte doseringen. Het is goed mogelijk dat de gebruikte dosis losartan geen volledige RAS-blokkade heeft geëffectueerd. Echter, de studies waren niet opgezet om een dosis-effect relatie te beschrijven. Wij wilden de interventies splitsen met de gebruikte doseringen, en werden ook, gezien de bloeddruk, slechts lage doseringen losartan en metoprolol verdragen.

Concluderend kunnen wij zeggen dat volledige correctie van de cardiorenale connectoren zinvol lijkt. Dit bracht de achteruitgang van de hartfunctie tot stilstand en gaf een sterke vermindering van de nierschade. Medicamenten die aangrijpen op de inflammatie en oxidatieve stress kunnen van toegevoegde waarde zijn in de behandeling van het ECRS, omdat ze niet direct op de bloeddruk ingrijpen maar wel de weefselschade verminderen.

Hepcidine: een mogelijke rol bij hart-dysfunctie?

In hetzelfde model onderzochten wij of de genexpressie van hepcidine was veranderd als gevolg van nierfalen, hartfalen of de combinatie. Hepcidine is een eiwit dat voornamelijk in de lever wordt geproduceerd, maar ook in andere organen waaronder het hart. Het is een belangrijke regulator van de ijzerstatus doordat het ferroportine inactiveert. Ferroportine is een kanaaltje in de celwand dat ijzer uit de cel transporteert. De remming van ferroportine door hepcidine zorgt voor een toegenomen ijzerconcentratie in de cel. In de darm zorgt dit voor een verminderde

ijzer-opname, en in de rode bloedcel een verminderde uitgifte van ijzer waardoor het gehalte van ijzer in het bloed wordt verlaagd. Ijzer is een belangrijk element voor de energiehuishouding en het afweersysteem, maar speelt ook een rol bij de vorming van zuurstofradicalen en oxidatieve stress. Verstoring van de ijzerregulatie in het hart kan consequenties hebben voor het ontstaan hartschade en een verlies van hartfunctie. De hepcidine-spiegels in het bloed zijn doorgaans verhoogd bij patiënten met CNF, maar hoe de regulatie op orgaanniveau is, is onbekend. Wij vonden een verhoogde expressie van het hepcidine-gen in het hart van ratten met nierfalen, hartfalen en de combinatie. In de lever was de expressie enkel verlaagd bij ratten met ECRS. De expressie in het hart was niet gerelateerd aan het hematocriet, maar wel aan markers van overbelasting en verlittekening (fibrose). Het lijkt er op dat hepcidine een beschermend effect heeft bij het ontstaan van schade of dat het juist daaraan bijdraagt. Wat de rol van hepcidine is bij de regulatie van ijzerconcentratie binnen en buiten de cel en de rol bij schade zal moeten worden onderzocht in celexperimenten.

Stikstofoxide (NO): een belangrijke modulator van de hartfunctie

In het tweede deel van het proefschrift onderzochten wij hoe een gebrek aan NO gedurende de progressie van CNF bijdraagt aan de ontwikkeling van hartdysfunctie. Een progressieve verlaging van de concentratie van NO is een veel voorkomend verschijnsel bij patiënten met CNF maar ook bij patiënten met HF. Naast dat NO een belangrijke vaatverwijder is speelt het een rol als anti-oxidant, bij de regulatie van transcriptie van genen, en bij de functie van hartspiercellen. De hartfunctie blijft echter bij ratten met SNX lange tijd relatief ongestoord, afgezien van dilatatie en hypertrofie, mogelijk als gevolg van behoud van voldoende NO. Onze hypothese was dat een achteruitgang van de hartfunctie secundair is aan een vermindering in de NO beschikbaarheid (hoofdstuk 6).

Wij vonden dat bij ratten waar CNF werd geïnduceerd middels SNX, een lage dosis van een NO synthase-remmer (L-NNA, dat de aanmaak van NO remt), gegeven tot 8 weken na SNX, een sterke daling in de systolische hartfunctie tot gevolg had. Verder verergerde de hoge bloeddruk en de proteïnurie, en was de NO productie sterk gedaald. De NO productie, gemeten als 24-uurs excretie van stabiele metabolieten van NO (NOx) was bij ratten met enkel SNX slechts iets

verminderd. Dezelfde dosis van L-NNA bij ratten zonder SNX gaf slechts een matige verhoging van de bloeddruk, die na beëindiging van de dosering volledig reversibel was, zonder aantasting van de nierfunctie. Bij de SNX ratten die met een lage dosis L-NNA werden behandeld, was de hartdysfunctie en de lage NO productie niet reversibel, evenals de verergerde proteïnurie.

Aan het einde van de studie bleek dat de mate van verlittekening (sclerose) was toegenomen in de glomeruli, maar ook in het hart. Een vijf keer hogere dosis van L-NNA veroorzaakte bij gezonde dieren hypertensie en hart-dysfunctie, maar niet zo erg als bij ratten met SNX en lage dosis L-NNA, ondanks dat de 24-uurs uitscheiding van NO metabolieten even sterk gedaald was. Daarbij ontstonden geen afwijkingen in de nierfunctie, en waren alle effecten zo goed als reversibel. Wij concludeerden dat het hart zeer gevoelig is voor verlaging van de NO beschikbaarheid als er CNF bestaat. De hartdysfunctie en de lage NO productie gingen gelijk op en waren irreversibel.

Het toedienen van een NO-donor verbetert de gestoorde hartfunctie

Deze resultaten deden vermoeden dat de hartfunctie en de NO beschikbaarheid een causaal verband hebben bij ratten met CNF. We herhaalden het bovenstaande experiment in hoofdstuk 7 voor de SNX+ L-NNA groep en stopten wederom de L-NNA op acht weken. Na een wash-out periode van 3 weken, verdeelden we de ratten in twee groepen met gelijke hart- en nierfunctie. De ene groep kreeg geen therapie, de andere groep kreeg molsidomine, en beide groepen werden voor vier weken vervolgd. Molsidomine (MOLS) is een pro-drug, die wordt omgezet in de lever tot de actieve metaboliet SIN-1. Dit maakt zonder verdere enzymatische omzetting NO vrij. Zodoende is MOLS vrij van tolerantie, een veel voorkomende bijwerking van organische nitraten waardoor patiënten ongevoelig worden voor deze medicamenten.

MOLS had weinig effect op de arteriële bloeddruk, maar de systolische hartfunctie, gemeten met de echo, verbeterde significant. Wij verrichten invasieve druk-volume metingen van de linkerkamer (linker-ventrikel) met een catheter aan het eind van de studie (week 16). Hiermee konden wij laten zien dat de diastolische functie ernstig gestoord was bij ratten met SNX en een tijdelijke lage dosis L-NNA behandeling, en dat deze verbeterde met de MOLS behandeling.

Daarnaast gaf MOLS een verbetering van de nierfunctie, gemeten als creatinine-klaring, en een lichte vermindering van de tubulo-interstitiele schade.

Neuronaal NOS: belangrijk voor de relaxatie van het hart

Bovendien vonden wij dat de genexpressie van een bepaald subtype van de NO-producerende enzymen, het neuronaal NO synthase (nNOS), in het hart verhoogd was bij ratten met SNX+ L-NNA, zeven weken na het stoppen van L-NNA. Gezien het positieve effect van NO therapie met MOLS hadden we verwacht dat NOS expressie misschien verlaagd zou zijn, en wij veronderstelden dat deze toegenomen genexpressie van nNOS een compensatoir effect is bij de verminderde hartfunctie (hoofdstuk 8). Daarom onderzochten wij de functie van het hart door middel van het meten van de druk-volume relaties van dieren met SNX+ L-NNA zeven weken naar het stoppen van L-NNA. Wij hebben deze relaties gemeten onder vier verschillende condities: op het basis-niveau, met stimulatie van de hartfunctie met dobutamine (de zogenaamde beta-adrenerge respons), met blokkade van het nNOS enzym, en met een combinatie van beiden.

Bij zowel CNF als HF was de beta-adrenerge respons sterk verminderd ten opzichte van gezonde dieren, zoals eerder beschreven door anderen. Tevens gaf de blokkade van het nNOS enzym een afname van de diastolische functie bij de zieke ratten, wat er op duidt dat nNOS de relaxatie en vulling van het hart ten dele onderhoud. Secundair was de pompfunctie van het hart verder afgenomen. Het blokkeren van nNOS had geen effect op de respons op dobutamine. Samenvattend kunnen we concluderen dat NO een belangrijke rol vervult voor het hart in chronisch nierfalen. Het hart is zeer gevoelig voor verlaging van de NO productie en er lijkt een causaal verband te bestaan tussen de algehele NO beschikbaarheid en de hartfunctie.

Medicamenten die NO bevatten kunnen van toegevoegde waarde zijn bij patiënten met CNF door de verminderde hartfunctie te ondersteunen. Vooral de vullingsfase van het hart is verstoord bij patiënten met nierfalen en kan therapie met NO donoren een gunstig effect hebben. Maar ook als er een verminderde pompfunctie is, kan MOLS deze helpen ondersteunen. Een groot voordeel van MOLS is dat het geen tolerantie opwekt en nauwelijks effect heeft op de bloeddruk.

Proefdier-modellen van het ECRS

De bevindingen uit dit proefschrift zijn gebaseerd op proefdiermodellen die ontwikkeld zijn om de interactie tussen CNF en dysfunctie van het hart te onderzoeken. In hoofdstuk 9 bespraken wij de resultaten van proefdier-onderzoeken naar de effecten van CNF op het hart, en naar de effecten van HF op de nier. Daarnaast beschreven we de resultaten van modellen van het gelijktijdig bestaan van CNF en HF, waaronder die van onze eigen modellen. Vervolgens bespraken wij de geschiktheid van diverse diermodellen om deze ziektebeelden na te bootsen. In het CNF-model van de rat werden reeds zeer veel afwijkingen aan het hart beschreven. Desondanks vinden veel onderzoekers, inclusief wijzelf, dat de systolische functie langere tijd bewaard blijft. Het modelleren van CNF in de muis is lastig en geeft een wisselende mate van nierfunctiestoornissen en lijkt daarom minder geschikt. Ten aanzien van hartfalen zijn bij verschillende modellen bij de rat en bij de hond functionele veranderingen in de nieren beschreven, zonder duidelijke aanwijzingen voor nierschade op weefselniveau. Slechts twee onderzoeken hebben eerder gekeken naar het effect van gecombineerd hart- en nierschade, maar deze lieten wisselende resultaten zien. We concludeerden dat er veel variabiliteit is tussen de verschillende diermodellen en dat er maar weinig onderzoeken zijn die gekeken hebben naar de effecten van de combinatie van langer bestaand CNF en HF. De twee modellen die wij in dit proefschrift presenteren geven een beginnend inzicht in de complexe veranderingen die optreden in het ECRS. Verder stelden wij een aantal criteria voor waaraan een proefdiermodel van cardiorenaal falen en het ECRS zou moeten voldoen. In het kort behelst dit een model waarin CNF wordt geïnduceerd van adequate duur met zowel kenmerkende functionele als structurele veranderingen, waarop vervolgens HF wordt toegevoegd, of vice versa.

In hoofdstuk 10 werden alle behaalde resultaten bediscussieerd en tegen een bredere, klinische achtergrond geplaatst.

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Allerliefste Mayke, in de afgelopen 4,5 jaar is mijn liefde voor jou alleen maar verder gegroeid. Je waardeert me zoals ik ben, maar laat me ook kritisch naar mezelf kijken. De manier waarop je altijd voor iedereen klaar staat en je bezieling waarmee je dingen organiseert werken aanstekelijk. De reis die we samen hebben gemaakt heeft ons dichter bij elkaar gebracht, en zorgde ervoor dat we uiteindelijk op Fiji elkaar symbolisch het ja-woord hebben gegeven. Eigenlijk is leven met jou een grote ontdekkingsreis.

Curriculum Vitae

Lennart Guido Bongartz werd geboren op 5 september 1980 te Duisburg als eerste zoon van Eric Bongartz en Brigitte van der Velden. Kort daarna verhuisde het kersverse gezin naar Leiden. Na afronding van het Stedelijk Gymnasium aldaar, ging hij in 1999 Geneeskunde studeren in Utrecht. In 2004 begon hij onder begeleiding van Maarten Jan Cramer, Branko Braam en Jaap Joles aan het opzetten van het model van de Cardiorenal Connection en dit betekende de start van het huidige promotie-onderzoek. Dit resulteerde eerst in het review-artikel dat in Hoofdstuk 2 wordt beschreven en het behalen van een “vrij doctoraal”. Daarna begon hij aan het opzetten van de proefdierkundige studies en startte hij in 2006 onder supervisie van Jaap, Maarten Jan, Branko en Pieter Doevendans aan zijn promotie-onderzoek. Samen met Branko Braam en Jaap Joles was hij coauteur en projectleider van de twee Wetenschapsbeurzen van de Nierstichting Nederland, die de studies in dit proefschrift hebben ondersteund. In 2010 ging hij samen met zijn vriendin, Mayke Treur, 3 maanden op reis in Nepal, Japan, Australië en Fiji. In oktober 2010 begon hij onder begeleiding van Carlo Gaillard met de vooropleiding Interne Geneeskunde in het Meander Medisch Centrum Amersfoort als start van de opleiding tot Cardioloog.

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Van der Putten K, Bongartz LG, Braam B, Gaillard CA. Letter: **The cardiorenal syndrome a classification into 4 groups?** *J Am Coll Cardiol* 2009;53:1339.

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Bongartz LG, Braam B, Verhaar MC, Cramer MJ, Goldschmeding R, Gaillard CA, Doevendans PA, Joles JA. **Transient nitric oxide reduction induces permanent cardiac systolic dysfunction and worsens kidney damage in rats with chronic kidney disease.** *Am J Physiol Regul Integr Comp Physiol.* 2010;298:R815-23.

Bongartz LG, Braam B, Verhaar MC, Cramer MJ, Goldschmeding R, Gaillard CA, Doevendans PA, Joles JA. **The nitric oxide donor molsidomine rescues cardiac function in rats with chronic kidney disease and cardiac dysfunction.** *Am J Physiol Heart Circ Physiol. 2010 Dec;299(6):H2037-45.*

Arslan F, Smeets MB, Bongartz LG, Peters J, Doevendans PA, Pasterkamp G, de Kleijn DP. **Lack of fibronectin-EDA promotes survival and prevents adverse remodeling and heart function deterioration after myocardial infarction.** *Circ Res 2011 In press.*

Appendix

Expanded Methods

In vivo measurements

Systolic blood pressure (SBP) was measured by the tail-cuff method as described previously.[1] Directly after each SBP measurement rats were placed in metabolism cages without food for 24 h, but with free access to water with 2% glucose, supplemented with L-NNA as appropriate, for determination of urinary protein, measured with Coomassie blue. Urine was collected on 1 mL of antibiotic/antimycotic solution (Sigma, St. Louis, MO; A5955) and stored at –80°C. Urinary excretion of stable NO metabolites NO_2 + NO_3 (NO_x) were determined by fluorometric quantification of nitrite content.[2] The rats were fasted during the 24h collection period to minimize the effect of dietary protein intake. Thus, analysis of the 24-h NOx excretion can provide a solid estimate of whole body NO production during 24 hours.[3] Creatinine clearance was calculated by the standard formula. Urinary sodium content was determined by flame photometry.

Echocardiography

Transthoracic echocardiography was performed as previously.[4] In short, animals were anesthetized with isoflurane and placed in a supine position on a warming pad. A three-lead ECG system was connected to the paws, the chest shaved and excess hair removed with depilatory cream. Two-dimensional B-mode cine-loops with continuous ECG-registration were recorded in the parasternal long axis (LAX) and the mid-papillary short axis (SAX) views, while isoflurane anesthesia was adjusted to the lowest possible level (1.75-1.85%) to minimize effects on heart rate and blood pressure. Typical study duration was 15 min. Acquisitions were coded and results were decoded after analysis. The recordings were analyzed off-line using the software present on the system, and the variables were measured in at least three heartbeats at end-diastole and corresponding end-systole.

Details on calculations:

Long axis: LV area (LVA) was measured by tracing the endocardial border; LV length (LVL) was measured from the apical trace border to the middle of the trace border on the LV outflow tract.

LV volume was calculated with the prolate ellipsoid area–length method:

V (mL) = [8*(LVA)2]/(3π*LVL)

at end-diastole (end-diastolic volume; EDV) and end-systole (end-systolic volume; ESV).[5]

Calculated variables:

Stroke volume (SV, mL) = EDV – ESV;

Ejection fraction (EF, %) = (EDV-ESV)/ESV * 100%;

Cardiac output (CO; mL/min) = SV*HR.

Cardiac index (CI; mL/min/100 g BW): CO/BW*100.

Short axis: the endocardial border was traced at end-diastole (LVEDa) and end-systole (LVESa) at the mid-papillary level. Trabeculae and papillary muscles were included in the LV lumen. Fractional area change of the LV (LVFAC) was calculated with the formula: LVFAC (%) = [(LVEDa-LVESa)/LVEDa] x 100%.

The internal anterior-posterior diameter was measured at end-diastole (LVEDd) and anterior wall and intraventricular septum thickness (AWT; IVST) were measured at end-diastole and used to compute LV mass by the standard cubical formula:

LV mass (g) = 1.04 x $[(LVEDd + PWT + IVST)^3 – (LVEDd)^3]$.

After that, a correction was applied, which was found to calculate LV mass more accurately in rats: LV mass (g) = 0.8*(cubical formula) + 0.14.[6]

End-organ damage and histology.

After exsanguination through the abdominal aorta under anesthesia, organs were harvested and weighed. The kidney was cut transversely and one part was formalin-fixed and embedded in paraffin for standard histology and one part was snap-frozen in liquid nitrogen and stored at – 80ºC. The heart was weighed after removing the atria and large vessels. The right ventricle was cut off and snap-frozen in liquid nitrogen and stored as a reference sample. The LV was weighed and the apex (~1-2 mm thick) was cut off, snap-frozen in liquid nitrogen, and stored at – 80ºC. The rest of the LV was fixed in formalin, embedded in paraffin, and cut

into three transverse sections. Slices of 3 µm thickness were cut from the paraffin-embedded tissues and mounted on glass slides for staining.

Glomerulosclerosis and tubulo-interstitial damage was scored on PAS-stained kidney sections.[4]

Cardiomyocyte circumference was measured on PAS-stained myocardial slices in sections with transversely cut myocardial fibers and was traced on the cellular border on photomicrographs of at least 50 different cardiomyocytes with a computer assisted image analysis system (OptiMas, Houston, TX).

Digital photomicrographs of transverse sections of the heart stained with Sirius Red were taken to measure collagen content of the heart. The extent of cardiac fibrotic patches was scored in a blinded manner on joined digital images acquired at 20x magnification using ImageJ software.[7] Images were converted to an RGB stack and collagen area fraction was measured on the green channel, where the red-stained areas appear black. The percentage of the collagen area was calculated by dividing the Sirius Red stained area by the total LV tissue area. Infarct scar size was measured on photomicrographs of transverse sections of the heart stained with Sirius Red, by dividing the length of the infarct scar by the circumference of the total LV section, traced in mid-wall using ImageJ software.[7] All measurements were performed by an experienced technician blinded to the group allocation.

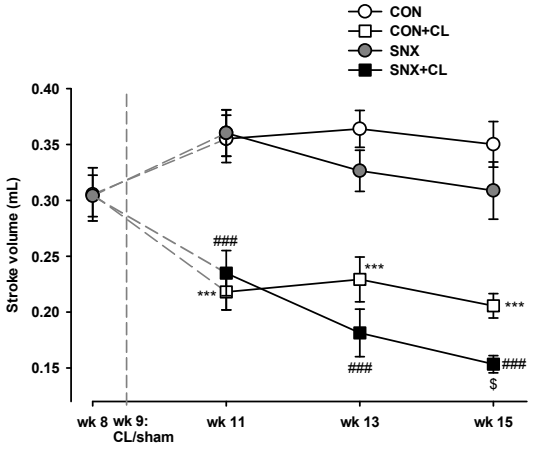
Supplementary data

Chapter 3

Supplementary Table 1. Kidney histological damage.

	CON (n=10)	**CON+CL** (n=9)	**SNX** (n=10)	**SNX+CL** (n=7)
LK/BW (g/100 g)	0.34 ± 0.01	0.32 ± 0.01	0.44 ± 0.02 ***	0.42 ± 0.02 †††
TI injury score (a.u.)	0.91 ± 0.16	0.61 ± 0.08	6.88 ± 0.50 ***	5.73 ± 0.35 †††

LK: left kidney; BW: body weight. TI: tubulo-interstitial.
Data as mean±SEM. *** *P*<0.001 vs. CON; ††† *P*<0.001 vs. CON+CL.



Supplementary Figure 1. Stroke volume (SV) in all groups. SV was lower in SNX+CL vs. CON+CL at wk 15. Mean ± SEM. *** *P*<0.001 vs. CON; $ *P*<0.05 vs. CON+CL; ### *P*<0.001 vs. SNX.

Chapter 4

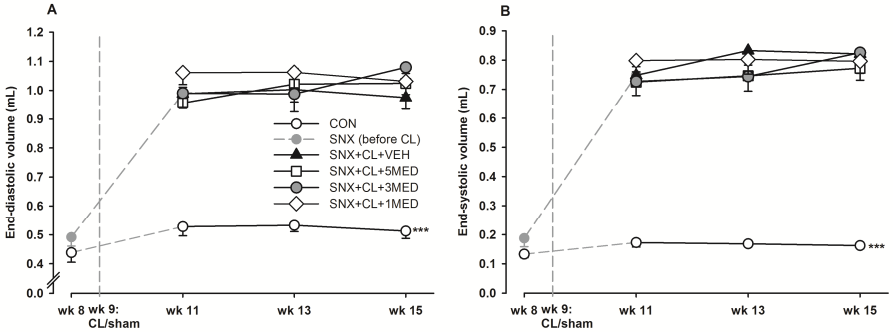
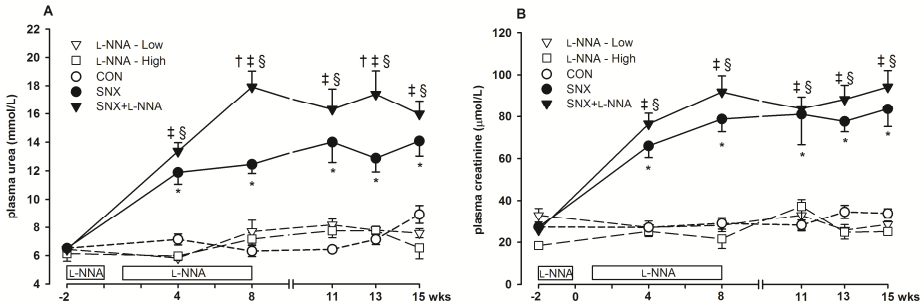


Figure S1. End-diastolic volume (A) and end-systolic volume (B), assessed by echocardiography.

Chapter 6



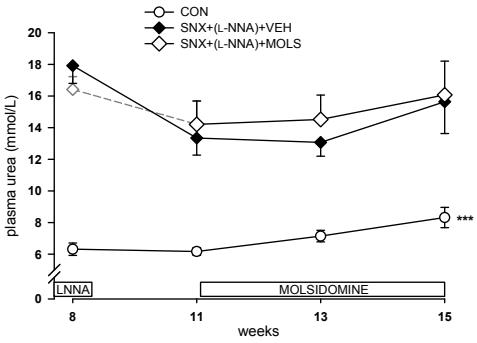
Supplementary Figure. (A) Plasma urea levels and (B) plasma creatinine levels in all groups at all time-points. Mean ± SEM, * p<0.05 vs. CON; † p<0.05 vs. SNX; ‡ p<0.05 vs. L-NNA-Low. § p<0.05 vs. L-NNA-High. For clarity different levels of significance are not indicated.

Chapter 7

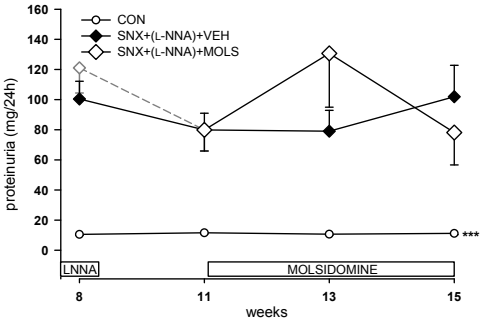
Table 1. Organ weights

	SNX (+L-NNA) +VEH	SNX (+L-NNA) +MOLS	CON
	n=20	n=12	n=12
RV weight (g)	0.21 ± 0.01	0.23 ± 0.02	0.21 ± 0.01
RV weight/100g BW (g/g)	0.057 ± 0.00	0.066 ± 0.01	0.049 ± 0.00
Wet lung weight (g)	1.73 ± 0.13	1.50 ± 0.05	1.34 ± 0.03 **
Wet lung weight/100g BW (g/g)	0.47 ± 0.04	0.43 ± 0.03	0.31 ± 0.01 ***

Mean ± SEM. ** *P*<0.01, *** *P*<0.001 vs. SNX(+L-NNA)+VEH.



Supplemental Figure 1. Plasma urea levels were not altered by MOLS treatment. Mean ± SEM. *** *P*<0.001 vs. SNX(+L-NNA)+VEH. Where error bars are not visible, they fall within the symbol.



Supplemental Figure 2. Proteinuria was not affected by MOLS therapy.
Mean ± SEM.
*** *P*<0.001 vs. SNX(+L-NNA)+VEH. Where error bars are not visible, they fall within the symbol.

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