Cardiac abnormalities in chronic kidney disease

- an investigation of pathophysiological mechanisms

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“The right question is usually more important than the right answer”

-Plato

Cardiac abnormalities in chronic kidney disease

- an investigation of pathophysiological mechanisms

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ABSTRACT

Chronic kidney disease (CKD) is a global health problem associated with increased risk of mortality and development of end-stage renal disease (ESRD). Cardiovascular diseases are the leading cause of morbidity and mortality even before the development of ESRD. The main purpose of this thesis is to elucidate pathophysiological mechanisms causing cardiac injury in patients with CKD. The specific aims were: 1) To examine the effects of two weeks of high NaCl diet on left ventricular (LV) morphology and serum levels of cardiac troponin-T (cTnT) in rats with adenine-induced chronic renal failure (ACRF). 2) To determine the effects of ACRF on cardiac morphology and function and to examine mechanisms causing cardiac abnormalities. 3) To identify early, sub-clinical, cardiac abnormalities by echocardiography in patients with CKD stages 3 and 4 and to investigate mechanisms that might cause these alterations. *Paper 1*. Rats with ACRF showed statistically significant increases in arterial pressure (AP), LV weight and fibrosis, and serum cTnT levels compared to controls. Two weeks of high-NaCl intake augmented the increases in AP, LV weight, fibrosis, and serum cTnT concentrations only in ACRF rats and produced LV injury with cardiomyocyte necrosis, scarring, and fibrinoid necrosis of small arteries. *Paper 2*. Cardiac function was assessed both by echocardiography and by LV catheterization. ACRF rats developed LV hypertrophy and showed signs of LV diastolic dysfunction but systolic function and cardiac output were preserved. *Paper 3*. In a cohort of patients with CKD stages 3 and 4, and matched controls, we performed comprehensive investigations including echocardiography and assessment of coronary flow velocity reserve (CFVR) in response to adenosine. CKD patients had normal systolic function but showed signs of LV diastolic dysfunction without fulfilling criteria for heart failure with preserved ejection fraction. In addition, CKD patients had significantly reduced CFVR versus controls suggestive of coronary microvascular dysfunction (CMD). In conclusion, ACRF rats developed LV hypertrophy and diastolic dysfunction while systolic performance was preserved. High-NaCl diet in rats with ACRF produced severe LV injury and aggravated increases in serum cTnT levels, presumably by causing hypertension-induced small artery lesions leading to myocardial ischemia. These results support the hypothesis that a high dietary intake of NaCl has deleterious effects on LV integrity in patients with kidney failure. Patients with CKD stages 3 and 4, without a diagnosis of heart disease, showed signs of LV diastolic dysfunction and a relatively large proportion had CMD suggesting that microvascular abnormalities may have a pathogenic role in the development of heart failure in this patient group.

Keywords: cardiovascular, chronic kidney disease, diastolic dysfunction

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SAMMANFATTNING PÅ SVENSKA

Kronisk njursvikt är en sjukdom som är både vanlig och allvarlig. De flesta patienter dör till följd av kardiovaskulära händelser innan de har hunnit utveckla terminal njursvikt.

Målet med denna avhandling är att studera mekanismer bakom hjärtskada hos patienter med kronisk njursvikt. Studie 1 och 2 är baserade på en djurexperimentell modell på råttor med adenin-orsakad njursvikt. Studie 3 baseras på patienter med kronisk njursjukdom.

Studie 1: Efter två veckor med högt saltintag, utvecklade råttor med njursvikt högre blodtryck, hjärtförstoring och mycket höga nivåer av TnT vilket indikerar skada av hjärtmuskelceller. Hjärtat visade uttalade skador med ärromvandling och väggförtjockning av små kärl. Studie 2: Hjärtultraljud visade att råttor med njursvikt hade hjärtförstoring och att vänster kammares relaxationsförmåga var nedsatt men att pumpfunktionen var intakt. Studie 3: Patienter med måttligt nedsatt njurfunktion visade tecken på avvikande fyllnadsförmåga i hjärtat medan hjärtats pumpförmåga var välbevarad. Patienter med njursvikt hade dessutom tecken på mikrovaskulär dysfunktion i hjärtat.

Försämrad relaxation av vänster kammare och hjärtförstoring verkar vara de första tecknen på hjärtskada vid nedsatt njurfunktion. De uttalade kardiella skadorna efter två veckor av högt saltintag indikerar att salt kan vara skadligt för patienter med njursvikt. Sannolikt skadar högt saltintag hjärtat genom att orsaka ett kraftigt förhöjt blodtryck. Det är möjligt att en försämrad mikrocirkulation i hjärtat hos patienter med kronisk njursjukdom kan bidra till utveckling till hjärtsvikt.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Kashioulis P, Hammarsten O, Marcussen N, Shubbar E, Saeed A, Guron G.
High-NaCl Diet Aggravates Cardiac Injury in Rats with Adenine-Induced Chronic Renal Failure and Increases Serum Troponin T Levels.
Cardiorenal Med. 2016 Aug;6(4):317-27

II. Kashioulis P, Lundgren J, Shubbar E, Nguy L, Saeed A, Guron CW, Guron G.
Adenine-Induced Chronic Renal Failure in Rats: A Model of Chronic Renocardiac Syndrome with Left Ventricular Diastolic Dysfunction but Preserved Ejection Fraction.
Kidney Blood Press Res. 2018;43(4):1053-1064

III. Kashioulis P, Guron CW, Svensson M, Hammarsten O, Saeed A, Guron G.
Patients with chronic kidney disease stages 3 and 4, without known heart disease, show echocardiographic abnormalities in left ventricular diastolic function and reduced coronary flow velocity reserve.
In manuscript.

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ABBREVIATIONS

ACRF	Adenine induced Chronic Renal Failure
ABP	Ambulatory Blood Pressure
AP	Arterial Pressure
APO	Apolipoprotein
APRT	Adenine Phosphoribosyltransferase
ASBP	Ambulatory Systolic Blood Pressure
BNP	Brain Natriuretic Peptide
BP	Blood Pressure
CFVR	Coronary Flow Velocity Reserve
CKD	Chronic Kidney Disease
CMD	Coronary Microvascular Dysfunction
CO	Cardiac Output
^{51}Cr-EDTA	Chromium-51 labelled Ethylene Diamine Tetra-Acetic acid
CRS	Cardiorenal Syndrome
cTnT	Cardiac Troponin T
CV	Coefficient of variation
CVD	Cardiovascular Disease
DAP	Diastolic Arterial Pressure
DAPI	4′, 6´-diamidino-2-phenylindole
DHA	2,8-Dihydroxyadenine
EF	Ejection Fraction
EPO	Erythropoietin
ESRD	End Stage Renal Disease
FITC	Fluorescein isothiocyanate
GFR	Glomerular Filtration Rate
HDL	High Density Lipoprotein
HF	Heart Failure

HFmrEF	Heart Failure with mid-range Ejection Fraction
HFpEF	Heart Failure with preserved Ejection Fraction
HFrEF	Heart Failure with reduced Ejection Fraction
HNa	High NaCl
HR	Heart Rate
hs TnI	High Sensitive Troponin I
LVEDd	Left Ventricular End Diastolic Diameter
LVEDs	Left Ventricular End Systolic Diameter
LV	Left Ventricle
LVH	Left Ventricle Hypertrophy
MDRD	Modification of Diet in Renal Disease
MI	Myocardial Infarction
NNa	Normal NaCl
NSTEMI	Non ST Elevation Myocardial Infarction
NT-proBNP	N-terminal prohormone of brain natriuretic peptide
RBC	Red Blood Cell
PASP	Pulmonary Artery Systolic Pressure
PCNA	Proliferating Cell Nuclear Antigen
PTH	Parathormone
RAAS	Renin Angiotensin Aldosterone System
RRT	Renal Replacement Therapy
SAP	Systolic Arterial Pressure
SCD	Sudden Cardiac Death
STEMI	ST Elevation Myocardial Infarction
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UAE	Urinary Albumin Excretion
WGA	Wheat Germ Agglutinin
XO	Xanthine Oxidase

1. INTRODUCTION

More than 11% of the adult population worldwide have chronic kidney disease (CKD)[1] and are facing an increased risk of end-stage renal disease (ESRD), cardiovascular (CV) disease and death [2]. Cardiovascular diseases are the leading cause of morbidity and mortality in patients with CKD [3, 4]. Already when glomerular filtration rate (GFR) falls below approximately 60 ml/min/1.73m^2 there is a graded and inverse relationship between kidney function and CV morbidity and mortality [4, 5].

The main purpose of this thesis was to elucidate pathophysiological mechanisms that cause cardiac injury in patients with CKD.

1.1 The kidneys

The kidneys are mainly responsible for maintaining a stable internal environment for optimal cellular function (homeostasis). The nephron is the structural and functional unit of the kidney and a healthy adult has about 1 million nephrons per kidney (figure 1). Each nephron consists of a capillary tuft called glomerulus, Bowman's capsule, and the tubular system. In the glomerular capillaries, plasma water is filtered across the capillary wall (the blood-urine barrier) and the primary urine is collected by Bowman's capsule and passed on to the tubule.

About 20-25% of cardiac output passes through the renal circulation producing about 150-180 liters of glomerular filtrate (primary urine) per day. In the tubular system almost all of the filtered water and electrolytes are reabsorbed while waste products are retained in the urine and excreted.

The flow rate at which fluid is filtered across all the glomerular capillaries is called glomerular filtration rate. GFR is used as a measure of kidney function and in clinical practice it is expressed in the unit ml/min per 1.73 m^2 of body surface area. It is usually measured by clearance techniques. A small exogenous marker that is freely filtered, and neither reabsorbed, nor secreted, by the tubules after filtration, is injected intravenously and a blood test is taken after a certain time in order to analyse the remaining level of the exogenous marker in the blood. Both chromium-51 labelled ethylene diamine tetra-acetic acid (^{51}Cr-EDTA) and iohexol are commonly used filtration markers. An easier, faster but less accurate method to evaluate kidney function is to estimate GFR (eGFR) by measuring endogenous filtrations

markers, such as creatinine or cystatin c, in the blood. In daily clinical practice eGFR is used more often due to its simplicity. Normally young adults have a GFR of approximately 125 ml/min/1.73 m². With increased age GFR falls gradually and at 80 years of age GFR is around 70 ml/min/1.73 m².

The glomerular capillary wall is a living ultrafiltration membrane and acts as the blood-urine barrier. It permits water and small solutes to pass readily into Bowman's space, while normally rejects albumin and other large proteins with great efficiency. Thus the presence of albumin in the urine (albuminuria) indicates a possible injury in the glomerular capillary wall [6].

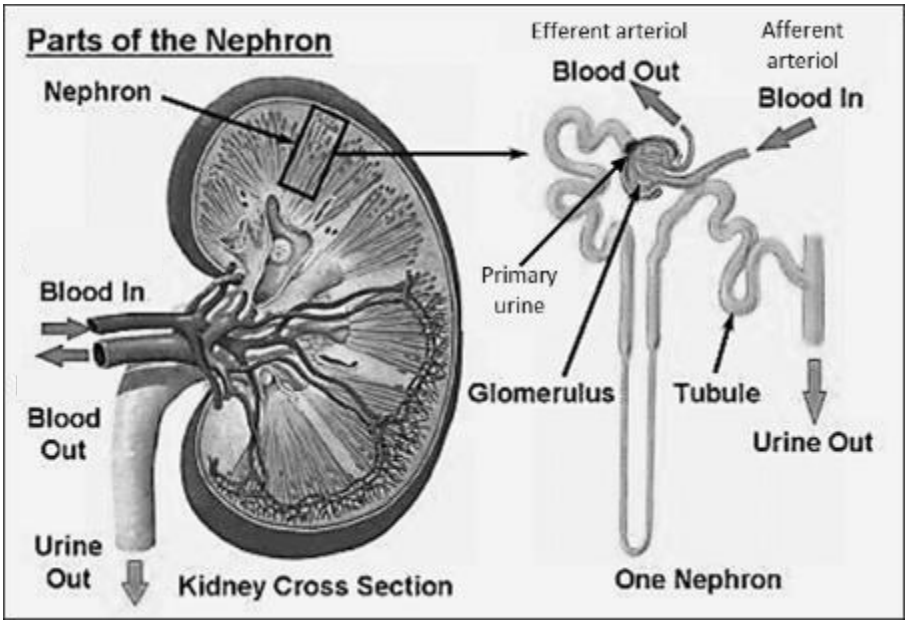

Figure 1. The kidney and the nephron. With the kind permission of www.unckidneycenter.org

1.1.1 Endocrine functions of the kidney

The kidneys produce several vital hormones, e.g. erythropoietin, 1,25-dihydroxyvitamin D3 and renin. Erythropoietin is essential for the production of red blood cells in the bone marrow. In ESRD erythropoietin synthesis is insufficient and this leads to renal anemia. 1,25- dihydroxyvitamin D3 (active form of vitamin D) is responsible for calcium and phosphate balance and promotes bone health.

Renin is an enzyme that is produced by the juxtaglomerular cells of the afferent arterioles of the kidneys. By catalyzing the conversion of angiotensinogen to angiotensin I, renin activates the renin-angiotensin-aldosterone system (RAAS). The RAAS has multiple functions but its main role is to maintain arterial blood pressure (BP) and extracellular fluid volume. In addition, the RAAS acts to preserve GFR. Renin release, and the activation of the RAAS, is stimulated by hypotension, hypovolemia and decreased GFR. Angiotensin II, which is the main effector peptide of the RAAS, causes vasoconstriction (increased peripheral resistance) and triggers aldosterone synthesis and its secretion from the adrenal glands [7]. Aldosterone stimulates tubular sodium and water reabsorption and potassium secretion. In addition, angiotensin II increases thirst and stimulates tubular water reabsorption through the release of antidiuretic hormone [7].

To maintain a normal GFR the kidneys are dependent on an appropriate renal perfusion pressure and blood flow. As the heart is the center of the circulatory system, a continuous communication between the kidneys and the heart is essential. This occurs at multiple levels including the central nervous system, the sympathetic nervous system, the RAAS, antidiuretic hormone, and the natriuretic peptides.

1.1.2 Kidney innervation

The kidneys are innervated with efferent and afferent nerves to communicate with the central nervous system. Major structural components of the kidneys, such as blood vessels, juxtaglomerular cells and tubules are innervated forming a two-way neural path to transmit sensory and sympathetic signals from and to the kidneys [8]. Stimulation of the renal sympathetic efferent nerves causes renin release, sodium reabsorption, and reduced renal blood flow. Elevated afferent renal sensory nerve signaling increases sympathetic outflow to the skeletal muscle vasculature, the kidneys, and the heart, thereby increasing peripheral vascular resistance and BP.

1.2 Chronic Kidney Disease

1.2.1 Definition and staging

Chronic kidney disease is defined as either kidney damage or decreased kidney function for a period longer than three months regardless the cause. Kidney damage refers to pathological findings, either on renal biopsy, imaging studies, or abnormal markers such as increased rates of urinary albumin excretion (UAE) or abnormalities on urinary microscopy (e.g. erythrocyte casts). Decreased kidney function refers to a GFR below 60 ml/min/1.73 m². This cutoff value represents a reduction by more than half of the normal value of 125 ml/min/1.73 m² in young men and women, and is associated with the onset of laboratory abnormalities characteristic of kidney failure and with a higher risk of complications of CKD [9].
The Kidney Disease: Improving Global Outcomes (KDIGO) guidelines recommend CKD classification based on cause, GFR category, and albuminuria category (CGA). Both the classification based on GFR (Table 1) and albuminuria (Table 2) are used to guide management, including stratification of risk for progression and complications of CKD. Designations 5D and 5T indicate end-stage renal disease patients who undergo chronic dialysis (5D) treatment or have undergone kidney transplantation (5T).

Table 1. *GFR stages.*

Stage	GFR (ml/min per 1.73 m^2)	Description
G1	≥90	Normal or high
G2	60 to 89	Mildly decreased
G3a	45 to 59	Mildly to moderately decreased
G3b	30 to 44	Moderately to severely decreased
G4	15 to 29	Severely decreased
G5	<15	Kidney Failure

Table 2. *Albuminuria categories*

Category	AER (mg/24 hours)	ACR (mg/mmol)	Description
A1	<30	<3	Normal
A2	30-300	3-30	Moderate increased
A3	>300	30	Severely increased

AER= albumin excretion rate; ACR= albumin-to-creatine ratio

1.2.2 Epidemiology

The prevalence of CKD stages 3-5 is between 1-6 % in European countries whereas in Scandinavia it is considered to be around 3.3-4.5%. Meanwhile, in the USA it varies from 5-12 % [10]. According to the Swedish Renal Registry there were approximately 4000 patients receiving dialysis in Sweden by the end of 2017 and around 6000 were living with a functional kidney transplant. Furthermore, the annual uptake of new patients on dialysis has been stable during the latest 20 years and is about 1000 patients per year.

Globally, the major causes of CKD in adults are diabetes and hypertension. Other common causes are glomerulonephritis and autosomal dominant polycystic kidney disease.

1.2.3 Clinical Manifestations

The early stages of CKD usually proceed with no symptoms, even though hypertension is common. Anemia and disorders of calcium and phosphate balance are less common and become more pronounced in the advanced stages of CKD [11]. Eventually, systemic manifestations due to accumulation of metabolic waste products (uremia) develop when GFR declines below 15 ml/min/1.73m^2 and ESRD is established (Figure 2). Nausea, vomiting, weight loss, pruritus, mental changes and fatigue are common uremic symptoms. As GFR declines below 6-8 ml/min/1.73m^2 kidney replacement therapy with dialysis or transplantation becomes a necessary life sustaining intervention.

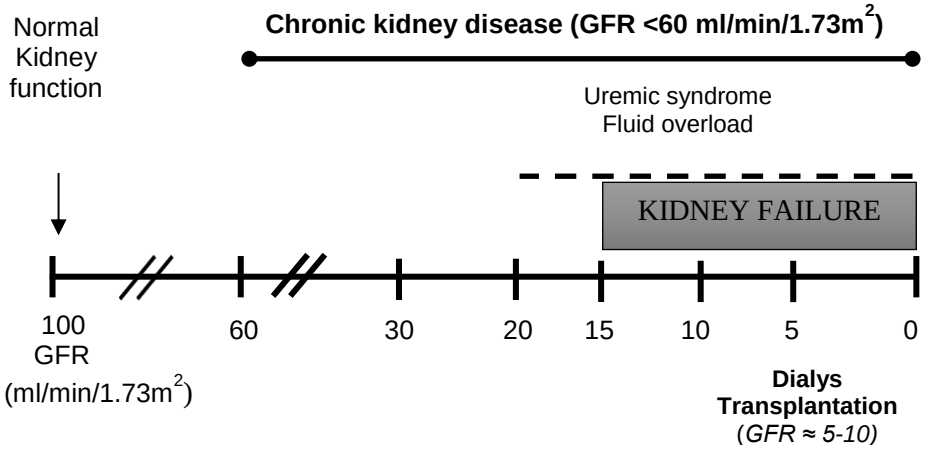

Figure 2. Chronic kidney disease

1.2.4 Mortality

Cardiovascular events are the main cause of death among CKD patients and increases as kidney function declines (figure 3). It appears to be twice as high in patients with CKD stage 3 and three times higher at stage 4 than in individuals with normal kidney function. Also albuminuria, already at the upper end of the normal range, increases CV risk independently of eGFR [4, 5] Moreover, sudden cardiac death (SCD) is the most common cause of death among patients with ESRD comprising approximately 25% of all-cause mortality[12].

Traditional CV risk factors such as smoking, obesity, hypertension, hyperlipidemia and diabetes cannot completely explain the increased CV risk in CKD [2, 5].

1.3 Why is the risk of cardiovascular events increased in chronic kidney disease?

1.3.1 Spectrum of cardiovascular diseases in CKD

A wide spectrum of CV diseases has been associated with CKD. The risk of heart failure is practically doubled in patients with eGFR below 60 mL/min per 1.73 m^2 compared to a healthy population. The risk is similarly increased

for stroke, peripheral artery disease, coronary heart disease, and atrial fibrillation [4, 13].

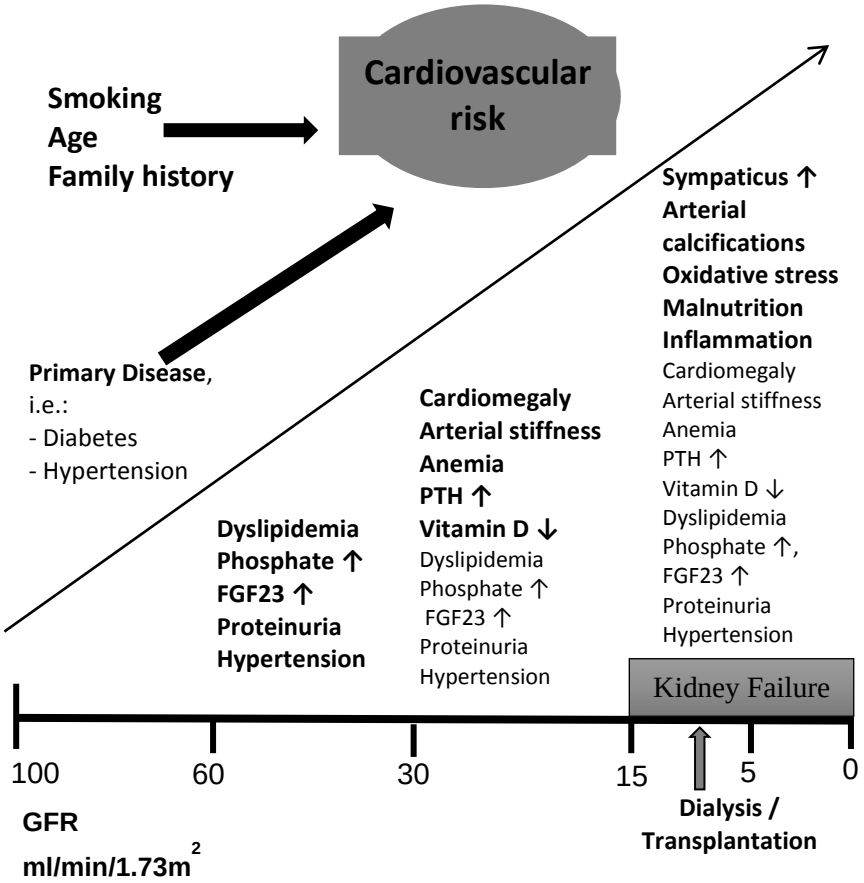

Figure 3. Cardiovascular risk and kidney function. The risk for cardiovascular events increases as renal function declines and further clinical signs associated to GFR appears (bold letters)

1.4 Coronary heart disease in CKD

Chronic kidney disease is associated with a high burden of coronary artery disease [14]. In patients with acute coronary syndromes (ACS) ≈40% of patients with non-ST-elevation myocardial infarction (NSTEMI), and 30% of those with ST-elevation myocardial infarction (STEMI) have CKD [15].

Furthermore, patients with more severe CKD have worse prognosis regardless the type of myocardial infarction (MI) [15]. Chronic kidney disease is the third strongest predictor of death after a MI and is only exceeded by cardiogenic shock and congestive heart failure [14, 16].

In clinical practice the diagnosis of ACS is based on ECG abnormalities and the levels of specific biomarkers of myocardial injury such as cardiac troponin-T (cTnT) and troponin-I (cTnI).

Increased serum levels of cardiac troponins are frequently observed in CKD patients even in the absence of acute coronary syndrome [17, 18]. Chronically elevated, stable, cTnT levels are associated with an increased risk of CV events and mortality [19-21]. Elevated serum levels of cTnT in asymptomatic CKD patients are partially explained by reduced renal clearance [22] but the underlying mechanisms are not fully understood.

1.5 Heart Failure and CKD

1.5.1 Heart failure; definition, terms and diagnosis

According to the European Society of Cardiology heart failure (HF) is a clinical syndrome characterized by typical symptoms (e.g. breathlessness, ankle swelling and fatigue) that may be accompanied by signs (e.g. elevated jugular venous pressure, pulmonary crackles and peripheral oedema) caused by a structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress.

Heart failure is categorized further based on left ventricular (LV) ejection fraction (EF) (LVEF). Patients with adequate LVEF (>50%) are considered to have HF with preserved EF (HFpEF), whereas patients with LVEF<40% have HF with reduced EF (HFrEF). Heart failure with mid-range EF (HFmrEF) refers to patients with EF ranging from 40-49%.

Left ventricular diastolic dysfunction is the hallmark of HFpEF. Left ventricular diastolic dysfunction is characterized by increased LV stiffness that impairs relaxation and leads to increased filling pressures.

Echocardiography is currently the most commonly used technique for diagnosing different types of HF. Systolic dysfunction is identified by estimation of global EF and regional wall motion. Diastolic dysfunction can be diagnosed indirectly based on signs of impaired LV relaxation, reduced

restoring forces and increased diastolic stiffness. For an exact determination of diastolic dysfunction LV catheterization is required [23]. Current evidence suggests that up to 30–50% of patients with HF have HFpEF [24]. Interestingly, patients with HFpEF have as high mortality rates as patients with HFrEF [24].

Natriuretic peptides are used widely as a tool in the detection and evaluation of HF. B-type natriuretic peptide (BNP) and N-terminal pro b-type natriuretic peptide (NT-proBNP) are produced in the cardiac ventricles in response to distention and stretching of the ventricular wall. Small amounts of a precursor protein, pro-BNP, are continuously produced. Pro-BNP is cleaved by the enzyme corin to release the active hormone BNP and an inactive fragment, NT-proBNP, into the blood. The release of BNP is increased in HF in response to high ventricular filling pressures and stretching of the ventricular wall. The main physiological actions of BNP are to reduce LV afterload by reducing systemic vascular resistance and to decrease preload by exerting natriuretic effects.

1.5.2 Heart failure and chronic kidney disease

The risk of developing heart failure (HF) increases considerably as GFR declines and CKD progresses [25, 26]. Interestingly, one community-based study found that CKD was a risk factor for HFpEF , but not for HFrEF [27]. Remarkably, HF patients face higher mortality risk regardless their EF, if CKD coexists [28]. In a longitudinal study where CKD patients were subjected to repeated echocardiographic examinations, it was found that EF declined as patients progressed to ESRD [28]. These findings support the hypothesis that patients with CKD initially develop HFpEF and that EF may decline as patients develop ESRD.

Both BNP and NT-proBNP levels in plasma may be elevated in CKD patients as both these peptides are partially cleared by the kidneys [29]. Nevertheless, NT-proBNP is used more widely, as it circulates at higher plasma concentrations and has a longer plasma half-life compared to BNP. When interpreting plasma concentrations of BNP and NT-proBNP in patients with CKD it is important to consider that increased levels may result from both reduced renal clearance, and fluid retention, in addition to impaired cardiac function. Hence, increased levels of natriuretic peptides in patients with CKD are difficult to interpret, and can be present even in the absence of HF.

1.6 Cardiorenal syndromes (CRS)

Cardiorenal syndromes are a group of disorders that are the result of the bidirectional interaction between the heart and the kidneys where acute or chronic dysfunctions of one organ induce acute or chronic dysfunctions of the other [30].

The different interactions that can occur led to the classification of CRS that was proposed by Ronco and colleagues in 2008 (Table 3) [31, 32]. Here the chronic renocardiac syndrome (CRS type 4) will be discussed as only this syndrome was investigated.

Table 3. *Types of CRS.*

Type	Primary event	Secondary disturbance
Type 1 or acute CRS	Acute HF	Acute kidney injury
Type 2 or chronic CRS	Chronic HF	Progressive kidney injury (CKD)
Type 3 or acute CRS	Acute kidney injury	Acute cardiac disorder (HF)
Type 4 or chronic CRS	Primary CKD	Cardiac dysfunction (coronary disease, HF, or arrhythmia)
Type 5 or secondary CRS	Acute or chronic systemic disorders	Both cardiac and renal dysfunction

1.6.1 Chronic renocardiac syndrome (CRS type 4)

Chronic renocardiac syndrome is defined as progressive morphological or functional cardiac abnormalities secondary to CKD. In real life it is often difficult, or impossible, to know which abnormality that developed first as CKD and HF share many risk factors, e.g. hypertension and diabetes.

As kidney function deteriorates the activity of the sympathetic nervous system (SNS) and renin angiotensin aldosterone system (RAAS) becomes maladjusted. Data indicate that activation of the RAAS, renal afferent stimulation, reduced nitric oxide (NO) concentrations and increased oxidative stress all contribute to sympathetic activation [33]. This results in numerous adverse consequences, e.g. a reduction of myocardial β-adrenergic receptor density, vasoconstriction, and renal sodium retention. Simultaneously, the RAAS causes vasoconstriction, excessive sodium reabsorption and extracellular fluid volume expansion [34]. Moreover, angiotensin II acts as a growth factor in the left ventricle and in the arterial wall through binding in specific receptors that are present in the heart [7] On the other hand, aldosterone, is known to promote cardiac fibrosis and cell death through inflammatory and oxidant signaling [35].

1.6.2 Hypertension and volume overload

More than 80% of CKD patients have hypertension [11] most likely due to an inappropriate activity of the sympathetic nervous system and the RAAS in combination with endothelial dysfunction and sodium retention [36].

Hypertension increases LV afterload, i.e. the pressure against which the heart must work to eject blood during systole. The LV adapts to the increased workload by developing LV hypertrophy (LVH). Moreover, hypertension contributes to remodeling and atherosclerosis of both small and large arteries. Increased stiffness of large arteries, including the aorta, is common in CKD [37] and can enhance LV afterload by elevating central aortic systolic pressure. Increased afterload leads mainly to concentric LVH (increased wall-to-lumen ratio). Volume overload on the other hand leads to eccentric hypertrophy where LV cavity size increases more than wall thickness.

1.6.3 Mineral metabolism and calcifications

Dysregulated mineral metabolism characterized by increased plasma levels of phosphate, parathyroid hormone (PTH), and fibroblast growth factor 23 (FGF23), and decreased levels of calcium and 1,25-dihydroxyvitamin D, is common in CKD patients even with moderately reduced GFR[11]. It is believed that reduced renal excretion of phosphate and elevated levels of FGF23 and PTH develop early in CKD. A consistent association between elevated FGF23 levels and CV events and LVH has been shown [38, 39].

Patients with kidney failure often have arterial media calcifications consisting of calcium-phosphate deposits [3, 40, 41]. Studies have shown direct effects of increased calcium and phosphate levels on vascular smooth muscle cells (VSMCs) leading to osteogenic differentiation and the formation of calcifications [42]. These media calcifications lead to increased stiffness of aorta in CKD patients and increased LV afterload as it is mentioned above.

1.6.4 Dyslipidemia

Renal dyslipidemia develops as GFR falls below 60 ml/min/1.73m^2 and is characterized by elevated levels of apoB-containing and apoC-containing lipoproteins [43]. The increase in apoC-III-containing, triglyceride-rich, lipoproteins is the hallmark of renal dyslipidemia. [44]. ApoC-III is a powerful inhibitor of lipoprotein lipase (LPL) resulting in impaired lipolysis. The prolonged presence of lipoproteins in the circulation, make them accessible for modifications that can further increase their atherogenecity [44].

1.6.5 Anemia

Anemia can be a burden for heart function through increased cardiac stress. Besides tachycardia and increased stroke volume it may reduce renal blood flow and cause fluid retention, adding further stress to the heart [45, 46]. Long term anemia regardless its cause, may result in LVH and progressively in HF [46]. As oxygen transportation capacity is reduced, anemia may also contribute to cardiac hypoxia in itself.

1.7 KNOWLEDGE GAP

Despite the scientific progress made during the past years in the understanding of the pathophysiology of cardiac injury in CKD, there are still questions to be answered.

How does HFpEF develop in CKD? Can we establish an experimental model to investigate this?

What are the initial cardiac abnormalities that occur before patients with CKD develop symptomatic heart disease?

Does a high NaCl intake cause cardiac injury in patients with kidney failure?

2. AIM

The overall aim of this thesis was to elucidate pathophysiological mechanisms that cause cardiac injury in patients with CKD.

The specific aims were:

I. To examine the effects of two weeks of high NaCl diet on LV morphology and serum levels of cTnT in rats with adenine-induced chronic renal failure (ACRF).

II. To determine the effects of chronic renal failure on cardiac morphology and function in rats and to establish an experimental model of HF in CKD.

III. To identify early, sub-clinical, abnormalities in cardiac morphology or function in patients with CKD stages 3 and 4 by echocardiography and to elucidate mechanisms causing these alterations.

3. METHODS

A combination of experimental animal studies (I, II) and clinical investigations in patients were performed (III). A brief overview of the methods used in this project follows. Detailed descriptions of materials and methods can be found in the manuscripts.

All studies were approved by the regional ethics committee in Gothenburg, Sweden. All the participants in the clinical investigations gave their written consent.

3.1 Experimental studies

Adenine induced chronic renal failure (ACRF)

To establish chronic renal failure in rats we developed a model of ACRF. We used male Sprague-Dawley rats weighing~300g. No female rats were studied, because we wanted to exclude any influence of the estrous cycle on our experiments. Adenine was administered by adding it to the chow. The adenine concentration was gradually reduced in order to prevent reductions in body weight that occur otherwise (figure 5) [47]. Control rats were pair-fed, i.e. they received the same amount of normal chow as ACRF rats had consumed.

Adenine is one of the four nucleobases used in the nucleic acid of DNA. It is primarily converted to harmless adenosine by an enzyme called adenine phosphoribosyltransferase (APRT) widely expressed in mammalians cells (figure 4). However, in situations of adenine excess, adenine degrades to 2,8-dihydroxyadenine (DHA) via the enzyme xanthine oxidase (XO). DHA is freely filtered through the glomeruli and due to its low solubility at the physiological urine pH it precipitates in renal tubules resulting in tubular obstruction and injury and finally causes renal failure.

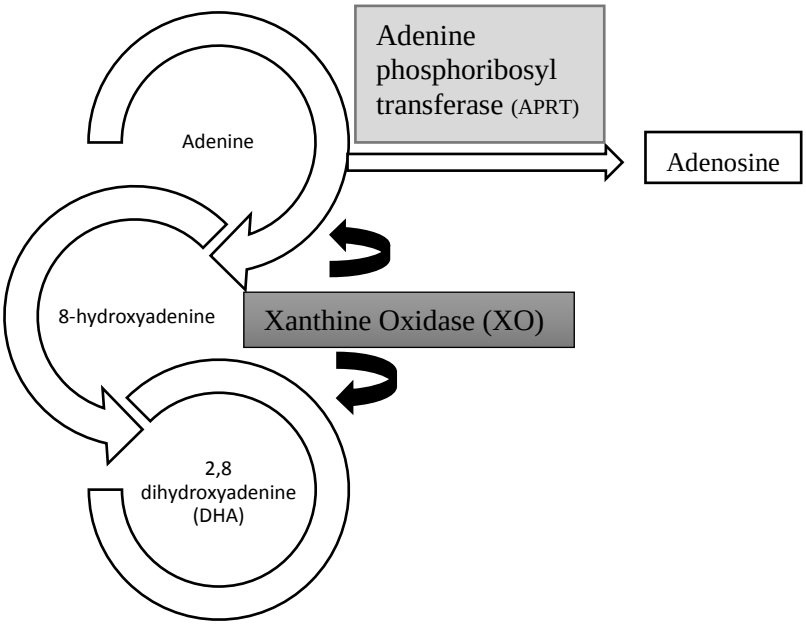

Figure 4. Metabolism of adenine. DHA causes renal failure through its precipitation in renal tubules.

Adenine induced renal failure versus 5/6 nephrectomy

The ACRF model has several advantages compared to the widely used method of 5/6 nephrectomy. No surgery is needed thereby reducing the risk of perioperative complications. Rats subjected to 5/6 nephrectomy typically develop only a modest decrease in GFR and consequently secondary metabolic changes such as alterations in mineral and bone metabolism are not as pronounced as in ACRF rats [48, 49]. In addition, severe hypertension is a characteristic feature of most 5/6 nephrectomy models [49, 50] which makes it more difficult to distinguish whether cardiovascular abnormalities are primarily caused by high blood pressure or reduced kidney function.

Feeding protocols

Study I

Rats either received chow-containing adenine or were pair-fed an identical diet without adenine [controls (C)]. Approximately 10 weeks after the beginning of the study, rats were randomized to either remain on a normal

NaCl diet (NNa; 0.6%) or to be switched to high-NaCl chow (HNa; 4%) for 2 weeks, after which acute experiments were performed (figure 5).

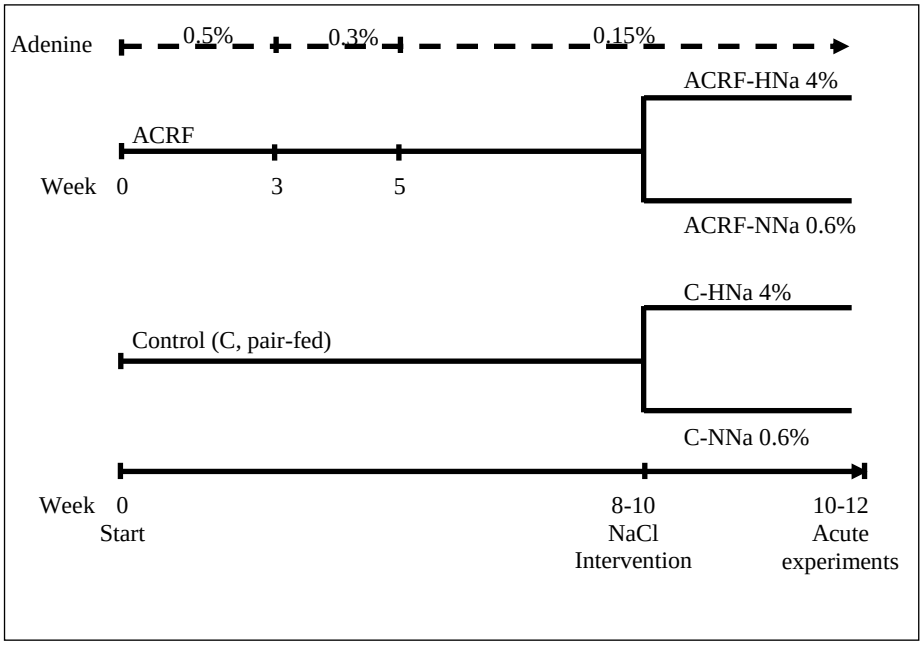

Figure 5. Schematic presentation of the feeding protocol and study groups. Figure from paper I.

Study II

Male Sprague-Dawley rats received either chow containing adenine or were pair-fed an identical diet without adenine (controls, C). After 9-13 weeks the experiments were performed (figure 6).

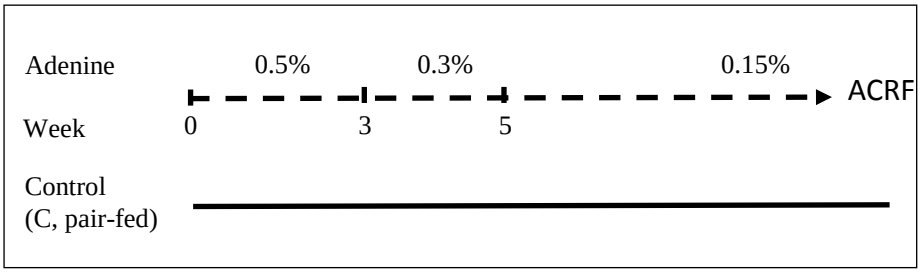

Figure 6. Feeding protocol in study II.

Measurements and tests (I, II)

Kidney function and arterial pressure measurements (I)

Glomerular filtration rate was measured by renal ^{51}Cr-EDTA clearance. Two consecutive 20-min renal clearance periods were performed under anesthesia, after a 45-min equilibration period. For induction and maintenance of anesthesia, isoflurane concentrations of 5 and 1.5% (vol/vol), respectively, were used. Rats were killed by an overdose of pentobarbital sodium after the second clearance period, and the heart and kidneys were immediately excised and weighed.

During the experiment arterial pressure (AP) and heart rate were recorded continuously via a polyethylene catheter in the femoral artery using the data acquisition program Biopac MP 150 (Biopac Systems, Santa Barbara, Calif., USA).

Biochemical analyses (I, II)

Plasma concentrations of creatinine and electrolytes were determined by a Modular P800 Cobas C 701/502 analyzer. Plasma BNP-32 concentrations were measured by a commercially available ELISA kit in duplicate and the values were averaged. cTnT was measured using the Elecsys hs-cTnT immunoassay.

LV histology (I, II)

An investigator blinded to the treatment group performed all assessments. Using routine techniques, 3-μm-thick transverse sections were prepared and stained with hematoxylin and eosin, picrosirius red (analysis of fibrosis), von Kossa (assessment of calcifications), or Miller's elastin. LV calcification was scored semi quantitatively as either present or absent.

Cardiomyocyte hypertrophy was determined on sections stained with fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA, Vector Laboratories, Burlingame, CA, USA) to delineate the cell membrane, and with 4′, 6´-diamidino-2-phenylindole (DAPI, Vectashield mounting medium, Vector Laboratories, Burlingame, CA, USA) to visualize cell nuclei. The cell diameter was measured through the cell nuclei and was used as a measure of cell size.

Proliferating cells were detected on paraffin-embedded sections by immunohistochemistry using a mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody.

Apoptotic cells were detected in situ on paraffin-embedded sections by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method using the ApopTag peroxidase in situ apoptosis detection kit according to the manufacturer's instructions (Merck KGaA, Darmstadt, Germany). To verify that TUNEL-positive cells were apoptotic we examined if these cells also expressed cleaved caspase-3 by double immunohistochemistry staining on the same section.

Morphometric analysis of LV fibrosis (I, II)

Images of sections stained with picrosirius red were derived using an Olympus BX60 microscope (camera Olympus DP72) and the imaging software cellSens (Olympus). The imaging software BioPix iQ 2.0 (BioPix, Gothenburg, Sweden) was used to objectively measure general and perivascular fibrosis.

Western blotting of the LV (II)

Western blotting was carried after tissue homogenization and protein preparation according to routine techniques. The primary antibodies employed were rabbit anti-collagen-1 alpha-1 (COL1A1), rabbit anti-intercellular adhesion molecule-1 (ICAM-1), rabbit anti-vascular cell adhesion molecule-1 (VCAM-1), rabbit anti-sodium-calcium exchanger-1 (NCX-1) and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), all from Santa Cruz Biotechnology, Texas, USA). Additional primary antibodies were rabbit anti-monocyte chemotactic protein-1 (MCP-1, Nordic Biosite AB, Stockholm, Sweden), and rabbit anti-bone morphogenetic protein-4 (BMP4) and mouse anti-sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2), both from Abcam, Cambridge, UK.

Echocardiography (II)

Echocardiography was performed while rats were anesthetized with isoflurane. A high-frequency 12-MHz phased-array transducer (P12-5, Philip Medical System, Best, The Netherlands) connected to an ultrasound system (HDI 5000 ATL, Philip Medical System) was used as previously described [51] .

LV pressures (II)

Under isoflurane anesthesia two ultra-miniature fiber optic pressure sensors (Samba preclin 420, sensor diameter 0.42 mm, Harvard Apparatus Ltd., Edenbridge, Kent, UK) were placed through the right femoral artery and left carotid artery in the distal abdominal aorta at the level of the aortic bifurcation, and in the ascending aorta immediately above the aortic valve. After a 15 min equilibration period, baseline recordings of aortic BPs were performed during 5 minutes. Subsequently, the proximal aortic pressure sensor was gently inserted into the LV for recording of pressure with a sampling frequency of 1000 Hz. The data were collected and analyzed by the Biopac MP 150 system using the data acquisition software Acq*Knowledge*. Left ventricular pressure variables were derived by post-processing of the data using the built-in routines in Acq*Knowledge*. Left ventricular end-diastolic pressure was determined by identifying the peak of the second derivative of the pressure curve during each pressure waveform. Results were derived from all pressure waveforms during 4-6 consecutive respiratory cycles (corresponding to approximately 25-40 pressure waveforms) for each animal and average values are presented.

3.2 Clinical study (III)

Subjects and protocol

Patients were recruited from the Nephrology outpatient clinic at the Sahlgrenska University Hospital, Gothenburg, Sweden, between February 2009 and December 2011.
Inclusion criteria were >18 years of age, and an estimated GFR (eGFR) of 15 to 59 ml/min/1.73m^2 according to the MDRD formula since at least 3 months (i.e. CKD stages 3 and 4). Exclusion criteria were previous organ transplantation, ongoing immunosuppressive medication, inflammatory systemic disease, endocrine disease aside from diabetes mellitus or substituted hypothyroidism, expected survival less than 12 months, expected need of renal replacement therapy (RRT) within 12 months, and pregnancy or current breast feeding. Overall, 122 patients were recruited. Of these 24 had a diagnosis of heart disease and were excluded. Of the remaining 98 patients, 91 accepted to undergo echocardiographic examinations and were included in the study. Forty-seven healthy individuals, matched for age and gender were recruited through an advertisement in local newspapers. Of them, 41 approved of echocardiographic examinations and were included as controls.

Hemodynamic assessments

Ambulatory blood pressure (ABP) was recorded during 24 hours. Nocturnal dipping of ambulatory systolic blood pressure (ASBP) was calculated as (nighttime ASBP - daytime ASBP) / daytime ASBP and expressed in percent. Carotid-femoral pulse wave velocity (cfPWV), digital reactive hyperemia, and ankle-brachial index (ABI) were measured under standardized conditions in the morning after an overnight fast.
Carotid-femoral pulse wave velocity, an indirect measure of aortic stiffness, was calculated by measuring the distance between the femoral and carotid pulse, using the suprasternal notch as reference measure point, divided by the pulse transit time between the two locations. SphygmoCor software was used. Digital reactive hyperemia was analyzed by EndoPAT2000, to assess endothelial function as previously described [52]. Reactive hyperemic index (RHI) was calculated as the mean flow response post-occlusion using the non-occluded arm as a reference. Ankle-brachial index was measured using a Doppler probe and a sphygmomanometer. The mean of the indices for the posterior tibial artery and dorsalis pedis artery for each foot was calculated and the average value of the left and right foot was determined.

Echocardiography

All examinations were performed by the same physician according the recommendations of the American Society of Echocardiography (ASE). Left ventricular hypertrophy (LVH) was defined as LVMI >115 g/m^2 in men or >95 g/m^2 in women [53]. Left ventricular hypertrophy was further classified as either concentric (RWT >0.42) or eccentric (RWT ≤0.42) [53]. Subjects with normal LVMI but RWT >0.42 were considered to have concentric remodeling. In subjects with normal EF, LV diastolic dysfunction was evaluated according to the guidelines by the ASE and based on the following variables and cut-offs: E/e´ >14, septal e´ velocity <7 cm/s or lateral e´ <10 cm/s, TR peak velocity >2.8 m/s, and LAVI >34 mL/m^2 [54].
To evaluate the coronary circulation we assessed coronary flow velocity reserve (CFVR) [55]. This gives an integrated measure of coronary microvascular function and was determined by the ratio of left anterior descending artery (LAD) blood flow velocity during maximum vasodilation to resting blood flow velocity [56]. Flow velocity at rest and during adenosine infusion (140 μg/min/kg) was measured over approximately 5 minutes by pulsed Doppler from the mid to distal part of LAD [57]. Measurements of CFVR were carried out on 49 CKD patients and 33 healthy controls who accepted to receive adenosine. Doppler echocardiography for assessment of

CFVR has been validated against positron emission tomography based measurements [56]. A CFVR <2.5 was considered abnormal and compatible with coronary microvascular dysfunction (CMD) based on prior studies [58, 59].

Statistics

Statistical analyses were performed using the SPSS Statistics Data Editor (IBM SPSS Statistics for Windows, Version 17.0, 20.0 and 22.0. Armonk, NY, USA). Reported values are means and standard deviations (SD) for continuous data and proportions (%) for categorical variables. Statistical significance was set at the level of $p<0.05$.

Study I; analyses were performed using two-factorial ANOVA. The degree of correlation between variables was analyzed by determining the Pearson correlation coefficient (r).

Study II; differences between means were analyzed using paired or un-paired Student's t-test. Chi-square test was used for categorical data.

Study III; correlations between continuous data were calculated using Pearson's or Spearman´s test when appropriate. The Mann-Whitney U-test was used for comparing differences in continuous data between groups. Differences in frequencies were analyzed using Fisher´s exact test.
Univariate regression analyses were designed to evaluate the relationship between clinical characteristics and measures of cardiac function. Only continuous variables that showed a statistically significant correlation with the dependent variable were included in regression models. Similarly, only categorical variables that were significantly different in the dependent variable were included in regression models.

4. REVIEW OF RESULTS

4.1 Experimental studies (I, II)

Study II

Kidney function and general characteristics (Table 4)

Plasma concentrations of creatinine and potassium were clearly elevated in ACRF rats. Left ventricular weight was significantly elevated in ACRF rats whereas there were no statistically significant differences between groups in body weight or right ventricular weight.

Table 4. *Organ weights and blood analyses 12-13 weeks after study start.*

	Controls (n=10)	A-CRF (n=10)
BW (g)	359 ± 19	343 ± 19
LVW/tibia (mg/mm)	22.5 ± 2.2	29.5 ± 2.4***
P-creatinine (µmol/L)	33 ± 5	323 ± 107 ***
P-potassium (mmol/L)	4.2 ± 0.3	6.3 ± 0.7 ***

BW, body weight; LVW, left ventricular weight; *** P<0.001.

LV morphology and function by echocardiography (Table 5)

Stroke volume (SV) and cardiac output (CO) were significantly elevated in ACRF rats vs. controls. Thickness of the LV anterior wall was significantly elevated in ACRF rats vs. controls indicating LV hypertrophy.

Rats with ACRF showed a significant decrease in e, and an increase in a, resulting in a marked decrease in the e/a ratio, vs. controls (table 6). In addition, the E/e ratio was significantly elevated in ACRF rats indicating LV diastolic dysfunction.

Table 5. *Echocardiographic data 9 weeks after study start.*

	Controls (n= 10)	A-CRF (n=10)
Heart rate (bpm)	346 ± 23	350 ± 27
Stroke volume (ml)	0.43 ± 0.07	0.61 ± 0.23*
Cardiac output (ml/min)	149 ± 24	211 ± 66*
EF (%)	82 ± 4	88 ± 6
LVEDd (mm)	7.98 ± 0.47	7.90 ± 0.39
LVESd (mm)	4.24 ± 0.49	3.65 ± 0.77
LA diameter (mm)	3.54 ± 0.41	4.80 ± 0.75***
LV anterior wall thickness (mm)	1.41 ± 0.09	1.89 ± 0.35***

EF, ejection fraction; LVEDd, left ventricular end diastolic diameter; LVESd, left ventricular end systolic diameter; LA, left atrium; LV, left ventricle. * P<0.05, ** P<0.01; *** P<0.001.

Table 6. *Echocardiographic indices of diastolic function and tissue-Doppler velocities 9 weeks after study start.*

	Controls (n=10)	A-CRF (n=10)
e (cm/s)	7.2 ± 1.4	5.7 ± 0.6 **
a (cm/s)	5.5 ± 1.4	7.9 ± 1.6 **
e/a	1.4 ± 0.5	0.8 ± 0.3 **
E/e	13.3 ± 2.5	17.8 ± 2.9**
IVRT (ms)	19.7 ± 1.5	20.5 ± 4.6
s (cm/s)	8.7 ± 1.3	8.8 ± 1.3

E, early diastolic filling velocity; e, early diastolic tissue velocity; a, diastolic tissue velocity at atrial contraction; IVRT, isovolumetric relaxation time; and s, systolic tissue velocity. * P<0.05, ** P<0.01; *** P<0.001.

Left ventricular and aortic pressures

Systolic pressure, and pulse pressure, in the ascending aorta were significantly elevated in ACRF rats vs. controls. Both LV end-diastolic pressure (LVEDP) and LV systolic blood pressure (LVSBP) were significantly elevated in ACRF rats vs. controls (table 7). Maximal rates of LV pressure change during systole (dp/dt max), and diastole (dp/dt min) were both significantly increased in ACRF rats vs. controls.

Table 7. *Left ventricular pressures 12-13 weeks after study start.*

	Controls (n=10)	A-CRF (n=8)
Heart rate (bpm)	353 ± 25	342 ± 24
LVEDP (mmHg)	8 ± 1	15 ± 5***
LVSBP peak (mmHg)	125 ± 6	138 ± 10**
LVDBP min (mmHg)	-1.7 ± 0.9	-3.2 ± 2.5
dp/dt max (mmHg/s)	7428 ± 624	9529 ± 2331*
dp/dt min (mmHg/s)	-9615 ± 890	-10637 ± 746*

LVEDP, left ventricular end diastolic pressure; LVSBP, left ventricular systolic blood pressure; LVDBP, left ventricular diastolic blood pressure; dp/dt max, maximal rate of pressure increase in the left ventricle ; dp/dt min, minimal rate of pressure increase in the left ventricle. * P<0.05, ** P<0.01, ***P<0.001.

Left ventricular histology (Table 8)

Cardiomyocytes in the LV of ACRF rats had an increased diameter compared to controls (figure 7). Likewise, the number of PCNA-positive and TUNEL-positive cells were increased in the LV of ACRF rats. Most of the PCNA – positive cells were identified in the perivascular interstitium and were most likely no cardiomyocytes. TUNEL-staining and cleaved caspase-3 co-localized in cardiomyocytes clearly indicating that these cells were undergoing apoptosis. No difference regarding fibrosis was seen between the groups.

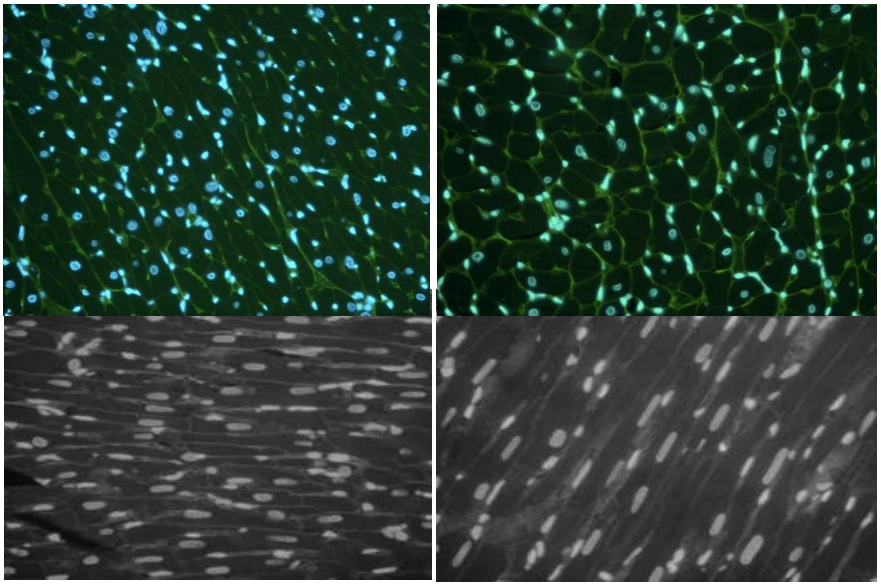

Figure 7. Left panels show LV tissue from pair-fed controls and right panels from rats with A-CRF. Upper panels show immunofluorescence staining with FITC-conjugated WGA (green) to delineate the cell membrane, and with DAPI (light blue) to visualize cell nuclei. Lower panels display longitudinally organized cardiomyocytes without immunostaining. Cardiomyocytes from A-CRF rats had an increased diameter indicating hypertrophy. Magnifications were x20. Figure from paper II.

Table 8. *Left ventricular histology 10 weeks after study start.*

	Controls (n=10)	A-CRF (n=10)
Cardiomyocyte diameter (μm)	14.58 ± 0.96	17.36 ± 2.17 **
Number of PCNA positive cells	8.30 ± 5.17	41.40 ± 35.29 **
Perivascular fibrosis/diameter (μm)	80.7 ± 14.9	88.5± 16.9
General fibrosis, %	2.25± 0.56	2.35 ± 0.91
Number of TUNEL positive cells (%)	0.34 ± 0.22	1.57 ± 1.20**

PCNA, proliferating cell nuclear antigen. * P<0.05; ** P<0.01

Study I

Effects of high-NaCl intake on arterial pressures

ACRF rats had higher blood pressure than controls, while high NaCl intake increased blood pressure only in ACRF rats (figure 9).

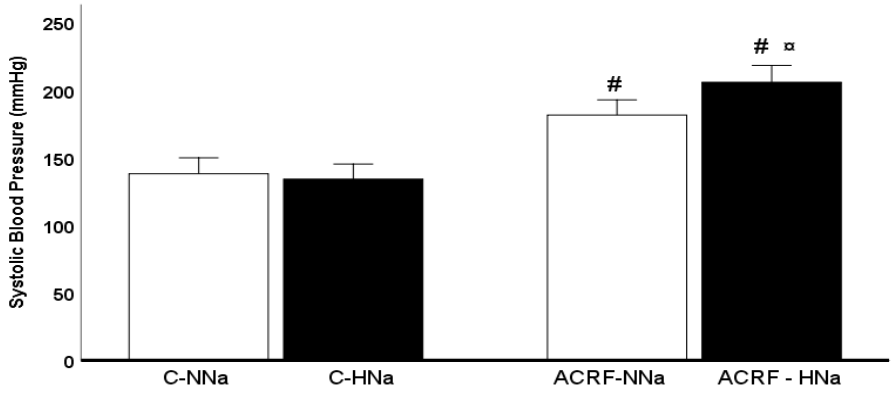

Figure 9. Main effects and between-factors interaction from two-factorial ANOVA are presented. # *P*<0.01 adenine *vs.* controls, ¤ P<0.05 interaction.

Effects of high-NaCl intake on cardiac weights, left ventricular fibrosis, serum levels of cardiac troponin-T and BNP-32 (Table 9)

ACRF as well as high NaCl intake produced marked increases in cTnT levels. Compared to group C-NNa, serum levels of cTnT were elevated approximately 6-fold in group ACRF-NNa and 24-fold in group ACRF-HNa (figure 10).

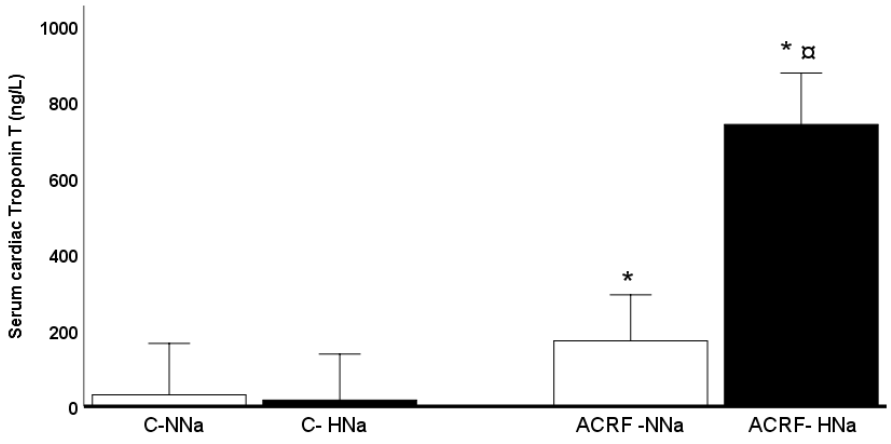

Figure 10. Main effects and between-factors interaction from two-factorial ANOVA are presented. ***** *P*<0.01 adenine *vs.* controls, ¤ P<0.01 interaction.

Both general and perivascular LV fibrosis were significantly elevated in ACRF rats versus controls (table 9). There were statistically significant between-factor interactions as a consequence of high NaCl intake producing increases in fibrosis only in ACRF rats.

There was a positive correlation between LV general fibrosis and cTnT levels in ACRF rats ($r = 0.81$, $p < 0.01$). In addition, cTnT concentrations were significantly correlated with systolic arterial pressure ($r = 0.69$, $p < 0.01$).

Table 9. Cardiac weights, left ventricular fibrosis and BNP-32.

	C-NNa (n=9)	C-HNa (n=10)	ACRF-NNa (n=10)	ACRF-HNa (n=8)	ANOVA effects: Adenine	NaCl intake	Interaction
LVW, g/kg BW	2.33±0.24	2.25±0.12	3.24±0.35	3.54±0.40	P<0.001	ns	P<0.05
RVW, g/kg BW	0.54±0.09	0.56±0.09	0.66±0.08	0.71±0.13	P<0.001	ns	ns
LV fibrosis, %	2.9±0.9	2.5±0.6	3.4±1.4	10.7±5.1	P<0.001	P<0.001	P<0.001
LV PV fibrosis, $\mu m^2/\mu m$	76±15	50±7	83±12	90±11	P<0.001	P<0.05	P<0.001
BNP 32, pg/ml	545 ± 116	1.061 ± 117	1.034 ± 160	1.314 ± 121	P<0.01	P<0.001	ns

Main effects and between-factors interaction from two-factorial ANOVA are presented LVW, left ventricular weight; RVW, right ventricular weight; BW, body weight; LV, left ventricle; and PV, perivascular.

LV histology

In ACRF rats that had received 2 weeks of high-NaCl (4%) chow (ACRF-HNa), the LV showed focal areas with inflammatory cell infiltration, fibrosis, necrotic cardiomyocytes, and perivascular erythrocytes, indicating hemorrhages (figure 11).

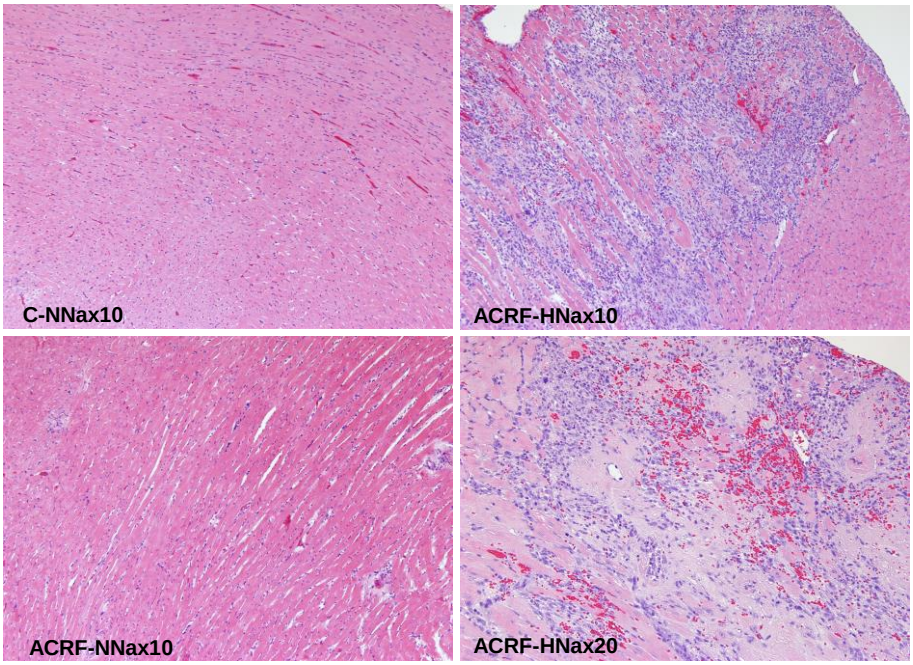

Figure 11. LV histology. Sections were stained with hematoxylin and eosin.Magnification x10 and x 20 as indicated. Figure from paper I.

A large proportion of the cells within the inflammatory infiltrate in ACRF-HNa rats were positive for CD68 using immunochemistry, indicating that these cells were macrophages/monocytes (figure 12).

In ACRF-HNa rats, arteries from non-injured areas of the myocardium showed alterations characterized by thickening of the medial layer (figure 13b). In myocardial areas with severe focal tissue injury, arteries (arrow) demonstrated fibrinoid necrosis with destruction of the internal elastic lamina and pronounced occlusion of the vessel lumen (figure 13c).

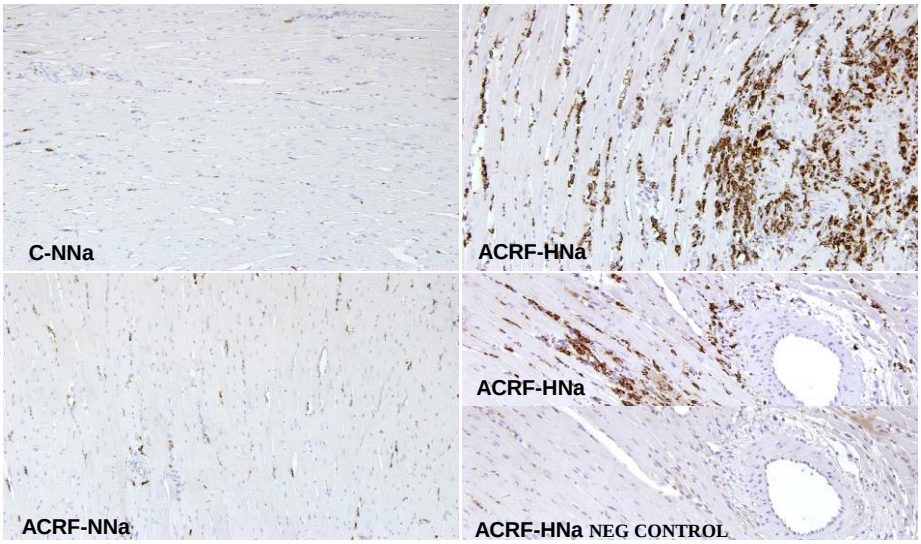

Figure 12. Immunohistochemistry identifying CD68-positive cells (monocytes and macrophages) in the LV of pair-fed controls (C) and rats with ACRF on a normal (0.6%; NNa) or high-NaCl (4%; HNa) diet. Magnificationx2. Figure from paper I.

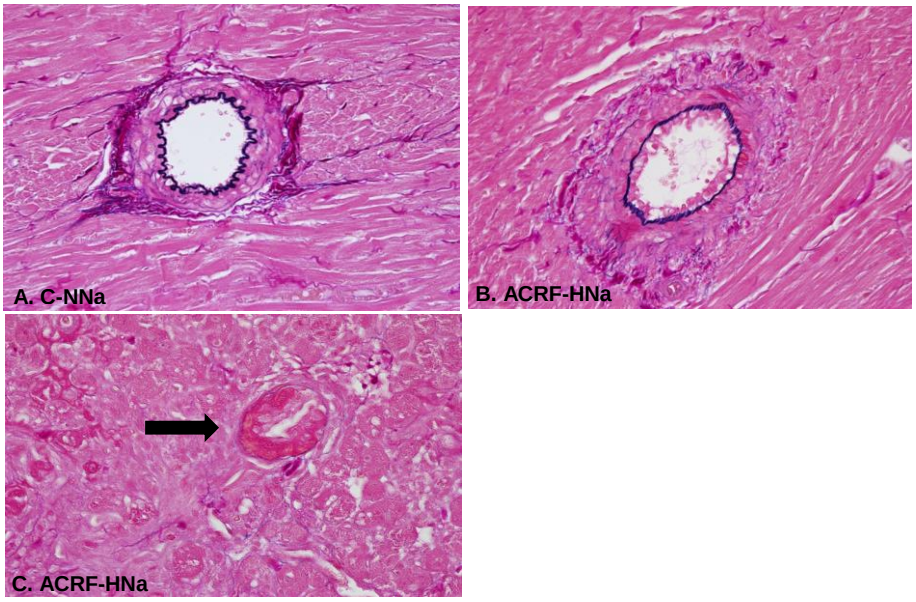

Figure 13. LV arteries from pair-fed controls on normal (0.6%) NaCl diet (C-NNa; **a**) and rats with ACRF subjected to 2 weeks of high-NaCl (4%) diet (ACRF-HNa; **b**, **c**) Sections were stained with Miller's elastin. Magnification×60. Figure from paper I

4.2 Clinical Study

Study III

General characteristics of study population

The primary cause of CKD was glomerulonephritis in 32% of patients, diabetic kidney disease in 20%, hypertension in 14%, autosomal dominant polycystic kidney disease in 9%, renovascular disease in 6%, and other causes in 19%.

Hemodynamic variables

Ambulatory blood pressure during 24 h, daytime or nighttime was not significantly different between the groups. However, nocturnal dipping of ASBP and ankle branchial index were significantly reduced in CKD patients.

Left ventricular morphology and function by echocardiography (table 10)

Ten CKD patients (11%), but no controls, met the criteria for LVH (p=0.027 between groups) even though there was no statistically significant difference between groups in LVMI. In the CKD group, 7 patients had eccentric LVH and 3 had concentric LVH. In addition, 10 CKD patients (11%) had concentric LV remodeling. No difference between the groups were seen in heart rate, cardiac output or cardiac index.

Table 10. *Left ventricular morphology and function.*

	CKD (n= 85-91)	Controls (n=39-41)
LV RWT	0.37±0.06*	0.32±0.05
Maximal septal wall thickness, mm	8.6±2.7 (n=75)*	7.5±1.8 (n=39)
LV ejection fraction (Simpson), %	64±7 (n=68)	65±5 (n=39)
LV diastolic volume/BSA, ml/m²	68.3±15.3 (n=70)	63.1±8.9 (n=39)
LV stroke volume, ml	85.4±16.9*	76.9±13.3
LV stroke volume/BSA, ml/m²	43.4±6.5*	40.5±5.3

Left ventricular ejection fraction according to Simpson, LV diastolic volume and maximal septal wall thickness were measured in a subset of patients using contrast enhancement (see Methods). Abbreviations: CKD = chronic kidney disease; LV = left ventricle; RWT = relative wall thickness; BSA = body surface area. * P< 0.05

Regression analyses in patients with CKD showed independent associations between nighttime ASBP (B=0.067, p=0.001), cfPWV (B=0.241, p=0.01) and BSA (B=2.947 p=0.047) with maximal septal wall thickness.

Doppler measures and indices of diastolic function (table 11)

Left ventricular tissue velocities e´, a´, and s´ were significantly elevated in CKD patients vs. controls. There was no statistically significant difference between groups in E, E/e´ or E/A. However, pulmonary vein flow velocity during atrial contraction was significantly elevated in patients with CKD. Only one patient with CKD met the criteria for LV diastolic dysfunction according to the ASE guidelines criteria but no control.

Table 11. *Doppler measures and indices of diastolic function.*

	CKD (n= 82-91)	Controls (n=38-41)
LV s´ mean, cm/s	9.3±1.8**	8.1±1.3
LV e´ mean, cm/s	9.7±2.5*	8.7±2.1
LV a´ mean, cm/s	11.1±2.4**	9.4±2.1
A, m/s	0.72±0.18*	0.61±0.13
LV IVRT, ms	85±16*	78±15
LAVI, ml/m²	36.3±9.7*	33.2±9.7
LA-RA area, cm²	4.0±2.5**	1.8±1.9

Abbreviations: CKD = chronic kidney disease, LV = left ventricle; s´ = systolic tissue velocity; e´ = early diastolic tissue velocity; a´ = late (atrial) diastolic tissue velocity; A = late (atrial) diastolic transmitral flow velocity; IVRT = isovolumic relaxation time; LAVI = left atrial volume index; LA = left atrium; RA = right atrium; In the LV tissue velocities were measured at the mitral annulus.* P< 0.05, **P<0.001.

Regression analyses in CKD patients showed that NT-proBNP (B=0.013, p=0.001) and LV diastolic volume/BSA (B=0.367, p<0.001) were independently associated with LAVI. Moreover, 24 h urinary Na excretion (B=0.008, p=0.013) and heart rate (B=0.042, p=0.022) were independent predictors of LV s´. Only age (B=-0.130, p<0.001) and LVMI (B=-0.029, p=0.026) showed independent associations with LV e´.

LAD blood flow velocity and CFVR

Baseline flow velocity in LAD was significantly elevated in CKD patients. However, in response to adenosine CFVR was significantly reduced in CKD patients. The number of subjects with a CFVR <2.5, indicating CMD, were 3 controls (9%) and 21 CKD patients (43%) (p=0.001).

Using a regression model including medical history of diabetes, age, 24 h ASBP and PASP, we found no variable that was significantly associated with baseline LAD flow velocity in CKD patients. Age (B=-0.027, p=0.015) and 24

h ASBP (B=-0.024, p=0.01) were independently associated with CFVR in CKD patients applying a regression model that also included TNI and LVMI. When baseline LAD flow velocity was added to this model only age (B=-0.023, p=0.011) and baseline LAD flow velocity (B=-3.927, p<0.001) were independently associated with CFVR.

5. DISCUSSION

Experimental model of ACRF

Rats with ACRF developed both CKD-associated metabolic abnormalities and cardiovascular alterations similar to humans with kidney failure. The main advantage of this model, compared to clinical studies in humans, is the ability to study the effects of kidney failure on the heart in the absence of comorbidities. Furthermore, the model enable us to perform advanced histological examinations of the heart which had been difficult in humans. The model of ACRF in rats was first applied in 1986 [60] and has been modified since then. Rats with ACRF (study I, II) had more advanced kidney disease (GFR approximately 10% of control values) compared to CKD patients (study III). The experimental and human studies in the current thesis complemented each other in the matter of our main question as we could study how the heart is affected at moderate CKD (CKD stages 3-4) and when severe kidney failure is established (animal studies).

Clinical study

Cardiac abnormalities in patients with CKD stages 3-4 (study III)

Even though the echocardiographic criteria for LV diastolic dysfunction are well defined, the diagnosis of HFpEF is still not easy to make. Four echocardiographic variables have to be calculated: e′, E/e′ ratio, LAVI, and peak TR velocity [54]. Diastolic dysfunction is present when more than 2 of these variables are abnormal. In our study, patients with CKD stages 3 and 4, without a prior diagnosis of heart disease, had normal EF but significantly elevated IVRT and increased LAVI and LA-RA area, similarly to patients with HFpEF. Moreover they had significantly elevated levels of NT-pro-BNP. However they did not meet the criteria for LV diastolic dysfunction or HFpEF [54]. Patients with CKD showed significant increases in both LV e´ and RV e´ compared to healthy controls. Most likely these changes were caused by systemic factors. Velocity e´ is known to be preload dependent [61]. Therefore, hypervolemia with increased preload, which is common in patients with renal insufficiency [62], could explain the increased tissue velocities. In support of this, CKD patients had also increased s´ velocities in both the LV and RV. We believe that the observed increase in LV e´velocities, regardless the cause, may complicate the diagnosis of HFpEF in patients with CKD. In addition the interpretation of plasma concentrations of NT-proBNP in patients with CKD is difficult , as increased levels can result from reduced renal clearance [29]. It

is possible that the current criteria for LV diastolic dysfunction and HFpEF may not be fully applicable to CKD patients. LV catheterization could be an alternative diagnostic approach but this procedure is not without risks and is also time and resource consuming. Magnetic resonance imaging has been shown to be an alternative diagnostic tool in the diagnosis of HFpEF [63].

Coronary flow velocity reserve

During recent years CFVR has increasingly been used to assess coronary microvascular function. Existing data [64, 65] indicate that CMD is an early feature of atherosclerosis and can predict future CV events and death. CFVR can be measured invasively using an intracoronary Doppler flow wire but this is associated with certain risks and increased costs [66]. Hence, noninvasive echocardiography with Doppler is to prefer. We interpret the significantly reduced CFVR of CKD patients in our study as a sign of CMD. This is interesting considering that CMD may contribute to the development of HFpEF [67]. CKD is associated with inflammation, dyslipidemia and increased oxidative stress and all these mechanisms can impair endothelial function [68] and produce CMD. However, the mechanisms causing CMD in patients with CKD need to be investigated further. We believe that our findings should be interpreted with some caution. LAD flow velocity at baseline, prior to adenosine, was significantly elevated in patients with CKD suggesting an increased metabolic demand [69]. We observed a negative correlation between baseline LAD flow velocity and CFVR in CKD patients suggesting that individuals with a high baseline flow velocity were unable to increase flow further in response to adenosine. Hence, in our study the reduced CFVR in CKD patients may not only have reflected CMD.

Experimental studies

Cardiac abnormalities in severe renal failure

In contrary to CKD patients (study III), ACRF rats (study II) developed signs of HFpEF. They had LV diastolic dysfunction but preserved systolic function. Velocity e´ was significantly reduced and the E/e´ ratio elevated compared to controls suggesting increased diastolic filling pressures. We confirmed significantly elevated LVEDP by invasive pressure recordings. Rats with ACRF also had increased left atrial diameter, indicative of chronic diastolic dysfunction [70]. As ACRF rats did not have increased LVEDd, the increase in LV filling pressure was most likely explained by decreased compliance of the LV.

The elevated CO found in ACRF rats was presumably not caused by hypervolemia. LVEDd was not elevated. However, ACRF rats tended to have a reduced LVESd compared to controls suggesting increased contractility. In addition, LV dp/dt max was significantly increased in ACRF rats supporting this interpretation. Possible explanations for the increased contractility could be the anemia seen in ACRF rats [47] and sympathetic activation that has been shown in chronic renal failure [71]. Anemia is a known cause of hyperdynamic circulation through increased cardiac output [72], reduced blood viscosity and general vasodilation [73]. As Converse et al have shown, CKD patients have elevated sympathetic nerve activity mediated by an afferent signal arising in the failing kidneys [71]. This overactivity may contribute to increased inotropy and elevated stroke volume.

Rats with ACRF had clear LVH. Both decreased LV compliance and diastolic dysfunction are linked to LV hypertrophy [74]. Different animal models of LVH, have shown impairments in cytosolic calcium handling in cardiomyocytes during diastole that may slow relaxation [74, 75]. Our group has shown that rats with ACRF have a decreased relaxation rate in thoracic aortas associated with altered intracellular Ca^{2+} handling in vascular smooth muscle cells [76]. However, in study II we could not see any association between diastolic dysfunction and altered expression in the LV of proteins involved in cytosolic Ca^{2+} handling. Moreover, we could not find any connection between diastolic dysfunction and LV fibrosis. We believe that LVH in ACRF rats is mainly caused by hemodynamic mechanisms. Hypertension results in elevated LV afterload and consequently LVH. Possible non-hemodynamic factors could also have an additive effect e.g. aldosterone [77, 78] and fibroblast growth factor-23 [79], which both are elevated in the ACRF model [47, 80]. In study III most of the CKD patients had a history of hypertension but only minor abnormalities on the echocardiogram. This finding is not surprising given the fact that CKD patients had a very well-controlled blood pressure during the study period.

Besides hypertrophy, cardiomyocytes in ACRF rats had increased apoptosis. This has been shown also in earlier studies of experimental CKD [81, 82] but the underlying mechanisms are still undefined. There was also, a noticeable increase of PCNA-positive cells compared to controls indicating increased proliferation. As these cells were localized mainly in the microvascular interstitium we believe that most of these cells were fibroblasts or leukocytes. This finding corroborates our results in study III where we detected CMD in patients with CKD.

The effects of high NaCl-diet on the heart in kidney failure

ACRF rats on normal NaCl diet had elevated cTnT levels compared to controls and a relatively normal LV histology. However, two weeks of high-NaCl diet led to dramatically increased levels of cTnT and severe LV injury. Histological examinations revealed focal areas in the LV with pronounced interstitial inflammatory cell infiltration, fibrosis, necrotic cardiomyocytes and perivascular erythrocytes indicating hemorrhages. Moreover, myocardial arteries showed wall thickening and fibrinoid necrosis with luminal narrowing.

Similar findings as in ACRF-NNa rats were found in CKD patients, where levels of cTnT are usually elevated without any clinical signs of myocardial injury [18, 83]. There is a vivid discussion whether this TnT elevation is due to decreased renal clearance or increased release from the myocardium. Studies have shown that elevated TnT has a low specificity for MI in patients with reduced GFR [84] and therefore the interpretation of TnT levels in CKD patients can be difficult. As hsTnI shows better specificity for this patient group [84] maybe this marker should be preferred in clinical practice. In agreement with this, our patients with CKD had significantly increased levels of TnT, but not hs-TnI, compared to controls. Still, hs-cTnI has lower specificity for CKD patients than for healthy controls and the right cut off value for CKD patients has not yet been defined [85, 86]. Nevertheless, it is helpful to know that troponin levels are usually stable in a patient with CKD without acute coronary syndrome [87]. Chronic, stable, mildly elevated levels of TnT can be explained by reduced renal clearance [88]. Therefore, when acute NSTEMI is suspected and elevated troponin levels are found, a second measurement after some hours, is helpful to distinguish between infarction and other causes of chest pain.

Completely different pathophysiological mechanisms seem to be responsible for the pronounced TnT elevation found in ACRF-HNa rats. Light microscopy of the LV revealed abnormal small arteries in areas of myocardial injury with marked medial thickening, fibrinoid necrosis and luminal narrowing, findings typical for malignant hypertension. Thus, we believe that hypertension-induced arterial lesions caused myocardial ischemia and hypoxic injury. The marked hypertension that ACRF- HNa rats had, together with increased LV afterload, likely increased tissue oxygen demand leading to even worse myocardial hypoxia. Similar abnormalities in LV histology have been found in salt-loaded, malignant hypertensive, double transgenic rats harboring human renin and angiotensinogen genes [89], even though the pathophysiology of hypertension between our model and double transgenic rats is different [90]. Interestingly similar findings to ours have also been shown in experimental

models of CKD without hypertension [91]. Although our results indicate that high NaCl caused cardiac injury via hypertension we cannot rule out a contribution from other, non-hemodynamic, mechanisms.

The salt-loading that was performed in our experimental model was pronounced and corresponded to a 7-fold higher NaCl intake compared to controls. The reason for the marked NaCl challenge was that we wanted to test whether NaCl-loading had the potential to cause cardiac injury. Our purpose was not to determine the dose-effect relationship. The fact that control rats with intact kidney function had normal TnT levels during NaCl-loading suggests that the dietary challenge was not extreme. The inability of ACRF rats to handle a salt load, probably led to malign hypertension and secondary cardiac injury. This is an interesting finding as a similar risk may apply to patients with CKD.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Patients with CKD stages 3 and 4, without a prior diagnosis of heart disease, displayed abnormalities in LV diastolic function without fulfilling the criteria for LV diastolic dysfunction. We hypothesize that the observed increase in LV e´ velocity in CKD patients might reduce the ability to detect early stages of LV diastolic dysfunction and lead to underdiagnosis of HFpEF in the CKD population. Our results highlight the difficulties in diagnosing HFpEF in patients with CKD. Interestingly, patients with CKD had a reduced CFVR indicating CMD. It is possible that CMD may be involved in the pathogenesis of HFpEF in patients with CKD. This is a field for further investigation.

ACRF rats, having more advanced kidney disease, developed LV hypertrophy and diastolic dysfunction with preserved EF. These abnormalities resemble LV dysfunctions in patients with HFpEF. LV hypertrophy and diastolic dysfunction seem to be the primary cardiac abnormalities that develop as kidney function declines.

Rats with ACRF had elevated serum levels of cTnT. Two weeks of high-NaCl diet enhanced the increase in serum cTnT concentrations and caused LV injury most likely through hypertension-induced small artery lesions and myocardial ischemia. Having demonstrated its resemblance to patients with CKD, the ACRF model could be used in future studies for examining the pathophysiology of cardiac injury, elevated serum cTnT and heart failure in CKD.

In addition, our results support the hypothesis that a high dietary intake of NaCl can have deleterious effects on LV integrity in patients with kidney failure. It would be interesting to examine whether a reduced NaCl intake, or a reduced dialysate sodium, could prevent cardiac disease and preserve cardiac function in CKD patients. In addition, it would be appealing to investigate if cardio-protective effects of a reduced NaCl intake is mediated only via hemodynamic mechanisms or if non-hemodynamic factors are also involved.

The main goal still remains; to reduce cardiovascular mortality in patients with CKD. There is an urgent need for treatments that can protect the heart.

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