RED CELL DYNAMICS AND HAEMODYNAMICS IN CARDIORENAL FAILURE

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DYNAMIEK VAN DE RODE BLOEDCEL EN HEMODYNAMIEK IN CARDIORENAAL FALEN

(met een samenvatting in het Nederlands)

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CONTENTS

ABBREVIATIONS

ACE	Angiotensin converting enzyme
ARAS	Atherosclerotic renal artery stenosis
AUC	Area under the curve for haemoglobin change over time
BP	Blood pressure
CHD	Coronary heart disease
CHF	Chronic heart failure
CKD	Chronic kidney disease
cMRI	Cardiac magnetic resonance imaging
CPET	Cardiopulmonary exercise test
CV	Cardiovascular
eCrCl	Estimated creatinine clearance by Cockroft Gault equation
EDV	Left ventricular end diastolic volume
EPO	Erythropoietin
ESA	Erythropoietin stimulating agent
ESRD	End stage renal disease
ESV	Left ventricular end systolic volume
GFR	Glomerular filtration rate
Hb	Haemoglobin level(s)
HF	Heart failure (both chronic and acute heart failure)
HFPEF	Heart failure with preserved left ventricular ejection fraction
HFREF	Heart failure with reduced left ventricular ejection fraction
hs-CRP	High sensitive C-reactive protein
IL-6	Interleukin-6
IRF	Immature reticulocyte fraction
LGE	Late gadolinium enhancement
LVEF	Left ventricular ejection fraction
MAP	Mean arterial pressure
MCV	Mean corposcular volume
MDRD	Estimated GFR by the Modified Diet in Renal Disease formula
MLHF	Minnesota living with heart failure questionnaire for quality of life
MRA	Magnetic resonance angiography
NGAL	Neutrophil gelatinase associated lipocalin
NT-proBNP	NT pro-brain natriuretic peptide
NYHA	New York Heart Association functional class
O/P ratio	Log observed/predicted erythropoietin ratio for degree of anaemia
pVO2	Peak oxygen uptake consumption
QoL	Quality of life
RAND-36	RAND-36 item health survey for quality of life
RAS inhibitor	Renin angiotensin system inhibitor
RBF	Renal blood flow
RDW	Red cell distribution width
Ret-He	Reticulocyte haemoglobin content
RPP	Renal perfusion pressure
sTfR	Soluble transferrin receptor
TSAT	Transferrin saturation

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INTRODUCTION

CHRONIC CARDIORENAL FAILURE

Both chronic heart failure (CHF) and chronic kidney disease (CKD) have become an increasing problem for public health systems. According to a recent report from the National Institute for Health and Environment (Rijksinstituut Volksgezondheid en Milieu, RIVM)1, about 130,000 people in the Netherlands (1% of the population) are currently diagnosed with CHF, while estimations are that his number will increase by 50% by the year 2025 due to ageing of the population[1]. CHF results into high health care costs mainly due to associated frequent hospitalizations. The prevalence of CKD is even higher; according to the Prevention of Renal and Vascular End-stage Disease (PREVEND) study up to 12% of the Dutch population has CKD according to the most recent CKD classification, whereas 5.3% has a markedly reduced estimated glomerular filtration rate (eGFR < 60 ml/min/1.73m^2, which matches the reported prevalence in the USA[2]. Besides the potential progression towards end-stage renal disease, requiring costly renal replacement therapy, CKD patients have a very high risk of cardiovascular comorbidity: according to the United States Renal Data system (USRDS) 12.8% of CKD patients get a myocardial infarction, 26.5% have cerebrovascular disease and 44.2% have CHF[3].

As a consequence, in many CKD patients the diagnosis CHF is also established. Conversely, about 50% of all patients with CHF are known to have impaired renal function[4-6]. The presence of CKD is one of the most important risk factors for mortality in patients with CHF[7, 8]. This "dangerous liaison", the combination of both CHF and CKD is thus highly prevalent and associated with an even higher morbidity and mortality than can be expected on solely the summation of both[9, 10]. Hence we previously defined the "Cardiorenal Syndrome"; a clinical condition where both CKD and CHF co-exist, and the failure of one organ accelerates progression of structural damage and failure of the other organ[9].

Several mechanisms have been proposed to explain this deadly interaction between CHF and CKD. The late professor Arthur Guyton was the first to extensively describe the physiological haemodynamic interactions between the kidney and the heart[11, 12] (Figure 1). However, the haemodynamic model of interaction between the extracellular fluid volume, regulated by the kidney, and the systemic circulation, predominantly regulated by the heart and peripheral resistance, seems to incompletely explain the pathogenesis of cardiorenal failure.

New evidence from epidemiological and clinical studies has revived the concept that an increased central venous pressure exerts a strong influence on renal perfusion[13-17]. In CHF, lower GFR is strongly related to elevated central venous pressure[18] and aggressive decongestion by ultrafiltration is associated with a decrease in creatinine levels[19, 20]. The alleged exact underlying mechanism is beyond the scope of this thesis[21]. Other factors involved in the interaction between heart and kidney that need to be further elucidated are renal autoregulation and the possible influence of renal artery stenosis.

In addition to these haemodynamic interactions, non-haemodynamic interactions are involved in cardiorenal interactions. A few years ago our research group proposed an alternative model in which the renin-angiotensin system, the balance between the nitric-oxide and reactive oxygen species, the sympathetic nervous

system and inflammatory mediators interact and form the cornerstones of the non-haemodynamic pathogenesis of cardiorenal failure. We hypothesized that disturbance of one of these mediators, called the cardiorenal connectors, activates a cascade of other connectors, leading to a vicious circle resulting in progressive failure of both organs (Figure 1)[9, 22].
Since there is an increasing awareness for the important role of anaemia [23-25], a disturbed iron metabolism[26, 27] and inflammation in both CHF and CKD[28, 29], we have extended this model to include anaemia, erythropoietin and red cell dynamics (Figure 1). Recently, anisocytosis, the heterogeneity of circulating red blood cells as measured by red cell distribution width (RDW), was recognized as a strong independent predictor for mortality and morbidity in CHF[30]. Collectively, anaemia, iron metabolism, inflammation and anisocytosis, are related to red cell dynamics: the production and decay of the circulating erythrocyte.

As such, two areas of research interest can be recognized regarding heart-kidney interactions. One is formed by the complex of cardiorenal connectors, that couple heart and kidney (dys)function, not necessarily by haemodynamic effects. The other is formed by haemodynamic coupling between heart and kidney function. In both of these areas, a number of uncertainties surface that form the basis of the investigations described in this thesis: the role of haemodynamics and red cell dynamics in the pathogenesis of cardiorenal failure. The next paragraphs provide some background information about these subjects.

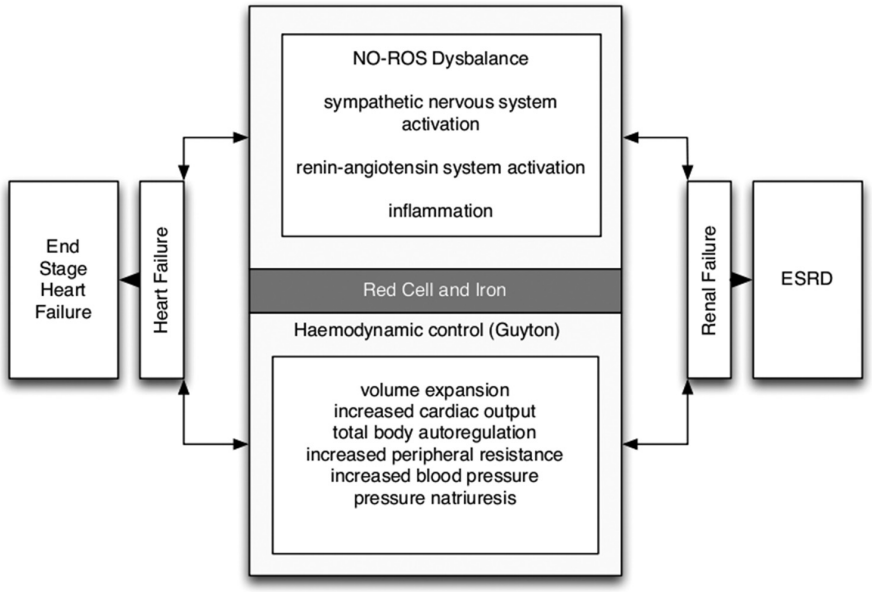

Figure 1 | The pathophysiological model of combined heart and renal failure, adapted from Bongartz et al.[9] Abbreviations: ESRD, end stage renal disease; NO-ROS, nitric-oxide reactive oxygen species

ANAEMIA IN CARDIORENAL FAILURE

Anaemia (defined by the World Health Organization as haemoglobin-levels < 13 g/dL in men and < 12 g/dL in women) is highly prevalent in both CHF[31, 32] and CKD[33, 34]. The presence of anaemia in cardiorenal failure further amplifies morbidity and mortality[24, 35-37]. The exact aetiology of anaemia in cardiorenal failure remains still incompletely resolved but appears to be multifactorial (Table 1). Potential causative mechanisms involve mechanisms affecting effective erythropoiesis, such as an impaired erythropoietin (EPO) production, EPO resistance, a disordered iron metabolism and the use of renin-angiotensin-system inhibitors. Other mechanisms affect the life span of the red cell, such as inflammation or haemolysis due to renal replacement therapy and prosthetic heart valves. Additional there is (occult) red blood cell loss due to the use of platelet inhibitors and warfarin and haemodilution (Table 1). Some mechanisms affecting the effective erythropoiesis and the life span of the red cell will be discussed in more detail.

One of the main causes of anaemia in CKD is an insufficient EPO production, being produced in the renal peritubular cells (renal anaemia)[38]. Although EPO levels appear to be elevated in patients with CKD and CHF compared to healthy controls, these levels are disproportionally low for the degree of anaemia[39, 40]. This impaired EPO production is partly due to the CKD, but also due to a direct inhibition of EPO production by circulating pro-inflammatory cytokines[41] and the use of renin-angiotensin-system inhibitors[42, 43].

Yet, the relatively elevated EPO levels in CHF and CKD also point towards a decreased responsiveness of the bone marrow to EPO. This decreased EPO responsiveness, also referred to as EPO resistance, has first been recognized in CKD patients based on the high variability in sensitivity to erythropoiesis stimulating agents (ESA)[44]. One of the explanations for this EPO resistance is the chronic inflammatory state characterized by increased levels of pro-inflammatory cytokines present in both patients with CKD and with CHF. Cytokines can directly inhibit the growth of erythroid precursor cells, can diminish EPO production and can have a direct toxic effect on progenitor cells[45, 46].

Table 1 | Potential causes of anaemia in chronic cardiorenal failure

Factors affecting effecting effective erythropoiesis / maturation
Impaired EPO production
EPO resistance
Disordered iron metabolism
The use of renin-angiotensin-system inhibitors
Vitamin B12/folate deficiency
(Myelodysplasia, bone marrow aplasia, leukemia, lymphoma, toxic drugs, defects in haem synthesis)
Factors affecting life span of red blood cell
Haemolysis (e.g. due to renal replacement therapy, valvular heart disease, prosthetic heart valves)
(Occult) blood loss
Inflammation
Haemodilution

As mentioned earlier, another causative factor for both anaemia and EPO resistance is a disordered iron metabolism. Both functional and absolute iron deficiency are prevalent in CHF and CKD and associated with a poor prognosis[26, 27]. Once again, inflammation plays a central role. The circulating pro-inflammatory cytokines directly induce the expression of divalent metal transporter 1 in macrophages and the intestine and down regulate ferroportin expression, the efflux channel for iron in macrophages and enterocytes. Furthermore, cytokines directly stimulate an increased transferrin-receptor mediated uptake of transferrin-bound iron into the macrophages. Probably more importantly, cytokines stimulate the synthesis of hepcidin, a central regulator of the iron metabolism. Hepcidin levels are higher in CKD and cardiorenal patients compared to healthy subjects[47, 48]. This acute phase protein inhibits the efflux of iron into plasma transferrin by down regulating ferroportin, thereby inhibiting iron absorption from the intestines and sequestering iron in macrophages. All these processes reduce intestinal iron absorption, promote intracellular iron storage and decrease the plasma concentration of iron, needed for an effective erythropoiesis[45], causing both an absolute and a functional iron deficiency. Interestingly, the first iron supplementation studies thus far show promising results regarding exercise capacity and quality of life[49]. In addition, hepcidin may contribute to EPO resistance through a direct inhibitory effect on erythroid progenitor proliferation[50]. Our group has shown that hepcidin levels decrease in response to ESA treatment and the magnitude in decrease correlates with the ESA response in cardiorenal patients[51].

Analogous to hepcidin is neutrophil gelatinase associated lipocalin (NGAL), a 25kd protein, also related to both inflammation and iron metabolism. NGAL is a natural bacteriostatic agent by interfering with bacterial iron uptake. It increases in response to inflammatory processes, especially in response to acute and chronic tubular injury. Subsequently it is mostly known and used as a biomarker for kidney injury[52-56]. However, recently two studies demonstrated that lower NGAL levels reflect reduced iron availability n haemodialysis patients; lower NGAL levels correlated with lower transferrin saturation and higher total hepcidin levels[57-59]. It is unknown whether NGAL levels also reflect iron metabolism in chronic cardiorenal patients and whether ESA treatment modulates NGAL levels, in correspondence with its effect on hepcidin levels.

Thus, anaemia is prevalent in both CHF and CKD. An impaired EPO production, EPO resistance and a disturbed iron metabolism are important causative mechanisms, associated with a poor outcome. Red cell distribution width (RDW), a parameter of anisocytosis, has recently been discovered as an important risk predictor, potentially incorporating aspects of both EPO resistance and a disturbed iron metabolism. It should be noted that all these processes are related to red cell dynamics, the production and decay of the circulating erythrocyte.

ERYTHROPOIETIN TREATMENT IN CARDIORENAL FAILURE

Until a few years ago, renal anaemia in CKD was routinely treated with ESA. Undeniably, multiple studies in CKD showed beneficial effects on quality of life[60], exercise capacity, reduced hospitalization rates[60] and regression of left ventricular

hypertrophy[61, 62]. These favourable results aroused the interest from the cardiologists. Subsequently, many smaller interventional trials with ESA treatment in CHF were performed, confirming beneficial effects on quality of life, exercise capacity and left ventricular function[63-68]. In addition, experimental animal studies showed that ESA treatment has many non-erythropoietic effects, such as vascular repair and anti-apoptosis in coronary heart disease models and CHF[69, 70]. Furthermore, anti-inflammatory effects were demonstrated, as summarized by our group[71].

However, in 2006 two large-scale randomized trials in CKD failed to demonstrate a beneficial effect of ESA on mortality or morbidity when targeting normalization of haemoglobin levels[72, 73]. The Correction of Haemoglobin and Outcomes In Renal insufficiency (CHOIR) was terminated due to an observed increased risk of death and cardiovascular hospitalizations in the group treated with EPO to a higher target for haemoglobin. A few years later, the Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT) study in patients with CKD and diabetes mellitus type 2 demonstrated an increased risk of stroke in the ESA-treated group[74]. In addition, a meta-analysis of all prospective randomized trials of ESA in CHF reported a neutral effect on mortality and heart failure events[75] and the randomized Studies of Anaemia in Heart Failure Trial (STAMINA-Heft) also failed to demonstrate beneficial effects of ESA in CHF[76]. These results dampened the initial enthusiasm about ESA treatment in both CHF and CKD.

The secondary analyses of the CHOIR study showed a few possible explanations for these unexpected negative results. One of these explanations is that the use of high-dosages of ESA to achieve the target haemoglobin level necessary to overcome an inadequate bone marrow response (i.e. EPO resistance) was related to the higher morbidity and mortality. This might possibly be due to the high-dose dependent adverse non-erythropoietic effects, such as trombogenic or direct vascular actions and pro-carcinogenic effects[77, 78]. Thus, the results of the ESA studies may be related to the attained high haemoglobin levels (erythropoietic effects), to (dose-related) non-erythropoietic effects, or due to unwanted effects of high-dose ESA occurring predominantly in those patients with EPO resistance[45].

In conclusion, EPO exerts a myriad of both erythropoietic and non-erythropoietic actions in the human body. So far, the clinical focus has been mainly on its erythropoietic effects in which treatment with ESA is associated with adverse outcome, when targeting normal haemoglobin levels. It is unknown which mechanisms of ESA treatment are responsible for these adverse effects, erythropoietic or non-erythropoietic, and at what dosages in what kind of patients. This might lead to a therapeutic nihilism of ESA and denial of its potential beneficial effects. Further research is necessary for the role of EPO in cardiorenal failure and to differentiate the erythropoietic versus the non-erythropoietic effects of ESA.

ANISOCYTOSIS IN HEART AND KIDNEY FAILURE

RDW is routinely performed as part of the complete blood count to gather information about the heterogeneity in the size of circulating erythrocytes. This parameter for anisosyctosis is defined as the standard deviation of erythrocyte size divided by the

mean corpuscular volume (MCV). It was originally introduced as an aid in the diagnostic work-up for anaemia[79, 80]; when MCV is low in the context of a high RDW, the most likely cause of the anaemia is iron deficiency, while a low MCV with a normal RDW points towards thalassemia. Conversely, a high MCV with a high RDW is suggestive of a folate and/or vitamin B12 deficiency, while a high MCV with a normal RDW value is more suggestive of an aplastic anaemia[79].

However, a few years ago, Felker et al., in search for novel biomarkers for cardiovascular risk in CHF, examined numerous laboratory markers in two large heart failure populations. Unexpectedly, the authors found that RDW was a very strong and independent predictor for morbidity and mortality. Importantly, this association was independent from other haematological variables, such as MCV or haemoglobin concentrations, and was found to be a stronger predictor for adverse outcome than classical predictors for outcome such as left ventricular ejection fraction, New York Heart Association functional class and renal function[30]. After this initial publication, many reports followed, confirming this association between higher RDW levels and increased mortality risk in both chronic and acute heart failure[81-84]. Other publications even extended this association to other cardiovascular populations, such as patients with coronary heart disease[85, 86], pulmonary hypertension[87], and even in community based, apparently healthy subjects[88, 89].

Unfortunately, the causal pathophysiological mechanisms linking anisocytosis with adverse outcomes are still unknown, limiting the use of RDW as a biomarker in daily clinical practice. A better understanding of the underlying biology determining the RDW-associated risk would support the plausibility of RDW as a biomarker and could possibly provide more insight in the pathophysiology of heart failure. As mentioned previously, RDW is a parameter for the heterogeneity of the size of circulating erythrocytes. This heterogeneity in size is mainly determined by red cell dynamics; on the one hand the production of (larger, young) erythrocytes and on the other hand the early decay of (older, smaller) circulating erythrocytes. Suggested possible causative factors are vitamin B12/folate deficiency, inflammation, a disturbed iron metabolism and EPO resistance[83, 90].

Although a few reports demonstrated an association between vitamin B12 and folate deficiency with higher RDW levels in the general population, a clear gradient across RDW quintiles was not seen[89, 91]. As previously mentioned, an inflammatory state plays an important role in the pathophysiology of CHF, CKD and other cardiovascular diseases, and is associated with adverse outcomes[28, 92, 93]. Many have speculated that the RDW associated risk might be a reflection of this underlying inflammatory state. Increased cytokines inhibit the growth of erythroid precursor cells, decrease EPO production and reduce iron availability for erythropoiesis by increasing hepcidin synthesis[45, 46], all potentially resulting in an increased anisocytosis. However, although reports demonstrated an association between RDW and inflammation, as determined by leucocytes, interleukin-6 or hs-CRP levels[84, 88, 89, 91, 94], several studies have shown that RDW predicts survival independent of inflammation,[91, 94, 95].

As discussed previously, EPO resistance to both endogenous and exogenous EPO is of prognostic importance. Hence, the presence of EPO resistance could explain the RDW-associated risk. However, so far, no direct data are available about the association between RDW and EPO resistance.

A disordered iron metabolism plays an important role in the pathophysiology and prognosis of both CHF and CKD and can lead to a higher anisocytosis due to the production of smaller erythrocytes. Hence it seems obvious to hypothesize that possibly iron metabolism links RDW to adverse outcome. However, outcome data with respect to the association between RDW and parameters for iron homeostasis are scarce.

In conclusion, anisocytosis as assessed by RDW is associated with adverse outcome in CHF and several other cohorts, but the underlying pathophysiological explanation is unclear. Plausible causative mechanisms would be inflammation, EPO resistance and iron deficiency. One aim of the present thesis was to attempt to shed more light on RDW in the context of non-haemodynamic coupling of heart and kidney failure.

HAEMODYNAMICS REVISITED

The role of blood pressure and renal artery stenosis in combined heart and renal failure

Although coupling of heart and kidney function in a haemodynamic way has been investigated thoroughly by Guyton, this was mainly done in the context of physiological manipulations, and not in pathophysiological models. Several issues, specific to cardiorenal disease are identified. One issue is, that the prevalence of atherosclerotic disease in another organ than the heart is extremely high if atherosclerotic coronary artery disease has been established[96, 97]. When this is combined with the notion that one of the main causes of CKD and CHF in the western society is atherosclerosis, immediately the question arises, whether the presence of atherosclerotic renal artery stenosis (ARAS)[98] could (at least in part) explain the high prevalence of CKD in CHF patients. Indeed, ARAS is prevalent in cardiovascular diseases; 12-15% of patients with coronary heart disease[99, 100], 24-59% of patients with peripheral arterial disease and up to 40% in patients with CKD have ARAS[101]. It can manifest itself by hypertension, progressive renal dysfunction, flash pulmonary oedema, CHF, but can also be diagnosed in the absence of clinical symptoms[102]. Without doubt, ARAS can affect the renal circulation. The kidney can experience a lower perfusion pressure behind the stenosis, possibly falling below the "lower limit of autoregulation". Furthermore, ARAS can amplify renin and angiotensin II generation, leading to sodium retention with increased extracellular fluid volume as a consequence, possibly further aggravating renal venous congestion and potentially further compromising GFR. However, data about the prevalence and the cardiac clinical profile of ARAS in chronic cardiorenal failure is still scarce.

Not only the presence of ARAS, but also the CKD per se and the many medications that are applied in CHF could potentially make the renal function more susceptible to fluctuations in blood pressure and thereby interfere with the fine regulation of volume balance. Certainly, many clinicians attribute a decrease in GFR in patients with CHF to a decrease in arterial blood pressure and/or a decrease in cardiac output. Yet, this concept is not well proven in a chronic setting of combined heart and renal failure. The presence of ARAS would reinforce this concept, making patients with the combination of CHF and ARAS even more vulnerable to a reduction in blood pressure or worsening heart failure. However, this concept

bypasses the observation that the healthy kidney is extremely capable of autoregulation[103]. Nonetheless, this ability to autoregulate could be impaired in patients with CKD[104]. Furthermore, the hypothesis that an increased central venous pressure also attributes to a decrease in GFR has regained clinical interest the last few years[15, 105]. Thus, the net effect of changes in blood pressure on renal function are unknown so far, as is the influence of ARAS[21].
Hence, two questions arise. The first is what the prevalence and associated clinical profile is of ARAS in patients with chronic cardiorenal failure. The second is whether GFR is dependent on fluctuations in blood pressure in patients with chronic cardiorenal failure and whether this is aggravated by the presence of ARAS.

OUTLINE OF THIS THESIS

As outlined, there are uncertainties regarding the characterization of combined heart and kidney failure with respect to non-haemodynamic and to haemodynamic interactions. The thesis focuses on red cell dynamics regarding the former, and ARAS and stabilization of GFR in cardiorenal failure regarding the latter. The combined studies hopefully shed more light on this devastating complex disease state.

PART I focuses on red cell dynamics in chronic cardiorenal failure. We hypothesize in this part that the production and anisocytosis of the circulating erythrocyte plays an important role in cardiorenal failure. We investigate this hypothesis by exploring the role of endogenous erythropoietin concentrations, treatment with fixed low-dose erythropoietin stimulating agent (ESA) and anisocytosis in chronic cardiorenal failure.
The treatment with ESA is surrounded by substantial controversies. It is unknown whether the adverse and/or favourable effects of ESA treatment are due to its direct erythropoietic effects or due to its non-erythropoietic effects. Therefore we designed the EPOCARES (**E**rythro**po**ietin in the **Ca**rdio**R**enal **S**yndrome) trial, to differentiate the erythropoietic versus the non-erythropoietic effects of fixed low-dose ESA treatment on cell studies, biomarkers, quality of life, exercise capacity and cardiac function in anaemic patients with stable chronic cardiorenal failure. The trial already has provided a number of new insights[51, 106]. Data from this study are also applied to address the questions that form the basis of this thesis. The objectives and design of the EPOCARES trial are outlined in **chapter 2**.
In **chapter 3** we explore whether RDW is associated with EPO resistance, which could explain the RDW associated risk. We study this using data from the EPOCARES trial. The design of the trial offers information about both the endogenous EPO levels and the response to ESA treatment.
An analysis of the effect of fixed low-dose ESA treatment, differentiated into erythropoietic and non-erythropoietic effects, on quality of life, exercise capacity and cardiac function, is presented in **chapter 4**. Moreover, we investigate in this chapter the associations between RDW and clinical parameters, such as exercise capacity and quality of life.
So far, multiple studies demonstrated the association between higher RDW levels and adverse outcome in multiple cardiovascular populations. In **chapter 5** we

explore whether higher RDW levels are associated with an increased risk of incident heart failure and coronary heart disease events in a large population of apparently healthy men and women. Moreover, we examine whether this association is independent of established cardiovascular risk factors, such as used in the Framingham Risk Score, inflammation or iron metabolism, to appreciate if RDW reflects a different underlying pathophysiological mechanism. For this purpose we use data from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk cohort.

NGAL is a natural bacteriostatic agent by interfering with bacterial iron uptake that increases in response to inflammatory processes, especially in response to tubular injury. Recent data showed that lower NGAL levels reflect reduced iron availability in haemodialysis patients[57-59]. In **chapter 6** we investigate whether serum NGAL levels also reflect iron availability in patients with chronic cardiorenal failure and whether ESA treatment influences NGAL levels, in agreement with its effect on hepcidin[51].

PART II of this thesis focuses on two aspects of the haemodynamics between heart and kidney: the relationship between chronic fluctuations in blood pressure and renal function and the prevalence and clinical consequences of ARAS.

Chapter 7 explores whether chronic fluctuations in blood pressure are related to chronic changes in GFR. Subjects from the EPOCARES trial are followed for 12 months, in which blood pressure and creatinine are measured monthly. From these data we derive individual relationships between fluctuations in blood pressure and GFR. Likewise, we investigate whether indicators of volume control, associated with central venous pressures, are associated with changes in GFR. In addition, we differentiate between patients with and without ARAS, in order to appreciate whether the presence of ARAS makes the kidney more susceptible for changes in blood pressure.

Hence, in **chapter 8**, we investigate the prevalence of ARAS in our small but well characterized group of chronic cardiorenal patients. All eligible subjects from the EPOCARES study undergo combined cardiac magnetic resonance imaging, including late gadolinium enhancement, with magnetic resonance angiography of the renal arteries. With one examination we can determine the presence and extent of ARAS, the cardiac function and volumes and the presence of myocardial fibrosis. We hypothesize that ARAS is highly prevalent in cardiorenal failure and that the presence of ARAS is associated with a specific clinical profile and more severe cardiac dysfunction and fibrosis.

In **chapter 9** the results of the aforementioned studies are summarized and discussed.

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PART I The role of erythropoietin and red cell dynamics in cardiorenal failure

2

Erythropoietin treatment in patients with combined heart and renal failure: Objectives and design of the EPOCARES study

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ABSTRACT

Background

Anaemia is common in patients with the combination of chronic heart failure and chronic kidney disease and is associated with increased mortality. Recent clinical studies suggest that recombinant human erythropoietin (EPO) treatment has desirable as well as undesirable effects, related to its haemapoietic or non-haemotopoietic effects. Therefore a translational study is needed to elucidate mechanistic aspects of EPO treatment.

Methods

In this open-label randomized 12-month trial (the Mechanisms of Erythropoietin Action in the Cardiorenal Syndrome [EPOCARES]), patients with the combination of chronic heart failure and chronic kidney disease (glomerular filtration rate 20-70 ml/min) and mild anaemia (haemoglobin 10.3-12.6 g/ dL in men, and 10.3-11.9 g/ dL in women) are being randomized into 3 groups: 1 group (n=25) receives a fixed dose of 50 IU/kg per week EPO to increase haemoglobin level to a maximum of 13.7 g/dL for men and 13.4 g/dL for women; another group (n=25) is treated with 50 IU/kg per week EPO maintaining baseline haemoglobin levels for the first 6 months by phlebotomy. The control group (n=25) receives standard care without EPO.

Results

Cardiac and renal function as well as a panel of biomarkers and iron parameters are being assessed. Furthermore, the effects of EPO on monocyte gene expression profiles and on endothelial progenitor cells are being evaluated.

Conclusion

This translational study is designed primarily to discern haematopoietic from non-haematopoietic effects of EPO in cardiorenal patients. The study will add insights into the mechanisms that could explain the fragile balance between desirable and undesirable effects of EPO

INTRODUCTION

Coexistence of chronic heart failure (CHF) and chronic kidney disease (CKD) has a worse prognosis than failure of either organ alone. We recently proposed a model of the cardiorenal syndrome in which cardiac and renal dysfunction mutually amplify progressive failure of both organs[1]. Observational data indicate that haemoglobin (Hb) levels are correlated with hospitalization and mortality in dialysis patients and in CHF patients[2, 3] which led to the belief that recombinant human erythropoietin (EPO) treatment of anaemia may improve outcome. In addition to well-documented haematopoietic effects, EPO can diminish inflammation, reduce renin-angiotensin system and sympathetic nervous system activity, and shift the nitric oxide (NO) / reactive oxygen species (ROS) balance toward NO. Moreover EPO has been reported to increase progenitor cells which may be important, since in CKD and in advanced stages of CHF, the number and function of endothelial progenitor cells (EPCs) is decreased[4, 5]. Based on these experimental data, it was hypothesized that EPO treatment exerts beneficial effects in patients with CHF and CKD. However, large-scale trials failed to demonstrate a beneficial effect of EPO when targeting normalization of Hb in CKD patients[6, 7]. This could be related to the Hb levels attained, or alternatively to unwanted non-haematopoietic effects of (high dosages of) EPO such as endothelial dysfunction and/or increased thrombogenicity. Also patient-related factors may play a role, since it is known that EPO resistance in itself is associated with worse outcomes.[3, 8] The objective of the Mechanisms of Erythropoietin Action in the Cardiorenal Syndrome (EPOCARES) study is to investigate haematopoietic as well as non-haematopoietic effects of EPO treatment in patients with CKD and CHF. Therefore, the study design is not intended to show differences in hard end points but specifically allows identification of desirable and undesirable effects of EPO as measured by biomarkers, genomics and cell studies.

METHODS

Overall study design

EPOCARES is an open-label randomized trial, including patients with the combination of CHF, CKD and anaemia. Complete inclusion and exclusion criteria are outlined in Table 1. The aetiology of both CHF and CKD and the sequence in which the 2 conditions arise is not important in the selection of the patients[1] but patients with active systemic disease as a cause of CHF or CKD are excluded. The study is being carried out in compliance with the Helsinki Declaration, and the protocol has been approved at each participating center by its internal review board. In all patients, standard treatment is started, comprising oral iron supplementation, aspirin when indicated and maximal tolerated dosages of a β-blocker, an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin receptor blocker, according to CHF guidelines. Patients are randomized once they have been clinically stable on standard treatment for at least 4 weeks. One group receives a fixed dose of 50 IU/kg per week of EPO (Neorecormon; Roche Pharmaceuticals) to increase the Hb level to a maximum of 13.7 g/dL for men and

13.4 g/dL for women. Another group receives 50 IU/kg per week EPO maintaining baseline Hb for the first 6 months by sequential blood withdrawal up to a maximum of 250 mL per 2 weeks. The third group does not receive EPO, but may receive a red blood cell transfusion in the unlikely event that Hb falls below 10.3 g/dL. In aggregate, 75 subjects will be enrolled. Randomization is stratified for EPO resistance (defined as an observed/predicted log[serum EPO] ratio less than 0.6), and allocation is performed in blocks of 6 patients (block randomization), using a computerized table of random numbers. In addition, for biomarkers, genomics and cell studies, 25 healthy, age- and sex-matched controls will be recruited. Hb level will be checked at least monthly in all patients. In the second group, Hb level will be measured every 2 weeks during the first 6 months of the study to assess the necessity of phlebotomy.

Differentiation between haemotopoietic effects and non-haemotopoietic effects of EPO

Figure 1 depicts the measurements that are performed throughout the study period. Since Hb level does not increase until about 4 weeks after starting EPO treatment, non-haemotopoietic effects of EPO treatment are assessed 2 weeks after initiation of treatment. Moreover, comparing the 2 active groups after 6 months provides an additional way to discern haematopoietic and non-haematopoietic effects of EPO.

Table 1 I Inclusion and exclusion critera

Inclusion criteria
Age >18 years, < 85 years
Hb between 10.3 and 12.6 g/dL in men and between 10.3 and 11.9 g/dL in women
Heart Failure (diastolic and systolic)
GFR by Cockcroft-Gault formula of 20-70 ml/min
Exclusion criteria
Erythropoietic therapy within 6 months before randomization
Uncontrolled hypertension (SBP >160 mm Hg, DBP >100 mm Hg)
Uncontrolled diabetes (HbA1c >8.0%)
Kidney transplantation
Proteinuria >3.5 g/L
Acute renal failure or rapidly progressive glomerulonephritis
Hyperparathyroidism (PTH >40 pmol/L)
Haemoglobinopathies, bleeding or haemolysis as a cause of anaemia
Deficiency of iron, folate and/or vitamin B12
Chronic inflammatory disease or clinically significant infection
Haematological malignancy of solid tumor < 3 years ago
Enrolment in another study
Alcohol and/or drugs abuse
Women with child-bearing potential

Abbreviations: GFR, glomerular filtration rate; Hb, haemoglobin; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycosylated haemoglobin; PTH, parathyroid hormone

Cardiac and renal function

CHF is defined as New York Heart Association (NYHA) class II or higher, based on symptoms, signs and objective evidence of an abnormality in cardiac structure or function according to the European Society of Cardiology (ESC) guidelines. Patients with heart failure with preserved left ventricular ejection fraction (LVEF) (HFPEF) or heart failure with reduced LVEF (HFREF) will be included. HFPEF is defined according to the recent ESC consensus. Echocardiography will be performed according to the recommendations of the American Society of Echocardiography. Diastolic function will be assessed using standard methods. Cardiovascular magnetic resonance imaging (CMR) and magnetic resonance angiography (MRA) of the renal arteries will be performed on a 1.5-tesla Philips Intera (Philips Medical Systems). In a 45-minute protocol, both cardiac function and the renal arteries are assessed. The cardiac function analysis will be performed using ECG-triggered multiphase, multislice steady-state free precession (SSFP) short axis scans. Volumes and ejection fraction will be acquired by manually tracing endocardial and epicardial contours on the stack of contiguous short-axis cine-images at end-diastole and endocardial contour at end-systole. The left ventricular mass will be calculated by multiplying the summed area between the endocardial and epicardial contour by the specific density of myocardial tissue. A bolus of 30-ml cyclic gadolinium-based contrast (Dotarem; Geurbet, France) will be administered intravenously to obtain delayed enhancement scans of the ventricles (inversion recovery T1 pulse) in 4-chamber, short axis and left 2-chamber view. At the time of injection, the renal arteries will be examined, while delayed enhancement of the heart will be acquired after 15 minutes. Assessment of segmental wall motion and late enhancement will be performed by 2 independent investigators. The left ventricle (LV) will be divided into 17 segments according to standardized nomenclature. Late enhancement will be estimated by using a 5-group classification according to the degree of LV wall involvement. Cardiopulmonary exercise performance will be measured up to the symptom-limited maximum. Exercise capacity will be evaluated by peak oxygen consumption (VO2max). CKD is defined as estimated creatinine clearance (Cockcroft-Gault formula) 20-70 ml/min. Albuminuria is assessed by 24-hour urinary collection.

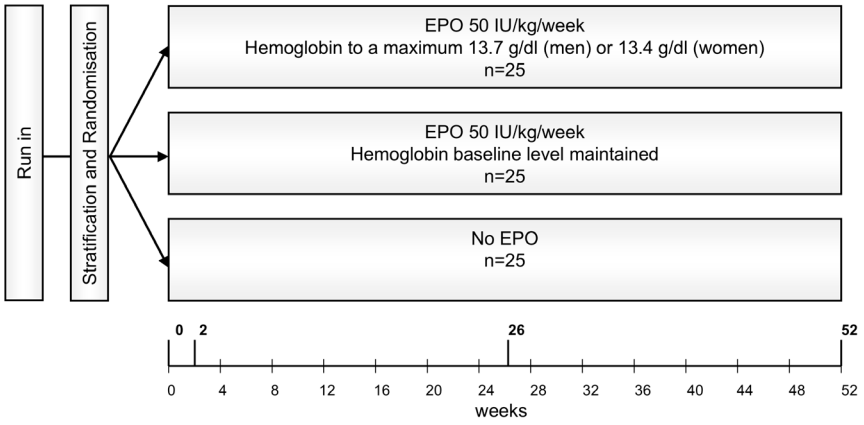

Figure 1 | Study design and main time points of measurements during the study period.
Abbreviation: EPO, recombinant human erythropoietin

Biomarkers

Blood samples are collected to allow a comprehensive panel of biomarkers to assess oxidative status, components of the renin-angiotensin-aldosterone system including prorenin, catecholamines and inflammation. In addition to routine iron parameters, hepcidin is measured, using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS).

Endothelial function/arterial stiffness

Global endothelial function will be assessed by measuring circulating endothelial dysfunction markers. Augmentation index (the difference between early and late pressure peaks divided by the pulse pressure amplitude) and aortic pulse wave velocity are determined using applanation tonometry (SphygmoCor).

Cellular mechanisms

Monocytes are both biosensors of the atherosclerotic environment and mediators of vascular damage. Monocyte gene expression profiles are determined by ILLUMINA bead-arrays with qPCR as confirmation. Circulating type I EPCs, defined as CD34+/KDR+ EPCs and CD34+ haematopoietic stem cells, are determined in peripheral blood by flow cytometry. Peripheral blood-derived mononuclear cells will be isolated by Ficoll density-gradient centrifugation. After 7 days of culture in specific medium rich in serum and growth factors, the amount of EPC outgrowth will be assessed. Migratory, proliferative, adhesive and angiogenic capacity of the EPC outgrowth is determined.

Statistics

The study is not powered for hard end points, but for intermediate end points as measured by biomarkers, imaging data and cell studies. We intend to study 25 subjects per group based on power calculations for several parameters. For instance, for ejection fraction, n=20 with 5% as a minimum relevant and measurable change and power = 0.8 (SD 5%; alpha=0.05); and for estimated glomerular filtration rate (eGFR), n=20 with a minimum relevant and measurable change in GFR per year of 5 ml/min and power = 0.8 (SD 5%; alpha=0.05). Power analysis of several biomarkers, such as plasma TBARS, has been determined in a previous study in our department in patients with CKD, and resulted in n=25 with power=0.8 and alpha=0.05. Power calculations for other variables mentioned were all around n=20. Similar trials had a comparable numbers of subjects. Validity of the statistical analysis will be monitored by the Center of Biostatistics of the University of Utrecht.

Safety concerns

Safety procedures regarding Hb levels and phlebotomies are described in an appendix of the protocol. Hb levels are measured frequently to assure maintenance of the appropriate Hb level. When necessary, EPO dose is reduced, but no dose escalation is performed. Blood is withdrawn in a way that minimizes the risk of rapid volume shifts. At any time during the study, EPO will be withheld for any patient who experiences an adverse event reported by the investigator to be related to the study drug.

DISCUSSION

The efficacy and safety of anaemia treatment with EPO in patients with CKD and/or CHF is under debate. It is uncertain to what extent the haematopoietic or non-haematopoietic effects of EPO are responsible for the unexpected outcomes of recent studies[6, 9]. The EPOCARES study is specifically designed to assess both the haematopoietic and non-haematopoietic effects of EPO in a patient group that, on the one hand, may benefit from a higher Hb level and, on the other hand, may be vulnerable to unwanted effects of EPO. The study will provide more insights into mechanisms that underlie why anaemia correction does not always lead to reduction of cardiovascular risk. This could help to define appropriate EPO doses and Hb targets, and may help us to appreciate the full range of positive and negative effects of EPO.

The study has a specifically devised study design that justifies discussion of potential safety concerns in detail. One group receives a fixed low dose of EPO to a maximum Hb of approximately 13.5 g/dL. Since the study protocol was devised, 2 large-scale studies in CKD using similar Hb targets were published that did not show beneficial effects[6] and maybe even harm[7]. As a result the anaemia guidelines in most countries were modified to restrict EPO treatment to a target of 11.0-12.0 g/dL with a maximum of 13.0 g/dL. Our study however differs from these large-scale studies in that a low fixed dose of EPO is used. So, no dose escalation is performed if targets are not achieved. In the CHOIR study, to achieve the high Hb targets, high doses of EPO were used. Indeed, in the secondary analysis of this trial, the inability to achieve a target Hb level and high dosages of EPO were each associated with increased risk of cardiovascular events[10] whereas achieving higher Hb levels was not associated with worse outcomes. The intervention in the other active group keeps the patients at their baseline level of anaemia. Since the lower level of inclusion is 10.3 g/ dL, all patients will remain above the predefined levels of the Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines for starting EPO treatment in CKD patients.

To accurately describe cardiac and renal abnormalities, among others, CMR and MRA are performed, which involves intravenous gadolinium. CMR provides accurate and reproducible information about cardiac function, structure and aetiology of heart failure. However, after the study was started, the use of some gadolinium contrast agents (predominantly Gadodiamide, Omniscan) in MRA was linked to nephrogenic systemic fibrosis/fibrosing dermopathy (NSF/NFD), specifically in patients with advanced CKD. In the EPOCARES study the cyclic gadolinium compound gadoterate meglumine Dotarem is used, which is more stable. No cases of NSF using Dotarem have been formally reported in The Netherlands to the adverse events database. Nonetheless, it was decided to withhold MRA studies in patients with an eGFR <30 ml/min.

In conclusion, EPOCARES is a (small) translational study that is uniquely designed to specifically look into haematopoietic and non-haematopoietic effects of EPO in patients with combined heart and renal failure at the level of organ function, circulating cellular and humoral mediators and markers of cardiorenal disease.

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PART I

3

Determinants of red cell distribution width (RDW) in cardiorenal patients: RDW is not related to erythropoietin resistance

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ABSTRACT

Background

Studies have shown that red cell distribution width (RDW) is related to outcome in chronic heart failure (CHF). The pathophysiological process is unknown. We studied the relationship between RDW and erythropoietin (EPO) resistance, and related factors such as erythropoietic activity, functional iron availability and hepcidin.

Methods and Results

In the Mechanisms of Erythropoietin Action in the Cardiorenal Syndrome (EPOCARES) study, which investigates the role of EPO in 54 iron supplemented anaemic patients with CHF and chronic kidney disease (CKD) (n=35 treated with 50 IU/kg/wk Epopoetin beta, n=19 control), RDW was not associated with EPO resistance. We defined EPO resistance by EPO levels (r=0.12, p=0.42), the observed/predicted log EPO ratio (r=0.12, p=0.42), the increase in reticulocytes after 2 weeks of EPO treatment (r=-0.18, p=0.31) and the increase of haemoglobin after 6 months of EPO treatment (r=0.26, p=0.35). However, RDW was negatively correlated with functional iron availability (reticulocyte haemoglobin content, r=-0.48, p<0.001 and transferrin saturation, r=-0.39, p=0.005) and positively with erythropoietic activity (soluble transferrin receptor, r=0.48, p<0.001, immature reticulocyte fraction, r=0.36, p=0.01) and positively with interleukin-6 (r=0.48, p<0.001). No correlation existed between hepcidin-25 and RDW.

Conclusions

EPO resistance was not associated with RDW. RDW was associated with functional iron availability, erythropoietic activity and interleukin-6 in anaemic patients with CHF and CKD.

INTRODUCTION

Red blood cell distribution width (RDW) is routinely performed as part of a complete blood cell count and quantifies the variability in size of circulating red blood cells (i.e. anisocytosis), defined as the standard deviation of erythrocyte size divided by the mean corpuscular volume (MCV). Recently, researchers have reported an independent association between RDW and the risk of adverse outcomes in patients with chronic and acute heart failure[1-4], in patients with stable coronary artery disease[5] and even in a community-based cohort[6-8]. The pathophysiological mechanism responsible for the association between RDW and adverse outcome is open to question. Anaemia is highly prevalent in chronic heart failure (CHF) and is associated with morbidity and mortality[9]. Most factors that cause anaemia through ineffective red cell production or increased red cell destruction could cause anisocytosis. Importantly however, RDW remained an independent predictor of outcome after adjusting for haemoglobin level[1].

Erythropoietin (EPO) resistance, that is the inadequate bone marrow response to *endogenous* and/or *exogenous* EPO leading to an impaired red blood cell line, plays an important role in anaemia of CHF and chronic kidney disease (CKD)[10]. Resistance to EPO is associated with morbidity and mortality[11]. Several authors hypothesize that EPO resistance could explain the association between RDW and outcome[2, 4, 12]. Inflammation and disordered iron metabolism are factors that can cause EPO resistance and indeed, recent studies have shown that inflammatory markers, EPO levels and decreased functional iron availability correlate with RDW[2, 4]. However, no direct data are available as to the association between RDW and EPO resistance. The Mechanisms of Eryhtropoietin Action in the Cardiorenal Syndrome (EPOCARES) study created an opportunity to investigate the association between RDW and EPO resistance in iron-supplemented, EPO naive patients with CHF and CKD[13]. Because a universally accepted definition of EPO resistance does not exist, we estimated EPO resistance in three ways using 1. the log observed/predicted ratio (O/P), which reflects the EPO level for the degree of anaemia[14], 2. the extent in increase of reticulocyte count, soluble transferrin receptor or immature reticulocyte fraction after two weeks of exogenous EPO treatment, and 3. the haemoglobin increase after 6 months of EPO treatment. In addition, we investigated the role of associated factors, such as inflammation, erythropoietic activity (rate of red cell production), functional iron availability and hepcidin.

METHODS

Study design and patients

The study design of the EPOCARES study (ClinicalTrials.gov number NCT 00356733) has been published elsewhere[13]. In short, the EPOCARES study is an open-label, prospective, randomized trial, in which patients with CHF, CKD (glomerular filtration rate by Cockroft-Gault equation of 20-70 ml/min) and mild anaemia (haemoglobin 10.3-12.6 g/dL for men and 10.3-11.9 g/dL for women) are included to test the haematopoietic and non-haematopoietic responses to EPO treatment. Exclusion criteria, amongst others, were erythropoietic therapy within 6 months, bleeding,

haemolysis, haemoglobinopathies, chronic inflammatory disease or malignancy. Haemoglobin (Hb) level for inclusion was measured after at least four weeks of oral iron supplementation, if tolerated. The diagnostic criteria for CHF were those recommended by the European Society of Cardiology guidelines[15]. Patients with heart failure with reduced left ventricular ejection fraction (HFREF) as well as patients with preserved left ventricular ejection fraction (HFPEF) were included[16]. The Medical-Ethical Committee approved the protocol of the study and informed consent was obtained from all subjects. Procedures were in accordance with the Helsinki Declaration and all patients gave written consent.

Patients were randomized on a 1:1:1 basis, after they had been clinically stable on maximal tolerated doses of a ß-blocker, an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker for at least four weeks. One group received 50 IU/kg/wk Epopoetin beta (Neorecormon®, Roche Pharmaceuticals) and their Hb was kept at baseline level by phlebotomies during the first 6 months. One group received 50 IU/kg/wk Epopoetin beta and their Hb could rise (to a certain safety level). The third group received standard treatment. Most biochemical measurements were performed at baseline, after two weeks and monthly thereafter. Blood samples were drawn between 8 and 9 AM in supine position and stored at -80°C until analysis.

Biomarker analysis

Levels of Hb, haematocrit, MCV, RDW, white blood cells, platelets, reticulocyte count and reticulocyte haemoglobin content (Ret-He) were measured with the use of a Sysmex XE-2100 haematology analyzer (Toa Medical, Kobe, Japan).

High sensitivity C-reactive protein (hs-CRP) was determined by particle-enhanced immunonephelometry using a standard Cardio-Phase hsCRP for BNII (Dade Behring Holding GmbH, Liederbach, Germany). Plasma interleukin-6 (IL6) levels (pg/ml) were measured in duplo using a commercially available high sensitive ELISA kit (R&D Systems, Minneapolis, USA).

As a marker of total iron stores[17], ferritin was determined using a sandwich immunoassay on an Acces®2 immunoanalyzer within a Dx automated system from Beckman Coulter (Brea, CA). Functional iron availability was determined by measuring transferrin saturation (TSAT), soluble transferrin receptor (sTfR) and Ret-He. TSAT was calculated from serum iron and transferrin estimates obtained with standard methods on a Beckman Coulter Dx. sTfR assay was performed with an immunoassay on a BNProSpec nephelometer from Siemens (Marburg, Germany). Ret-He was performed using flow cytometric analysis with Ret-Search (II)® dye on a Sysmex XE-2100 haematology analyzer (Toa Medical, Kobe, Japan).

Erythropoietin levels, erythropoietic activity and EPO resistance

Serum EPO levels were measured by a two-site sandwich chemiluminescent immunoassay on an IMMULITE 2000 platform (Siemens Healthcare Diagnostics, Breda, the Netherlands). As markers of erythropoietic activity, we measured sTfR and assessed the ratio of young immature reticulocytes (IRF). Reticulocytes have variable amounts of RNA, which correlates with their maturation. The fluorescent intensity of a reticulocyte, measured by using a fluorescent polymethine dye, is proportional to the quantity of RNA. Reticulocytes are thus divided in the most

immature, moderately immature (together comprising IRF) and mature reticulocytes. An increase in IRF precedes the increase in reticulocyte count and is therefore used as a marker of erythropoietic activity [18, 19].

EPO resistance was measured in multiple ways. *Endogenous* EPO resistance was determined by defining the EPO levels for the degree of anaemia, by calculating the observed/predicted log (EPO) ratio (O/P ratio). EPO levels were defined as inappropriate at an O/P ratio <0.80. The O/P ratio can be calculated as follows: O/P ratio=[log(observed Epo)]/[log(predicted Epo)]. To predict EPO levels, we used the regression equation as defined by Opasich et al: log(Epo)=4.746–(0.275xHb)[14]. Patients were stratified by O/P ratio at inclusion. *Exogenous* EPO response was measured as the increase of reticulocyte count, sTfr and IRF after 2 weeks of EPO treatment and by assessing the Hb response after 6 months of EPO treatment.

Hepcidin-25

Serum hepcidin-25 measurements were performed by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry[20]. Serum hepcidin-25 concentrations were expressed as nmol/l. The lower limit of detection of this method was 0.5 nM; average coefficients of variation were 2.7% (intra-run) and 6.5% (inter-run). The median reference level of serum hepcidin-25 as measured at 08.30 am is 2.9 nM, range 0.5-8.2 nM[20-22].

Statistical analysis

Data are presented as medians with inter-quartile ranges (IQR) for non-normally distributed variables and means ± standard deviation (SD) for normally distributed continuous variables. Unpaired data were compared with the unpaired t-test or the Mann-Whitney U test. For paired data we used the paired t-test or Wilcoxon Rank test. Pearson correlation coefficient was used to test univariate correlation with RDW in normally distributed variables. Skewed variables were log-transformed. Analysis of variance (ANOVA) was applied on parametric variables. Differences were considered significant when $P<0.05$. Following univariate correlations, variables with a P-value<0.05, were entered into a multivariable linear regression model with stepwise forward selection process, to identify independent predictors of RDW. For statistical analyses the Statistical Package for Social Sciences (SPSS, Chicago, Illinois, USA) version 17 was used.

RESULTS

Population characteristics

The original study population of the EPOCARES study comprised of 62 patients. Five patients withdrew their informed consent and 1 patient was excluded because of malignancy (diagnosed on routine X-ray). Baseline RDW data were missing for 2 patients. Baseline characteristics of the 54 patients for this study are presented in Table 1, divided according to tertiles of RDW. Univariate linear correlations are listed in Table 2. In Table 3, the multivariable regression analysis is shown.

All patients had CKD, CHF and anaemia, as shown by the decreased estimated glomerular filtration rate (eGFR) using the modified diet in renal disease formula

(MDRD), the higher NT-proBNP, the lower LVEF and the lower Hb-levels. Vitamin B12 and folate levels were normal and haemolysis was absent, as measured by lactodehydrogenase levels. CRP and hs-CRP levels were only slightly elevated, showing that the study involved chronic stable patients without evident inflammation. At baseline there was no significant differences in all above-mentioned variables among the 3 study groups.

Table 1 | Baseline characteristics of patients from the EPOCARES study, stratified by RDW values

Characteristics*	All patients n=54	1st tertile RDW < 13.4%	2nd tertile RDW 13.4-14.7%	3rd tertile RDW ≥ 14.8 %	P value
Age (yrs)	74 [69-80]	72 [68-75]	77 [71-82]	77 [66-81]	0.27
Male sex, n (%)	35 (65%)	12 (67%)	12 (67%)	11 (61.1%)	0.49
Haemoglobin (g/dL)	11.8 ± 0.9	11.6 ± 0.9	12.1 ± 0.9	11.8 ± 0.9	0.36
Haematocrit (%)	0.35 ± 0.03	0.35 ± 0.03	0.32 ± 0.12	0.34 ± 0.09	0.71
MCV (/μm^3)	90 ± 4	90 ± 3	91 ± 5	89 ± 4	0.60
MDRD (ml/min/1.73m^2)	34.7 ± 13.8	30.5 ± 13.8	39.0 ± 15.3	34.7 ± 11.5	0.18
Cockroft Gault (mL/min)	36.7 ± 14.9	33.9 ± 11.6	38.4 ± 17.2	37.8 ± 15.7	0.62
Cystatin C (mg/L)	1.72 [1.36-2.47]	2.03 [1.50-2.72]	1.49 [1.08-2.45]	1.76 [1.48-2.37]	0.43
NT-proBNP (pg/mL)	1453 [718-265]	1128 [482-1887]	1306 [718-2162]	2352 [926-6688]	0.08
LVEF (%)	43.8 ± 11.7	45.7 ± 11.8	47.1 ± 10.3	37.9 ± 10.5	0.97
Iron (µmol/L)	10 [8.8-14]	12 [10-14]	10 [9-15]	9 [8-12]	0.08
Ferritin (ng/mL)	126 [75-175]	140 [68-198]	126 [90-195]	106 [55-141]	0.46
Transferrin (g/dL)	2.2 [2.0-2.5]	2.1 ± 0.23	2.3 ± 0.32	2.4 ± 0.51	0.19
TSAT (%)	20 [15.8-25.0]	24 [18.8-29.0]	20 [16.5-25.3]	17 [14.0-20.3]	**0.020**
Ret-He (fmol)	1.9 ± 0.14	1.94 ± 0.09	1.93 ± 0.14	1.80 ± 0.14	**0.003**
Hepcidin-25 (nM)	5.9 [3.6-7.9]	6.9 [3.5-10.0]	5.8 [4.3-7.4]	5.0 [2.9-8.5]	0.64
sTfR (mg/L)	1.40 ± 0.47	1.19 ± 0.31	1.35 ± 0.42	1.67 ± 0.53	**0.003**
Erythropoietin (IU/L)	13.0 [7.0-16.0]	11.5 [6.8-15.3]	14.5 [10.0-18.0]	12.5 [7.0-16.8]	0.59
O/P ratio	0.78 ± 0.19	0.74 ± 0.18	0.80 ± 0.19	0.80 ± 0.20	0.55
Reticulocytes (1012/L)	0.046 ± 0.02	0.038 ± 0.01	0.050 ± 0.01	0.049 ± 0.02	**0.026**
IRF (%)	8.8 [5.4-11.4]	5.6 [3.8-9.2]	9.2 [6.4-11.2]	11.0 [7.4-14.7]	**0.003**
CRP (mg/L)	6 [2-12]	2.5 [1-5]	7.0 [2-12]	7.0 [4-20]	0.03
hs-CRP (mg/L) (n=37)	5.8 [2.0-10.4]	2.4 [0.9-6.4]	6.2 [2.2-10.2]	7.2 [3.8-20.2]	0.09
IL-6 (pg/mL)	3.7 [1.9-5.6]	2.5 [1.6-3.3]	3.7 [1.8-4.8]	7.1 [3.6-10.7]	**<0.001**
Vitamin B12 (pg/mL)	277 [214-408]	277 [232-468]	239 [171-362]	329 [259-515]	0.16
Folate (ng/mL)	17.5 [12.5-40.7]	20.2 [11.6-45.0]	16.5 [11.2-29.7]	21.7 [15.4-45.0]	0.48
LDH (U/L)	408 [353-487]	397 [326-428]	422 [370-574]	411 [373-534]	0.19
Albumin (g/L)	36.7 ± 3.0	37.0 ± 2.3	37.8 ± 2.3	35.4 ± 3.6	**0.045**
Cholesterol (mmol/L)	4.1 ± 1.1	4.6 ± 1.26	4.1 ± 0.98	3.6 ± 0.94	**0.044**

MCV=mean corpuscular volume, MDRD=estimated glomerular filtration rate by modified diet in renal disease formula, NTproBNP=N-terminal pro-brain natriuretic peptide, LVEF= left ventricular ejection fraction, TSAT= transferrin saturation, Ret-He= reticulocyte haemoglobin content, sTfR= soluble transferrin receptor, O/P ratio= log observed/predicted erythropoietin ratio, IRF=immature reticulocyte fraction, CRP=C-reactive protein, hs-CRP=high sensitive CRP, IL-6=Interleukin-6, LDH= lactate dehydrogenase
*values in mean ± standard deviation or median [interquartile range]

RDW at baseline

The median RDW value at baseline was 14.0% (interquartile range 13.3-15.1), and 26% of the patients had a level above the upper limit (>15%) which corresponds with data from other studies[1-5, 23]. In our normocytic anaemic population, a higher RDW was not associated with baseline Hb levels, haematocrit or MCV. RDW showed no correlation with renal function, as measured by creatinine, Cockroft-Gault equation, MDRD or cystatin C, nor with cardiac function as measured by left ventricular ejection fraction or NTproBNP levels at baseline (Tables 1 and 2).

Table 2 I Univariate Correlation Coefficients of clinical and biochemistry variables with RDW, EPOCARES study, n=54

Variable	RDW	
	r	P
Age	0.25	0.076
Haemoglobin	-0.05	0.72
Haematocrit	0.09	0.52
MCV	-0.08	0.58
MDRD	0.13	0.37
Cockroft Gault	0.11	0.43
Cystatin C	-0.26	0.85
NT-proBNP	0.24	0.10
LVEF	-0.17	0.22
Ferritin	-0.21	0.16
Transferrin	0.34	0.012
Transferrin saturation	-0.39	0.005
Ret-He	-0.48	<0.001
Hepcidin	-0.25	0.07
Soluble transferrin receptor	0.48	<0.001
Erythropoietin	0.12	0.42
O/P ratio	0.11	0.42
Reticulocyte count	0.13	0.36
IRF	0.36	0.01
CRP	0.23	0.11
hs-CRP (n=37)	0.27	0.13
IL-6	0.48	<0.001
Vitamin B12	0.25	0.073
Folate	0.07	0.61
Lactatedehydrogenase	0.21	0.13
Albumin	-0.17	0.23
Total cholesterol	-0.27	0.055

MCV=mean corpuscular volume, MDRD=estimated glomerular filtration rate by modified diet in renal disease formula, NT-proBNP=N-terminal pro-brain natriuretic peptide, LVEF= left ventricular ejection fraction, Ret-He= reticulocyte haemoglobin content, O/P ratio= log observed/predicted erythropoietin ratio, IRF= immature reticulocyte fraction, CRP=C-reactive protein, hs-CRP=high sensitive CRP, IL-6=Interleukin-6
All non-parametric variables were considered for analysis after logarithmic transformation

Table 3 I Stepwise multivariate linear regression for RDW from the EPOCARES study*, n=54.

Variable	Step no.	Multiple r^2	β coefficient	P
Soluble transferrin receptor	1	0.218	0.483	<0.001
Interleukin 6	2	0.356	0.394	<0.001
Reticulocyte haemoglobin content	3	0.434	-0.309	<0.001
Immature reticulocyte fraction	4	0.529	0.278	<0.001

* all non-parametric variables were considered for analysis after logarithmic transformation

Iron metabolism

In these patients on oral iron supplementation (if tolerated) without overt inflammation, ferritin at baseline was < 100 ng/ml in 22 of the patients (41%), indicating that some patients may have been (relatively) iron deficient despite oral supplementation. Indeed, also baseline TSAT levels were low in some patients (< 20% in 26 of the patients or < 15% in 8 patients). However, in the 35 patients that received EPO, there was no significant decrease in Ret-He after two weeks of EPO treatment (p=0.32). This indicates that there was no iron-restricted erythropoiesis in these patients. Therefore no direct evidence of decreased functional iron availability in the patients that received EPO was observed[24]. At baseline there was no difference in the variables for iron metabolism between the EPO treated groups versus the control group, therefore we conclude that there was no iron-restricted erythropoiesis in the whole study population.

Total iron store variables at baseline did not correlate with RDW (Tables 1 and 2). However, functional iron availability was negatively associated with RDW; patients in the highest RDW-tertile had both significant lower TSAT level and Ret-He levels at baseline, as demonstrated by univariate correlation in Table 2 and further depicted in Figure 1A. Baseline hepcidin-25 showed no significant correlation with RDW.

Inflammation

A positive correlation was observed between RDW and IL6 at baseline, but this was not the case with CRP, nor with hs-CRP. There was a negative correlation between IL6 with Ret-He (p=0.03, r= -0.30). As previously described, there was no correlation between both IL6 and hs-CRP with hepcidine-25 (respectively p=0.93, r=-0.12 and p=0.27, r=0.20) in our population[25].

EPO levels, erythropoietic activity and EPO resistance

There was a positive correlation between RDW and erythropoietic activity as measured by sTfR and IRF at baseline (univariate correlation Table 2 and scatter plot in Fig. 1B and 1C), but this was apparently not related to higher endogenous EPO levels, because there was no correlation between baseline EPO levels and RDW (Table 2). The EPO level was defined for the degree of anaemia by the baseline O/P ratio. Given that the average O/P ratio was only slightly below 0.80, the endogenous EPO production was partly preserved in this population with CKD and CHF. We found no correlation with RDW and endogenous EPO resistance as measured by the O/P ratio (Table 2).

Of the 54 patients, 35 patients received EPO treatment. After two weeks of EPO

treatment, the reticulocyte count significantly increased (p<0.0001) as did sTfR (p<0.0001) and IRF (p= 0.027). Also, after 2 weeks of EPO treatment, RDW was significantly increased (p<0.001). In the control group, without EPO treatment, the reticulocyte count (p= 0.21) did not change, nor did IRF (p= 0.62) or RDW (p=0.80). The magnitude of increase in reticulocyte count in the EPO treated group, however, did not correlate with the baseline RDW values. Neither did the extent in increase of sTfR and IRF after two weeks EPO treatment correlate with baseline RDW. These correlations between the increase in reticulocyte count, sTfR and IRF after two weeks with log-transformed RDW at baseline are depicted in Figure 2A, 2B and 2C.
In 17 of the 35 patients who received EPO treatment, the Hb was let to increase. After 6 months of EPO treatment the Hb in these patients was significantly increased compared to baseline Hb (p=0.001). The magnitude in increase of Hb after 6 months, showed no correlation with RDW at baseline (p=0.35, r= 0.26). Neither was there any correlation with baseline RDW and the magnitude of increase in reticulocyte count, sTfR and IRF after 6 months EPO treatment (resp. p=0.54, p=0.39 and p=0.36). These results show that there is neither a correlation between baseline RDW and response to exogenous EPO.

Multivariable regression model

After entering all baseline biomarkers with a significant univariate correlation with baseline RDW in a multivariable regression model, on a stepwise forward selection, sTfR, IL6, Ret-He and IRF proved to be independent predictors of RDW (Table 3).

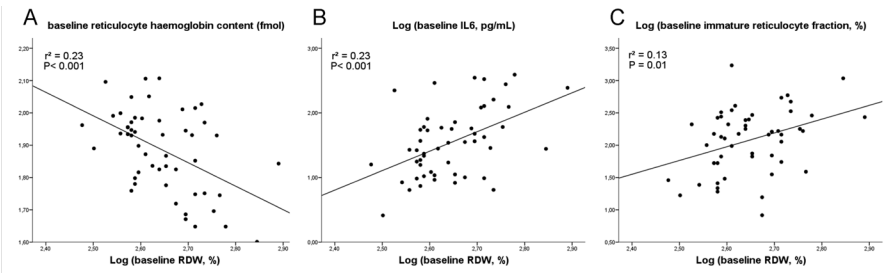

Figure 1 | The correlation between log-transformed baseline red cell distribution width (RDW) values and baseline levels of (A) reticulocyte haemoglobin content, (B) log transformed interleukin-6 and (C) log transformed immature reticulocyte fraction in 54 cardiorenal patients.

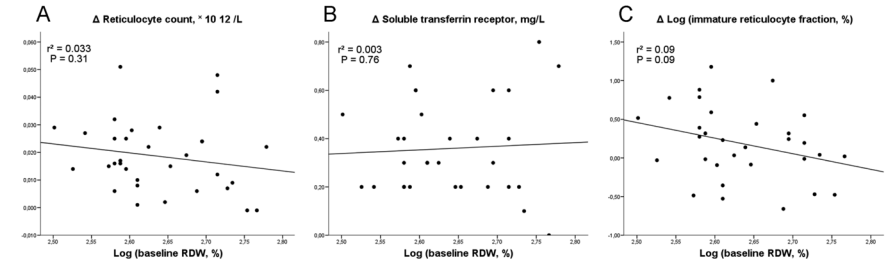

Figure 2 | The correlation between log-transformed baseline red cell distribution width (RDW) values and (A) the reticulocyte increase, (B) the increase in soluble transferrin receptor and (C) the increase in immature reticulocyte fraction, after two weeks of erythropoietin treatment in 35 cardiorenal patients.

DISCUSSION

A strong independent association exists between RDW, a measure of anisocytosis, and adverse outcomes in cardiovascular disease (e.g. CHF). The underlying process that links RDW to outcome is unknown. One of the main findings of this study is that EPO resistance as measured by several different methods was not associated with RDW. In this stable patient group with both heart and renal failure, RDW was associated with functional iron availability and erythropoietic activity. After multivariate analysis, markers of functional iron availability, erythropoietic activity and IL-6 were independent predictors of RDW. However, hepcidin levels were not significantly associated with RDW. This underscores earlier findings that, in low inflammatory patient groups, hepcidin levels are not associated with markers of inflammation[25, 26].

Erythropoietin (EPO) resistance, defined as an inadequate bone marrow response to *exogenous* or *endogenous* EPO, contributes to anaemia[11] and is associated with increased mortality in patients with heart and/or kidney failure [27, 28]. Indeed, EPO levels in patients with CKD and/or CHF are higher as compared to healthy controls, but inappropriately low for the degree of anaemia [10, 14]. This observation indicates a relative EPO deficiency as well as a reduced bone marrow response to endogenous EPO [11]. Approximately, 10% of CKD patients treated with *exogenous* EPO have an inadequate response, which leads to therapy with higher doses of exogenous EPO. In several studies, such as the Correction of Haemoglobin and Outcomes in Renal Insufficiency (CHOIR) study, the use of high-dose EPO in EPO-resistant patients was associated with increased morbidity and mortality[27]. Subsequently it was hypothesized by several authors that a disturbed bone marrow response to erythropoietin could explain the association between morphologic changes in the red blood cell (RDW) and cardiovascular risk[2, 4, 12]. However, in the current study, none of the markers of EPO resistance, estimating both the response to *endogenous* EPO as well as the response to *exogenous* EPO, was associated with RDW. Furthermore, in our EPO-naïve patient group treatment with exogenous EPO induced an increase in RDW. This contradicts an association between resistance to EPO and RDW.

We did find a significant correlation between RDW and markers of erythropoietic activity at baseline (IRF and sTfR). It is important to note that increased erythropoietic activity not necessarily results in increased haemoglobin levels since the level of haemoglobin is determined by the red cell production *and* maturation rate and by the rate of red cell destruction. IRF is defined as the ratio of young, immature reticulocytes to the total number of reticulocytes. IRF is used to assess the degree of erythropoietic activity (e.g. after chemotherapy)[18]. Circulating reticulocytes shed the soluble transferrin receptor during their maturation sequence. This sTfR level correlates more strongly with corpuscular indices than with iron parameters and is used as a biomarker of increased erythropoietic activity[29]. Förhécz et al. also reported a positive correlation between sTfR and RDW in a non-anaemic cohort of patients with systolic heart failure[2]. This concept is further strengthened by our results, in which RDW is positively related to erythropoietic activity, as measured by both IRF and sTfR, in anaemic patients with heart and kidney failure.

RDW is most commonly used in the differential diagnosis of iron deficiency anaemia, in which MCV is decreased and RDW is increased. Decreased functional iron

availability plays a role in anaemia of CHF and CKD. We demonstrate a negative correlation between baseline TSAT, Ret-He, sTfR and RDW in our iron-supplemented patients. Ret-He is considered to be a very sensitive indicator reflecting iron availability for erythropoiesis[30] and is an indicator for iron-restricted erythropoiesis in patients receiving EPO[24]. Compared with the use of ferritin or TSAT, it compared better in sensitivity and specificity[17]. Therefore, although 41% of our orally iron-supplemented patients had a ferritin level < 100ng/ml based, indicating possible lower iron stores, the lack of decrease in Ret-He after EPO therapy suggests that there was no overtly iron-restricted erythropoiesis. Because we found no association between ferritin levels and RDW, our data suggest that decreased functional iron availability but not iron stores play a role in higher RDW values. Our results correspond with recently published results[2, 4], describing a positive correlation between RDW and TSAT and/or sTfR, in symptomatic heart failure patients. In addition to these studies, we determined Ret-He, a more sensitive marker of functional iron availability, which further substantiated the contention that increased RDW values are associated with decreased functional iron availability.

As mentioned earlier, in our study, RDW was associated with markers of both erythropoietic activity and functional iron availability. Furthermore, a strong correlation was observed between RDW and IL-6. It has been hypothesized that the correlation between RDW and functional iron availability is mediated by hepcidin[4]. Hepcidin is upregulated by a number of stimuli (e.g. anaemia and inflammation, IL6). Hepcidin thus integrates input from erythropoietic and inflammatory pathways[31]. In patients with CKD[32] it has been shown that hepcidin levels are higher compared to healthy controls, but in patients with CHF and anaemia this was not confirmed [26, 33]. In our patients with the combination of heart- and kidney failure and anaemia hepcidin-25 levels, were higher. Thus, hepcidin seems an obvious "candidate-linking factor" between inflammation and decreased functional iron availability, leading to higher RDW levels. However, our data show no clear correlation between hepcidin-25 and RDW. It should be noted that, since this is a small study and the p-value (p=0.07) approached significance, a weak association between RDW and hepcidin couldn't be ruled out. Also, there was no correlation between hepcidin-25 and inflammation in our patient group. This finding was confirmed in another study and is in keeping with our earlier finding that in our group of stabile cardiorenal patients, with relatively low levels of inflammatory biomarkers, increased hepcidin levels were associated with markers of iron load (ferritin) rather than with markers of inflammation [25 26]. Thus, in our study RDW was associated with IL-6 but not with other markers of inflammation. Although data exist that IL-6 can influence iron absorption during the hypoxic exposure, via a mechanism independent of hepcidin[34], at this point it is unclear how IL-6 is related to an increase in RDW.

RDW is elevated in conditions of increased erythropoiesis/ineffective erythropoiesis and in conditions of increased red cell destruction. RDW increases when the relative number of larger and/or smaller red blood cells increases. Since RDW is not correlated to MCV in our patient group, it can be hypothesized that the changes in RDW are caused by both an increase in the relative number of large red cells as well as an increase of the relative number of small red cells in the peripheral blood. Indeed, MCV positively correlated with both Ret-He (r=0.387, p=0.004) as well as with IRF (r=0.245, p=0.08). The absence of a correlation between RDW and MCV

in our anaemic patients thus may be due to a balanced net effect of increased erythropoietic activity leading to a higher MCV and decreased iron availability, leading to a lower MCV.

Finally, limitations of the study as result of sample size and selection bias need to be acknowledged. The cohort size of the two center EPOCARES study is rather small. Studying simple associations and constructing a multivariate model using a relatively small sample size is of limited value, although most of the associations we report are robust despite the small sample size. However, we cannot fully exclude the possibility that the lack of association between some parameters, e.g. hepcidin, and RDW, are due to lack of power. This patient group comprised anaemic patients with both CHF and CKD, therefore these results should be carefully interpreted and cannot be generalized to all patients with CHF, especially those patients without renal dysfunction. Also, the EPOCARES patients were receiving multiple drugs, including oral iron supplementation, throughout the study, and were in a relatively low inflammatory state. This might not fully represent daily clinical practice on which data of RDW as biomarker for outcome are based.

In conclusion, EPO resistance was not associated with RDW in these iron supplemented anaemic patients with CKD and CHF. However, RDW was associated with erythropoietic activity, decreased functional iron availability and IL-6. We found no significant correlation between hepcidin and RDW. In our view, as also pointed out by Allen et al., the association of RDW with outcome may imply that the erythrocyte may be viewed as a "barometer" of overall cardiovascular health [4]. Therefore mechanisms that cause changes in relative distribution of red cell size such as increased erythropoietic activity, increased red cell destruction and reduced red cell half life should be investigated.

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PART I

Does the erythropoietic effect of low-dose erythropoietin account for its effect on quality of life and cardiac function in cardiorenal patients?

4

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In preparation

ABSTRACT

Background

The EPOCARES study was designed to differentiate erythropoietic from non-erythropoietic effects of low-dose erythropoiesis stimulating agent (ESA) in combined chronic heart and kidney failure (CHF/CKD). Here we report cardiac function, quality of life (QoL) and exercise capacity.

Methods and results

Fifty-six anaemic patients (median age 74) with CKD (Cockroft-Gault 36 ± 15 ml/min) and CHF, on iron supplementation, were randomized into three groups: Two groups received 50 IU/kg/week ESA during 6 months. In one group haemoglobin levels (Hb) were allowed to increase (ESA-Hb-rise); in the other group Hb-levels were maintained at baseline level by phlebotomy (ESA-Hb-stable). The control group did not receive ESA. The area under the curve (AUC) for the Hb-change in time was calculated to assess the Hb-response. The AUC after 6 months was 19.8 ± 17.1 in the ESA-Hb-rise group, 5.9 ± 9.6 in the ESA-Hb-stable group and -2.2 ± 9.4 in the control group (rise vs stable group, p=0.009). Only the ESA-Hb-rise group demonstrated an increase in QoL and ejection fraction (from 41.8 ± 8.2 to 45.2 ± 10.4 %, p= 0.048) and a decrease in end systolic volume (from 69 ± 35.9 to 61 ± 38.0 ml, p= 0.028). RDW levels at baseline correlated negatively with QoL and exercise capacity (RAND-36 r=-0.363, p=0.008; pVO2/kg r = -0.586, p< 0.001).

Conclusions

Positive effects of a low, fixed dose of ESA on cardiac function and QoL in cardiorenal patients is dependent upon red cell levels, given the abrogation of the effects by phlebotomy. Together with the negative correlation between RDW, a marker of anisocytosis, and QoL this underscores the central role of red cell dynamics in cardiorenal patients.

INTRODUCTION

Anaemia in patients with heart and/or renal failure is associated with reduced exercise tolerance and quality of life (QoL)[1, 2]. Although impaired erythropoietin (EPO) production and EPO resistance causes anaemia in these patients[3-6], studies investigating the use of erythropoiesis stimulating agents (ESAs) in patients with chronic kidney disease (CKD) failed to demonstrate a favourable effect on cardiovascular outcome, when targeting normal haemoglobin (Hb) levels[7, 8]. In chronic heart failure (CHF) we are awaiting the results of the Reduction of Events with Darbepoetin Alfa in Heart Failure Trial (RED-HF)[9]. It is unknown why treating anaemia with high-dose ESA results in unfavourable outcomes. The actions of the glycoprotein hormone EPO are not limited to erythropoiesis but also include protection to ischemia by anti-apoptosis and a wide array of effects on the cardiovascular system[10, 11]. Thus, the results of the ESA studies may be related to the attained high Hb-levels (erythropoietic effects), to (dose-related) non-erythropoietic effects, or due to unwanted effects of high-dose ESA occurring predominantly in patients with EPO resistance[3]. The lack of success of the ESA studies has shifted the focus from erythropoietin deficiency to iron deficiency as a causative factor in the association between anaemia and outcome[12, 13]. Yet it remains to be established which exact mechanisms interfere with red cell production and decay in these patients leading to anaemia and adverse outcomes.

Interestingly, recent studies have shown that red cell distribution width (RDW; the standard deviation of mean corpuscular volume), a marker of red cell turnover, is a strong predictor of mortality and morbidity in CHF and other chronic diseases, independently of anaemia [14, 15]. Moreover, Craenenbroeck et al. showed in CHF that RDW levels are independently and inversely related to exercise tolerance, as measured with peak oxygen uptake consumption (pVO_2)[16].

We hypothesized that the red blood cell production and decay plays a pivotal role in cardiorenal failure and that low dose ESA improves cardiac function, QoL and exercise performance through its effect on erythropoiesis. Furthermore, we tested whether RDW is a determinant of QoL, cardiac function and exercise capacity in cardiorenal failure. The Erythropoietin in the Cardiorenal Syndrome (EPOCARES) study created an opportunity to compare the erythropoietic effects of ESA to the non-erythropoietic effects in iron-supplemented, ESA-naive patients with cardiorenal failure[17].

METHODS

Study design and patients

This study is part of the EPOCARES study (ClinicalTrials.gov number NCT 00356733), which is an open-label, prospective, randomized trial that is designed to investigate the erythropoietic versus the non-erythropoietic effects of low-dose ESA in patients with cardiorenal failure and anaemia. The study design is not intended, nor powered, for hard end points, but for intermediate end points as measured by biomarkers, imaging and cell studies[17]. Stable patients of the age >18 and <85 years with CKD (Cockroft-Gault equation 20-70 ml/min), CHF and mild anaemia (Hb 10.3 to 12.6 g/

dL for men and 10.3 to 11.9 g/dL for women) were included. Patients with an active systemic disease, malignancy, uncontrolled hypertension/diabetes (respectively SBP>160mmHg, DBP>100mmHg or HbA1C>8.0%), previous erythropoietic therapy < 6 months, anaemia due to bleeding, haemolysis, vitamin B_{12}, folate or iron deficiencies were excluded. Iron deficiency was assessed by measurement of serum iron, ferritin and transferrin saturation (TSAT) levels. Both patients with heart failure with reduced left ventricular ejection fraction (LVEF) as well as patients with heart failure with preserved LVEF were included[18].

All eligible patients started with a standard run in treatment, at least 4 weeks prior to inclusion and randomization, consisting of oral iron supplementation and maximal tolerated dosages of a β-blocker, an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin receptor blocker, according to CHF guidelines[19]. If the subjects were still anaemic after at least 4 weeks oral iron supplementation, they were included and randomized into three groups. One group received a fixed dose of 50 IU/kg per week of ESA (Neorecormon®; Roche Pharmaceuticals) to increase the Hb level to a maximum of 13.7 g/dL for men and 13.4 g/dL for women (the ESA-Hb-rise group). The second group also received 50 IU/kg per week ESA, but the Hb levels in these patients were maintained at baseline level by sequential blood withdrawal (the ESA-Hb-stable group). The third group, the control group, received standard care, without ESA. Randomization was stratified for EPO resistance (defined as an observed/predicted log[serum EPO] ratio less than 0.6)[20]. All baseline measurements were performed after randomization. The safety procedures regarding Hb levels and phlebotomies are described in an appendix of the protocol. In the patients treated with EPO, blood was withdrawn if the Hb exceeded 14.0 g/dL in men or 13.8 g/dL in women, while the low dose (50 IU/kg) of EPO was maintained. Blood was withdrawn up to a maximum of 250 ml per session, to a maximum of 250 mL per 2 weeks. In this way, the risk of rapid volume shifts was minimal. When necessary, the ESA dose was reduced, but no dose escalation was performed. The Medical-Ethical Committee approved the protocol of the study and informed consent was obtained from all subjects. Procedures were in accordance with the Helsinki Declaration and all patients gave written consent.

Biomarker measurements

All blood samples were drawn between 8 and 9 AM. As a marker of total iron stores, ferritin was determined. Functional iron availability was determined by transferrin saturation (TSAT), soluble transferrin receptor (sTfR) and reticulocyte haemoglobin content (Ret-He). As markers of erythropoietic activity, we measured sTfR and IRF. Ret-He is considered to be an accurate measure of functional iron availability for erythropoiesis over the previous 3-4 days, due to the 4-day life span of a reticulocyte[21]. sTfR is a marker for both functional iron availability as well as erythropoietic activity, due to the shedding of transferrin receptors by circulating reticulocytes during their maturation[22]. IRF is the ratio of young immature reticulocytes (IRF), which precedes the increase in reticulocyte count. Ret-He was measured with the use of a Sysmex XE-2100 haematology analyzer (Toa Medical, Kobe, Japan). sTfR assay was performed with an immunoassay on a BNProSpec nephelometer from Siemens (Marburg, Germany).

The area under the haemoglobin change curve

Two study groups received identical dosages of ESA treatment during the study period. In the ESA-Hb-stable group the Hb levels were to be kept stable at baseline level using phlebotomies. To assess the necessity of a phlebotomy, a preceding increase in Hb was required, for which the Hb levels were measured every 2 weeks, which renders comparison of single time-point measurements unreliable. We therefore assessed the Hb response by calculating the area under the curve for Hb change over time (Hb AUC). The AUC is a more reliable estimate of Hb response to ESA treatment and better reflects the haemoglobin level a patient a patient is exposed to throughout the study [23]. The Hb AUC was calculated by linear trapezoidal integration, in which the total area under the Hb change versus time curve is obtained by summation of each individual area between two consecutive time points, as extensively described elsewhere[23]. The Hb AUC was based on monthly Hb measurements in all patients.

Cardiopulmonary exercise testing

Maximal exercise performance was measured with cardiopulmonary exercise testing (CPET) on an incremental cycle ergometer (Excalibur; Lode, Groningen, the Netherlands). Measurements of oxygen uptake (VO_2), carbon dioxide production (VCO_2) and minute ventilation (VE) were taken breath-by-breath (Oxycon pro; Mijnhart-Jaeger, Bunnik, the Netherlands) and averaged over 15-s intervals. The peak VO_2 (pVO_2) was calculated as the mean of the values during the final 30s of exercise. The anaerobic threshold was defined using the V-slope method. The slope of the relationship between VE and VCO_2 (VE/VCO_2) was determined.

Quality of life

We used two questionnaires to assess the QoL at baseline and after 6 months. The Minnesota living with heart failure questionnaire (MLHF) is a 21-item questionnaire evaluating different components of quality of life during the last month[24]. The RAND 36-item Health Survey (RAND-36) consists of several subscales, each generating a score ranging from 0 to 100[25]. Completeness of the questionnaire was checked.

Echocardiography and cardiac magnetic resonance imaging

Echocardiography was performed at baseline and after 6 months, using an iE33 (Philips Medical systems, Best, the Netherlands). LVEF was quantitatively assessed in the two- and four chamber views following recommendations of the European Society of Echocardiography[26], by two experienced clinical investigators (ME and MC), blinded for clinical data. Cardiac magnetic resonance imaging was performed on a 15 Tesla Philips Intera (Philips Medical Systems, Best, the Netherlands) and the protocol and assessment have been described elsewhere[27]. A trained investigator (ME) performed the quantitative image analyses.

Statistical analysis

Data are presented as means ± standard deviation (SD) or median with inter-quartile ranges (IQR) as appropriate. Normality of data was evaluated using the Kolmogorov-Smirnov test. Non-normally distributed variables were log transformed, after which normality was checked again. Differences between groups were compared with

the unpaired student's t-test, Mann-Whitney U test or χ^2 –test where appropriate. Paired data were compared with the paired student's t-test or the paired Wilcoxon-Rank test. Pearson correlation was used for bivariate correlations. Following bivariate correlations, variables with a $p < 0.10$, were entered into a multivariable linear regression model with stepwise forward selection process. Differences were considered significant when $P<0.05$, two-sided. For statistical analyses the Statistical Package for Social Sciences (IBM, Chicago, Illinois, USA) version 18 for OS-X was used.

RESULTS

Baseline demographic and clinical characteristics

Of the 62 included patients for the EPOCARES study, five patients withdrew their informed consent and 1 patient was excluded because of a suspected malignancy. Baseline characteristics of the 56 patients, divided into the three study groups, are presented in Table 1. The study groups were not different at baseline. During the course of the study, 6 patients died; 3 patients due to terminal heart failure, 1 due to an abdominal sepsis, 1 due to an out-of-hospital-cardiac-arrest and 1 died due to ventricular fibrillation (3 in control, 2 in ESA-Hb-rise and 1 in ESA-Hb-stable).

The effect of ESA on haematological variables and RDW

Based on the single time point Hb levels after 6 months, Hb levels increased significantly compared to baseline in the ESA-Hb-rise group ((from 11.8 ± 1.1 to 13.3 ± 1.4 g/dL, $p< 0.001$) and more moderately in the ESA-Hb-stable group (from 11.7 ± 0.8 to 12.6 ± 0.7 g/dL, $p=0.001$). The Hb levels in the control group did not change (from 11.8 ± 0.8 to 12.1 ± 1.3 g/dL, $p= 0.71$). The Hb AUC, depicting the cumulative Hb change over time and a better reflection of the overall exposure to higher Hb level than a comparison at 1 time point, was significantly higher in the ESA-Hb-rise group compared to the ESA-Hb-stable group (respectively 19.8 ± 17.2 vs 5.9 ± 9.6, $p= 0.009$; Figure 1). Furthermore, the Hb AUC in the control group (-2.2 ± 9.4) did not differ from the ESA-Hb-stable group ($p= 0.99$).

Levels of inflammatory markers were, as expected, low in this stable carefully selected patient population. Baseline hs-CRP levels were numerically, but not significantly, higher in the ESA-rise group, yet the opposite was true for levels of IL-6.

Although there was a decrease in ferritin values in both ESA groups (ESA-Hb-rise group, $p=0.008$ and in the ESA-Hb-stable group, $p<0.001$) suggesting an iron deficiency, there was no decrease in TSAT or Ret-He in both ESA groups after 6 months compared to baseline, indicating that there was no iron-restricted erythropoiesis under oral iron supplementation (TSAT levels in the ESA-Hb-rise group, $p=0.70$ and in the ESA-Hb-stable group, $p=0.15$, for Ret-He levels $p=0.82$ in the ESA-Hb-rise and $p=0.86$ in the ESA-Hb-stable group). sTfR increased in both ESA groups due to the increased erythropoiesis ($p< 0.001$) and remained stable in the control group ($p= 0.24$).

The effect of ESA on quality of life

After 6 months, the ESA-Hb-rise-group demonstrated an improvement in QoL; both in the MLHF and the RAND-36 score (Fig 2 AB). The MLHF decreased (from 35.3 ± 18.2 to 25.1 ± 17.4, p=0.01) and the RAND-36 increased (from 54.9 ± 13.8 to 63.6 ± 15.3, p=0.03), both indicating an improved QoL. In the ESA-Hb stable-group and the control group, there were no significant differences in the total scores of MLHF and RAND-36 at 6 months compared to baseline (Figure 2 AB).

The effect of ESA on exercise capacity

Of 56 patients, 46 patients performed CPET at baseline of which 38 achieved the anaerobic threshold. Ten patients were incapable to perform CPET. The respiratory exchange ratio at maximal exercise at baseline and after 6 months did not change. ESA treatment was not associated with an increase in pVO_2/kg values after 6 months in the ESA-Hb-stable group (from 16.4 ± 3.9 to 16.4 ± 4.1 ml/kg/min, p=ns), the ESA-Hb-rise group (from 14.9 ± 3.9 to 15.2 ± 4.0 ml/kg/min, p=ns) or the control group (from 14.9 ± 3.0 to 15.1 ± 2.9 ml/kg/min, p=ns), nor was this the case with pVO_2 absolute values. No significant differences were either observed in VE/VCO_2 values.

Table 1 | Baseline characteristics of the patients

Characteristic	ESA-Hb-stable n=18	ESA-Hb-rise n=19	Control n=19	p- value
Age, years	78 [69-81]	74 [70-80]	72 [66-77]	0.31
Male sex, no. (%)	10 (55.6)	13 (68.4)	14 (73.7)	0.49
BMI (kg/m^2)	26.1 ± 4.9	25.7 ± 3.6	27.4 ± 4.3	0.54
Cockcroft Gault (ml/min)	35 ± 10	36 ± 13	39 ± 20	0.88
NT-proBNP (pg/mL)	1767 [762-3127]	1373 [524-2151]	1680 [659-2610]	0.78
hs-CRP (mg/L)	2.8 [1.1-10.9]	6.8 [1.7-11.4]	4.3 [1.7-6.9]	0.44
Interleukin-6 (pg/mL)	4.1 [1.8-5.5]	2.9 [2.2-4.9]	3.2 [2.3-7.0]	0.94
RAS inhibitor, n (%)	17 (94.4)	18 (94.7)	19 (100.0)	0.59
Aetiology of heart failure				0.80
Ischemic, no. (%)	10 (55.6)	12 (63.2)	13 (68.4)	
Hypertensive, no. (%)	4 (22.2)	2 (10.5)	3 (15.8)	
Valvular, no. (%)	3 (16.7)	3 (15.8)	1 (5.3)	
Other, no. (%)	1 (5.6)	3 (15.8)	2 (10.5)	
Haemoglobin (g/dL)	11.7 ± 0.84	11.8 ± 1.07	11.8 ± 0.79	0.95
Haematocrit (%)	0.36 ± 0.025	0.35 ± 0.036	0.35 ± 0.026	0.81
MCV (/μm^3)	90.1 ± 4.2	90.6 ± 4.2	89.2 ± 4.3	0.50
Ferritin (ng/mL)	127 [87-179]	136 [71-307]	128 [76-164]	0.81
Transferrin saturation (%)	19 [15-24]	23 [17-26]	20 [18-29]	0.63
Erythropoietin (IU/L)	13 [7-15]	14 [10-19]	15 [5-17]	0.64
RDW (%)	14.3 ± 1.06	14.2 ± 2.14	14.3 ± 1.53	0.48

Mean ± standard deviation or median [interquartile range] are shown. Abbreviations: BMI, body mass index; NT-proBNP, N-terminal pro-brain natriuretic peptide; hs-CRP, high sensitivity CRP; RAS inhibitor, renin-angiotensin-system inhibitor; MCV, mean corpuscular volume; RDW, red cell distribution width

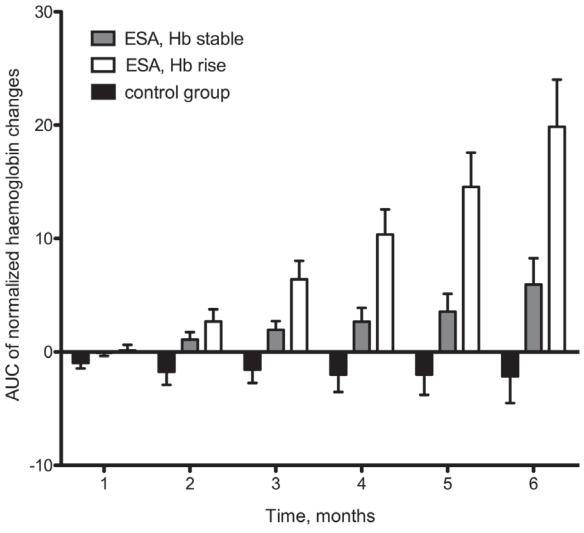

Figure 1 | Development of haemoglobin levels relative to baseline, depicted as the area under the curve of haemoglobin changes over time in months. Abbreviations: AUC, area under the curve Bars represent mean and standard error of the mean

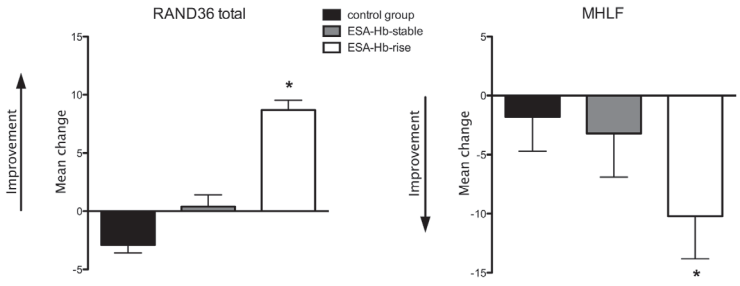

Figure 2 | Shown are mean changes at 6 months compared to baseline for quality of life scores. Panel A: MLHF, Minnesota living with heart failure questionnaire; the overall score ranges from 0 to 105, where higher scores indicate poorer quality of life. Panel B: RAND-36, RAND 36-item Health Survey; it consists of several subscales where each generates a score ranging from 0 to 100. Higher scores indicate a better health related quality of life. Bars represent mean change and standard error of the mean, * $p < 0.05$

The effect of ESA on cardiac function

Mean changes in NT-proBNP levels, LVEF and end systolic volume (ESV) after 6 months compared to baseline are shown in Figure 3. The NT-proBNP levels decreased in the ESA-Hb-rise group (from 1373 [524-2151] to 941 [452-1721] pg/mL, p= ns), although not significant, while in the ESA-Hb-stable group a even more moderate decrease was observed (from 1767 [762-3127] to 1091 [747-2251] pg/mL, p= ns, Fig 3A). In the ESA-Hb-rise group, the LVEF significantly increased (from 42 ± 8.2 to 45 ± 10.4%, p= 0.048) with a decrease in ESV (from 69 ± 35.9 to 61 ± 38.0 ml, p= 0.028). The end diastolic volume (EDV) did not change (from 112 ± 48.9 to 105 ± 51.7 ml, p=ns). The ESA-Hb-stable and the control group showed no significant changes in LVEF, ESV or EDV (ESA-Hb-stable group; LVEF from 45 ± 13.9 to 46 ± 10.9 %, p=ns, ESV from 58 ± 33.8 to 56 ± 35.9 ml, p=ns).

Cardiac MRI studies were only performed in those patients without cardiac

implantable electronic devices and with eGFR > 30 ml/min/1.73m^3. Unfortunately, there was a skewed randomization; 17 patients in the ESA-Hb-stable group, only 8 patients in the ESA-Hb-rise group and 9 in the control group. However, the MRI data were consistent with the echocardiographic data; after 6 months, the LVEF in the ESA-Hb-stable group showed no change (from 43 ± 11.2 to 42 ± 13.6 %, p=ns), nor did the ESV (from 111 ± 57 to 117 ± 63 ml, p=ns). In the ESA-Hb-rise group the LVEF increased (from 43 ± 11.3 to 49 ± 13.7%, p=ns), although this was not significant in this small cohort. The ESV decreased, but not significantly (from 106 ± 24 to 89 ± 57 ml, p=ns). There were no changes in the control group.

Associations at baseline with quality of life and exercise performance

At baseline a higher RDW correlated negatively with QoL, as measured by both the MHLF and the RAND-36 (resp. p=0.022, r=0.317 and p=0.008, r= -0.363, Figure 4A). Furthermore, exercise capacity (MLHF with peak VO_2/kg p=0.04, r=-0.309, RAND-36 with peak VO_2/kg; p=0.01, r=0.364), parameters for functional iron availability (TSAT with MLHF; p=0.002, r= - 0.432, TSAT with RAND-36; p= 0.006, r= 0.382, sTfR with MLHF; p<0.001, r= 0.469, sTfR with RAND-36; p=0.002, r= -0.402) and inflammation (CRP with MLHF p=0.014, r=0.330, and interleukin-6 with MLHF p=0.044, r= 0.276) correlated strongly with QoL. Remarkably, ventilator efficiency (VE/VO_2), Ret-He and hs-CRP showed no correlation with QoL (data not shown).

A multiple linear regression analysis was performed with QoL as the dependent variable, correcting for all variables with a p-value < 0.10 in bivariate correlations. The independent predictors for the MLHF were sTfR and CRP (resp. β=0.369, p<0.001 and β=0.289, p=0.014), while for the RAND-36 the sole independent determinant was pVO2 absolute (β= 0.411, p=0.08). When parameters for exercise capacity were excluded from this model, sTfR and CRP were the main dependent associates for both the MLHF and the RAND-36. RDW proved not to be an independent determinant of QoL. Thus, inflammation, functional iron availability and/or red cell turnover and exercise capacity are independently associated with QoL in these anaemic patients with cardiorenal failure.

Additionally, RDW correlated negatively with parameters of maximal exercise capacity (pVO_2/kg; p< 0.001, r = -0.586, Figure 4B) and positively with ventilator efficiency (VE/VCO_2 slope; p=0.04, r= 0.345), corresponding with the results of Craenenbroeck et al.[16].

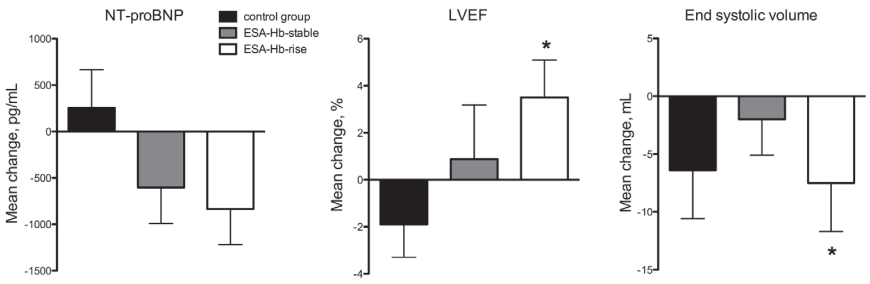

Figure 3 I Shown are mean changes at 6 months compared to baseline for NT-proBrain natriuretic peptide levels (NT-proBNP, panel A), left ventricular ejection fraction by echocardiography (LVEF, panel B) and end systolic volume by echocardiography (panel C). Bars represent mean change and standard error of the mean, * p < 0.05

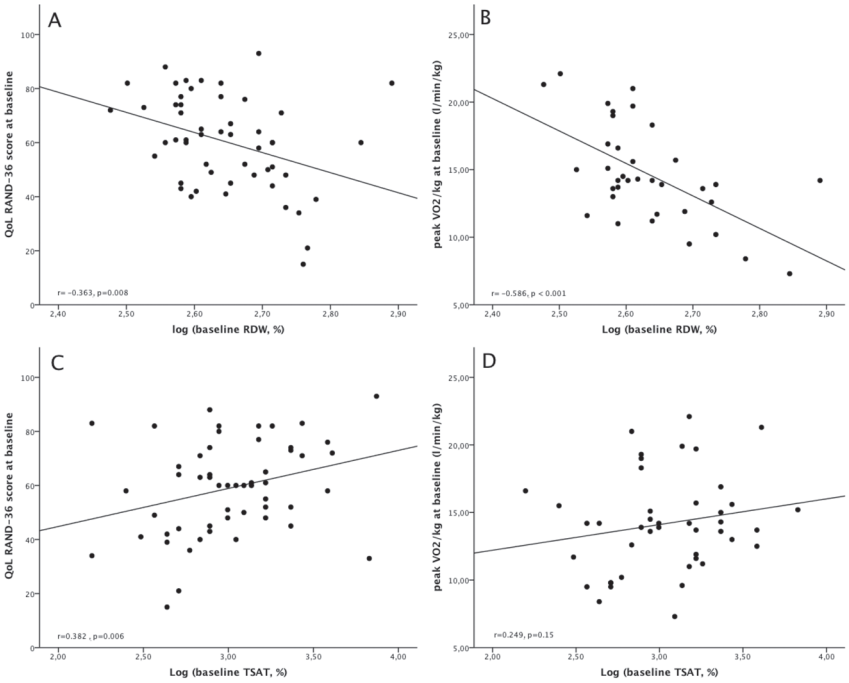

Figure 4 | Shown are the correlations between A. log-transformed baseline RDW values and baseline RAND-36 score, B. log-transformed baseline RDW values and baseline peak VO2/kg values, C. log-transformed baseline TSAT levels and baseline RAND-36 score, D. log-transformed baseline TSAT levels and baseline peak VO2/kg values. Abbreviations: RDW, red cell distribution width; pVO2/kg, peak oxygen uptake consumption/kg; TSAT, transferrin saturation score

DISCUSSION

This prospective randomized study compared the erythropoietic and the non-erythropoietic effects of low-dose ESA treatment on cardiac function, quality of life (QoL) and exercise capacity in anaemic patients with cardiorenal failure. In this cohort, ESA-induced improvement of cardiac function and QoL was related to the erythropoietic effects of ESA. The notion that erythropoiesis is important in cardiorenal failure, was further supported by the negative correlation at baseline between RDW levels, a parameter of red cell turnover, and QoL and exercise capacity.

Our unique study design allowed us to demonstrate that the beneficial effects of low, fixed dose ESA treatment, on cardiac parameters and QoL are specifically related to its erythropoietic effects. QoL, as assessed by two validated questionnaires, and LVEF increased, while ESV levels decreased in those patients whose Hb was allowed to rise, while this was not the case in patients receiving similar ESA dosages without increasing Hb levels. As shown in Figure 1, we succeeded in creating a large separation in haemoglobin levels between the groups. The ESA stable group also displayed a small increase in Hb and AUC at the time point of 6 months after initiation of treatment. As opposed to single time point estimates (e.g. at 6 months),

the AUC reflects the time a patient is exposed to a Hb level and thus better corresponds with long-term modulation of erythropoiesis.

Despite these beneficial effects on cardiac function and QoL, we found no effect on exercise capacity of low-dose ESA treatment. In contrast, Mancini et al. demonstrated an increase in peak VO2 with higher dosages of ESA compared to placebo in 15 patients with CHF[28]. Our data are in accordance with the report by Ponikowski et al.[29] demonstrating an improved health-related QoL, but no increase in peak VO_2 during (lower dosages) ESA treatment in CHF. Moreover, in the STAMINA-Heft study, in which ESA was dosed to reach pre-set Hb-targets, no improvement of peak VO2 was observed[30]. Importantly, a post hoc analysis of the STAMINA-Heft study showed that the increase in exercise duration was associated with the attained Hb-levels, which is in keeping with the notion that the effect of ESA on exercise capacity is related to its erythropoietic effect[30].

RDW, a routine measurement of anisocytosis, has emerged within the last few years as a powerful predictor of poor outcome in patients with chronic and acute heart failure[14, 31], independent of anaemia. Recently, Craenenbroeck et al. demonstrated that higher RDW values in CHF are independently related to impaired exercise capacity, as measured by pVO_2[16]. In this study we confirm and extend this association between exercise capacity and RDW values to anaemic patients with cardiorenal failure (Figure 4B). Moreover, we demonstrate a clear association between RDW values and QoL (Figure 4A). The observed association between RDW and both QoL and exercise capacity underscores the notion that red cell production and decay is of major importance in cardiorenal failure. Since exercise capacity in heart failure is linked to iron deficiency one may hypothesize that iron deficiency accounts for the association between RDW and exercise capacity and QoL. Although we confirmed the association between iron deficiency and exercise capacity, this association was weak as compared to RDW and exercise capacity (Figure 4B and 4D). In multiple linear regression analysis QoL was linked to RDW via a complex of factors associated with inflammation, iron homeostasis and erythropoiesis. These data confirm and point at the significance of a disordered iron homeostasis and red cell turnover in anaemic patients with cardiorenal failure.

It should be noted that, as a consequence of the complexity of the study design, this is a single blind study with relatively small sample size. Nevertheless, our study results are robust and consistent throughout the different methodologies used; QoL and cardiac function increased in parallel, as assessed by echocardiography and cardiac MRI. Furthermore, we need to acknowledge that studying associations and constructing a multivariate model using a small patient cohort comes with some limitations, although our data are in accordance with recent published studies. Finally, our patient cohort was carefully selected and comprised stable ambulant mildly anaemic patients with cardiorenal failure. Therefore interpretation of these results should be done carefully and cannot be generalized to other populations, especially to those patients with CHF without CKD or acute heart failure.

Thus, red cell production may play a pivotal role in cardiorenal failure. Treatment with low fixed dose ESA may not necessarily elicit identical results to the use of higher ESA dosages, targeting normalization of Hb-levels. Indeed, in our small cohort low-dose ESA seems to have favourable actions. We therefore propose further research using low fixed doses of ESA treatment, without targeting for certain Hb

levels, in CKD, CHF or the combination of these two. The importance of erythropoiesis in cardiorenal failure is further emphasized by the association between RDW, a marker of red cell turnover, and QoL and exercise capacity.

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CHAPTER 4

EPO AND QUALITY OF LIFE AND CARDIAC FUNCTION

PART I

Red cell distribution width associates with physical activity and heart failure and coronary heart disease events, independent of established risk factors, inflammation or iron homeostasis; the EPIC-Norfolk study

5

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ABSTRACT

Aims

Red cell distribution width (RDW) is associated with increased risk of heart failure (HF) and coronary heart disease (CHD). We examined in a healthy population (1) whether this association is independent of established cardiovascular risk factors and iron metabolism and (2) whether RDW associates with physical activity.

Methods and results

Hazard ratios (HRs) for the risk of CHD and HF by quartiles of RDW were calculated in 17,533 participants from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk cohort. During a follow up of 11.2 ± 2.2 years, 1987 participants developed a CHD and 640 a HF event. The HR for CHD events was 1.89 (95%CI 1.66-2.14, p<0.001) and 2.21 (95%CI 1.77-2.76, p <0.001) for HF events. Adjustment for established risk factors (sex, age, diabetes, smoking, systolic blood pressure, total and high-density lipoprotein cholesterol) attenuated the HR for CHD to 1.34 (95%CI 1.17-1.53, p<0.001) and for HF to 1.40 (95%CI 1.11-1.77, p=0.001). Adjustment for CRP, iron and ferritin levels did not affect the HR for CHD or HF. RDW levels inversely associated with physical activity (per category β= -0.37, 95%CI -0.053 - -0.021, p<0.0001), independent of iron and ferritin levels.

Conclusions

RDW levels associated with physical inactivity and the risk of CHD and HF events, independent of established risk factors or inflammation. The risk of HF and the association with physical inactivity was also independent of iron metabolism. This suggests that RDW reflects a different pathophysiological mechanism than represented by established risk factors, inflammation or iron metabolism.

INTRODUCTION

Red cell distribution width (RDW), a measure of anisocytosis, is a strong risk factor for increased all-cause mortality, cardiovascular death, symptomatic heart failure and coronary events in patients with heart failure (HF)[1-3], coronary heart disease[4, 5] (CHD) and even in the general population[6-8]. In patients with chronic HF this association was independent of risk factors for HF, such as anaemia and renal function[1]. The underlying pathophysiology linking increased anisocytosis with both incident HF and CHD events is incompletely unravelled. However, there is a growing body of evidence that inflammation, disordered iron homeostasis and red cell turnover are involved in the pathophysiology of HF and that RDW possibly reflects these processes[9-12]. RDW might therefore represent a different underlying pathophysiological process for cardiovascular risk than that represented by well-established risk factors, such as those included in the Framingham risk score.

Furthermore, recently high RDW levels were shown to be related to quality of life and impaired exercise capacity as measured by cardiopulmonary exercise testing in patients with chronic HF[13]. Impaired exercise capacity is related to both inflammation and iron deficiency in chronic HF[14, 15], suggesting that inflammation and iron homeostasis might link RDW with exercise capacity. It is unknown whether the association between RDW and exercise capacity or physical activity holds in the general population and whether the association between RDW and risk of HF is influenced by established risk factors, inflammation or iron homeostasis.

It was our objective to explore (1) whether the relationship between RDW and risk of both CHD and HF is independent of established cardiovascular risk factors, such as those included in the Framingham risk score and inflammation, (2) whether the relationship between RDW and the risk of HF is independent of iron metabolism and (3) whether RDW is inversely associated with physical activity in an apparently healthy middle-aged population and the effect of iron metabolism or inflammation on this association. We addressed these questions in the EPIC-Norfolk study, a large cohort of apparently healthy men and women.

METHODS

Study design

The European Prospective Investigation into Cancer and Nutrition (EPIC) Norfolk is a prospective population study of 25 639 men and women aged between 40 and 79 years, resident in Norfolk, United Kingdom. Details of the recruitment process, study design and population characteristics have been published earlier[16]. The study was originally designed to investigate risk factors for cancer but additional questions were asked to study risk factors for other diseases. The EPIC-Norfolk population is broadly similar to the UK population in terms of the distribution of anthropometric and cardiovascular risk factors, but with a lower rate of smokers. The EPIC-Norfolk study was approved by the Norfolk Local Research Ethics Committee and participants gave signed informed consent at each contact. The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology[17]

At the baseline survey between 1993 and 1997, participants completed a detailed health and lifestyle questionnaire including questions on history of diabetes, heart attack, stroke, cancer, smoking (current, former, never) and occupational status (manual, non-manual). The level of physical activity was assessed by a self-reported composite measure of leisure time and work related physical activity based on four questions, as described previously[18]. The EPIC physical activity questions refer to activity during the past year. The first question is a four point, mutually exclusive, ordered category concerning physical activity at work. The second question asks about the amount of time spent in hours per week for summer and winter separately in each of the following activities; walking, cycling, gardening, do-it-yourself, physical exercise and housework. The third question asks whether any of the activities in question 2 were engaged in such that it caused sweating or faster heartbeat and, if so, for how many hours during a typical week. The fourth question asks about stair climbing. The simple classification of self-reported occupational activity and categorization of time spent in cycling and other physical exercise were combined to form a physical activity index. Individuals were allocated into four categories of increasing physical activity: very inactive, moderately inactive, moderately active and very active. Specific definitions for the four categories are presented in Table 1. This index was validated against repeated individually calibrated heart rate monitoring in 173 individuals in 1 year[18]. Briefly, the volunteers wore a heart rate monitor continuously during the walking hours of the following 4 days. The energy expenditure data were summed over the day to create an estimate of daytime expenditure. After adjustment for age and sex, the self-reported physical activity index was positively correlated with mean daytime energy expenditure ($r=0.28$, $p< 0.001$). The unadjusted ($r=0.44$, $p< 0.001$) and adjusted ($r=0.45$, $p<0.001$) indices were more strongly correlated[18].

Biochemical analysis

Non-fasting blood samples were taken by venepuncture into plain and citrate bottles. Blood samples were stored for assay at the Department of Clinical Biochemistry, University of Cambridge, or stored at -80°C. Blood samples for haematology analysis were stored overnight at room temperature and were collected each morning by

Table 1 | Description of physical activity categories

Category	Description
Inactive	Sedentary job and no recreational activity
Moderately inactive	Sedentary job with < 0.5h recreational activity per day or Standing job with no recreational activity
Moderately active	Sedentary job with 0.5-1 h recreational activity per day or Standing job with 0.5h recreational activity per day or Physical job with no recreational activity
Active	Sedentary job with >1 h recreational activity per day or Standing job with > 1h recreational activity per day or Physical job with at least some recreational activity or Heavy manual job

The estimated average energy expenditure in MJ/day for each category, adjusted for age and sex, was as follows: inactive 9.9 (0.6); moderately inactive 10.9 (0.3); moderately active 11.5 (0.4) and active 12.8 (0.5)

EPIC-Norfolk technicians and transported to the EPIC-Norfolk laboratory in Attleborough, UK. A MD18 haematology analyser (Coulter Corporation, Miami, FL, USA) was used for analysis of RDW, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and leucocyte concentrations. RDW was calculated as (standard deviation of MCV / mean MCV) x 100. Serum concentrations of total cholesterol, HDL-C and triglycerides were measured with the RA 1000 (Bayer Diagnostics, Basingstoke, UK). When additional funding became available in 2010, serum concentrations of C-reactive protein (CRP) were measured in all participants with available frozen baseline serum samples using a full-range, high-sensitivity assay on an Olympus AU640 clinical chemistry analyser (Olympus UK Ltd). Serum iron and serum ferritin were also measured with an Olympus AU640 clinical chemistry analyser (Olympus UK Ltd) in 13,285 subjects. Estimated glomerular filtration rate was calculated by using the "Modification of Diet in Renal Disease (MDRD) formula[19].

Ascertainment of outcome definitions

Prevalent heart failure was defined by self reported intake of drugs that were recommended for the treatment of HF in clinical practice at the time of the baseline survey (loop diuretics in combination with digitalis or angiotensin-converting enzyme-inhibitors), as described previously.[20, 21] Ninety per cent of the patients in the UK general practices received one of these treatment regimes within 6 months after diagnosis of heart failure in 1996. This definition of prevalent HF proved to be highly specific when validated against B-type natriuretic peptide levels, which were measured in a random subgroup of 926 participants.[20, 22]

All participants are flagged for death at the UK Office of National Statistics. Death certificates are coded by trained nosologists using International Classification of Disease revision 10 (ICD-10). Participants are also linked to National Health Service hospital information systems so that hospital admissions anywhere in the UK are reported to EPIC-Norfolk through routine annual record linkage. Participants were identified as having a CHD event (e.g., unstable angina, stable angina and myocardial infarction) during follow-up if they were hospitalized or died and the underlying cause of hospital admission or death was coded as ICD-10 I20-I25. Patients were identified as having a HF event if they were hospitalized or died and the underlying cause of hospital admission or death was coded as ICD-10 I50. The current study is based on the follow-up through March 31st 2008.

Statistical analysis

Baseline characteristics are reported as mean ± standard deviation (SD) for continuous variables with normal distribution, median and interquartile range for continuous variables with non-normal distribution, or percentage (number) for categorical variables. The population was divided into RDW quartiles based on pooled sexes. Baseline characteristics were compared between RDW quartiles using one-way ANOVA for continuous variables and χ^2 test for categorical variables. Triglycerides, CRP and albumin/creatinin ratio were not normally distributed and therefore log transformed prior to testing. A Cox proportional hazards model was used to calculate hazard ratios (HR) and corresponding 95% confidence intervals (95% CI) for the risk of incident HF or incident CHD events by quartiles of RDW,

using the lowest quartile as a reference. Hazard ratios were calculated according to an unadjusted regression model and according to models that adjusted for established cardiovascular risk factors (sex, age, diabetes mellitus, smoking, systolic blood pressure, total cholesterol and high-density lipoprotein), established risk factors and estimated GFR, established risk factors and CRP, established risk factors, estimated GFR and CRP, established CV risk factors and serum iron and ferritin, as well as just serum iron and ferritin. To test whether the association between RDW and physical activity was influenced by inflammation or iron homeostasis, a linear regression model was used with RDW as dependent variable and physical activity as independent variable controlling for CRP, serum iron and ferritin separately. Analyses were performed using SPSS version 17.0.

RESULTS

Baseline characteristics

RDW data were available in 18,202 study participants. A total of 166 participants were excluded because they reported taking medical heart failure treatment at baseline and 503 participants were excluded because at baseline they reported having suffered a myocardial infarction, leaving 17,533 individuals for the current analysis. Baseline characteristics according to quartiles of RDW are shown in Table 2. Mean ± SD age of the study population was 58.8 ± 9.4 years. Mean RDW levels were 13.3 ± 1.0%. Higher quartiles of RDW were associated with higher age, higher body mass index, lower haemoglobin levels, higher systolic and diastolic blood pressure, hyperlipidaemia, worse renal function, lower serum iron and ferritin levels, higher CRP levels and physical inactivity. Smoking and micro albuminuria were only moderately associated with RDW levels, while the presence of diabetes mellitus showed no association with RDW levels.

Physical activity

In linear regression analysis performed on the entire population, physical activity categories were significantly inversely associated with RDW levels (per category β= -0.37, 95%CI -0.053- -0.021, p<0.0001). This association was still significant after adjustment for CRP (β= -0.024, 95%CI -0.040- -0.008, p<0.0001) and after adjustment for serum ferritin and serum iron levels (β= -0.31, 95%CI -0.047 - -0.015, p<0.0001).

RDW levels and risk of heart failure events

Among the 17,533 men and woman included in this analysis, 640 developed a HF event during a follow-up of 11.2 ± 2.2 years. In the highest RDW quartile, 251 people (5.5%) developed a HF event whereas in the lowest RDW quartile 113 people (2.6%) developed a HF event. The HR for incident HF was 2.21 (95% CI 1.77-2.76) for the highest RDW quartile (Table 3, Figure 1A). Adjustment for established risk factors (sex, age, diabetes, smoking, systolic blood pressure, total cholesterol and high-density lipoprotein) attenuated the HR for incident HF to 1.40 (95%CI 1.11-1.77, p for linearity 0.001, Figure 1B). Additional adjustment for estimated GFR, CRP or both, further attenuated this association (HR 1.34, 95%CI 1.01-1.79, p for linearity

0.019). Adjustment for serum iron and serum ferritin levels did not attenuate the association between RDW quartiles with incident HF (HR 2.66, 95% CI 1.99-3.56, p for linearity < 0.001, Figure 1D) compared to the unadjusted HR. We performed a sensitivity analysis excluding all CHF events occurring < 2 years from baseline in order to avoid the possibility that results were explained by latent CHF being present sub clinically at baseline. The results did not differ substantially from those reported above (data not shown).

Table 2 | Baseline characteristics by quartiles of red cell distribution width

	RDW quartiles				
	1	**2**	**3**	**4**	**P linearity**
n	4300	4171	4459	4603	
RDW (%)	12.3 ± 0.3	12.9 ± 0.1	13.4 ± 0.2	14.5 ± 1.1	
Male sex	38.4 (1652)	45.1 (1883)	49.2 (2193)	44.3 (2040)	<0.001
Age mean (years)	57.0 ± 9.0	58.4 ± 9.1	59.3 ± 9.3	60.6 ± 9.6	<0.001
Diabetes mellitus	1.9 (82)	1.8 (76)	2.0 (88)	2.2 (100)	0.67
Smoking:					0.046
Current	10.8 (462)	12.5 (516)	12.0 (531)	12.3 (560)	
Former	40.8 (1745)	41.2 (1704)	41.6 (1841)	42.5 (1941)	
Never	48.4 (2069)	46.3 (1913)	46.4 (2056)	45.2 (2062)	
Haemoglobin (g/dL)	13.8 ± 1.0	13.9 ± 1.2	14.0 ± 1.3	13.6 ± 1.6	<0.001
MCV (fL)	91 ± 3.3	90 ± 3.8	89 ± 3.8	87 ± 5.2	<0.001
White blood count (10*3/mcl)	6.3 ± 1.6	6.4 ± 1.7	6.6 ± 1.7	6.8 ± 2.2	<0.001
BMI (kg/m^2)	25.7 ± 3.6	26.2 ± 3.8	26.5 ± 3.9	26.7 ± 4.2	<0.001
SBP (mmHg)	133 ± 18	135 ± 18	136 ± 18	137 ± 19	<0.001
DBP (mmHg)	82 ± 11	83 ± 11	83 ± 11	83 ± 12	<0.001
Total cholesterol (mmol/l)	6.1 ± 1.1	6.2 ± 1.1	6.2 ± 1.2	6.2 ± 1.2	<0.001
LDL cholesterol (mmol/l)	3.9 ± 1.0	3.9 ± 1.0	4.0 ± 1.0	4.0 ± 1.1	<0.001
HDL cholesterol (mmol/l)	1.5 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.5	<0.001
Triglycerides (mmol/l)	1.5 [1.1-2.2]	1.6 [1.1-2.3]	1.6 [1.1-2.3]	1.5 [1.1-2.2]	<0.001
Creatinin (g/dL)	84 ± 23	85 ± 19	86 ± 20	86 ± 20	<0.001
eGFR (ml/min/1.73m^2)	88 ± 28	86 ± 27	85 ± 26	84 ± 25	<0.001
Albumin/creatinin ratio	0.6 [0.3-1.3]	0.6 [0.3-1.2]	0.6 [0.3-1.3]	0.6 [0.3-1.5]	0.024
Serum iron (umol/L)	17 ± 6	16 ± 6	15 ± 6	14 ± 6	<0.001
Ferritin (ng/ml)	93 ± 78	91 ± 75	88 ± 72	77 ± 69	<0.001
CRP (mg/L)	1.3 [0.6-2.7]	1.4 [0.7-2.9]	1.5 [0.8-3.1]	1.8 [0.9-4.0]	<0.001
Physical activity:					<0.001
Very inactive	28.3 (1219)	29.2 (1220)	30.9 (1376)	35.4 (1628)	
Moderately inactive	29.2 (1255)	29.0 (1209)	28.1 (1254)	27.3 (1258)	
Moderately active	23.5 (816)	23.3 (971)	22.2 (991)	21.1 (969)	
Active	19.0 (816)	18.5 (771)	18.8 (838)	16.2 (747)	

Data are presented as mean ± standard deviation, median [interquartile range] or percentage (number).
Abbreviations: RDW, red cell distribution width; MCV, mean corpuscular volume; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate calculated by the Modification of Diet in Renal Disease equation; CRP, C-reactive protein.

RDW levels and risk of coronary heart disease events

During follow-up, 1987 study participants developed a CHD event. The incidence of CHD events was 14.8% (n=682) in the highest quartile versus 8.5% (n=365) in the lowest RDW quartile. People in the highest RDW quartile had a HR for CHD events of 1.89 (95%CI 1.66-2.14, p for linearity < 0.001) compared to those in the lowest quartile (Table 3, Figure 2A). Adjustment for established risk factors reduced the HR for CHD events to 1.34 (95%CI 1.17-1.53, p for linearity <0.001, Figure 2B). The HR attenuated upon additional adjustment for eGFR and CRP (HR 1.25, 95%CI 1.06-1.47, p for linearity 0.009). Adjustment for serum iron and serum ferritin levels did not affect the HR for CHD events (HR 1.97, 95% CI 1.68-2.31, Figure 2D) compared to the unadjusted HR.

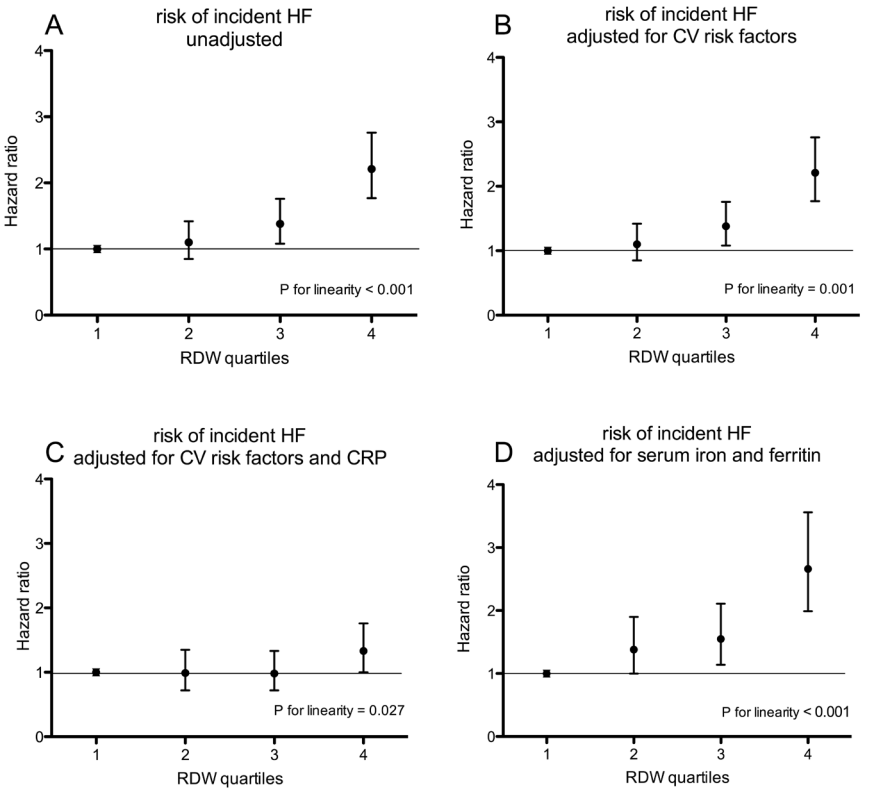

Figure 1 | Risk of incident heart failure event according to quartiles of RDW; A. unadjusted, B. adjusted for CV risk factors, C. adjusted for CV risk factors and CRP and D. adjusted for serum iron and ferritin levels. Panels show hazard ratio and 95% confidence intervals.
Abbreviations: HF, heart failure; RDW, red cell distribution width; CV risk factors, cardiovascular risk factors including sex, age, diabetes, smoking, systolic blood pressure, total and high-density lipoprotein cholesterol; CRP, C-reactive protein.

Table 3 | Hazard ratios for incident heart failure and coronary heart disease by quartiles of red cell distribution width

	RDW quartiles				
	1	**2**	**3**	**4**	**P linearity**
Heart failure events (n/N)	113/4187	119/4052	157/4302	251/4352	
- unadjusted	1.0	1.10 [0.85-1.42]	1.38 [1.08-1.76]	2.21 [1.77-2.76]	< 0.001
- adjusted for established CV risk factors*	1.0	0.92 [0.70-1.21]	0.98 [0.76-1.27]	1.40 [1.11-1.77]	0.001
- adjusted for established CV risk factors and estimated GFR‡	1.0	0.99 [0.72-1.36]	1.00 [0.74-1.36]	1.43 [1.08-1.90]	0.005
- adjusted for established CV risk factors and CRP	1.0	0.99 [0.72-1.35]	0.98 [0.72-1.33]	1.33 [1.00-1.76]	0.027
- adjusted for established CV risk factors, estimated GFR and CRP	1.0	0.97 [0.70-1.33]	0.97 [0.71-1.32]	1.34 [1.01-1.79]	0.019
- adjusted for serum iron and ferritin	1.0	1.38 [1.00-1.90]	1.55 [1.14-2.11]	2.66 [1.99-3.56]	< 0.001
- adjusted for established CV risk factors and serum iron and ferritin	1.0	1.07 [0.77-1.48]	1.08 [0.78-1.49]	1.45 [1.07-1.96]	0.010
Coronary heart disease events (n/N)	365/3935	426/3745	514/3945	682/3921	
- unadjusted	1.0	1.23 [1.07-1.41]	1.41 [1.23-1.61]	1.89 [1.66-2.14]	< 0.001
- adjusted for established CV risk factors*	1.0	1.05 [0.91-1.21]	1.05 [0.91-1.21]	1.34 [1.17-1.53]	< 0.001
- adjusted for established CV risk factors and estimated GFR‡	1.0	1.08 [0.91-1.28]	1.05 [0.89-1.24]	1.30 [1.10-1.52]	0.002
- adjusted for established CV risk factors and CRP	1.0	1.08 [0.91-1.28]	1.05 [0.89-1.24]	1.25 [1.07-1.47]	0.008
- adjusted for established CV risk factors, estimated GFR and CRP	1.0	1.07 [0.90-1.27]	1.03 [0.87-1.22]	1.25 [1.06-1.47]	0.009
- adjusted for serum iron and ferritin	1.0	1.27 [1.07-1.50]	1.36 [1.16-1.61]	1.97 [1.68-2.31]	< 0.001
- adjusted for established CV risk factors and serum iron and ferritin	1.0	1.06 [0.99-1.26]	1.04 [0.88-1.24]	1.27 [1.07-1.50]	0.007

Presented data are hazard ratios and corresponding 95% confidence intervals y RDW quartiles, using the lowest quartile as reference. *established cardiovascular risk (CV) factors; sex, age, diabetes, smoking, systolic blood pressure, total cholesterol and high-density lipoprotein. ‡ estimated glomerular filtration rate (GFR) calculated by the Modification of Diet in Renal Disease equation

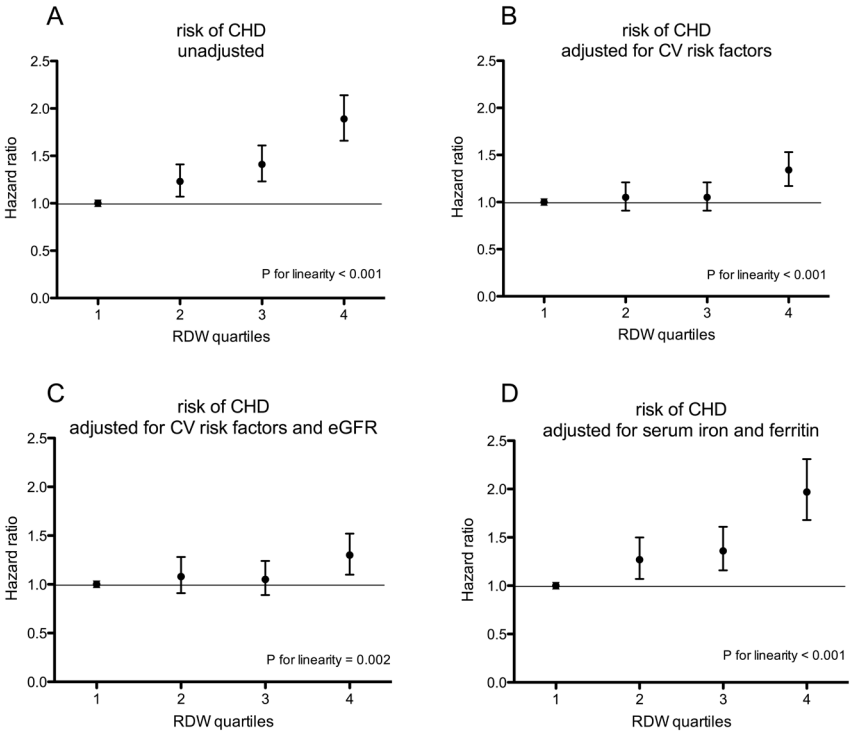

Figure 2 | Risk of incident coronary heart disease event according to quartiles of RDW; A. unadjusted, B. adjusted for CV risk factors, C. adjusted for CV risk factors and D. CRP and adjusted for serum iron and ferritin levels. Panels show hazard ratio and 95% confidence intervals.
Abbreviations: CHF, coronary heart disease; RDW, red cell distribution width; CV risk factors, cardiovascular risk factors including sex, age, diabetes, smoking, systolic blood pressure, total and high-density lipoprotein cholesterol; CRP, C-reactive protein

DISCUSSION

The present study confirms in a large population of apparently healthy middle-aged men and women, that higher RDW levels are associated with an increased risk of CHD and HF events. Furthermore, we demonstrate that higher RDW levels are associated with physical inactivity, independent of parameters for iron metabolism or inflammation. Finally, we show that the RDW associated risk of CHD and HF events is independent of established cardiovascular risk factors (age, sex, diabetes mellitus, smoking, systolic blood pressure, total and high-density lipoprotein cholesterol), renal function and inflammation and that the RDW associated risk of HF events is independent of parameters of iron metabolism.

The observation that the RDW associated risk of CHD events is independent of established cardiovascular risk factors and inflammation, suggests that the underlying pathophysiology linking CHD events with anisocytosis differs from that reflected by conventional risk factors. This also holds for the risk of HF, although additional adjustment for known risk factors for HF such as estimated GFR, inflammation and parameters of iron metabolism slightly attenuated the association between RDW

and the risk of incident HF. Hence, RDW might be of additional value for predicting the risk of both CHD and HF events, as opposed to many new biomarkers[23, 24].

Importantly, our study shows that RDW levels are associated with physical inactivity in a large population of apparently healthy middle-aged men and women. Craenenbroeck et al. recently demonstrated, that high RDW levels are a strong independent predictor for impaired exercise capacity in a small cohort of patients with chronic HF, as assessed by peak aerobic capacity. In addition, RDW decreased after exercise training and this decrease was related to the change in peak aerobic capacity[13]. We explored whether this relationship between higher RDW levels and impaired exercise capacity was also present in a large population without HF. As a substitute for exercise capacity, we used physical activity as assessed by a questionnaire. The used questionnaire involved both leisure time activity as well as work-related physical activity and we believe it represents a good approximation of cardiorespiratory fitness. This physical activity questionnaire has previously been validated against heart rate monitoring in 173 individuals over one year[18]. We demonstrate a clear inverse relationship between RDW levels and physical activity. This is in concordance with data from another general cohort, in which physical activity was negatively related with RDW levels[6].

The fact that the underlying pathophysiological mechanism linking anisocytosis with the risk of both CHD and HF is unknown, precludes the design of therapeutic interventions[25, 26]. To explain the association between RDW and HF, a number of causative mechanisms such as disordered iron metabolism, inflammation and erythropoietin resistance[7, 27, 28] have been hypothesized, but studies show equivocal results [2, 9]. Recently the pivotal role of iron in HF has been emphasized[29] and there is ample evidence that impaired exercise capacity is related to iron deficiency in chronic HF[14, 15]. Therefore it seems obvious to hypothesize that iron metabolism links RDW with exercise tolerance in HF. However, outcome data with respect to the association between RDW and parameters for iron homeostasis are scarce. We studied iron metabolism as an underlying mechanisms linking anisocytosis with increased risk of HF events in an apparently healthy population. Despite a clear association between increased RDW levels and serum iron and serum ferritin, the risk of incident HF with higher RDW levels remained more or less unchanged after adjustment for these parameters.

Increased levels of cytokines are associated with adverse outcomes in both CHD and HF [21, 30, 31]. Indeed, cytokines inhibit the growth of erythroid precursor cells, erythropoietin production and reduce iron availability for erythropoiesis by increasing hepcidin synthesis[32, 33], which could explain the link between RDW and adverse outcome. Several studies demonstrated an association between RDW and inflammation[8, 28]. However, despite a clear association between increased RDW levels and CRP in our study, the risk of both incident HF and CHD events with higher RDW levels only slightly attenuated after adjustment for these parameters. This finding concurs with Borne et al. and Rhodes et al., demonstrating that RDW predicts survival independent of inflammation, as quantified by interleukin-6 or CRP levels[7, 34].

Thus, our study fails to explain the link between anisocytosis and incident HF and CHD events and physical activity. Since RDW is a measure of red cell size properties, it is obvious that disturbed red cell turn over partakes in the causal mechanism.

It remains to be established what processes are responsible for this disturbed red cell turn over. Erythropoietin resistance has frequently been implicated in this respect. Yet, recently we demonstrated in a cohort of patients with combined heart- and renal failure, that RDW levels were not related to erythropoietin resistance, as assessed by multiple measurements[9]. It may be speculated that other processes, regulating red cell production and decay, such as eryptosis are involved. Enhanced eryptosis, the suicidal death of erythrocytes, is observed in common diseases, such as renal failure, diabetes mellitus and iron deficiency[35-37] and recently in HF[38]. It leads to enhanced formation of new, larger erythrocytes, thereby possibly causing anisocytosis. Similarly, recently RDW levels were shown to be related to cholesterol content of erythrocytes membranes[39]. This is a novel marker for clinical instability in CHD, in which erythrocytes play an active role in atherosclerotic plaque growth and rupture. It would be interesting to investigate whether these processes are also related to increased risk of both CHD and HF events, thereby possibly explaining the link between RDW levels and adverse outcome.

A number of limitations should be acknowledged. Firstly, we used a questionnaire to assess both leisure time and work-related physical activity, as a surrogate for cardio-respiratory fitness. Although, this questionnaire has been validated with calibrated heart rate monitoring[18], for obvious reasons it is less reliable than assessing exercise performance with peak aerobic capacity. Secondly, we ascertained incident HF and CHD events by hospital records and death certificates, thereby possibly selecting more severe cases of HF and CHD. This could limit the generalization of our results to milder CHD and HF. Also, we lack information about the etiology of incident HF and, it should be noted that events were not independently adjudicated. Finally, in an ideal world we would have had access to data about erythropoietin levels, reticulocyte haemoglobin content, soluble transferrin receptor, reticulocyte count and immature reticulocyte fraction, which would add to the insight on the link between iron metabolism, red cell turn over and RDW levels.

In conclusion, our results confirm the association between higher RDW levels and increased risk of both incident HF and CHD events in a large population of apparently healthy men and women. This association with both CHD and HF events was not affected upon adjustment for established risk factors such as included in the Framingham risk score or inflammation. Also, the association with HF events was not affected by adjustment for iron metabolism, implying that RDW levels reflect a different underlying pathophysiological mechanism than those represented by known risk factors. Importantly, RDW levels were related to physical inactivity in this population. However, also this association with physical inactivity remained largely similar after adjustment for serum iron, ferritin and CRP, Therefore, further research is required to determine which underlying pathophysiological mechanisms link anisocytosis with both HF and CHD events and physical inactivity.

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PART I

Neutrophil Gelatinase-Associated Lipocalin (NGAL) in chronic cardiorenal failure is correlated with endogenous erythropoietin levels and decreases in response to low-dose erythropoietin treatment

6

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Accepted Kidney Blood Pressure Research

ABSTRACT

Background

Neutrophil-gelatinase associated lipocalin (NGAL) is known as a marker for tubular injury. However, in two recent studies, NGAL levels were associated with disordered iron metabolism in haemodialysis patients. We investigated whether serum NGAL levels reflect iron metabolism in combined chronic heart failure and chronic kidney disease (CHF/CKD) and whether treatment with erythropoietin stimulating agent (ESA) modulates NGAL levels.

Methods

The EPOCARES trial (ClinTrialsNCT00356733) investigates the role of erythropoietic and non-erythropoietic effects of ESA in 56 oral iron supplemented anaemic patients with combined CKD (estimated creatinine clearance (eCrCl) 36±15 ml/min) and CHF. Subjects were randomized into three groups: two groups received 50 IU/kg/week ESA (the ESA-group, n=37). The third group received no ESA (no-ESA group, n=19). After two weeks the ESA group divided into two groups; in the ESA-Hb-rise group the haemoglobin levels (Hb) were allowed to increase and in the ESA-Hb-stable group Hb-levels were maintained at baseline level by phlebotomies. Serum NGAL, hepcidin-25, transferrin saturation (TSAT), reticulocyte haemoglobin content (Ret-He) and endogenous erythropoietin (EPO) levels were measured at baseline, after two weeks and 6 months.

Results

At baseline, serum NGAL levels correlated positively with cystatin C ($r=0.767$, $p<0.001$) and were inversely correlated with eCrCl ($r=-0.571$, $p<0.001$) and baseline EPO levels ($r=-0.395$, $p=0.003$). There was no correlation with TSAT ($r=-0.316$, $p=0.32$), Ret-He ($r=-0.119$, $p=0.39$) and hepcidin-25 levels ($r=0.147$, $p=0.28$). After two weeks, NGAL levels decreased in the ESA-group ($p=0.02$), while there was no change in the no-ESA group ($p=0.62$). The magnitude in NGAL decrease in the ESA-group correlated with baseline EPO levels ($r=0.431$, $p=0.01$). However, NGAL values after 6 months showed no significant changes compared to baseline levels in all three groups.

Conclusions

In contrast to in haemodialysis patients, in combined CKD and CHF, elevated serum NGAL levels did not correlate with parameters of iron metabolism, hence NGAL might reflect tubular damage in these patients. Interestingly NGAL levels inversely correlated with baseline EPO levels and decreased in response to short-term low-dose ESA treatment, which might possibly reflect an effect of ESA on tubular damage. However, this findings needs to be confirmed and alternative explanations should be evaluated.

INTRODUCTION

Neutrophil-gelatinase associated lipocalin (NGAL or lipocalin-2), a 25kDa protein of the lipocalin family, acts as a natural bacteriostatic agent by interfering with bacterial iron uptake and also increases in response to inflammation. In the setting of acute renal tubular injury, human serum NGAL levels increase 7 to 16-fold and urinary NGAL levels increase 25-100 fold[1]. Consequently, NGAL has been proposed as a biomarker for tubular damage to detect acute kidney injury at an early stage in various conditions[2-4]. However, NGAL has also been shown to be elevated in chronic conditions, such as chronic kidney disease (CKD)[5, 6] and chronic heart failure (CHF)[7], presumably reflecting chronic damage to tubular cells, irrespective of the glomerular filtration rate[8, 9].

In addition, NGAL levels might also reflect iron metabolism since the NGAL pathway acts as an alternative to the transferrin-mediated iron delivery pathway by cytoplasmic iron delivery into target cells[10]. Recently, Bolignano et al. and Malyszko et al. suggested that low NGAL levels in haemodialysis patients reflect reduced iron availability and transport; lower NGAL levels were associated with lower transferrin saturation (TSAT), lower ferritin levels and higher hepcidin levels[11-13].

Thus, NGAL levels might reflect inflammation, tubular damage or reduced iron availability. Iron deficiency plays an important role in the pathophysiology of CKD and CHF[14] and is related to a reduced quality of life and increased mortality[15]. In combined CHF/CKD, it is unknown whether NGAL reflects iron metabolism or tubular damage. Since ESA treatment induces increased iron utilization and reduces hepcidin one could hypothesize that during ESA treatment NGAL levels increase[16, 17]. Therefore, we investigated the hypothesis that (1) serum NGAL levels reflect iron availability in anaemic patients with combined chronic heart and kidney failure and (2) that ESA treatment increases serum NGAL levels in accordance with its effect on hepcidin. To this end we measured NGAL and assessed markers of inflammation and iron metabolism including transferrin saturation (TSAT), reticulocyte haemoglobin content (Ret-He) and hepcidin-25 levels, using a mass spectrometry based assay, in the EPOCARES study.

METHODS

Study design and patients

The study design of the EPOCARES study (ClinicalTrials.gov number NCT 00356733) has been published elsewhere[18]. In short, the EPOCARES study is an open-label, prospective, randomized trial, in which patients with CHF, CKD (estimated creatinine clearance (eCrCl) by Cockroft-Gault equation of 20-70 ml/min) and mild anaemia (haemoglobin 10.3-12.6 g/dL for men and 10.3-11.9 g/dL for women) are included to test the erythropoietic and non-erythropoietic responses to low-dose ESA treatment. Exclusion criteria, amongst others, were ESA therapy within 6 months, bleeding, chronic inflammatory disease or malignancy. Haemoglobin (Hb) level for inclusion was measured after at least four weeks of oral iron supplementation, if tolerated. The diagnostic criteria for CHF were those recommended by the European Society of Cardiology guidelines[19]. Patients with heart failure with reduced left

ventricular ejection fraction as well as patients with preserved left ventricular ejection fraction were included[20].

The subjects were randomized into 3 groups. The first two groups received a fixed dose of 50 IU/kg per week of ESA (Neorecormon; Roche Pharmaceuticals), After two weeks, the Hb level was let to increase to a maximum of 13.7 g/dL for men and 13.4 g/dL for women in one group (ESA-Hb-rise group) whereas in the other group the Hb levels were maintained at baseline level for 26 weeks by sequential blood withdrawal to a maximum of 250 mL per 2 weeks (ESA-Hb-stable group). The third group, the control group, did not receive ESA (the no-ESA group). In addition, a group of 25 healthy, age- and sex-matched controls were recruited for comparison of NGAL levels. The Medical-Ethical Committee approved the protocol of the study and informed consent was obtained from all subjects. Procedures were in accordance with the Helsinki Declaration and all patients gave written consent.

Biomarker analysis

All blood samples were drawn between 8 and 9 a.m. and stored at -80°C until analysis. As a marker of total iron stores, ferritin was determined using a sandwich immunoassay on an Acces®2 immunoanalyser within a Dx automated system from Beckman Coulter (Brea, CA). Functional iron availability was determined by measuring transferrin saturation (TSAT), soluble transferrin receptor (sTfR) and reticulocyte haemoglobin content (Ret-He). TSAT was calculated from serum iron and transferrin estimates obtained with standard methods on a Beckman Coulter Dx. sTfR assay was performed with an immunoassay on a BNProSpec nephelometer from Siemens (Marburg, Germany). Ret-He was performed using flow cytometric analysis with Ret-Search (II)® dye on a Sysmex XE-2100 haematology analyser (Toa Medical, Kobe, Japan).

Serum hepcidin-25 measurements were performed by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (TOF MS)[21]. An internal standard (synthetic hepcidin-24; Peptide International Inc.) was used for quantification[22]. Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionisation TOF MS platform (Bruker Daltonics). Serum hepcidin-25 concentrations were expressed as nmol/l. The lower limit of detection (LLOD) of this method was 0.5 nM; ranges for the coefficients of variation were 2.2-3.7% (intra-run) and 3.9-9.1% (inter-run). The median reference level of serum hepcidin-25 is 4.5 nM for men, 2.0 nM for premenopausal women, and 4.9 nM for postmenopausal women. The reference levels for the WCX-TOF MS method were derived from those of a competitive ELISA method[23], based on the regression line between the data of both methods on the same samples[21].

Serum NGAL levels were measured on the Triage® NGAL test (Alere Inc. San Diego, CA, USA), an immunoassay in a single-use plastic cartridge that contains a fluorescently labelled monoclonal antibody against NGAL labelled with a fluorescent dye and NGAL. Measurements of NGAL concentration in the range from 60 to 1300 ng/ml. Calibration information is relayed to the meter via a lot-specific EPROM chip[4].

Assessment of changes in haemoglobin levels in time

Two study groups received similar dosages of ESA treatment during the study period (together the ESA group). After two weeks the ESA group was split into the ESA-

Hb-stable and the ESA-Hb-rise group. In the ESA-Hb-stable group the Hb levels were be kept at baseline level using phlebotomies. To assess the necessity of a phlebotomy, a preceding increase in Hb was required, for which the Hb levels were measured every 2 weeks, which makes comparison of single time-point measurements unreliable. We therefore assessed the Hb response by calculating the area under the curve for Hb change in time (Hb AUC) in the groups. Furthermore, the Hb AUC is more informative to asses the Hb response to ESA treatment and its clinical significance on end points[24]. The Hb AUC was calculated by linear trapezoidal integration, in which the total area under the Hb change versus time curve is obtained by summation of each individual area between two consecutive time points, as extensively described elsewhere[24]. The Hb AUC was based on monthly Hb measurements in all patients.

Statistical analysis

Data are presented as means ± standard deviation (SD) for normally distributed variables and median with inter-quartile ranges (IQR) for non-normally distributed variables. Normality of data was evaluated using the Kolmogorov-Smirnov test. Non-normally distributed variables were log transformed, after which normality was tested again. Differences between groups were compared with the unpaired student's t-test, Mann-Whitney U test or χ^2 –test where appropriate. Paired data were compared with the paired student's t-test using a Bonferroni adjusted alpha level. Pearson correlation or Spearman's rho were used for bivariate correlations (resp. normally and non-normally distributed variables). The one-way ANOVA was used for multiple group comparisons. Multivariable linear regression models with stepwise forward selection process were performed. Differences were considered significant when $P<0.05$, two-sided. For statistical analyses the Statistical Package for Social Sciences (IBM, Chicago, Illinois, USA) version 18 for Mac was used.

RESULTS

Baseline characteristics

The study population of the EPOCARES study comprised of 62 patients, of whom 5 patients withdrew their informed consent and 1 patient was withdrawn because of malignancy. Baseline characteristics of the remaining 56 patients are displayed in Table 1, divided by ESA group and no-ESA group. All patients had CKD, CHF and were anaemic, as shown by the decreased eCrCl, LVEF, Hb-levels and the higher NT-proBNP levels. Despite oral iron supplementation, TSAT levels were low in some patients (< 20% in 26 of the patients or < 15% in 8 patients). However, in the 37 patients that received ESA, Ret-He did not decrease, indicating that there was no iron-restricted erythropoiesis. At baseline, there were no differences between the ESA group and the no-ESA group. The NGAL levels in the EPOCARES cohort were increased in comparison to healthy age- and sex-matched volunteers (207 [132-287] ng/mL vs. 77 [60-116] ng/mL, n=25; p<0.001). When divided according to baseline NGAL levels below and above the median (high ≥ 207 ng/mL vs. low < 207 ng/mL), patients with high NGAL values had a higher cystatine C, NT-proBNP and interleukin-6 and lower eCrCl, serum iron and endogenous EPO levels, as shown in Table 2.

Table 1 | Baseline characteristics

Characteristic	ESA n=37	No ESA n=19	p- value
Age, years	76 [70-81]	72 [66-77]	0.52
Male sex, n (%)	23 (62.2)	14 (73.7)	0.39
BMI (kg/m^2)	26.1 ± 4.2	27.4 ± 4.2	0.68
Diabetes, n (%)	12 (32.4)	7 (36.8)	0.74
Hypertension, n (%)	27 (80.0)	16 (84.2)	0.35
NGAL (ng/mL)	189 [133-265]	238 [129-313]	0.86
NT-proBNP (pg/mL)	1400 [744-2631]	1680 [659-2610]	0.81
LVEF (%)	42.1 ± 9.1	44.1 ± 12.3	0.22
hs-CRP (mg/L)	5.4 [1.3-10.9]	4.3 [1.7-6.9]	0.55
RAS inhibitor, n (%)	35 (94.6)	19 (100.0)	0.30
Haemoglobin (g/dL)	11.7 ± 0.95	11.8 ± 0.79	0.74
Haematocrit (%)	0.35 ± 0.031	0.35 ± 0.026	0.96
Ferritin (ng/mL)	130 [75-209]	128 [76-164]	0.43
Transferrin saturation (%)	20 [15-25]	20 [18-29]	0.76
Erythropoietin (IU/L)	13 [9-16]	15 [5-17]	0.95

Mean ± standard deviation or median [interquartile range] are shown. Abbreviations: BMI, body mass index; NGAL, neutrophil gelatinase-associated lipocalin; NTproBNP, N-terminal pro-brain natriuretic peptide; LVEF, left ventricular ejection fraction; hs-CRP, high sensitivity C-reactive protein; RAS inhibitor, renin-angiotensin-system inhibitor

Table 2 | Baseline variables according to serum NGAL levels below or above median.

Characteristic	Low NGAL n=28	High NGAL n=28	p- value
Diabetes, n (%)	10 (35.7)	9 (32.1)	0.50
Hypertension, n (%)	19 (67.9)	24 (85.7)	0.10
eCrCl (ml/min)	44 ± 16	30 ± 10	**<0.001**
Cystatin C (mg/L)	1.45 [1.10-1.68]	2.40 [1.87-2.87]	**<0.001**
NT-proBNP (pg/mL)	1280 [531-2139]	1742 [894-5115]	0.020
hs-CRP (mg/L)	5.4 [1.3-10.9]	4.3 [1.7-6.9]	0.21
Interleukin-6 (pg/mL)	2.8 [1.7-4.7]	4.1 [2.3-7.2]	**0.044**
Haemoglobin (g/dL)	11.8 ± 0.86	11.8 ± 0.94	0.74
Serum iron (µmol/L)	12 [10-16]	10 [8-14]	**0.027**
Ferritin (ng/mL)	133 [78-216]	117 [75-167]	0.53
Transferrin saturation (%)	22 [18-26]	18 [14-25]	0.19
sTfR (mg/L)	1.28 ± 0.36	1.48 ± 0.55	0.11
Ret-He (fmol)	1.90 ± 0.14	1.88 ± 0.14	0.11
Hepcidin-25 (nM)	5.4 [3.2-7.4]	7.0 [4.4-8.8]	0.14
Erythropoietin (IU/L)	15 [12-19]	10 [6-15]	**0.005**

Mean ± standard deviation or median [interquartile range] are shown. Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; eCrCl, estimated creatinine clearance by Cockroft Gault; NT-proBNP, N-terminal pro-brain natriuretic peptide; hs-CRP, high sensitivity C-reactive protein; sTfR, soluble transferrin receptor; Ret-He, reticulocyte haemoglobin content

Table 3 | Univariate correlations with serum NGAL levels at baseline

Variable	NGAL	
	r	p-value
Age	- 0.184	0.18
Haemoglobin	- 0.112	0.41
NT-proBNP	0.219	0.11
Urea nitrogen	0.725	**< 0.001**
eCrCl	- 0.571	**< 0.001**
Cystatin C	0.767	**< 0.001**
Microalbuminuria	0.515	**< 0.001**
CRP	0.133	0.33
hs-CRP	0.123	0.37
Interleukin-6	0.194	0.16
Erythropoietin	- 0.395	**0.003**
Transferrin	- 0.387	**0.003**
Ferritin	- 0.110	0.42
TSAT	- 0.136	0.32
Soluble transferrin receptor	0.148	0.28
Reticulocyte haemoglobin content	- 0.119	0.39
Hepcidin-25	0.147	0.28

Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; NT-proBNP, N-terminal pro-brain natriuretic peptide; eCrCl, estimated creatinine clearance by Cockroft Gault; CRP, C-reactive protein; hs-CRP, high sensitivity CRP; TSAT, transferrin saturation

Univariate correlations of baseline NGAL and multiple regression analysis

Table 3 shows the univariate correlations with serum NGAL levels at baseline. Serum NGAL levels correlated positively with urea nitrogen, cystatin C levels and microalbuminuria and negatively with eCrCl. Furthermore, there was a negative correlation between baseline NGAL levels and endogenous EPO levels (Fig 1A). This correlation between endogenous EPO levels and serum NGAL remained significant after adjustment for eCrCl and cystatin C (adjusted r= -0.358, p=0.008), as for microalbuminuria (adjusted r= -0.338, p=0.020) and for Hb-levels (adjusted r= -0.400, p=0.003). There was no significant association between serum NGAL levels and age, Hb levels or with parameters for iron stores and iron availability such as ferritin, TSAT, sTfR and Ret-He. Nor did we find a correlation with hepcidin-25 levels (Figure 1B) or inflammation.

After multiple regression analysis, stepwise including variables with univariate correlation with NGAL with p<0.1, cystatin C (β=0.804, p<0.001), endogenous EPO levels (β= -0.249, p=0.004) and urea (β= 0.325, p=0.016) were the predictors for baseline serum NGAL levels (explaining 74.0% of NGAL variations).

Acute response to ESA treatment

Table 4 shows the effect of 2 weeks ESA treatment versus no treatment on the variables. Hb levels, reticulocytes and sTfR significantly increased in the ESA group compared to baseline. Individual NGAL levels at baseline and after two weeks are shown in Figure 2. NGAL levels significantly decreased after two weeks in the ESA

group (p= 0.02) whereas there was no change in the no-ESA group (p=0.62).
The magnitude of decrease in NGAL levels after two weeks ESA treatment correlated positively with baseline endogenous EPO levels (r=0.431, p=0.01, Figure 3A) and Ret-He (r=0.396, p=0.02, Figure 3B) and negatively with baseline serum NGAL levels (r= -0.615, p<0.001). There was no correlation between the magnitude in decrease in NGAL levels and baseline eCrCl, cystatin C, baseline Hb-levels or the magnitude in increase of reticulocyte count and sTfR.

Response to ESA treatment after 26 weeks

After two weeks the ESA group split into the ESA-Hb-stable and the ESA-Hb-rise group. Based on the single time point Hb levels after 26 weeks, the Hb increased significantly compared to baseline in the ESA-Hb-rise group (resp. 13.3 ± 1.40 vs 11.8 ± 1.08, p< 0.001) and the ESA-Hb-stable group (resp. 12.6 ± 0.74 vs 11.7 ± 0.84 g/dL, p=0.001). The Hb AUC, depicting the cumulative Hb change over time

Table 4 | Effects of 2 weeks ESA treatment versus no treatment

Characteristic	ESA (n=37)			ESA (n=37)		
	t=0	t= 2 weeks	p-value	t=0	t= 2 weeks	p-value
Hemoglobin (g/dL)	11.7 ± 0.95	12.0 ± 1.12	**0.018**	11.8 ± 0.79	11.7 ± 0.86	0.50
Reticulocytes (x1012/L)	0.046 ± 0.015	0.064 ± 0.018	**<0.001**	0.044 ± 0.016	0.047 ± 0.017	0.30
sTfR (mg/L)	1.39 ± 0.48	1.77 ± 0.53	**<0.001**	1.35 ± 0.47	1.44 ± 0.49	0.12
Ret-He (fmol)	1.90 ± 0.15	1.82 ± 0.23	0.24	1.88 ± 0.12	1.84 ± 0.15	0.30
eCrCl (ml/min)	35 ± 12	35 ± 11	0.40	39 ± 20	39 ± 18	0.87
Cystatin C (mg/l)	2.0 ± 0.83	1.9 ± 0.76	0.78	2.0 ± 0.77	2.0 ± 0.72	0.64
hs-CRP (mg/L)	5.4 [1.3-10.9]	2.2 [1.1-4.8]	0.08	4.3 [1.7-6.9]	6.6 [2.1-14.2]	0.34
Interleukin-6 (pg/mL)	3.7 [1.8-4.9]	3.7 [2.2-5.8]	0.28	3.2 [2.3-7.0]	3.3 [2.0-5.8]	0.93

Mean ± standard deviation or median [interquartile range] are shown. Abbreviations: sTfR, soluble transferrin receptor; Ret-He, reticulocyte haemoglobin content; eCrCl, estimated creatinine clearance by Cockroft Gault; hs-CRP, high sensitivity CRP

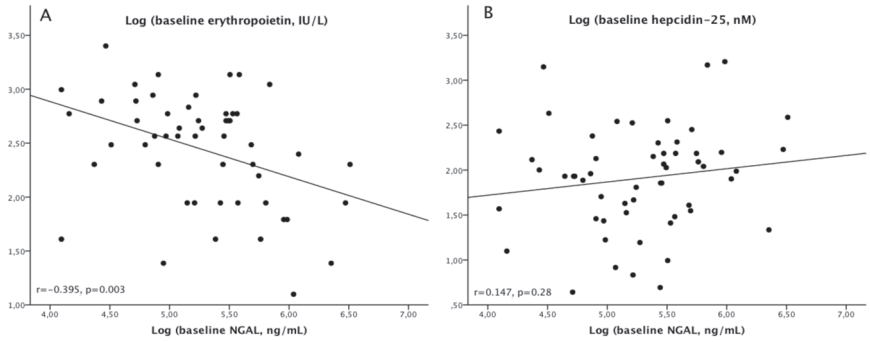

Figure 1 | The correlation between log-transformed baseline serum NGAL levels and baseline log-transformed erythropoietin levels (A) and baseline log-transformed hepcidin-25 levels (B). NGAL levels positively correlate with baseline erythropoietin levels and show no correlation with hepcidin-25 levels. Abbreviations: NGAL, neutrophil gelatinase associated lipocalin.

and a better reflection of the overall exposure to higher Hb level than a comparison at 1 time point, was significantly higher in the ESA-Hb-rise group compared to the ESA-Hb-stable group (resp. 19.8 ± 17.2 vs 5.9 ± 9.6, p= 0.009). Furthermore, the Hb AUC in the control group (-2.2 ± 9.4) did not differ from the ESA-Hb-stable group (p= 0.99).

NGAL values after 26 weeks showed no significant changes compared to baseline levels in all three the study groups (ESA-Hb-rise, 168 [144-259] vs 195 [146-266] ng/mL, p= 0.27; ESA-Hb-stable 152 [128-211] vs 187 [126-280] ng/mL, p= 0.97; control group 244 [115-319] vs 238 [129-313], p= 0.99). Nor were there any significant changes in cystatin C or eCrCl values after 26 weeks compared to baseline (data not shown).

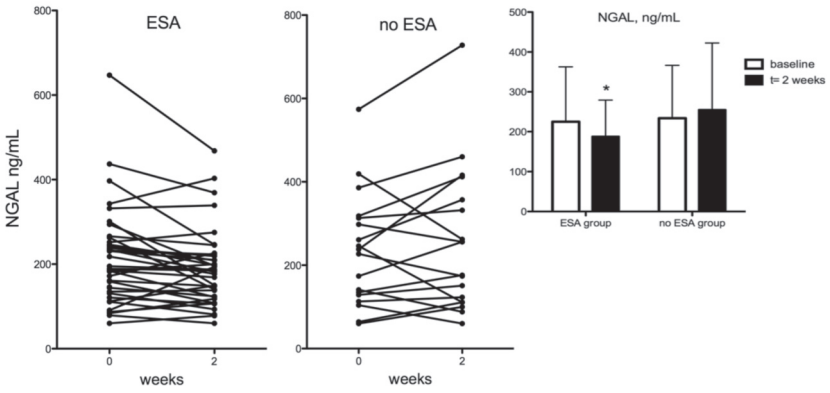

Figure 2. I Serum NGAL levels at baseline and after two weeks of ESA treatment; individual serum NGAL levels at baseline and after two weeks of ESA treatment (n=37) versus no ESA treatment (n=19). Two weeks of ESA treatment decreased log-transformed serum NGAL levels in patients with combined CHF and CKD, as depicted in the bar graph. Abbreviations; NGAL, neutrophil gelatinase associated lipocalin; ESA, erythropoiesis-stimulating agent. Error bars represent SD; * p<0.05

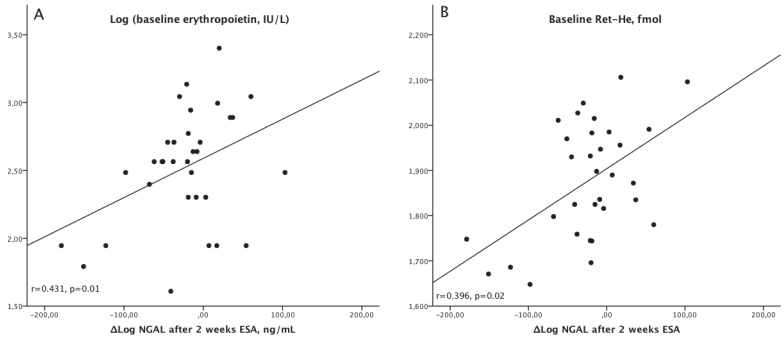

Figure 3 I The correlation between the magnitude in log-transformed serum NGAL decrease after two weeks of ESA treatment and baseline log-transformed erythropoietin levels (A) and baseline reticulocyte haemoglobin content levels (B). Abbreviations: NGAL, neutrophil gelatinase associated lipocalin; ESA, erythropoiesis-stimulating agent; Ret-He, reticulocyte haemoglobin content.

DISCUSSION

The main finding of the present study is that in combined CKD and CHF elevated serum NGAL levels inversely correlate with baseline EPO levels, independent of renal function. Concurrently low dose ESA treatment induced a moderate decrease in serum NGAL levels. However, there was no long-term effect of 26 weeks ESA treatment on NGAL levels. Lastly, although our study confirms that baseline serum NGAL levels are elevated in mildly anaemic patients with combined CKD and CHF, NGAL levels did not correlate with parameters of iron metabolism.

Recent reports show that decreased serum NGAL levels in haemodialysis patients correlate with higher (total) hepcidin levels and lower TSAT[11, 12], suggesting that low serum NGAL levels are associated with reduced iron utilization. Both CKD and CHF are chronic inflammatory conditions in which iron metabolism is disturbed. Therefore, we further explored the findings of Bolignano et al[12]. and Malyszko et al[11]. in a cohort of oral iron supplemented, mildly anaemic patients with combined chronic CHF and CKD. In this cohort we found, unexpectedly, no association between serum NGAL levels and parameters for iron availability, as assessed by hepcidin-25 levels (determined by mass spectrometry), reticulocyte haemoglobin content (Ret-He), soluble transferrin receptor (sTfR) and TSAT. In addition, we detected a moderate association between serum NGAL levels and inflammation, as estimated by hs-CRP and IL-6, However, it should be noted that the median hs-CRP levels in this stable, low–inflammatory cohort were only mildly elevated.

An important finding of our study is that serum NGAL levels inversely correlated with endogenous EPO levels, independent of renal function, and that NGAL levels, contrary to our hypothesis, acutely decreased in response to low-dose ESA treatment, albeit that the response was modest and variable. To our knowledge, this is the first study that demonstrates an effect of low-dose ESA treatment on serum NGAL levels. A stronger decrease in NGAL levels correlated with higher baseline EPO levels and Ret-He. However, this effect was no longer present after 6 months treatment, regardless of maintained Hb-levels by phlebotomies or increased Hb-levels.

It is unlikely that in our study NGAL levels reflected iron metabolism as baseline NGAL levels and the ESA induced decrease in NGAL were not associated with markers of iron metabolism such as hepcidin-25. A possible explanation for our findings is that in our population serum NGAL is a marker of on-going tubular damage. This finding is in agreement with studies showing that NGAL predominantly reflects tubular damage in acute conditions[25, 26]. A comparable association was already explored about ten years ago for IgA nephropathy; urinary N-acetyl-β-D-glucosaminidase (NAG), a marker of tubular injury, correlated with EPO levels27. Also, several animal studies show a direct cytoprotective effect of ESA on intrinsic renal cells[28, 29]. In a rat model of ischemic injury, low doses of darbepoietin, not resulting in increases in haematocrit levels, significantly reduced glomerulosclerosis and tubulointerstitial damage, and rarefaction of peritubular capillaries was prevented[28]. It should be pointed out that, although initial studies suggested a beneficial effect of fix-dose ESA administration on renal function [28, 30], this was not confirmed in later studies that used variable doses of ESA to reach preset

haemoglobin targets [31, 32]. Furthermore, in order to corroborate a role for NGAL as tubular marker in our study, urinary NGAL levels and other urinary markers, might have better reflected tubular damage[33].
The present study admittedly has some limitations. First, the study size is relatively small which is due to the complexity of the study design. Studying univariate correlations is of limited value is a small cohort. However, we believe that the lack of association between serum NGAL levels and iron metabolism is unambiguous, due to its assessment by multiple parameters for iron metabolism, including hepcidin-25 levels. However, we cannot exclude the possibility that the lack of univariate association between NGAL and inflammation is due to lack of power.

Conclusion

Serum NGAL levels in combined CHF/CKD do not reflect iron homeostasis, as assessed by hepcidin-25 levels, Ret-He, sTfR and TSAT and therefore may reflect the tubular damage. Short-term low-dose ESA treatment discreetly decreases serum NGAL levels, which might possibly reflect an effect of ESA on tubular damage. Furthermore, baseline EPO levels correlate with serum NGAL levels, independent of eCrCl or Hb-levels. Further research is required to investigate these acute effects of low-dose ESA treatment on serum NGAL levels in CHF, CKD or the combination of both.

Competing interests and funding

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CHAPTER
6

PART II The role of blood pressure fluctuations and renal artery stenosis in cardiorenal failure

Stability of glomerular filtration rate upon chronic fluctuations in blood pressure in patients with combined heart and renal failure

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ABSTRACT

Background

In combined chronic heart failure (HF) and kidney disease (CKD), GFR could be affected by low renal perfusion pressure (RPP). However, healthy kidneys stabilize GFR upon fluctuations in RPP. We tested the hypothesis that GFR is susceptible to fluctuations in blood pressure (BP).

Methods

In patients with combined HF, CKD and anaemia (ClinTrials; NCT00356733), creatinine and BP were measured monthly for 12 months and mean arterial pressure (MAP)/GFR relationships were studied (n=48). Slopes and degree of stabilization (% change in GFR upon a change in BP related to ideal and absent autoregulation) were calculated.

Results

Baseline GFR was 38±15 ml/min.1.73m^2, N-terminal pro-brain natriuretic peptide (NT-proBNP) was 1280 [640-2286] pg/ml and age 74 [69-80] yrs. MAP was 98±13 mmHg and stable over 12 months; the range within patients was 28±7 mmHg. The slope of the MAP/GFR curves at MAP of 95 mmHg was low: 0.07 (-0.12 - 0.65) (ml/min.1.73m^2)/mmHg, stabilization was 75% (1-147%). Some patients displayed a slight increase in GFR upon a decrease in MAP. The 24 patients with the lowest slopes were not different from those 24 with the highest slopes regarding age, smoking behaviour, baseline GFR, MAP, NT-proBNP, or prevalence of atherosclerotic renal artery stenosis (ARAS defined as>50% by MRA).

Conclusion

The results do not support that BP is a strong determinant of renal function in stable patients with combined chronic HF and CKD. Basic mechanisms stabilizing GFR upon changes in BP seem to be intact, even in the face of a very high prevalence of ARAS.

INTRODUCTION

Impairment of renal function is an important determinant of outcome in heart disease[1-3]. In the setting of acute heart failure (HF), worsening renal function defined as decrease in estimated glomerular filtration rate (GFR) of more than 15 ml/min/1.73m^2 has been associated with an odds ratio of >4 for all cause mortality[4]. In more chronic settings, epidemiological reports have associated GFR and renal blood flow (RBF) with arterial pressure, central venous pressure and cardiac index[5] and with brain natriuretic peptide (BNP)[5, 6]. Nevertheless, the pathophysiology of these associations remains ill defined[7]. A decrease in RBF and GFR in patients with HF has been classically attributed to a decrease in arterial blood pressure and/or a decrease in cardiac output. Recently, the hypothesis of increased renal venous pressure[8, 9] as explanation of a decrease in RBF and GFR in HF has resurfaced[5, 10]. Most associations are derived from cross-sectional data. It would be interesting to study whether GFR indeed shows dependency on arterial pressure within individual subjects with HF. Dependency of GFR and RBF upon renal venous pressure within an individual is harder to study.

In healthy subjects, GFR is not very sensitive to acute changes in renal perfusion pressure, because of the intrinsic ability of the kidneys to stabilize both RBF and GFR by adjustments in afferent arteriolar resistance (renal autoregulation)[7, 11]. However, in patients with chronic kidney disease (CKD)[12] and in experimental models of renal disease[13, 14], this ability to autoregulate can be substantially impaired, making GFR sensitive to changes in renal perfusion pressure. Combined HF and CKD, could cause GFR to be sensitive to renal perfusion pressure due to functional issues such as poor autoregulation, but also due to structural issues such as the presence of atherosclerotic renal artery stenosis (ARAS).

The EpoCaReS study (ClinTrials.Gov NCT 00356733) is a translational study originally designed to investigate haematopoietic versus non-haematopoietic consequences of exogenous erythropoietin in patients with combined stable chronic HF (CHF) and CKD. During the time frame of observation, patients had no exacerbations of their HF and no acute deteriorations of their CKD. In this study, GFR (derived from estimated creatinine clearance) and arterial blood pressure was measured at baseline, and then monthly for 1 year. From these data, individual relationships between slow fluctuations in arterial pressure and GFR can be studied to define whether GFR is dependent upon blood pressure. We tested the hypothesis that GFR is susceptible to fluctuations in arterial blood pressure in patients with combined CHF and CKD. We also investigated whether indicators of volume control, supposedly associated with lower central venous and renal venous pressures, were associated with better or worse long-term stabilization of GFR.

METHODS

Patient recruitment and study design

In an open-label, randomized trial, 48 patients with the combination of CHF, CKD and mild anaemia were included in the current study, which is part the EPOCARES trial of which a detailed protocol description is available (ClinicalTrials.gov NCT

00356733). The medical ethics committee of the Univ. Medical Center Utrecht, The Netherlands approved the protocol and all patients gave their written informed consent. All procedures were in accordance with the Helsinki Declaration. Anaemia was defined as haemoglobin (Hb) between 10.3-12.6 g/dl in men and between 10.3-11.9 g/dl in women, assessed after 4 weeks of oral iron supplementation. CKD was defined as a GFR of 20-70 ml/min/1.73m^2 according to the Cockroft-Gault equation. CHF was defined as NYHA class II or more, based on symptoms, signs and objective evidence of an abnormality in cardiac structure or function assessed by echocardiography[15, 16]. Patients with heart failure with reduced ejection fraction (EF) or heart failure with preserved EF were included. The last was defined as EF >50%, left ventricular end diastolic volume index <97 ml/m^2 by echocardiography and evidence of diastolic left ventricular dysfunction[17]. Run-in treatment comprised oral iron supplementation, aspirin when indicated, and maximum tolerated dosages of a β-blocker, an angiotensin converting-enzyme (ACE) inhibitor and/or an angiotensin receptor blocker. Exclusion criteria included uncontrolled hypertension, kidney transplantation and acute renal failure. Hypertension was defined as the presence of a persistent systolic blood pressure >140 mmHg, a diastolic blood pressure >90 mmHg or the use of medication for the treatment of elevated blood pressure in combination with a previous diagnosis of hypertension by general practitioner, cardiologist or nephrologist.

Since the EPOCARES study was designed to test haematopoietic and non-haematopoietic consequences of EPO treatment, patients were randomized 1:1:1 to EPO (50 IU/kg/wk; Neorecormon, Roche Pharmaceuticals, Woerden, Netherlands), EPO with maintenance of baseline haemoglobin or standard treatment without EPO for one year. In one subgroup of EPO-treated patients, Hb level was allowed to increase up to 13.7 g/dl for men and 13.4 g/dl for women. In the other subgroup of EPO-treated patients, Hb levels were kept stable at baseline levels by Hb monitoring and blood withdrawal for the initial 6 of the 12 months of follow up, as described in detail elsewhere[18]. All treated and untreated patients were followed monthly for 12 months.

Measurements

All blood samples were drawn between 8 and 9 AM with patients in a supine position; serum and plasma samples were stored at -80 ˚C until analysis. Serum urea nitrogen, creatinine and Hb were assessed using routine methods. NT-proBNP was measured using an electro-chemiluminescense immunoassay on a Cobas CA6000 from Roche (Mannheim, Germany). At baseline, creatinine clearance was calculated from plasma creatinine and 24-hour urine creatinine excretion using standard formula. Creatinine clearance was estimated using the Cockcroft equation at every monthly visit. The latter was used for the MAP-GFR analyses. Blood pressure was measured in duplicate every visit after a 5 min resting period in seated position using an automated device (Dinamap Pro 100V2; Eindhoven, The Netherlands). Body weight was measured at every visit using a calibrated digital Seca scale.

Imaging

The heart and the renal arteries were studied by magnetic resonance (MR) imaging on a 1.5 Tesla Philips Intera (Philips Medical Systems, Best, the Netherlands) at

baseline and at 6 and 12 months. Patients with cardiac implantable electronic devices and/or claustrophobia did not undergo this investigation. At the time the study started, no concerns about potentially severe consequences of gadolinium in the form of Nephrogenic Systemic Fibrosis had been reported. No complications have been observed in the study population. In a 45-min protocol, cardiac function and both renal arteries were assessed. The number of renal arteries as well as patency and presence or absence of stenosis was assessed. Stenosis was graded as: no stenosis, <50% stenosis, 50-70% stenosis, 70-99% stenosis, 100% occlusion or previous stent placement. ARAS was defined as having a stenosis > 50% or a previous renal revascularization.

Analysis of GFR-arterial pressure relationships

The study yielded 13 measurements of MAP and GFR. For each individual, these 13 pairs were plotted, and subjected to formal outlier detection performed using median ± 3x median average deviation as cut-off. Most of the curves did not visibly show a lower limit of autoregulation[14] as can be expected when using only spontaneous MAP fluctuations. This was verified for each curve by applying a logistic regression equation developed previously to analyse GFR-Pressure relationships to search for an inflection point at lower pressure, which could indicate a lower limit of autoregulation[14]. In the cases that a lower limit could be calculated (n=8), the inferred GFR, the slope of the curve at each pressure and the fractional stabilization, the equivalent of the autoregulatory index at mean arterial pressure of 70-120 mmHg were estimated with steps of 10 mmHg from the logistic regression equation[14]. If not lower limit could be determined a linear regression was used to calculate slopes and fractional stabilization. A fractional stabilization of 0% means that the observed slope was similar to the passive (linear) slope, a fractional stabilization of 100% indicates that upon changes in renal perfusion pressure, no change in calculated GFR was observed. For further analysis of clinical determinants, the group was dichotomized into subjects having fractional stabilization above and below the median at 95 mmHg of renal perfusion pressure.

Statistical analysis

Data analysis was performed using SPSS version 15.0 for Mac (IBM) and Prism 5.0 (GraphPad). Data are expressed as mean ± standard deviation for parametric data and as median (interquartile range) for non-parametric data. Multiple group comparisons were performed using ANOVA with LSD as post-hoc test. Correlations were assessed by Pearson's or Spearman's correlation coefficient where appropriate. $P<0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Baseline characteristics of the 48 patients for whom we had sufficient data for MAP/GFR analysis can be found in Table 1; data are depicted for all subjects, and for the subjects that had most stable and least-stable GFR. Most subjects were over 60 years of age, had significant renal dysfunction, had near-controlled blood pressure

on anti-hypertensive therapy, but displayed a tendency for increased pulse pressures and isolated systolic hypertension. Almost all of the patients used inhibitors of the renin angiotensin system; the majority also used beta-blockers and diuretics.

GFR and blood pressure throughout the follow up

Figure 1A displays average mean arterial pressure (MAP) and figure 1B shows GFR of all patients throughout the year. There were no consistent changes in either variable throughout the year of observation. The swings in blood pressure were considerable within each individual and averaged 28 ±7 mmHg.

Table 1 | Baseline characteristics of the patients

Characteristic	All patients n=48	Less stable n=24	More stable n=24	P- value
Age, years	74 [69-80]	73 [67-81]	74 [70-80]	0.78
Male sex, no. (%)	31 (65)	17 (71)	14 (58)	
Creatinine, umol/L	184 ± 79	200 ± 83	169 ± 73	0.21
Cockroft, ml/min	38 ± 14.9	38 ± 16.6	39 ± 13.4	0.52
eGFR (MDRD) (ml/min/1.73m^2)	36 ± 13.8	33 ± 12.4	39 ± 14.9	0.18
SBP, mmHg	143 ± 21.4	144 ± 16.6	142 ± 25.7	0.85
DBP, mmHg	75 ± 10.7	76 ± 9.4	75 ± 12.0	0.46
MAP, mmHg	98 ± 13.0	98 ± 10.8	97 ± 15.1	0.70
Pulse pressure, mmHg	68 ± 16.3	68 ± 12.4	67 ± 19.7	0.50
RAS inhibitor use	98	100	96	0.31
β-blocker use, %	73	63	83	0.10
Diuretic use, %	81	83	79	0.71
Diabetes, %	35	33	38	0.76
Hypertension, %	79	83	75	0.48
Smoking, % (active and/or previous)	66	62	71	0.62
NT-proBNP (pg/mL)	1280 [640-2286]	1586 [688-2642]	1038 [614-2273]	0.46
LVEF (%)	43 ± 11.0	44 ± 11.9	43 ± 10.3	0.92
NYHA class heart failure				0.75
Class II, no. (%)	35 (73)	17 (71)	18 (75)	
Class III/IV, no.(%)	13 (27)	7 (29)	6 (25)	
MRI/MRA results (n=36)				
ARAS, no (%)	20 (56)	7 (44)	13 (65)	0.20
Unilateral ARAS, no (%)	13 (36)	4 (25)	9 (45)	
Bilateral ARAS, no. (%)	7 (19)	3 (19)	5 (20)	

Mean ± standard deviation or median [interquartile range] are shown. Abbreviations: eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; RAS inhibitor, renin-angiotensin-system inhibitor; NT-proBNP, N-terminal pro-brain natriuretic peptide; LVEF, left ventricular ejection fraction by echocardiography; NYHA, New York Heart Association, cMRI/MRA, combined cardiac magnetic resonance imaging with MR angiography of the renal arteries; ARAS, atherosclerotic renal artery stenosis. * cMRI/MRA was performed in 36 subjects, absolute numbers and percentages of these 36 subjects are shown.

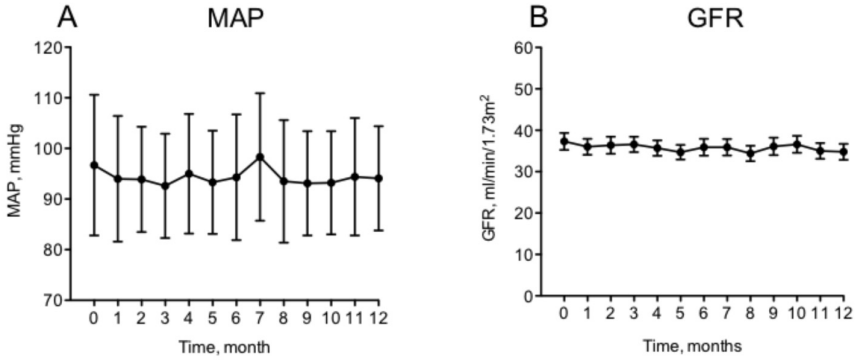

Figure 1 | Average mean arterial pressure (MAP, A) and glomerular filtration rate estimated using Cockroft Gault (GFR, B) throughout the 12 months of follow-up in all 48 patients.

Overall relation between MAP, NT-proBNP and GFR

The first approach to analyse these relations was to plot MAP versus GFR and NT-proBNP, and to plot GFR versus NT-proBNP of all data points (Figure 2). MAP was negatively related with GFR, (Figure 2A), and positively related to NT-proBNP (Figure 2C). Interestingly, GFR was negatively related to NT-proBNP, (Figure 2B), but at low GFR values, the curve was fan-shaped. This might indicate that subgroups behaved differently. It should be emphasized that all correlation coefficients were quite low.

Individual GFR-MAP relationships

The next step was to calculate the MAP-GFR relationship for each individual using the methods outlined above. Figure 3A shows the GFR recalculated at MAP of 70-110 mmHg in 5 mmHg bins. As can be appreciated from the figure, GFR changes on average were quite modest upon these subacute changes in blood pressure. This becomes even more evident, when the average slope (Figure 3B) and the degree of stabilization (Figure 3C) are inspected. The low slopes indicate that variations in MAP resulted in very small changes in GFR. This is also reflected in the stabilization index of about 80%. Since a stabilization index of 100% means that GFR remains completely stable upon variations in MAP, the observed stability can be interpreted as being high.

Clinical parameters of subjects with best versus least maintained GFR upon MAP changes

The patient data were divided into those with the most stable and least stable GFR upon changes in MAP, defined by the slope of the MAP-GFR relationship for each individual. Figure 4 shows that the group with the most stable GFR, had, if anything, a marginal, non-significant increase in GFR when MAP declined. The group with the least stable GFR, showed a decrease of about 2 ml/min/1.73m^2 per 10 mmHg decline, which is still low when related to the average GFR in this group of about 35 ml/min/1.73m^2.

When comparing the baseline parameters of the more stable and less stable GFR groups, as depicted in Table 1, no significant differences could be identified. In particular, there were no significant differences between baseline GFR, SBP, DBP, MAP, EF, NYHA class or NT-proBNP levels. Neither were there any differences in medication profile, e.g. the use and dose of inhibitors of the renin angiotensin system, Moreover, it should also be mentioned that the distribution of the patients

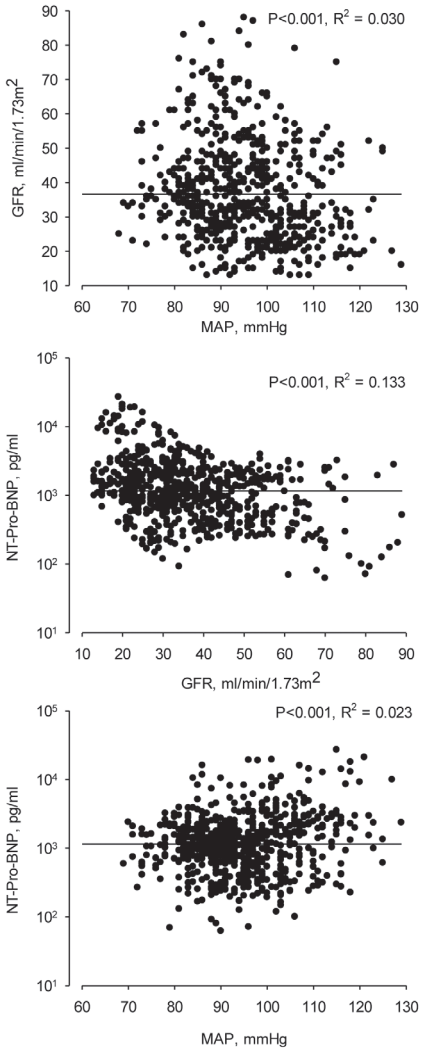

Figure 2 I Scatter plots and correlations are displayed between mean arterial pressure (MAP), and glomerular filtration rate estimated using Cockroft Gault (A), scatter plots and correlation between plasma NT-proBNP and glomerular filtration rate estimated using Cockroft Gault (B) and the relation between MAP and plasma NT-proBNP (C). Please note the logarithmic scale for NT-proBNP levels.

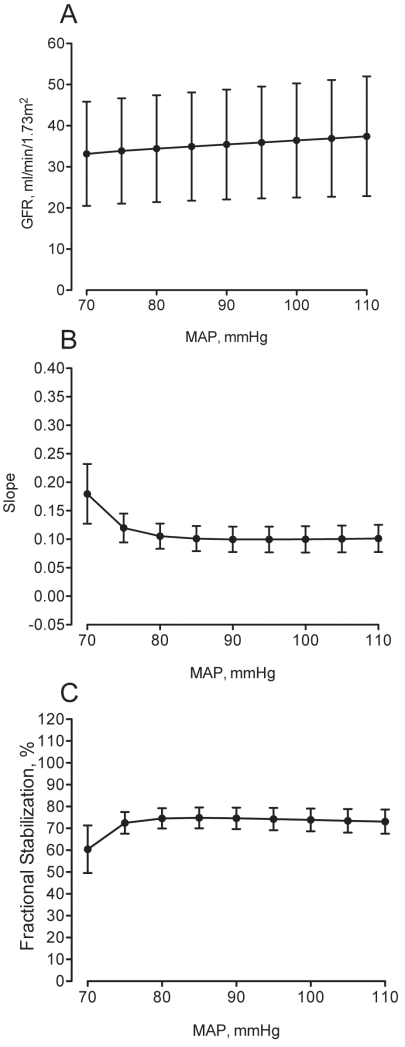

Figure 3 I Average of recalculated GFR values at 5 mmHg steps of MAP for each individual (A), average slopes (B) and average degree of stabilization (C).

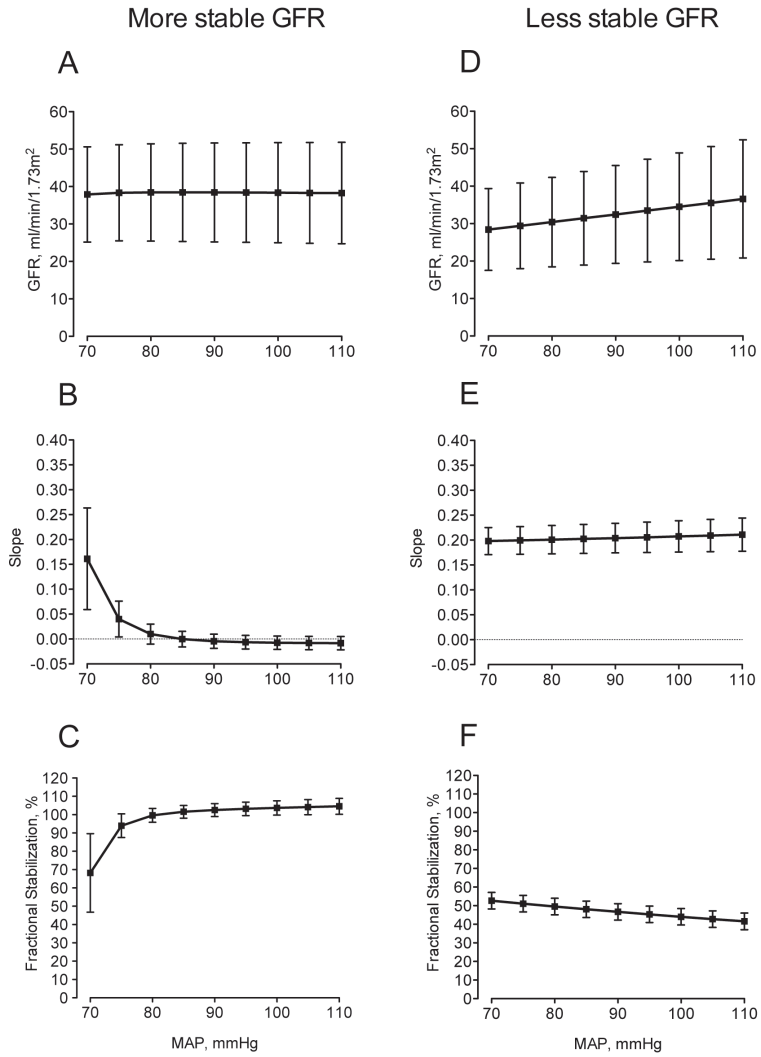

Figure 4 | Average of recalculated GFR values, slopes and degree of stabilization per 5 mmHg step of MAP for the patients with more stable (A-C) versus the less stable (D-F) GFR.

between those with less stable and more stable GFR was similar for the different study groups: The number of more stable GFR patients was 9 in the control group, 8 in the EPO group and 9 in the EPO group with Hb-levels maintained at phlebotomy. In the less stable GFR patients this was 8 in the control group, 10 in the EPO group and 7 for the EPO group with maintained Hb-levels.

Medication changes during the follow up

During the study period diuretics were increased in 11 (23%) patients and decreased in 4 (8%). There was no correlation between changes of diuretic dose and stabilization of GFR (p=0.35). RAS inhibition was increased in 5 (10%) patients and decreased in 5 (10%); this was also not correlated to stabilization indexes.

Presence of unilateral or bilateral RAS

In 36 of the 48 patients used for analysis, a combined cardiac MRI/MRA was performed. Twenty (55.6 %) of these 36 patients were diagnosed with an ARAS of >50%, versus 16 patients without ARAS. Of these 20 patients, 13 had an unilateral and 7 a bilateral ARAS. Between those patients undergoing cMRI/MRA and those that did not undergo this MR study, were no significant differences (data not shown). The presence of ARAS did not correlate with stabilization of GFR, irrespective of whether the ARAS was unilateral or bilateral (Table 1).

Relating monthly changes in GFR and changes in NT-proBNP

To further study whether GFR changes and NT-proBNP changes were related, the per cent change in GFR and NT-proBNP were calculated for each individual on a monthly basis. Out of the 48 relations, 10 individuals showed a significant positive correlation, and 7 a negative correlation, while the relation between GFR and NT-proBNP did not reach significance in the others. Examples of positive and negative correlations are shown in Figure 5.

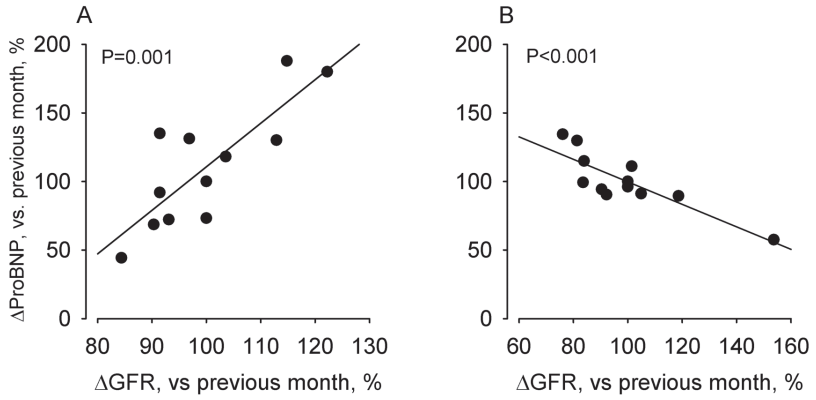

Figure 5 | Examples of a patient with a clearly positive and a patient with a clearly negative relation between GFR and NT-proBNP levels.

DISCUSSION

In the present study we studied patients with combined chronic heart and renal failure followed longitudinally for 12 months. We failed to confirm the hypothesis that blood pressure is an important determinant of GFR under these chronic conditions. Conversely, GFR was remarkably stable upon variations in MAP, and no clinical characteristics could be associated with more or less stabilization of GFR, including the presence of ARAS and NT-proBNP levels. Therefore, this study shows within this group of subjects with combined CKD and CHF that both neurohumoral control and autoregulatory mechanisms mediating stabilization of GFR appear to be largely intact.

A decrease in GFR in HF patients can be the consequence of a change in the primary driving forces for glomerular filtration, failure of renal autoregulation, neurohumoral control mechanism causing renal vasoconstriction in response to a reduction in

cardiac output or some combination of these factors. A long-held proposition is that HF causes a decrease in arterial pressure, which could decrease renal perfusion pressure and glomerular capillary pressure leading to a decrease in GFR[19]. This would also evoke activation of the renin-angiotensin system (RAS) and the sympathetic nervous system, leading to renal vasoconstriction and consequently to reduction of RBF and GFR[19]. The present data challenges this. First, within subjects, fluctuations in GFR upon fluctuations in blood pressure were very small. Second, between subjects, blood pressure did not differ in patients with less or more stable GFR. Thus, arterial blood pressure per se does not seem to be the key determinant of GFR in the present study group. The data are consistent with cross-sectional studies in humans with HF that were not able to identify lower arterial pressure as potential determinant of GFR[5, 20]. Regarding activation of the RAS, virtually all subjects were treated with ACE inhibitors or angiotensin receptor blockers. Therefore, the study is not able to confirm or reject the proposition that the RAS can mediate a decrease in GFR at a lower perfusion pressure.

Renal autoregulation of GFR and RBF is the well-recognized phenomenon that GFR and RBF remain stable over a wide range of renal perfusion pressures[11, 21, 22]. Renal autoregulation is generally considered in an acute context. Nevertheless, it also forms part of the mechanisms that stabilize GFR over more prolonged periods of time. In experimental animals, chronic failure of autoregulation has been associated strongly with the development of hypertensive renal injury in a variety of models[23, 24]. There are few studies on stability of GFR, or lack thereof, in CKD patients. We have reported that after cessation of ACE inhibitor or angiotensin receptor blocker therapy in patients with moderately severe CKD (eGFR ~40-45 ml/min/1.73m^2), MAP increased more than 10 mmHg on average, however, GFR was unchanged[25]. Two separate studies in diabetic nephropathy showed some more pronounced changes in GFR upon cessation of medication and consequent elevation of blood pressure, which was explained as a decrease in the ability to autoregulate, however, here blood pressure was reduced using clonidine, which makes a comparison difficult[26, 27]. Interestingly, the current data show that these systems are operational in patients with moderately severe renal dysfunction, at least in the light of slow fluctuations in renal perfusion pressure.

It has been documented that the prevalence of ARAS is approximately 15% in patients with coronary artery disease and up to 40% in patients with end-stage renal disease[28]. The prevalence of unilateral or bilateral ARAS (defined as >50% assessed by MR angiography) in this group of patients with combined CHF and CKD was very high: about 55%. In several study subjects, this might well have resulted in substantially lower pressure along the preglomerular vascular tree potentially leading to exhaustion of the capacity of autoregulation to stabilize GFR. If one considers that kidneys with impaired function were generally stabilizing GFR in the face of slow fluctuations in arterial pressure quite well, this might at first glance seem paradoxical. However, in experimental animals it has been shown that renal autoregulation can reset to align around prevailing perfusion pressures[29, 30]. Therefore, the mechanisms that are able to align autoregulation systems with prevailing pressures also seem to be intact. Clinically, this might have implications for the therapeutic approach to blood pressure management in this patient group, in that very gradual changes in arterial pressure might be well tolerated.

When considering the basic driving forces of glomerular filtration in the situation of HF with venous congestion, increase in central venous pressure is associated with lower levels of GFR[5, 8-10]. Direct dependency of GFR and RBF on renal venous pressure within an individual is hard to study. However, in line with these observations, the correlations between MAP on the one hand and estimated creatinine clearance and NT-proBNP on the other hand were negative, albeit weak. Moreover, in some subjects we observed increases in GFR upon decreases in MAP. This led us to investigate whether indicators of volume control, supposedly associated with lower central venous and renal venous pressures, were associated with GFR in individual subjects. Body weight, BMI and changes in diuretic doses could not be related to changes in GFR. Similarly, changes in NT-proBNP were related to GFR in only a minority of patients and even then both positive and negative correlations were observed. As such, we have not been able to correlate indicators of volume status to GFR in this small translational study, which was observation based. The data do not necessarily argue with such a correlation, however.

There are several limitations to the current study. First, one could argue that the assessment of GFR based on monthly plasma creatinine levels is suboptimal. Although there are potentially more precise methods available (creatinine and inulin clearance and radioisotope techniques), repeated application of these are limited by costs, discomfort and safety for the patient. Nevertheless, since the data indicate stability of GFR, we doubt that this is relevant for the overall conclusions of this work. Next, patients in the study were, in general, stable in the sense that only minor medication adjustments were necessary during the course of the one year protocol. As such, this might not be the same patient group as the group that is frequently reported in the literature, with exacerbations of acute HF and the necessity for aggressive diuretic therapy. Moreover, medications may have obscured potential pathophysiological mechanisms that might be involved in impairment of GFR stability, such as the RAS, however the study group was on therapy that is considered standard of care. Despite this, the data are consistent with the idea that in patients with established chronic heart and renal failure the capacity to stabilize GFR is maintained. Last but not least, one could argue that a larger group of patients would have been preferable, with more complete imaging. We do not consider it likely that this would change the key finding of the paper.

Altogether, the present study does not support that arterial blood pressure is a strong determinant of renal function in chronic patients with combined heart and renal failure. Basic mechanisms stabilizing glomerular filtration upon changes in arterial pressure seem to be intact, even in the face of a very high prevalence of ARAS. Although the data hint at a potential role for volume status, and indirectly for venous pressure, as a determinant for GFR, the present study does not allow firm conclusions in this respect.

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PART II

Atherosclerotic renal artery stenosis is prevalent in cardiorenal patients but not associated with left ventricular function and myocardial fibrosis as assessed by cardiac magnetic resonance imaging

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ABSTRACT

Background

Atherosclerotic renal artery stenosis (ARAS) is common in cardiovascular diseases and associated with hypertension, renal dysfunction and/or heart failure. There is a paucity of data about the prevalence and the role of ARAS in the pathophysiology of combined chronic heart failure (CHF) and chronic kidney disease (CKD). We investigated the prevalence in patients with combined CHF/CKD and its association with renal function, cardiac dysfunction and the presence and extent of myocardial fibrosis.

Methods

The EPOCARES study (ClinTrialsNCT00356733) investigates the role of erythropoietin in anaemic patients with combined CHF/CKD. Eligible subjects underwent combined cardiac magnetic resonance imaging (cMRI), including late gadolinium enhancement, with magnetic resonance angiography of the renal arteries (MRA).

Results

MR study was performed in 37 patients (median age 74 years, eGFR 37.4 ± 15.6 ml/min, left ventricular ejection fraction (LVEF) 43.3 ± 11.2%), of which 21 (56.8%) had ARAS (defined as stenosis >50%). Of these 21 subjects, 8 (21.6%) had more severe ARAS > 70% and 8 (21.6%) had a bilateral ARAS >50% (or previous bilateral PTA). There were no differences in age, NT-proBNP levels and medication profile between patients with ARAS versus those without. Renal function declined with the severity of ARAS (p=0.03), although this was not significantly different between patients with ARAS versus those without. Diabetes mellitus was more prevalent in patients without ARAS (56.3%) against those with ARAS (23.8%) (p=0.04). The presence and extent of late gadolinium enhancement, depicting myocardial fibrosis, did not differ (p=0.80), nor did end diastolic volume (p=0.60), left ventricular mass index (p=0.11) or LVEF (p=0.15). Neither was there a difference in the presence of an ischemic pattern of late enhancement in patients with ARAS versus those without.

Conclusions

ARAS is prevalent in combined CHF/CKD and its severity is associated with a decline in renal function. However, its presence does not correlate with a worse LVEF, a higher left ventricular mass or with the presence and extent of myocardial fibrosis. Further research is required for the role of ARAS in the pathophysiology of combined chronic heart and renal failure.

INTRODUCTION

The combination of chronic heart failure (CHF) and chronic kidney disease (CKD) is prevalent and associated with a high cardiovascular mortality and morbidity[1-3]. Both CHF and CKD can be caused by atherosclerosis. Renal artery stenosis is often of atherosclerotic aetiology and can manifest itself by hypertension, progressive renal dysfunction, flash pulmonary oedema as well as congestive heart failure[4-6]. Furthermore, it is often diagnosed without evident clinical symptoms. The prevalence of atherosclerotic renal artery stenosis (ARAS) is prevalent in cardiovascular diseases; its prevalence is approximately 15% in patients with proven coronary artery disease and it is up to 40% in patients with end stage renal disease[7]. However, only very few studies have determined the prevalence of ARAS in patients with combined CHF and CKD.

In CHF, in several observational studies, patients with ARAS had prolonged hospital admissions and a higher mortality rate[8-10]. This finding is in agreement with reports that the presence of ARAS is associated with systemic atherosclerosis[11], reduced renal filtration and perfusion and with cardiac abnormalities. However, few data are known about its association with cardiac abnormalities. In an echocardiography study the majority of patients with proven ARAS had cardiac abnormalities, mostly consisting of diastolic dysfunction and left ventricular hypertrophy[12]. To our knowledge, no data are known about its possible association with the presence and extent of myocardial fibrosis, potentially reflecting an increased risk of sudden cardiac death. Although the concurrence of ARAS and cardiac abnormalities may play a role in the cardiorenal syndrome, there are few data to substantiate this and the prevalence of ARAS in combined CKD and CHF is unknown.

We hypothesized that ARAS is prevalent in patients with combined CKD and CHF and that its presence is associated with a worse renal function, more severe cardiac dysfunction and the presence and extent of myocardial fibrosis. Therefore we assessed, in anaemic patients with combined heart and renal failure: 1. The prevalence of ARAS and 2. The association of ARAS with renal and cardiac dysfunction and the presence of myocardial fibrosis. We used cardiac magnetic resonance imaging (cMRI) for this purpose, as the quantitative values for cardiac volumes, left ventricular mass and function are more accurate than with echocardiography. In addition, intravenous gadolinium contrast material can be used both for magnetic resonance angiography (MRA) of the aorta and the renal arteries, as well as for late gadolinium enhanced (LGE) assessment of myocardial fibrosis, thereby providing information about the underlying aetiology of the cardiac dysfunction.

METHODS

Patient population

This study is a sub study of the EPOCARES study (ErythroPOietin in the CArdioREnal Syndrome, ClinTrials.Gov NCT 00356733). The study design has been published elsewhere[13]. In short, the EPOCARES study is an open-label, prospective, randomized trial, in which patients with CHF, CKD (glomerular filtration rate (GFR)

by Cockcroft-Gault equation of 20-70 ml/min) and mild anaemia (haemoglobin 10.3-12.6 g/dL for men and 10.3-11.9 g/dL for women) were included to test the erythropoietic and non-erythropoietic responses to erythropoietin (EPO) treatment. At baseline all patients without cardiac implantable electronic devices underwent combined cMRI and magnetic resonance angiography (MRA) of the renal arteries. During the study period, the concern about the association between certain gadolinium agents and nephrogenic systemic fibrosis surfaced, which led us to the decision to perform cMRI/MRA only in patients with a GFR > 30 ml/min. All patients were on maximal tolerated dosages of a β-blocker, angiotensin-converting enzyme (ACE) inhibitor and/or an angiotensin receptor blocker according to CHF guidelines. CHF was defined as NYHA class II or III, based on symptoms and signs[14]. Both patients with heart failure with reduced ejection fraction (HFREF) and patients with heart failure with preserved ejection fraction (HFPEF) were included. HFPEF is defined according to recent guidelines[15]. Hypertension was defined as the presence of a persistent systolic blood pressure > 140 mmHg, a diastolic blood pressure > 90 mmHg or the use of medication for the treatment of elevated blood pressure in combination with a previous made diagnosis of hypertension. The aetiology of heart failure was divided in ischemic, hypertensive, valvular or other. Ischemic aetiology was defined as having previously had a myocardial infarction, a percutaneous coronary intervention, a surgical coronary artery revascularization, a stenosis of > 70% in an epicardial vessel on coronary angiography or the presence of ischemia on nuclear testing. The Medical-Ethical Committee of both the University Medical Centre Utrecht and the Meander Medical Centre (no. 05/220) approved the protocol of the study. Procedures were in accordance with the Helsinki Declaration and all patients gave written informed consent.

Magnetic Resonance Imaging: acquisition protocol

Cardiovascular Magnetic Resonance Imaging and magnetic resonance angiography (MRA) of the renal arteries were performed on a 1.5 Tesla Philips Intera (Philips Medical Systems, Best, the Netherlands). Both the heart and the renal arteries were assessed in a 45-minute protocol. The patient was placed in the supine position with a five-channel phased array coil for the cardiac analysis and a circularly polarized spine coil in longitudinal direction over the area of the kidneys below the cardiac coil for MRA of the renal arteries.

ECG-triggered breath hold multiphase steady-state free procession (SSFP) images were acquired in the four-chamber, short-axis, and two- chamber view scans of the left ventricle. The short axis plane covered both ventricles from apex to base using 8-mm slices without interslice gap with the following scan parameters: TR/TE 4.0/2.0ms, flip angle 50, FOV 350-400, matrix 256x256, voxel size 1.6x1.6x8.0mm.

Gadolinium-based contrast (Dotarem, Geurbet, France) was administered intravenously to first obtain MRA of the renal arteries at the time of injection (first pass) and 15 minutes later delayed enhancement scans of the heart. For MRA of the renal arteries a standard breath hold 3D T1 contrast-enhanced MRA technique was used. Scan parameters: TR/TE 3.7/1.33ms, flipangle 25, FOV 430x430x75, voxel size 0.8x0.8x1.5mm.

For the delayed enhancement of the heart, breath hold inversion recovery T1 pulse images were acquired in four-chamber, short axis and left two-chamber view. Scan parameters: TR/TE 4.4/1.3ms, flipangle 15, FOV 410x410x80, matrix 300x169, voxel size 1.4x1.4x5.0mm. There were no complications related to the MRI procedures, and all patients tolerated the procedure well.

Magnetic Resonance Imaging: analysis

The SSFP cine short axis scans were used to acquire measurements of the left ventricle end diastolic volume (EDV), end systolic volume (ESV), ejection fraction (EF) and wall mass (EasyVision release 4 cardiac package, Philips Medical Systems). Endocardial and epicardial contours were traced manually on the stack of contiguous short-axis cine-images at end-diastole. This technique has been validated, with high accuracy and reproducibility[16]. A trained investigator (ME) performed the quantitative image analyses.

Assessment of segmental wall motion and late gadolinium enhancement (LGE) was performed by two independent investigators, blinded for clinical data (BV and YA). The left ventricle was divided in 17 segments according to standardized nomenclature[17] and described as either normal, hypokinetic, akinetic, dyskinetic or aneurysmatic. Enhancing areas of the myocardial wall were identified as fibrotic areas, and separated into probable previous myocardial infarctions (subendocardial to transmural location) or non-ischemic enhancing wall abnormalities (midwall or subepicardial location). To quantify the extent and/or transmurality of the scar tissue, we used the following definitions; a. spatial extent; the number of affected segments; b. transmurality; the number of affected segments with hyperenhancement score of 3 or higher and; c, the total scar score; the summed segmental scores per patient divided by 17 (reflecting the damage for each patient)[18]. Late enhancement was estimated by using a 5-group classification according to the degree of left ventricle wall involvement with 0, absence of hyperenhancement, 1, hyperenhancement of 1% to 25% of left ventricle wall thickness; 2, hyperenhancement of 26% to 50%; 3, hyperenhancement extending from 51% to 75%; 4, hyperenhancement extending 76% to 99% and 5, hyperenhancement extending 100%.

The number of renal arteries as well as patency and presence or absence of stenosis was assessed by two independent investigators, blinded for clinical and cMRI data (JV and LM). Stenosis was graded as: no stenosis, <50% stenosis, 50-70% stenosis, 70-99% stenosis, occlusion 100% or previous stent placement. The presence of a renal artery stenosis was defined as having a stenosis > 50% or previous renal revascularization, conform criteria from the Stenting in Renal dysfunction caused by atherosclerotic Renal Artery Stenosis[19].

Statistical analysis

Data are presented as medians with inter-quartile ranges (IQR) for non-normally distributed variables and means ± standard deviation (SD) for normally distributed continuous variables. Differences between groups were compared with the χ^2 test, Mann-Whitney U test or the Kruskal-Wallis one-way ANOVA when appropriate. Differences were considered significant when $P<0.05$. For statistical analyses the Statistical Package for Social Sciences (IBM, Chicago, Illinois, USA) version 18 was used.

RESULTS

Clinical characteristics

The original study population of the EPOCARES study comprised of 62 patients. Five patients withdrew their informed consent and one patient was excluded due to a suspected malignancy (diagnosed on routine X-ray at baseline). Of the 56 patients that eventually participated in the study, 37 patients underwent a cMRI/MRA. Nineteen patients did not undergo cMRI/MRA due to presence of cardiac implantable electronic devices (n=15), orthopnoea (n=2), claustrophobia (n=1) or a GFR < 30 ml/min (n=1). These data are obtained after run in treatment with optimal medical therapy for CHF and oral iron supplementation, but before treatment with EPO. Clinical characteristics of these 37 patients are shown in Table 1. The clinical characteristics of the patients that underwent cMRI/MRA did not differ from those patients that did not undergo cMRI/MRA. Overall, patients had markedly reduced eGFR and LVEF. The majority of patients were using a renin-angiotensin blocker and a beta-blocker. A substantial fraction had hypertension and/or diabetes mellitus.

Renal artery stenosis

Of the 37 patients that underwent cMRI/MRA, 21 patients (56.8%) had a renal artery stenosis defined as > 50% stenosis. A more severe stenosis, defined as > 70%, was present in 8 (21.6%) patients. A bilateral ARAS (>50%) was present in 7 (18.9%) patients. All stenosis were of atherosclerotic origin. One patient (2.7%) was previously treated bilaterally by angioplasty with stent placement. Baseline demographic, clinical and laboratory characteristics of the patients, divided up by the presence or absence of ARAS, defined as > 50% stenosis, are provided in Table 1. There were no differences in age, sex, smoking, the amount of pack years, and the aetiology and severity of heart failure (NYHA class and NTproBNP levels) between patients with ARAS versus those without ARAS. Although there seems to be a tendency for a higher systolic blood pressure based on the office measurements, there is no statistically significant difference in the averaged 24-hour ambulatory blood pressure measurements. The number of antihypertensive drugs in patients with and without ARAS did not differ. Diabetes mellitus was significantly more prevalent in patients without ARAS (Figure 1). We did not find a significant difference in renal function when patients with and without ARAS were compared. Nonetheless, the renal function did significantly decline as the severity of ARAS increased, (ANOVA; p=0.03); patients with a unilateral ARAS of > 70% or with bilateral ARAS had a lower GFR (Table 2 and Figure 2). However, there were no other differences in clinical profile between patients with a moderate ARAS (>50%, less than 70% stenosis) versus those with a more severe ARAS (>70% stenosis) (data not shown). The doses/day for RAS inhibition and loop diuretics were not different between patients with and without ARAS. There were also no apparent differences in medication profile between patients with more or less severe ARAS.

Cardiac magnetic resonance imaging

Data regarding cMRI are shown in Table 3. Although the average LVEF by cMRI was 43.3% in the study population, 8 patients (21.6%) had an LVEF > 50%, underlining the fact that many patients with combined CHF and CKD have HFPEF.

Table 1 | Clinical characteristics of patients with combined chronic heart failure and chronic kidney disease that underwent MR study.

		Atherosclerotic renal artery stenosis		
Variable	**All patients (n=37)**	**Present (n=21)**	**Absent (n=16)**	**p-value**
Age, years	74 [68-80]	74 [70-79]	74 [60-82]	0.83
Male sex, no. (%)	23 (62.2)	13 (61.9)	10 (62.5)	0.97
Body mass index (kg/m2)	26.5 ± 4.2	26.3 ± 3.5	26.8 ± 5.1	0.84
Creatinine, umol/L	190 ± 81	204 ± 83	172 ± 77	0.19
Cockcroft Gault (ml/min)	37.4 ± 15.6	33.1 ± 12.3	42.9 ± 18.1	0.07
Haemoglobin (g/dL)	11.7 ± 0.82	11.7 ± 0.81	11.7 ± 0.86	0.58
CRP (mg/L)	5 [1.0-10.5]	5 [1.5-9.5]	4.5 [1.0-11.3]	0.95
hsCRP (mg/L)	4.0 [1.3-9.9]	3.8 [1.3-8.3]	5.8 [0.7-10.4]	0.89
NTproBNP (pg/mL)	1400 [621-2499]	1680 [653-2229]	1360 [503-2853]	0.71
Micro albuminuria (mg/24h)	21.0 [10.3-218.0]	41.5 [14.5-230.0]	12.5 [8.5-93.8]	0.25
SBP, mmHg	145 ± 21.8	150 ± 21.6	138 ± 20.9	0.12
DBP, mmHg	75 ± 11.5	76 ± 13.2	72 ± 8.6	0.23
24-h SBP, mmHg	127 ± 15.3	126 ± 13.1	128 ± 18.3	0.73
24-h DBP, mmHg	66 ± 8.2	66 ± 8.3	66 ± 8.3	1.00
No. of antihypertensive drugs	3.5 [3.0-4.0]	3.3 [3.0-4.0]	3.8 [2.3-5.0]	0.37
RAS inhibitor				
n (%)	36 (97.3)	21 (100.0)	15 (93.8)	0.25
% of recommended dose/day	50 [38-100]	50 [50-100]	75 [25-138]	0.80
β-blocker use, no. (%)	30 (81.1)	16 (76.2)	14 (87.5)	0.38
Diuretic use, no. (%)	29 (78.4)	16 (76.2)	13 (81.3)	0.71
Loop diuretic, no. (%)	24 (64.9)	12 (57.0)	12 (75.0)	0.26
Loop diuretic, dose/day*	40 [0-80]	40 [0-40]	40 [20-110]	0.17
Aldosterone antagonist, no. (%)	5 (13.5)	3 (23.8)	2 (12.5)	0.42
Statin use, no. (%)	28 (75.7)	15 (71.4)	13 (81.3)	0.49
Diabetes, no. (%)	14 (37.8)	5 (23.8)	9 (56.3)	0.04
Hypertension, no. (%)	29 (78.4)	18 (85.7)	11 (68.8)	0.21
Smoking history, no. (%)	24 (64.9)	15 (71.4)	9 (56.3)	0.38
Pack years	16.4 [0-31]	16.4 [0-33]	11.5 [0-30]	0.34
Cerebrovascular disease, no. (%)	7 (18.9)	6 (28.6)	1 (6.3)	0.11
Peripheral arterial disease, no. (%)	14 (37.8)	11 (52.4)	3 (18.8)	0.05
Kidney length (cm) (n=34)	10.9 ± 4.7	10.3 ± 2.2	11.5 ± 6.5	0.85
Aetiology of heart failure:				0.58
Ischemic, no. (%)	22 (59.5)	13 (61.9)	9 (56.3)	
Hypertensive, no. (%)	5 (13.5)	3 (14.3)	2 (12.5)	
Valvular, no. (%)	4 (10.8)	3 (14.3)	1 (6.3)	
Other, no. (%)	6 (16.2)	2 (9.5)	4 (25.0)	
NYHA class				0.21
II, no. (%)	27 (73.0)	17 (80.9)	10 (62.5)	
III/IV, no. (%)	10 (27.0)	4 (19.0)	6 (37.5)	

Mean ± standard deviation or median [interquartile range] is shown.
Abbreviations; CRP, C-reactive protein; hsCRP, high sensitive CRP; NTproBNP, N-terminal pro-brain natriuretic peptide; SBP, systolic blood pressure; DBP, diastolic blood pressure; RAS inhibitor, renin-angiotensin-system inhibitor; NYHA, New York Heart Association class for heart failure
• Loop diuretic dose/day of furosemide, bumetanide was converted to 1 mg bumetanide=40mg furosemide

There were no differences between patients with versus those without ARAS regarding LVEF, left ventricular volume or the left ventricular mass index. LGE was present in 27 of the 37 patients (73%). In 24 of these 27 patients the LGE was subendocardial or transmural, determined as ischemic LGE. Non-ischemic LGE (midwall or subepicardial) was present in 6 patients; 4 patients had hypertensive heart failure, 1 patient had severe valvular disease and 1 patient had previously had a myocarditis. In 2 of the 4 patients with hypertensive heart failure, there was both midwall and subendocardial LGE present, as was the case in the patient with myocarditis. Based on baseline clinical data, only 22 of the 24 patients with ischemic LGE were known with CHF with ischemic aetiology.

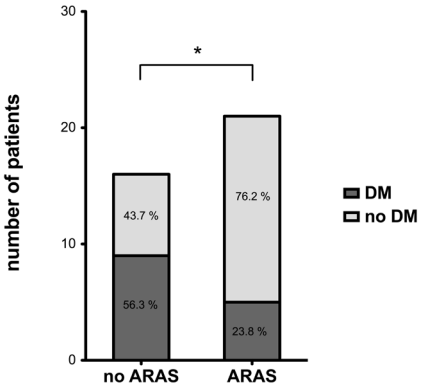

Figure 1 | Number of patients with and without diabetes mellitus (DM), divided by the presence of atherosclerotic renal artery stenosis (ARAS) in patients with combined chronic heart failure and chronic kidney disease. *p = 0.04

Table 2 | Clinical variables of patients with combined chronic heart failure and chronic kidney disease, according to severity of atherosclerotic renal artery stenosis by magnetic resonance angiography

	No stenosis (n=16)	Unilateral stenosis 50-70% (n=5)	Unilateral stenosis > 70% (n=8)	Bilateral stenosis > 50% or previous bilateral PTA (n=8)	p-value
Cockcroft Gault (ml/min)	42.9 ± 18.1	46.4 ± 10.2	30.1 ± 11.3	27.9 ± 8.9	0.01
RAS inhibitor:					
No. (%)	15 (94)	5 (100)	8 (100)	8 (100)	0.72
% of recommended dosage/day	75 [25-138]	50 [38-125]	58 [31-100]	50 [50-100]	0.99
diuretic use, no. (%)	13 (81)	3 (60)	7 (88)	6 (75)	0.68
Loop diuretic, dosage/day*	40 [20-110]	40 [0-120]	40 [10-40]	10 [10-70]	0.43

Mean ± standard deviation or median [interquartile range] is shown. Abbreviations: RAS inhibitor, renin-angiotensin-system inhibitor. * loop diuretic dose/day of furosemide, bumetanide was converted to 1 mg bumetanide=40mg furosemide

There was no difference in the presence and the extent of LGE in patients with ARAS when compared to those without ARAS (Table 3). Nor was there a difference between the two groups regarding the presence and the extent of ischemic LGE versus non-ischemic LGE.

Table 3 | Cardiac magnetic resonance imaging findings in patients with combined chronic heart failure and chronic kidney disease

		Atherosclerotic renal artery stenosis		
Cardiac MRI parameter	**All patients (n=37)**	**Present (n=21)**	**Absent (n=16)**	**p-value**
LVEF (%)	43.3 ± 11.2	46.1 ± 9.1	40.2 ± 12.7	0.15
LVESV (ml)	101 [81.8 -127.9]	96 [81.7 – 114.4]	115 [81.4 – 177.0]	0.19
LVEDV (ml)	186 [153.8 - 206.2]	173 [156.4 - 202.3]	197 [138.5 - 279.0]	0.60
LV mass index (g/m^2)	49 [43.0 - 59.7]	46 [43.2 - 53.3]	60 [41.7 - 69.0]	0.11
Cardiac output (l/min)	5.4 ± 1.85	5.3 ± 1.16	5.5 ± 2.44	0.72
Cardiac index (l/min/m^2)	2.7 ± 0.75	2.7 ± 0.62	2.6 ± 0.90	0.65
Late gadolinium enhancement				
Spatial extent	3.5 [1.0 - 6.0]	4.0 [0.5 - 6.0]	3.0 [2.0 - 6.5]	0.65
Transmurality	2.0 [0 - 4.0]	2.0 [0.0 - 4.0]	2.0 [0.0 - 4.5]	0.89
Total scar score	0.56 [0.12 - 0.99]	0.71 [0.09 – 1.03]	0.35 [0.18 - 0.88]	0.80

Mean ± standard deviation or median [interquartile range] is shown. Abbreviations; LVEF, left ventricular ejection fraction; LVESV, left ventricular end systolic volume; LVEDV, left ventricular diastolic volume; LV mass, left ventricular mass

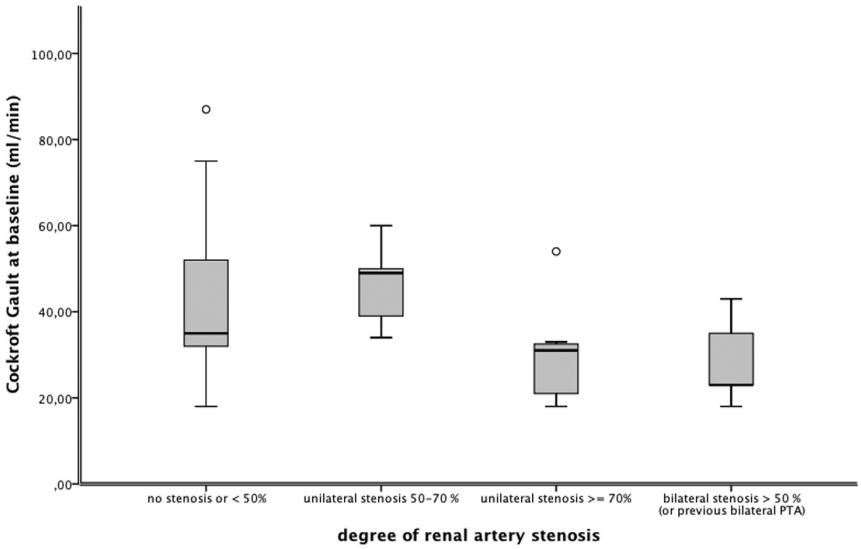

Figure 2 | Comparison of Cockcroft Gault equation (ml/min) in patients with combined chronic heart failure and chronic kidney disease, divided by increasing severity of atherosclerotic renal artery stenosis. (Shown: median and interquartile range)

DISCUSSION

Our study shows a high prevalence (56.8%) of atherosclerotic renal artery stenosis (ARAS) in patients with combined heart and renal failure, defined as having a stenosis > 50%. When taken a stricter cut-point (> 70 % stenosis) the prevalence of ARAS was still as high as 21.8% in this population. The presence of ARAS was not associated with the extent in abnormalities in left ventricular function or myocardial fibrosis based on cardiac MRI findings with late gadolinium enhancement (LGE), when compared to patients with combined CHF/CKD without ARAS. Neither did we observe a difference in the presence of an ischemic pattern of LGE. Furthermore; we found a weak association between eGFR and the severity of ARAS and observed a negative association between diabetes mellitus and ARAS. This could indicate that our small cohort may consist of two different groups of cardiorenal patients: non-diabetic patients in which ARAS is highly prevalent and diabetic patients with a much lower ARAS prevalence.

ARAS is very common in patients with manifestations of non-renal atherosclerosis, particularly in patients with peripheral arterial and aortic disease. A recent literature review found a pooled prevalence of 25.3% in patients with peripheral arterial disease and 33.1% in patients with aortic aneurysm[7]. Only few, small, studies determined the prevalence of ARAS in patients with CHF. McDowall et al. reported a prevalence of 34% of ARAS in patients with CHF and deSilva et al. found a prevalence of up to 54%[9, 20]. In both studies, ARAS was defined as a stenosis of >50% by magnetic resonance angiography (MRA). About the prevalence of ARAS in combined CHF/CKD, even fewer studies are published. DeSilva et al. reported a prevalence of 68% in 97 patients with CHF that had renal dysfunction. The study by deSilva et al. included only patients with HFREF. However, HFPEF is known to have a similar poor prognosis as HFREF and to be more prevalent in older patients and in patients with diabetes and/or hypertension[21, 22]. Our study included an ambulant stable outpatient clinic patient population with combined chronic heart failure and chronic kidney disease and mild anaemia. We included patients both with HFREF and HFPEF, treated with renin angiotensin (RAS) inhibitors and β- blockers according to present guidelines. In this cohort we demonstrate a high prevalence of ARAS (56.8%). A more severe degree of ARAS, defined as a unilateral stenosis of > 70% and/or bilateral stenosis > 50% was found in 43.2% of the patients. These results are similar to those of deSilva et al., confirming this high prevalence of ARAS in combined CHF/CKD in patients with both HFREF and HFPEF.

According to the present American Heart Association guidelines for the management of patients with peripheral arterial disease, the indication for percutaneous renal revascularization with stent placement (PTA) of ARAS is limited to "flash pulmonary oedema, recurrent episodes of unexplained congestive heart failure or unstable angina"[23]. There is debate however whether PTA could benefit CHF patients with ARAS. In patients with stable CKD and/or hypertension it has been reported several times that PTA does not affect renal function[5, 19, 24]. On the other hand, a small retrospective study showed that in patients referred for renal revascularization close to one-third had CHF (mainly HFPEF) and that revascularization was associated with better control of heart failure[25]. The results of the sub analysis of the Angioplasty for Renal Artery Lesions (ASTRAL) study of a predefined group of

patients with CKD and reduced ejection fraction are not yet available.
Nonetheless, diagnosing ARAS can be important for more reasons than to find patients suitable for PTA. One could interpret the presence of ARAS as a marker of "atherosclerotic burden" associated with a high risk of cardiovascular events, which would warrant more aggressive medical therapy[8]. Indeed, in a follow up study of elderly people with ARAS, the annual incidence of coronary events, heart failure and death were as high as 30%, 19% and 17% respectively[26]. A recent retrospective study in elderly patients with ARAS presented a very high morbidity and mortality (49% suffered a primary event and 37% died during median follow-up of 3.3 years), which was negatively associated with the use of statins.
In addition to determining the prevalence of ARAS in patients with both CHF and CKD, we determined whether there is an association between left ventricular structure and function and the presence of ARAS. Although one could hypothesize that ARAS would be associated more often, and to a greater extent, with cardiac abnormalities, such as left ventricular hypertrophy, we found no differences in left ventricular mass index, left ventricular volumes and LVEF in patients with and without ARAS. We also hypothesized that the existence of ARAS represents an "atherosclerotic burden", representing one of the mechanisms of combined CHF/CKD. However, we found no difference in the presence of ischemic aetiology of CHF. Moreover, we could not demonstrate a difference in the presence, the location and the extent of fibrosis, as depicted by LGE. The study by Wright et al. showed more diastolic dysfunction and left ventricular hypertrophy in patients with ARAS when compared with a matched control group with similar renal dysfunction[12]. In contrast, our study only included patients with known CHF and CKD. In those patients with combined CHF and CKD, the presence of ARAS was not associated with a more severely impaired cardiac function, more severe left ventricular hypertrophy or the presence of fibrosis.
Coincidentally we found a negative association between diabetes and ARAS; the patients with combined CHF/CKD without ARAS were markedly more likely to have diabetes mellitus than those patients with ARAS (Figure 1). Some studies identified diabetes mellitus as a predictor for ARAS[27], whereas others showed that diabetes mellitus was not associated with ARAS[28, 29]. The negative association between diabetes and ARAS may point to a mechanistic difference in the pathophysiology of combined CHF/CKD in patients with and without diabetes. However, alternatively, the difference may result from survival bias.
A point of debate is the definition of ARAS, since no uniform definition exists. Previously, most studies defined ARAS as > 50% stenosis [19, 24, 30-32]. Indeed, the American Heart Association Guidelines [23] are based on studies using this definition. However, more recent guidelines from the European Society of Cardiology define ARAS as having a stenosis > 60%[33], based on the fact that MRA (and CT angiography) tend to overestimate the degree of stenosis. More recent PTA studies often combine this definition with additional haemodynamic measurements, e.g. a systolic pressure gradient[34], measurement of the fractional flow reserve[35] or use a more strict cut-point of > 70%. In this study we used the definition of ARAS as defined > 50%, in accordance with the STAR study[19]. However, if we also apply the more stringent definition of > 70% stenosis we still find a prevalence of 21.8% of ARAS. Except for a significant decline in renal function in patients with a more severe ARAS versus

a moderate ARAS, we found no other differences in clinical profile between a moderate or severe ARAS.

Some limitations of this study need to be acknowledged. The small study population, due to the complexity of the study design, consists of a selected group of stable ambulant patients, almost all using RAS inhibitors which, in addition to the exclusion of uncontrolled hypertension and patients with flash pulmonary oedema, may have led to underestimation of the prevalence of ARAS in CRS. However, despite this small study population, we believe that these data are valuable and robust, due to a paucity of data in the present literature about this subject and the reliable assessment with cardiac MRI. Furthermore, the data are baseline data from a randomized intervention study, which precludes spontaneous follow-up.

Conclusion

In conclusion, ARAS is prevalent in patients with combined CHF and CKD and its presence does not correlate with worse left ventricular function, left ventricular volumes, mass, nor myocardial fibrosis as assessed by MRI. However, the severity of ARAS is weakly associated with renal dysfunction and ARAS is remarkably negatively associated with diabetes mellitus in this cohort. Further research is needed to investigate its role in the pathophysiology of combined chronic heart failure and chronic kidney disease and subsequent therapeutic consequences.

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SUMMARY

The combination of chronic heart failure (CHF) and chronic kidney disease (CKD) is prevalent and associated with a higher mortality and morbidity than can be expected solely on the combination of both conditions[1, 2]. This combination leads to a "dangerous liaison", also called the cardiorenal syndrome, in which failure of the one organ accelerates the progression of structural damage and failure of the other organ[1]. The responsible pathophysiological interactions that couple CHF and CKD are still incompletely identified. These underlying pathophysiological mechanisms can be differentiated in non-haemodynamic and haemodynamic interactions between heart and kidney. This thesis addressed red cell dynamics regarding the non-haemodynamic coupling. Regarding the haemodynamic coupling between heart and kidney failure we addressed atherosclerotic renal artery stenosis (ARAS) and stabilization of glomerular filtration rate (GFR) upon changes in blood pressure.

PART I RED CELL DYNAMICS

The first part of this thesis focused on erythropoiesis and anisocytosis in cardiorenal failure

Anaemia[3-6], disordered iron metabolism[7, 8], erythropoietin (EPO) resistance[9] and anisocytosis[10] are of prognostic value in cardiorenal failure. Collectively these factors are related to red cell dynamics, the production and decay of the circulating erythrocyte. One of the causes of the anaemia in cardiorenal failure is an impaired EPO production, for which supplementation with erythropoietin stimulating agent (ESA) would be a plausible treatment. However, the studies investigating ESA administration in both CKD and CHF have provided many controversies. Multiple favourable effects of ESA have been demonstrated, both erythropoietic and non-erythropoietic[11-13]. However, adverse effects have also been demonstrated in larger randomized studies, when targeting normal haemoglobin levels[14-16]. The responsible mechanisms for these adverse effects, erythropoietic or non-erythropoietic, are unknown.

Therefore we designed the **E**rythro**po**ietin in the **Ca**rdio**Re**nal **S**yndrome (**EPOCARES**) trial to investigate the role of fixed low-dose ESA in chronic cardiorenal failure, thereby differentiating erythropoietic as opposed to non-erythropoietic effects. The objectives and design of the EPOCARES trial are described in **chapter 2**; mildly anaemic patients with CKD and CHF, who were still anaemic after at least 4 weeks oral iron supplementation, were randomized into three groups; one group received a fixed dose of 50 IU/kg per week ESA to increase the haemoglobin level to a maximum of 13.7 g/dL for men and 13.4 g/dL for women (the ESA-Hb-rise group). The second group also received 50 IU/kg per week ESA, but the haemoglobin levels in these patients were maintained at baseline level by sequential blood withdrawal (the ESA-Hb-stable group). The third group, the control group, received standard care. Patients were followed during 12 months during which cell studies, a panel of biomarkers, quality of life, exercise capacity, cardiac and renal functions were assessed, at baseline, after two weeks, and after 6 and 12 months. The

combination of ESA treatment with simultaneous phlebotomies to maintain Hb levels at baseline level, makes this an unique study design. Data from this trial are used to address questions in this thesis.

In **chapter 3** we investigated whether red cell distribution width (RDW), a parameter of anisocytosis, was associated with EPO resistance, which could explain the strong RDW-associated risk for adverse outcome[17-20]. To this purpose, data from the EPOCARES trial were used, in which endogenous EPO levels and the response of ESA treatment in patients with stable chronic cardiorenal failure were available. EPO resistance was assessed by 1. Calculating the log observed/predicted EPO ratio (O/P ratio), which reflects the endogenous EPO level for the degree of anaemia[21], 2. Measuring the extent in increase of reticulocyte count, soluble transferrin receptor and immature reticulocyte fraction (depicting the young, newly produced erythrocytes) after two weeks of ESA treatment and 3. Measuring the increase in haemoglobin levels after 6 months of ESA treatment in those patients whose haemoglobin levels were allowed to rise. Additionally, we investigated the role of associated factors on RDW, such as inflammation, functional iron availability, erythropoietic activity and hepcidin-25 levels. RDW levels were not associated with EPO resistance in chronic cardiorenal failure. However, RDW did associate with functional iron availability, erythropoietic activity and inflammation in cardiorenal failure, as assessed by reticulocyte haemoglobin content, transferrin saturation, soluble transferrin receptor, immature reticulocyte fraction and interleukin-6 levels. Surprisingly however, there was no association between hepcidin-25 levels and RDW. In conclusion, these data suggest that the RDW-associated risk in chronic cardiorenal failure is not a reflection of EPO resistance, but relates to functional iron availability, erythropoietic activity and inflammation.

The effect of low-dose ESA treatment on quality of life, exercise capacity and cardiac function in the EPOCARES trial are discussed in **chapter 4**. In addition, we investigated whether RDW is associated with quality of life and exercise capacity in cardiorenal failure, based on a recent report showing that RDW is a determinant of exercise capacity in a group of CHF[22]. Only the group of those patients whose haemoglobin levels were allowed to increase, demonstrated an increase in quality of life, left ventricular ejection fraction and end systolic volume. There were no significant changes in those patients whose haemoglobin levels were kept stable (despite the same fixed low-dose ESA treatment) and in the control group. Moreover, we found a clear negative association between baseline RDW levels and quality of life and exercise capacity. However, RDW proved not to be an independent determinant of quality of life. The independent predictors for quality of life were baseline soluble transferrin receptor and CRP, suggesting that the quality of life in cardiorenal failure is determined by functional iron availability, red cell turnover and inflammation. Hence, the positive effects of a low, fixed dose of ESA on cardiac function and quality of life in cardiorenal patients is dependent upon red cell levels, given the fact that there was no improvement in the phlebotomy group. Together with the negative correlation between RDW and quality of life this underscores the central role of red cell dynamics in cardiorenal patients.

Chapter 5 demonstrated that higher RDW levels are associated with a higher risk of incident heart failure and coronary heart disease events in a large cohort of apparently healthy men and women, using data from the EPIC-Norfolk study. To

address whether RDW reflects a different underlying pathophysiological mechanism than that represented by established risk factors, we corrected this RDW associated risk of heart failure and coronary heart disease events for the risk factors used in the Framingham risk score (e.g. age, sex, diabetes mellitus, smoking, systolic blood pressure, total and high-density lipoprotein cholesterol). Furthermore, we also corrected for "newer" risk factors such as renal function, inflammation and parameters of iron metabolism. The RDW associated risk of both heart failure and coronary heart disease proved to be independent of the established risk factors, renal function, iron metabolism and inflammation, which suggests that RDW reflects a different (new) pathophysiological mechanism. Additionally, we demonstrated that higher RDW levels are associated with physical inactivity in this large apparently healthy cohort. By doing so, previous findings were extended from a recent small study in patients with chronic heart failure[22] and our findings in cardiorenal patients (Chapter 4), that RDW is associated with physical fitness (as assessed by cardiopulmonary exercise test) to a large cohort of apparently healthy subjects (as assessed by an activity questionnaire). This association with physical inactivity was also independent of parameters of iron metabolism and inflammation, implying that the association between higher RDW levels and physical inactivity depends on more than solely an underlying inflammation or iron deficiency.

The role of NGAL, a protein part of the innate immunity system by interference of bacterial iron uptake by sequestering iron, in chronic cardiorenal failure is being addressed in **chapter 6**. Currently, NGAL is being promoted as a biomarker for kidney injury[23-25], since serum and urinary NGAL levels increase strikingly in response to inflammatory processes and especially in response to kidney injury. However, NGAL might also reflect iron metabolism since it acts as an alternative iron delivery pathway[26]; two recent studies showed a correlation between NGAL levels and iron availability in haemodialysis patients[27, 28]. We hypothesized that serum NGAL levels also reflect iron availability in chronic cardiorenal failure and that ESA treatment decreases NGAL levels, in correspondence with the ESA effect on hepcidin levels[29]. However, in contrast to haemodialysis patients, elevated NGAL levels did not correlate with parameters of iron availability in patients with cardiorenal failure from the EPOCARES study, as assessed by transferrin saturation, hepcidin-25 and reticulocyte haemoglobin content levels. Hence NGAL levels might reflect tubular damage in these patients. Interestingly, NGAL levels did inversely correlate with baseline EPO levels and decreased in response to short-term low-dose ESA treatment. This might possibly reflect an effect of ESA treatment on tubular damage. However, this finding needs to be confirmed and alternative explanations should be evaluated.

PART II HAEMODYNAMICS IN CARDIORENAL FAILURE

The second part of this thesis focused on aspects of the haemodynamic coupling between heart and kidney in chronic cardiorenal failure

In **chapter 7** we addressed whether chronic fluctuations in blood pressure are related to chronic changes in GFR. In clinical practice, a decrease in GFR is often attributed to a decrease in arterial blood pressure, a decrease in cardiac output or

the use of renin-angiotensin-system inhibitors. However, we do not know whether this theory holds in a chronic setting of cardiorenal failure. Thus, we tested the hypothesis that GFR is susceptible to chronic fluctuations in blood pressure in ambulant patients with chronic cardiorenal failure. In subjects from the EPOCARES study, creatinine and blood pressure were measured monthly in a similar way during one year. We studied relationships between mean arterial blood pressure and GFR, of which slope, degree and stabilization were calculated. In general, changes of GFR upon sub acute changes in blood pressure were very modest; variations in mean arterial blood pressure resulted only in small changes of GFR. After dividing the data into two groups, a group of patients with the most stable and a group of patients with the least stable GFR upon changes in mean arterial blood pressure, no differences in baseline GFR, blood pressure, left ventricular ejection fraction or NT-proBNP levels were seen. Even in those patients that had an atherosclerotic renal artery stenosis (ARAS) (> 50% stenosis by magnetic resonance angiography), there was no correlation with stabilization of GFR, irrelevant of the presence of a unilateral or bilateral ARAS. These results suggest that blood pressure is not a strong determinant of renal function in stable ambulant patients with chronic cardiorenal failure. Therefore, mechanisms responsible for stabilizing GFR upon changes in blood pressure seem to be intact, even in the presence of ARAS.

There is a paucity of data about the prevalence of ARAS and its clinical consequences for the interaction between heart and kidney in chronic cardiorenal failure. Therefore, in **chapter 8**, we assessed the prevalence of ARAS in our small, but well characterized cohort of chronic cardiorenal patients from the EPOCARES study. All patients without a cardiac implantable electronic device and a GFR > 30 ml/min, underwent a combined cardiac magnetic resonance angiography with magnetic resonance angiography of the renal arteries at baseline. By combining these two studies in one investigation, we made optimal use of the gadolinium enhancement contrast by using its first pass effect for depicting the renal arteries and its late phase for assessing the presence and extent of myocardial fibrosis. There was a prevalence of ARAS of 56.8% in this cohort, defined as having a stenosis > 50%. A bilateral stenosis was present in 21.6% of the patients, as was a severe unilateral stenosis of > 70% also present in 21.6%. Between patients with and without ARAS, we found no differences in age, NT-proBNP levels or medication profile. The renal function declined with the severity of the ARAS, albeit this was not statistically significant between those patients with or without ARAS. Surprisingly, there was more diabetes mellitus in those patients *without* ARAS versus those with ARAS. In contrast to our hypothesis, there were no differences in left ventricular ejection fraction, end diastolic volume, left ventricular mass index, or in the presence or extent of myocardial fibrosis. Neither did we see a difference in the aetiology of the myocardial fibrosis. In conclusion, ARAS is highly prevalent in patients with cardiorenal failure, but its presence does not correlate with worse cardiac dysfunction or myocardial fibrosis as assessed by MRI. However, the severity of ARAS is weakly associated with renal dysfunction and there is a remarkably negative association with diabetes mellitus in this cohort. Further research is needed to investigate its role in the pathophysiology of chronic cardiorenal failure and subsequent therapeutic consequences.

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Nederlandse samenvatting

CHAPTER
9

Achtergrond

Chronisch hart- en nierfalen

Zowel chronisch hartfalen als chronisch nierfalen komen vaak voor en vormen een toenemend gezondheidszorgprobleem; beide ziekten gaan gepaard met een verminderde kwaliteit van leven, kostbare ziekenhuisopnames en vroegtijdig overlijden. Volgens een recent rapport van het RijksInstituut Volksgezondheid en Milieu (RIVM) hebben ca. 130.000 mensen in Nederland chronisch hartfalen en dit aantal zal in het jaar 2025 zijn toegenomen met ca. 50% als gevolg van de toenemende vergrijzing. Chronisch nierfalen komt zelfs nog meer voor. Volgens de PREVEND studie uit Groningen (Prevention of Renal and Vascular End-Stage Disease) heeft 5.3% van de Nederlandse bevolking een verminderde nierfunctie (uitgedrukt in een verminderde glomerulaire filtratiesnelheid). Patiënten met chronisch nierfalen hebben een opvallend hoog risico op hart- en vaatziekten, zoals een hartinfarct en/of hartfalen, hetgeen in deze groep de belangrijkste doodsoorzaak vormt. Omgekeerd krijgen patiënten met chronisch hartfalen heel vaak te maken met chronisch nierfalen. De combinatie van deze beide ziekten wordt het *cardiorenale syndroom* genoemd. Het primaire falen van één van beide organen leidt tot het falen van het andere orgaan, waarop er een neerwaartse vicieuze spiraal ontstaat met progressieve schade aan beide organen. Deze interactie tussen hart en nieren leidt uiteindelijk tot meer schade dan te verwachten is enkel op basis van de optelsom van beide ziekten.

De onderliggende oorzaken zijn nog onvoldoende bekend, maar de interacties tussen hart en nieren kunnen onderverdeeld worden in twee mechanismen, nl. 1) de veranderingen in de bloeddruk en weerstand in de bloedvaten (de *hemodynamische mechanismen*) en 2) de *niet-hemodynamische mechanismen*, die o.a. bestaan uit de zogenaamde "*cardiorenale connectoren*". Deze connectoren zijn factoren die verstoord zijn bij patiënten met hart- en nierfalen en bestaan uit; een teveel aan ontstekingseiwitten (inflammatie), oxidatieve stress, een over geactiveerd renine-angiotensine systeem en een overactief sympathisch zenuwstelsel. Daarnaast is het de laatste jaren duidelijk geworden dat bloedarmoede (*anemie*), en processen die daarmee verband houden zoals een *verstoord ijzermetabolisme* en een *ongelijke grootte van de circulerende rode bloedcellen (anisocytose)* een belangrijke rol spelen in hart- en nierfalen. Anisocytose wordt weergegeven door de *"red cell distribution width" (RDW)*. Een hogere RDW (dus een grotere ongelijkheid in de grootte van de circulerende rode bloedcellen) blijkt een sterke onafhankelijke voorspeller te zijn van vroegtijdige sterfte en ziekenhuisopnames. Waarom een ongelijke grootte van circulerende rode bloedcellen gerelateerd is aan een slechtere prognose is tot op heden nog onbekend. Zowel anemie, ijzermetabolisme, inflammatie als RDW zijn gerelateerd aan de *aanmaak en de (vroegtijdige) afbraak van de circulerende rode bloedcel*, ook wel de *dynamiek van de rode bloedcel* genoemd.

Dit proefschrift behandelt studies die betrekking hebben op 1. De rol van de dynamiek van de circulerende rode bloedcel in gecombineerd hart- en nierfalen (deel I) en 2. Enkele aspecten van de hemodynamische interactie tussen hart en nieren; de relatie tussen bloeddruk en nierfunctie en de rol van een vernauwing in de nierslagaders in gecombineerd hart- en nierfalen (deel II).

Anemie	Bloedarmoede
Anisocytose	Ongelijke grootte in circulerende rode bloedcellen
EPO resistentie	Verminderde beenmergrespons op EPO
Erytropoëse	Bloedaanmaak
Erytropoëtine	Hormoon dat het beenmerg stimuleert tot de aanmaak van rode bloedcellen
Hemodynamiek	Veranderingen in de bloeddruk en de weerstand in de bloedvaten
Inflammatie	Ontsteking zonder infectie
Nierarteriestenose	Vernauwing van de nierslagaders
RDW	Maat voor anisocytose

Anemie en behandeling met erytropoëtine in hart- en nierfalen

Bij patiënten met gecombineerd hart- en nierfalen komt bloedarmoede frequent voor, hetgeen leidt tot een verdere achteruitgang in de kwaliteit van leven en vroegtijdig overlijden. De oorzaak van deze bloedarmoede kent meerdere oorzaken. Enerzijds zijn er factoren die betrekking hebben op een effectieve *bloedaanmaak (erytropoëse)* zoals een tekort aan *erytropoëtine (EPO)* als gevolg van de chronische nierschade. EPO betreft een (voornamelijk) door de nieren geproduceerd hormoon dat het beenmerg stimuleert tot de aanmaak van rode bloedcellen. Verder is de erytropoëse minder effectief door een verminderde respons van het beenmerg op het aanwezige EPO, de zogenoemde *EPO resistentie*. Hierbij speelt een verstoord *ijzermetabolisme* een grote rol; ijzer speelt een essentiële rol bij de erytropoëse. Echter, bij patiënten met hart- en/of nierfalen is er sprake van een verminderde opname van ijzer via de voeding. Daarnaast wordt het ijzer afkomstig uit afgebroken rode bloedcellen uit de bloedbaan "gestapeld" in de witte bloedcellen (macrofagen). Beide processen worden gereguleerd door *hepcidine*, een door de lever onder invloed van o.a. inflammatie geproduceerd eiwit. Dit heeft tot resultaat dat er onvoldoende ijzer beschikbaar is voor de aanmaak van nieuwe rode bloedcellen. Anderzijds zijn er mechanismen die betrekking hebben op de levensduur van de rode bloedcel, zoals (latent) bloedverlies en versterkte afbraak door bijv. inflammatie.

Suppletie met kunstmatig EPO lijkt een voor de hand liggende oplossing voor de anemie. Vele (kleinere) studies met EPO bij patiënten met nierfalen toonden een gunstig effect op kwaliteit van leven, inspanningscapaciteit en het aantal ziekenhuisopnames. Hierdoor werd de interesse van de cardiologen gewekt, waarop studies bij patiënten met hartfalen ook deze gunstige effecten van EPO aangaven. Groot was dan ook de verbazing in 2006 toen twee groots opgezette, harde eindpunt, EPO studies in patiënten met nierfalen geen daling in sterfte aantoonden. Er bleek zelfs sprake van een ongunstig effect bij die patiënten die hogere doseringen EPO nodig hadden voor een volledige normalisatie van het bloedgehalte. De verklaring van deze teleurstellende bevinding is vooralsnog onbekend. EPO heeft ook effecten die geen betrekking hebben op de erytropoëse, de niet-erytropoëtische effecten, zoals een toegenomen vaatgroei, verbeterde wondgenezing en anti-

inflammatoire werking. Zowel de erytropoëtische als de *niet-erytropoëtische* effecten van EPO kunnen gunstige en ongunstige effecten hebben. Het netto effect van uitwerkingen op de individuele patiënt is met de huidige kennis onvoorspelbaar en wordt wellicht bepaald door de hoeveelheid EPO en de specifieke karakteristieken van de patiënt.
De EPOCARES (Erytropoietin in the CArdioREnal Syndrome) studie had tot doel de rol van EPO in gecombineerd chronisch hart- en nierfalen te onderzoeken met speciale aandacht voor (1) de pathofysiologie van hart- en nierfalen en (2) de erytropoëtische en de niet-erytropoëtische effecten van EPO. De resultaten van de EPOCARES studie vormen de basis van dit proefschrift.

Anisocytose en hart- en nierfalen

RDW, een maat voor anisocytose (de variatie in grootte van de rode bloedcel), wordt standaard berekend bij elke bloedbeeldbepaling. Het betreft de standaarddeviatie van het gemiddelde volume per rode bloedcel. Vooralsnog wordt RDW gebruikt voor de nadere diagnostiek van bloedarmoede. Echter, vrij recent hebben verschillende studies aangetoond dat RDW een onafhankelijke voorspeller is voor ziekenhuisopnames en sterfte bij patiënten met hartfalen. De onderliggende verklaring waarom een grotere ongelijkheid in grootte van rode bloedcellen verband houdt met een slechte prognose, is nog onbekend. RDW wordt bepaald door de dynamiek van de rode bloedcel; enerzijds de aanmaak van jonge (grotere) rode bloedcellen en anderzijds de (vroegtijdige) afbraak van oudere (kleinere) rode bloedcellen. Mogelijke verklarende factoren zijn dan ook een verstoord ijzermetabolisme, inflammatie en EPO resistentie. Eén van de doelen van dit proefschrift was om meer inzicht te krijgen in de rol van anisocytose in hart- en nierfalen, als zijnde een niet-hemodynamisch mechanisme.

Hemodynamiek in hart- en nierfalen; de rol van bloeddruk en/of een nierslagader vernauwing

Slagaderverkalking (*atherosclerose*) is een van de meest voorkomende oorzaken van hartfalen in de westerse wereld en wordt gezien als een systemische (potentieel in het gehele lichaam aanwezige) aandoening. Derhalve is het logisch te veronderstellen dat een *vernauwing van de nierslagader (nierarteriestenose) door atherosclerose* (mede) een oorzaak is van nierschade bij patiënten met hartfalen. Patiënten met hart- en vaatziekten, zoals vernauwingen van de kransslagaders en/of beenslagaders, hebben inderdaad vaak een nierarteriestenose. De symptomen hiervan zijn moeilijk te herkennen en kunnen bestaan uit een hoge bloeddruk, progressieve nierschade, vocht rondom de longblaasjes (longoedeem) of hartfalen. De aanwezigheid van een nierarteriestenose kan de bloedsomloop van de nieren beïnvloeden; achter de vernauwing in de nierslagaders kan de druk van het bloed zo laag worden dat deze te laag wordt voor het zelfregulerende vermogen van de nieren ("lower limit of autoregulation"). Hierdoor wordt het renine-angiotensine systeem geactiveerd met vochtophoping in het lichaam als gevolg. Het is echter onbekend hoe vaak een nierarteriestenose voorkomt bij patiënten met chronisch hart- en nierfalen en of dit in een chronische setting verband houdt met een slechtere nier- of hartfunctie.

De aanwezigheid van nierschade maakt de nier mogelijk gevoeliger voor schommelingen in de bloeddruk. In de dagelijkse praktijk wordt geregeld gesteld dat een daling van de nierfunctie (gemeten met de glomerulaire filtratiesnelheid) wordt veroorzaakt door een lage bloeddruk, bijv. als gevolg van hartfalenmedicatie. Hier bestaat vooralsnog geen bewijs voor, maar deze veronderstelling leidt vaak wel tot het verlagen van de hartfalenmedicatie. Daarbij zou de aanwezigheid van een nierarteriestenose deze patiënten met hartfalen mogelijk nog gevoeliger kunnen maken voor een daling in de bloeddruk met progressie van de nierschade tot gevolg.

Dit proefschrift behandelt twee aspecten van de hemodynamische interactie tussen hart en nieren betreffende; 1. Het voorkomen en het bijbehorend klinische profiel van nierarteriestenose in patiënten met chronisch hart- en nierfalen en 2. Het verband tussen schommelingen in de bloeddruk en de nierfunctie in chronisch hart- en nierfalen

RESULTATEN BESCHREVEN IN DIT PROEFSCHRIFT

DEEL I DYNAMIEK VAN DE RODE BLOEDCEL

Het eerste deel van dit proefschrift concentreert zich op de rol van erytropoëse en anisocytose in gecombineerd chronisch hart- en nierfalen.

In **hoofdstuk 2** wordt de opzet en de hypothesen van de EPOCARES studie beschreven. Deze studie onderzoekt de rol van EPO in hart- en nierfalen, waarbij met name onderscheid is gemaakt tussen de erytroëpotische en de niet-erytropoëtische effecten van EPO. Hiervoor werden mild anemische patiënten met stabiel chronisch gecombineerd hart- en nierfalen gerandomiseerd in drie groepen, indien ze nog immer anemisch waren na 4 weken ijzerpillen. Eén groep kreeg een vaste lage dosis EPO (50 IU/kg/week), waarbij het bloedgehalte mocht stijgen. De tweede groep ontving dezelfde dosis EPO, maar daarbij werd het bloedgehalte op het uitgangsniveau gehouden door middel van aderlatingen. De derde groep ontving geen EPO behandeling (de controle groep). Deze patiënten werden vervolgd gedurende 12 maanden waarbij meerdere aspecten werden beoordeeld bij de start van de studie, na 6 en na 12 maanden zoals; een panel van biomarkers, de kwaliteit van leven met vragenlijsten, de inspanningscapaciteit en de cardiale- en nierfunctie. De data van deze unieke studie vormen de basis van dit proefschrift.

Hoofdstuk 3 beschrijft of er een verband bestaat tussen RDW en EPO resistentie, hetgeen een mogelijke verklaring zou kunnen zijn voor het feit dat RDW een voorspeller is van een slechte prognose. In de EPOCARES studie hebben we EPO resistentie bepaald met verschillende methoden: de beenmergrespons op endogeen (lichaamseigen) EPO werd gemeten door de geobserveerde/voorspelde log EPO verhouding, hetgeen de hoeveelheid endogeen EPO in verhouding tot de mate van bloedarmoede weergeeft. De exogene EPO resistentie werd bepaald door de stijging van jonge rode bloedcellen, verschillende markers van erytropoëtische activiteit en het bloedgehalte te meten als reactie op EPO behandeling. Het bleek dat RDW niet gecorreleerd is aan EPO resistentie in deze populatie met chronisch hart- en nierfalen. Er bleek echter wel een verband te bestaan tussen RDW en

inflammatie (interleukin-6, een onstekingseiwit), een verhoogde erytropoëtische activiteit (soluble transferrin receptor en immature reticulocyte fraction) en een betere beschikbaarheid van ijzer (reticulocyte haemoglobin content en soluble transferrin receptor). Opvallend genoeg was er geen verband tussen RDW en hepcidine. Een verhoogde RDW wordt dus mogelijk bepaald door een verminderde beschikbaarheid van ijzer (een functioneel ijzergebrek, dit geeft kleinere rode bloedcellen) en een verhoogde erytropoëtische activiteit (grotere rode bloedcellen). Nader onderzoek naar deze factoren en het verband met een slechte prognose is geïndiceerd.

In hoofdstuk 4 wordt het effect beschreven van een lage dosis EPO op de kwaliteit van leven, inspanningscapaciteit en hartfunctie. Tevens hebben wij in deze studie gekeken naar een mogelijk verband tussen RDW en de kwaliteit van leven en inspanningscapaciteit. Dit laatste n.a.v. een recent gepubliceerde studie in een kleine groep van patiënten met hartfalen waaruit bleek dat RDW een voorspeller is van de inspanningscapaciteit. Uit onze studie bleek dat enkel die patiënten wiens bloedgehalte onder EPO behandeling mocht stijgen, een verbetering toonden in kwaliteit van leven en hartfunctie (uitgedrukt in linkerventrikel ejectiefractie en eindsystolisch volume). Er waren geen significante veranderingen in de groep wiens bloedgehalte stabiel werd gehouden, noch in de controle groep. Verder was er sprake van een negatief verband tussen RDW waarden en kwaliteit van leven en inspanningscapaciteit. Echter, RDW bleek geen onafhankelijke voorspeller te zijn voor de kwaliteit van leven, d.w.z. dat er nog andere factoren van invloed waren op dit verband tussen RDW en kwaliteit van leven. De onafhankelijke voorspellers voor de kwaliteit van leven waren CRP (een onstekingseiwit) en soluble transferrin receptor (een parameter voor erytropoëtische activiteit en ijzerbeschikbaarheid). Dit suggereert dat de kwaliteit van leven in patiënten met chronisch hart- en nierfalen, bepaald wordt door inflammatie, erytropoëtische activiteit en ijzerbeschikbaarheid. Dit, gecombineerd met het feit dat de hartfunctie en kwaliteit van leven enkel verbeterde in die patiënten met een stijging van het bloedgehalte, onderstreept het belang van de rol van de dynamiek van de rode bloedcel in chronisch hart- en nierfalen.

Hoofdstuk 5 toont aan dat verhoogde RDW waarden ook in een grote populatie van ogenschijnlijk gezonde mannen en vrouwen geassocieerd zijn met een verhoogd risico op hartfalen en kransslagaderlijden. Hiervoor hebben we data van de grote EPIC-Norfolk studie gebruikt. Daarbij hebben wij gekeken of RDW een ander onderliggend pathofysiologisch mechanisme weerspiegelt dan de klassieke risicofactoren, zoals leeftijd, geslacht, suikerziekte, roken, bloeddruk en cholesterol. De associatie tussen RDW en het risico op hartfalen en/of kransslagaderlijden hebben we gecorrigeerd voor deze risicofactoren (zoals gebruikt in de Framingham risico score). Tevens hebben we gecorrigeerd voor "nieuwere" risicofactoren, zoals nierschade, inflammatie en parameters van ijzermetabolisme. Het bleek dat het risico op hartfalen dat verband houdt met RDW, onafhankelijk is van bovenstaande risicofactoren. Dit suggereert dat RDW een ander, nog onbekend, onderliggend pathofysiologisch mechanisme vertegenwoordigt. Daarnaast tonen wij aan dat in deze populatie een hoger RDW verband houdt met fysieke activiteit. Dit versterkt de waarneming uit hoofdstuk 4, dat RDW waarden geassocieerd zijn met fysieke fitheid, zowel in patiënten met hartfalen, gecombineerd hart- en nierfalen als in ogenschijnlijk gezonde mannen en vrouwen.

Neutrophil gelatinase associated lipocalin (NGAL) is een eiwit dat deel uitmaakt van het afweersysteem van het lichaam doordat het ijzer "weg vangt" als voedingsbron voor bacteriën. Hierdoor stijgt de NGAL spiegel als reactie op inflammatoire processen. Deze stijging is met name opvallend aanwezig na nierschade. Om deze reden is NGAL de laatste jaren steeds meer in gebruik als biomarker voor acute en chronische nierschade, bijv. na een hartcatheterisatie of een grote operatie. Recent hebben echter twee studies aangetoond dat NGAL ook een verminderde ijzerbeschikbaarheid weerspiegelt in dialyse patiënten. Het is onbekend of dit ook het geval is voor patiënten met chronisch hart- en nierfalen. Tevens is het effect van EPO behandeling op NGAL waarden onbekend. In de EPOCARES studie hebben wij beiden onderzocht, waarvan de resultaten beschreven staan in **hoofdstuk 6**. Opvallend genoeg bleek er geen verband te bestaan tussen NGAL waarden en parameters van ijzerbeschikbaarheid, gemeten m.b.v. transferrine verzadiging, hepcidine en reticulocyte haemoglobin content. Dit suggereert dat NGAL waarden in patiënten met chronisch hart- en nierfalen de nierschade weergeven en niet zozeer het ijzermetabolisme. Opvallend genoeg was er een duidelijk verband tussen de NGAL waarden met de lichaamseigen EPO waarden bij de start van de studie (voor de EPO therapie) en daalden de NGAL waarden in de acute fase na EPO behandeling. Mogelijk geeft dit aan dat EPO behandeling een gunstig effect heeft op de nierfunctie. Echter, dit effect was niet meer aanwezig na 6 maanden EPO behandeling en vereist nader onderzoek.

DEEL II HEMODYNAMIEK IN HART- EN NIERFALEN

In het tweede deel van dit proefschrift behandelen we twee aspecten van de hemodynamische interactie tussen hart en nieren in chronisch hart- en nierfalen

Hoofdstuk 7 beschrijft het verband tussen chronische schommelingen in de bloeddruk en nierfunctie. In de dagelijkse praktijk wordt een daling in nierschade vaak geweten aan een daling van de bloeddruk t.g.v. het gebruik van renine-angiotensine-systeem remmers (bloeddrukverlagers), waarop deze hartfalenmedicatie verminderd wordt. Echter, het is nog onbekend of deze veronderstelling klopt in de setting van stabiele patiënten met chronisch hart- en nierfalen. In de EPOCARES studie is gedurende een jaar maandelijks het kreatinine (hetgeen de nierfunctie weergeeft) bepaald en de bloeddruk op dezelfde manier gemeten in patiënten met stabiel chronisch hart- en nierfalen. We hebben de relaties bestudeerd tussen de bloeddruk (gemiddelde arteriële bloeddruk) en de nierfunctie (geschatte glomerulaire filtratiesnelheid). Hieruit blijkt dat in het algemeen de veranderingen in nierfunctie als reactie op bloeddrukschommelingen zeer bescheiden waren; schommelingen in de bloeddruk hadden slechts zeer geringe veranderingen van de nierfunctie tot gevolg. Als we vervolgens de patiënten onderverdeelden in een groep met de meest stabiele nierfunctie en een groep met de minst stabiele nierfunctie, zagen we geen verschillen in bloeddruk of hartfunctie tussen de beide groepen. Zelfs in die patiënten die een nierarteriestenose hadden (zie hoofdstuk 8), was er geen evidente verband tussen de stabiliteit van de nierfunctie en chronische bloeddrukschommelingen. Dit impliceert dat bloeddruk geen evidente bepalende factor is van de nierfunctie in patiënten met chronisch hart- en nierfalen en dat de mechanismen van het zelfregulerend vermogen van de nieren dus grotendeels nog intact zijn.

Er bestaan weinig data over het voorkomen van nierarteriestenose in patiënten met chronisch hart- en nierfalen. Tevens is onbekend of de aanwezigheid hiervan mede verantwoordelijk is voor de neerwaartse spiraal van het falen van beide organen. Hieruit rijst de vraag of de aanwezigheid van een nierarteriestenose geassocieerd is met meer hart- en/of nierschade. Daarom hebben we in de EPOCARES studie gekeken naar het voorkomen van nierarteriestenose, beschreven in **hoofdstuk 8**. De patiënten ondergingen een gecombineerde MRI van het hart met een MRA van de nierslagaders. Door deze twee onderzoeken te combineren, werd er optimaal gebruik gemaakt van het benodigde contrastmiddel (gadolinium); het "first-pass effect" werd gebruikt voor de visualisatie van de nierslagaders en de late fase werd gebruikt om de aanwezigheid van myocardiale fibrose (littekens in het hart) aan te tonen. In deze, weliswaar kleine maar goed gedefinieerde, groep patiënten was er sprake van een nierarteriestenose in 56.8% van de patiënten, gedefinieerd als een vernauwing van > 50%. Er was sprake van een vernauwing in beide nierslagaders (bilaterale nierarteriestenose) in 21.6% van de patiënten en een ernstige vernauwing in 21.6% (> 70% vernauwing). Echter, in tegenstelling tot hetgeen wij veronderstelden, bleek er geen verschil te bestaan in hartfunctie (linker ventrikel ejectiefractie , -volumina of massa) of de aanwezigheid en mate van littekens in het hart tussen die patiënten met nierarteriestenose versus die zonder. Noch was er een verschil in de oorzaak van de littekens in het hart. Naarmate de nierarteriestenose ernstiger was, daalde wel de nierfunctie enigszins, echter dit was niet statistisch significant verschillend tussen de groep met en zonder nierarteriestenose. Verrassend genoeg was er meer suikerziekte in die patiënten die geen nierarteriestenose hadden dan in de patiënten met een nierarteriestenose. Concluderend komt een vernauwing van de nierslagaders door slagaderverkalking veel voor in patiënten met hart- en nierfalen, maar de aanwezigheid ervan houdt geen verband met een slechtere hartfunctie of meer littekens in het hart. De ernst van de vernauwing houdt wel licht verband met de nierfunctie en er is een opmerkelijk negatief verband met suikerziekte in deze populatie. Verder onderzoek is dan ook nodig om de rol van nierarteriestenose te onderzoeken in de pathofysiologie van hart- en nierfalen en de eventuele therapeutische consequenties.

Samenvattend bespreekt dit proefschrift enkele belangrijke factoren van de interactie tussen hart en nieren in gecombineerd chronisch hart- en nierfalen. Enerzijds is het belang van de dynamiek van de rode bloedcel aangetoond, waarbij de variatie in grootte van de rode bloedcel, beschikbaarheid van ijzer, erytropoëtische activiteit en inflammatie een belangrijke rol spelen. Anderzijds zijn er twee aspecten van de hemodynamische factoren in hart- en nierfalen besproken, zoals het veelvuldig voorkomen van een vernauwing van de nierslagaders, hetgeen echter niet gepaard gaat met een slechtere hartfunctie. Verder blijken schommelingen in de bloeddruk weinig verband te houden veranderingen in de nierfunctie, hetgeen impliceert dat de nier, ondanks de aanwezige schade, nog in staat is tot het reguleren van de eigen bloedvoorziening.

Dankwoord

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Curriculum Vitae

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Mireille (Maria Elisa) Emans was born on February 28th 1977 in Weert, the Netherlands. After graduating cum laude from high school in 1995 at the Bisschoppelijk College in Weert, she studied medicine at the Katholieke Universiteit Leuven, Belgium and at the Maastricht University, the Netherlands. During medical school, she worked for a research project at the department of cardiology at the Academical Hospital Maastricht for two years (Dr. E. de Muinck en Dr. W. Spiering). In 2000 she interrupted her study to travel during one year in Middle-and South America and the Middle East.

She obtained her medical degree cum laude in 2003, after which she started working as a resident cardiology in the Jeroen Bosch Hospital in Den Bosch and the Academical Hospital Utrecht. Subsequently, her cardiology training started in 2004 at the Academical Hospital Utrecht (Dr. J.H. Kirkels and Prof. dr. Doevendans), for which she worked as a resident internal medicine at the Meander Medical Center in Amersfoort from 2005 until 2007. During this period, she started combining her cardiology training with research work for the EPOCARES study (Erythropoietin in the CArdioRenal Syndrome), performed at the Meander Medical Center and the Academical University Utrecht (Prof. dr. Gaillard and Prof. dr. Doevendans). The results of this study form the basis for this thesis.

In February 2012 she registered as a cardiologist. Mireille lives with her partner Tabe van der Ploeg and together they have a daughter, Janna (born August 2012).