Anemia and response to erythropoietin in the cardiorenal syndrome

Karien van der Putten

ISBN: 978-90-5335-388-2

Printed by: Ridderprint BV, Ridderkerk, the Netherlands
Lay out: Simone Vinke, Ridderprint BV, Ridderkerk, the Netherlands

Anemia and response to erythropoietin in the cardiorenal syndrome

Anemie en respons op erythropoietine in het cardiorenaal syndroom

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 18 mei 2011 des middags te 4.15 uur

door Karien van der Putten

geboren op 12 april 1978 te Nijmegen

Promotoren: Prof. dr. P.A.F.M. Doevendans
Prof. dr. C.A.J.M. Gaillard

Co-promotor: Dr. B. Braam

Financial support for the publication of this thesis by the Netherlands Heart Foundation is gratefully acknowledged.

Additional support for the publication of this thesis by Amgen B.V., Genzyme B.V., Merck Sharp & Dohme B.V., Novartis Pharma B.V., Roche Nederland B.V. and Servier Nederland Farma B.V. is gratefully acknowledged.

CONTENTS

CHAPTER 1

Introduction

ANEMIA AND RESPONSE TO ERYTHROPOIETIN IN THE CARDIORENAL SYNDROME

In this thesis, three different aspects of anemia and response to erythropoietin (EPO) in patients with (cardio) renal failure will be discussed, e.g. response to endogenous/ exogenous EPO, the concept of Hb variability, and the role of circadian body functions. This first chapter provides background information on these different aspects.

THE CARDIORENAL SYNDROME

Chronic kidney disease (CKD) is a major public health problem, of which the incidence and prevalence are still increasing.[1] According to the Third National Health and Nutrition Examination Survey (NHANES III), up to 11% of the adult US population has some degree of CKD.[2] In Europe, the prevalence of CKD is similar to that in the US.[3] CKD is associated with a high risk of cardiovascular disease and death. Importantly, the association between high risk of cardiovascular disease and death also holds for patients with mild to moderate CKD.[4]

Whereas cardiovascular disease is common in CKD, patients with cardiac failure often have renal dysfunction. In chronic heart failure (CHF), around 50% of patients has some impairment in kidney function.[5] The presence of renal failure in these patients is independently associated with an increased risk for all-cause mortality, cardiovascular death and hospitalization.[6,7]

The combination of kidney disease and heart failure is currently known as the cardiorenal syndrome (CRS). We proposed a model for the pathogenesis of the cardiorenal syndrome, in which cardiac and renal dysfunction mutually amplify progressive failure of both organs.[8] Inflammation, the balance between nitric oxide (NO) and reactive oxygen species (ROS), the sympathetic nervous system and the renin-angiotensin system were proposed as the 'cardiorenal connectors'; key components in the pathophysiology of the CRS. Increased activity results in a vicious circle, leading to progressive loss of cardiac and renal function (Figure 1).

In 2008, Ronco et al. presented a classification of the CRS with 5 subtypes.[9] This classification focuses on two aspects of the process: duration (acute onset versus chronic disease), and the sequence of events (kidney failure first versus heart failure first). In response to this classification, we published a comment (see appendix 1, page 155) in which we argue the relevance of the distinction between CRS type 2 (CHF leading to progressive CKD) and CRS type 4 (CKD leading to CHF). According to the model published by Bongartz et al., similar pathophysiological interactions explain 'heart-kidney' as well as 'kidney-heart' failure.[8] Therefore, in this thesis, CRS is defined as the combination of CKD and CHF, i.e. CRS type 2 and 4 according to the classification by Ronco et al.

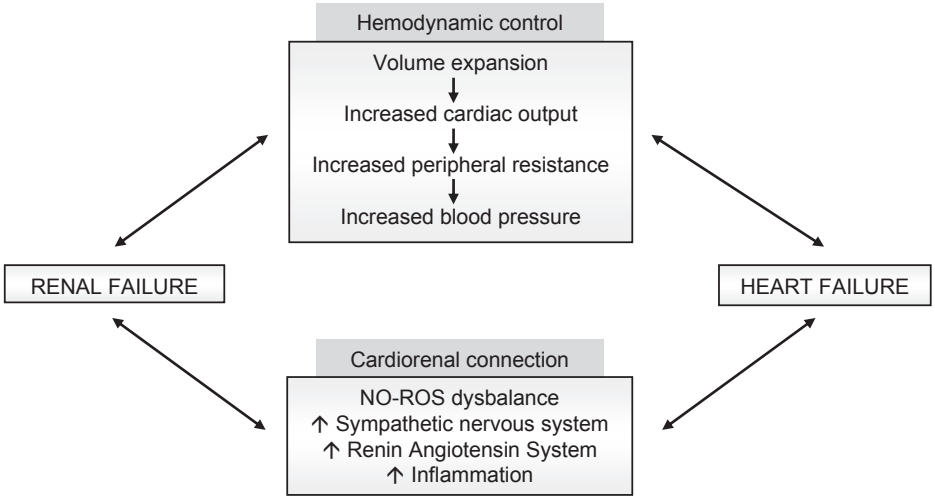

Figure 1 The pathophysiology of the cardiorenal syndrome.[8]

ANEMIA AND ERYTHROPOIETIN TREATMENT IN THE CARDIORENAL SYNDROME

Anemia is common in CKD. The main causative factor is deficient production of erythropoietin (EPO), with serum levels of EPO being disproportionately low for the degree of anemia ('renal anemia').[10] In addition, 'anemia of chronic disease' (i.e. the anemia that occurs in acute or chronic inflammation, caused by an inadequate bone marrow response to endogenous and/or exogenous EPO) has an important role in CKD.[11] Anemia is also a common problem in CHF, and as in CKD, 'anemia of chronic' disease is an important contributor.[12] In addition, approximately 50% of CHF patients has some degree of CKD, implying that 'renal anemia' also has an important role in CHF.[13] All together, it is not surprising that anemia is common in patients with CRS (Figure 2). The combination of CKD, CHF and anemia is known as the cardiorenal-anemia syndrome.[14]

Observational data indicate that hemoglobin (Hb) levels are correlated with hospitalization and mortality in dialysis patients and in CHF patients.[15,16] The role of treatment of anemia with recombinant human EPO has been the subject of many studies in the last decade. Small interventional studies showed that treatment of anemia with EPO has positive effects on quality of life[17] and improves exercise capacity[18] and left ventricular function.[19] Experimental studies showed that, in addition to its well known hematopoietic effects, EPO promotes vascular reparative processes in both CKD[20] and CHF[21], and exerts anti-apoptotic effects.[22,23] Moreover, EPO has anti-inflammatory properties and possibly also beneficial effects on the renin-angiotensin system and the NO-ROS balance, thereby interrupting the vicious circle underlying the CRS.[24]

In response to these findings, large-scale clinical trials were conducted to investigate whether treatment of anemia with recombinant human EPO reduces the risk of cardiovascular events in CKD patients. Surprisingly, none of these trials could demonstrate

a beneficial effect of EPO when targeting normalization of Hb in CKD patients.[25-27] Secondary analysis of the Correction of Hemoglobin and Outcomes in Renal Insufficiency (CHOIR) study revealed several possible explanations for these paradoxical results. It was shown that in the patients who were allocated to the high Hb group, the inability to achieve the target Hb was associated with increased risk of death and cardiovascular events, while achieving the target Hb was not. Furthermore, the use of high-dose EPO was associated with an increased risk.[28] In other words, the presence of EPO resistance (an inadequate bone marrow response to endogenous as well as exogenous EPO) appears to have a central role; patient factors *causing* EPO resistance as well as escalating dosages of EPO, being the *consequence* of EPO resistance, are associated with worse outcomes.[16,28,29]

The association between high dosages of EPO and negative outcomes can be explained by the fact that EPO exerts several dose-dependent non-hematopoietic effects. For example, both experimental and clinical data show that EPO has dose-dependent thrombogenic effects.[30,31] In addition, EPO has been shown to stimulate angiogenesis, thereby exerting pro-carcinogenic effects.[32,33] Unwanted effects of high dosages of EPO have not only been demonstrated in CKD patients, but also in cancer-associated anemia[34] and in critically ill patients.[35] In response to the trial by Corwin et al.[35] we published a comment in which we emphasize the consequences of high EPO dosages (see appendix 2, page 159).

Following the findings of the recent studies that failed to show a beneficial effect of (targeting for) normalization of Hb levels[25-27], the Anemia Working Group of ERBP maintains its view that 'Hemoglobin values of 11-12 g/dL should be generally sought in the CKD population without intentionally exceeding 13 g/dL' and that the doses of EPO therapy to achieve the target hemoglobin should also be considered.[36] It appears that EPO treatment should be tailored to the individual patient. However, no evidence is available as to which patients will encounter benefits or complications from EPO.

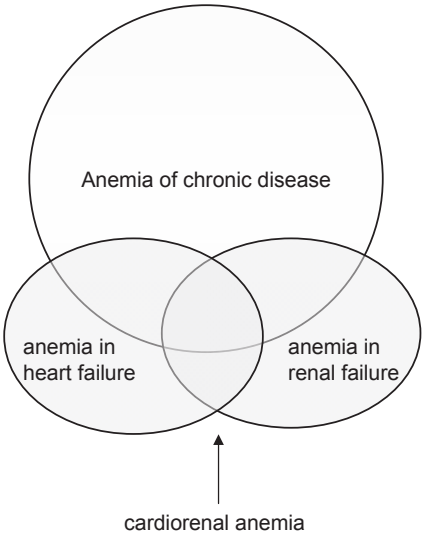

Figure 2 Anemia in the cardiorenal syndrome. Causes of anemia in renal failure and in heart failure partially overlap, with a main role for anemia of chronic disease.

HEMOGLOBIN VARIABILITY IN CKD PATIENTS

As mentioned above, the Anemia Working Group of ERBP recommends a target Hb level of 11-12 g/dL and advises against Hb levels above 13 g/dL.[36] This is in agreement with the most recent National Kidney Foundation's Kidney Disease Outcomes Quality Initiative (NKF-K/DOQI) guideline.[37] However, maintaining Hb levels within this target range is more difficult than anticipated. For example in a large sample of US hemodialysis patients, considerable variability was shown, with only 10% of patients maintaining stable Hb levels over time.[38] Also in predialysis patients, including patients not receiving EPO treatment, Hb levels fluctuate.[39]

The oscillations or fluctuations of an individual patient's Hb over time are referred to as 'Hb variability'. With regard to the concept of Hb variability, two issues are of importance: the identification of factors that cause variation, and the clinical consequences. There are several factors that could have a role in the development of Hb variability. Some data suggest that prescribing patterns of EPO and the type of EPO affect variability.[40-42] Also patient factors, e.g. hospitalization and infections, influence Hb variability.[43]

The clinical consequences of Hb variability are still a matter of debate. Several observational studies have shown that Hb variability is associated with increased mortality.[39,44,45] A recent study by Eckardt et al., however, demonstrated that Hb variability does not predict mortality.[46] In this respect it should be noted that different measures to define the degree of Hb variability were used in these studies and that different CKD populations were studied.

Up until now, no randomized trials have been performed with regard to the concept of Hb variability. Therefore, it is not possible to determine cause and-effect relationships.

ANEMIA AND CIRCADIAN BODY FUNCTIONS IN CKD PATIENTS

Circadian rhythms are fluctuations in body functions within a period of around 24 h. They are driven by the biological 'clock' located in the hypothalamic suprachiasmatic nucleus. Circadian misalignment can have important consequences on the pathology of cardiovascular disease and sleep-wake disturbances.[47,48] Patients with CKD exhibit a variety of disrupted circadian body functions. For example, the diurnal blood pressure rhythm is disturbed in renal patients, showing a nocturnal non-dipping profile[49], which is associated with increased cardiovascular mortality.[50] Furthermore, the nocturnal endogenous melatonin rise, which is associated with the onset of nocturnal sleep propensity, is absent in hemodialysis patients.[51,52] In addition to a role in sleep-wake rhythm, other effects of endogenous melatonin have been shown. For example, endogenous melatonin was found to have anti-oxidative effects[53,54], as well as anti-inflammatory effects.[55,56] Therefore, in addition to being a consequence, it could also be that dysregulation of melatonin secretion has a causative role in (cardio)renal failure,

With respect to the pathophysiology of anemia in cardiorenal failure, circadian misalignment of pro-and anti-erythropoietic factors could have a role. As mentioned earlier, anemia in CKD is frequently caused by an inadequate bone marrow response to endogenous and/or exogenous EPO. Pro-inflammatory cytokines, e.g. IL-6 and TNF, are

associated with an inadequate response to exogenous EPO in CKD.[57] These cytokines are regulated in a circadian fashion in healthy individuals.[58] In patients with insomnia, this circadian rhythm is absent.[58] Whether this also accounts for patients with CKD is unknown. A circadian rhythm of EPO itself could also have a role, as the time of administration of exogenous EPO modulates its therapeutic effect in hemodialysis patients.[59] Furthermore, it has been shown that exogenous administration of EPO can recover the defective melatonin rhythm in dialysis patients.[60,61] It can be hypothesized that endogenous EPO, in concordance with melatonin, is regulated in a circadian rhythm under normal conditions and that this rhythm is absent in CKD. However, little data on circadian body functions in CKD are available.

OUTLINE

This thesis focuses on several aspects of anemia and response to erythropoietin in patients with heart and renal failure. In **part I**, the role of endogenous as well as exogenous EPO in the cardiorenal syndrome will be discussed. Anemia is common in patients with combined cardiac and renal failure, and there is an association between anemia and progression of both diseases.[62,63] EPO has been used for over a decade to treat anemia in patients with CKD. However, up to 10% of patients receiving EPO are hyporesponsive to therapy and require large doses of the agent.[64] As resistance to EPO is associated with an increased risk of death,[16,28,65] it is important to understand how cardiorenal failure affects EPO production and function. **Chapter 2** reviews mechanisms underlying endogenous as well as exogenous EPO resistance in patients with CRS.

The exogenous effects of EPO were investigated in the EPOCARES ('Erythropoietin in the CardioRenal Syndrome') study. This is a randomized trial that was designed to discern hematopoietic from non-hematopoietic effects of EPO. The objectives and design of the EPOCARES study are described in **chapter 3**.

Hepcidin is an acute-phase protein that is upregulated by a number of stimuli, e.g. anemia and inflammation. Hepcidin thus integrates input from erythropoietic and inflammatory pathways, suggesting it could play a central role in the development of EPO resistance.[66] In CRS patients from the EPOCARES study we investigated whether and to what extent EPO treatment influences hepcidin levels, thereby assessing a potential role of hepcidin in EPO resistance. **Chapter 4** shows the results of this study.

Red cell distribution width (RDW), a measure of anisocytosis, is associated with adverse outcomes in patients with CHF.[67,68] It has been hypothesized that EPO resistance could explain the association between RDW and outcome.[69] 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.[69,70] However, no direct data are available as to the association between RDW and EPO resistance. In **chapter 5**, we study the relation between RDW and EPO response in CRS patients from the EPOCARES study.

The cardiorenal syndrome is associated with dysregulation of erythropoietin levels and inflammation. Both have been associated with the development of cancer.[71,72] We

hypothesized that the incidence of cancer is increased in patients with CRS. **Chapter 6** shows the results of a retrospective case-control study in which we examined the incidence of cancer in CRS patients.

Part II focuses on various aspects of the concept of hemoglobin variability in CKD patients. The guidelines for managing anemia in CKD patients recommend a target hemoglobin level between 11-12 g/dL, up to a maximum of 13 g/dL. However, several studies have shown that in CKD patients, Hb levels fluctuate over time and are often not maintained within this (narrow) range.[38,40] This is the case for both patients with and without EPO treatment.[39] This in clinically important, as many,[39,44,45] but not all[46] studies show that Hb variability is associated with increased mortality. With exception of several recent studies[39,42,73], until now most studies on Hb variability have focused on hemodialysis patients. In **chapter 7**, we describe a retrospective pilot study that was carried out in a single center to describe patterns of Hb variability and investigate factors associated with Hb variability comparing four different patient groups: hemodialysis and peritoneal patients treated with EPO, predialysis patients treated with EPO and predialysis patients not treated with EPO. In **chapter 8** we aim to identify patient-related, treatment-related as well as dialysis center-related factors associated with hemoglobin variability using several different measures of Hb variability. For this purpose, we used data from 20 centers in the Netherlands to construct multivariate regression models.

In **part III**, we present and discuss the results of the CREAM (Circadian Rhythm of Erythropoietin and Melatonin in CKD)-study. Patients with chronic kidney disease exhibit a variety of disrupted circadian body functions, which can have important consequences on the pathology of cardiovascular disease and sleep-wake disturbances.[47] The pineal hormone melatonin, which is normally only secreted during the night, is an important marker of the circadian timing system.[74] In hemodialysis patients, the nocturnal endogenous melatonin rise is absent.[51] In **chapter 9**, we tested whether melatonin secretion is also dysregulated in CKD patients with eGFR 30-80 ml/min.

It has been shown that the defective melatonin rhythm in dialysis patients can be restored by exogenous administration of EPO.[60,61] Therefore, we hypothesized that a relationship exists between melatonin rhythm and endogenous erythropoietin levels. In **chapter 10**, we investigate this hypothesis. Furthermore, we studied the circadian (mis) alignment of Insulin-like Growth Factor-1 (IGF-1, co-factor in erythropoiesis) and inflammatory markers (related to EPO resistance).

In **chapter 11**, the studies described in this thesis are summarized and discussed.

REFERENCES

1. U S Renal Data System, USRDS 2010 Annual Data Report. *Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD,* 2010.
2. Coresh J, Astor BC, Greene T, *et al.* Prevalence of chronic kidney disease and decreased kidney function in the adult US population: Third National Health and Nutrition Examination Survey. *Am J Kidney Dis* 2003;41:1-12.
3. Hallan SI, Coresh J, Astor BC, *et al.* International comparison of the relationship of chronic kidney disease prevalence and ESRD risk. *J Am Soc Nephrol* 2006;17:2275-2284.
4. Go AS, Chertow GM, Fan D, *et al.* Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 2004;351:1296-1305.
5. McAlister FA, Ezekowitz J, Tonelli M, *et al.* Renal insufficiency and heart failure: prognostic and therapeutic implications from a prospective cohort study. *Circulation* 2004;109:1004-1009.
6. Smith GL, Lichtman JH, Bracken MB, *et al.* Renal impairment and outcomes in heart failure: systematic review and meta-analysis. *J Am Coll Cardiol* 2006;47:1987-1996.
7. Hillege HL, Nitsch D, Pfeffer MA, *et al.* Renal function as a predictor of outcome in a broad spectrum of patients with heart failure. *Circulation* 2006;113:671-678.
8. Bongartz LG, Cramer MJ, Doevendans PA, *et al.* The severe cardiorenal syndrome: 'Guyton revisited'. *Eur Heart J* 2005;26:11-17.
9. Ronco C, Haapio M, House AA, *et al.* Cardiorenal syndrome. *J Am Coll Cardiol* 2008;52:1527-1539.
10. Fehr T, Ammann P, Garzoni D, *et al.* Interpretation of erythropoietin levels in patients with various degrees of renal insufficiency and anemia. *Kidney Int* 2004;66:1206-1211.
11. Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med* 2005;352:1011-1023.
12. Opasich C, Cazzola M, Scelsi L, *et al.* Blunted erythropoietin production and defective iron supply for erythropoiesis as major causes of anaemia in patients with chronic heart failure. *Eur Heart J* 2005;26:2232-2237.
13. Anand IS. Pathogenesis of anemia in cardiorenal disease. *Rev Cardiovasc Med* 2005;6 Suppl 3:S13-21.
14. Silverberg D, Wexler D, Blum M, *et al.* The cardio-renal anaemia syndrome: does it exist? *Nephrol Dial Transplant* 2003;18 Suppl 8:viii7-12.
15. Foley RN, Parfrey PS, Harnett JD, *et al.* The impact of anemia on cardiomyopathy, morbidity, and and mortality in end-stage renal disease. *Am J Kidney Dis* 1996;28:53-61.
16. Regidor DL, Kopple JD, Kovesdy CP, *et al.* Associations between changes in hemoglobin and administered erythropoiesis-stimulating agent and survival in hemodialysis patients. *J Am Soc Nephrol* 2006;17:1181-1191.
17. Jones M, Ibels L, Schenkel B, *et al.* Impact of epoetin alfa on clinical end points in patients with chronic renal failure: a meta-analysis. *Kidney Int* 2004;65:757-767.
18. Mancini DM, Katz SD, Lang CC, *et al.* Effect of erythropoietin on exercise capacity in patients with moderate to severe chronic heart failure. *Circulation* 2003;107:294-299.
19. Frank H, Heusser K, Hoffken B, *et al.* Effect of erythropoietin on cardiovascular prognosis parameters in hemodialysis patients. *Kidney Int* 2004;66:832-840.
20. Bahlmann FH, DeGroot K, Duckert T, *et al.* Endothelial progenitor cell proliferation and differentiation is regulated by erythropoietin. *Kidney Int* 2003;64:1648-1652.
21. George J, Goldstein E, Abashidze A, *et al.* Erythropoietin promotes endothelial progenitor cell proliferative and adhesive properties in a PI 3-kinase-dependent manner. *Cardiovasc Res* 2005;68:299-306.
22. Calvillo L, Latini R, Kajstura J, *et al.* Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. *Proc Natl Acad Sci U S A* 2003;100:4802-4806.
23. Vesey DA, Cheung C, Pat B, *et al.* Erythropoietin protects against ischaemic acute renal injury. *Nephrol Dial Transplant* 2004;19:348-355.

24. Jie KE, Verhaar MC, Cramer MJ, *et al.* Erythropoietin and the cardiorenal syndrome: cellular mechanisms on the cardiorenal connectors. *Am J Physiol Renal Physiol* 2006;291:F932-944.
25. Drueke TB, Locatelli F, Clyne N, *et al.* Normalization of hemoglobin level in patients with chronic kidney disease and anemia. *N Engl J Med* 2006;355:2071-2084.
26. Singh AK, Szczech L, Tang KL, *et al.* Correction of anemia with epoetin alfa in chronic kidney disease. *N Engl J Med* 2006;355:2085-2098.
27. Pfeffer MA, Burdmann EA, Chen CY, *et al.* A trial of darbepoetin alfa in type 2 diabetes and chronic kidney disease. *N Engl J Med* 2009;361:2019-2032.
28. Szczech LA, Barnhart HX, Inrig JK, *et al.* Secondary analysis of the CHOIR trial epoetin-alpha dose and achieved hemoglobin outcomes. *Kidney Int* 2008;74:791-798.
29. Pisoni RL, Bragg-Gresham JL, Young EW, *et al.* Anemia management and outcomes from 12 countries in the Dialysis Outcomes and Practice Patterns Study (DOPPS). *Am J Kidney Dis* 2004;44:94-111.
30. Fuste B, Serradell M, Escolar G, *et al.* Erythropoietin triggers a signaling pathway in endothelial cells and increases the thrombogenicity of their extracellular matrices in vitro. *Thromb Haemost* 2002;88:678-685.
31. Stohlawetz PJ, Dzirlo L, Hergovich N, *et al.* Effects of erythropoietin on platelet reactivity and thrombopoiesis in humans. *Blood* 2000;95:2983-2989.
32. Hardee ME, Cao Y, Fu P, *et al.* Erythropoietin blockade inhibits the induction of tumor angiogenesis and progression. *PLoS One* 2007;2:e549.
33. Yasuda Y, Fujita Y, Matsuo T, *et al.* Erythropoietin regulates tumour growth of human malignancies. *Carcinogenesis* 2003;24:1021-1029.
34. Bennett CL, Silver SM, Djulbegovic B, *et al.* Venous thromboembolism and mortality associated with recombinant erythropoietin and darbepoetin administration for the treatment of cancer-associated anemia. *Jama* 2008;299:914-924.
35. Corwin HL, Gettinger A, Fabian TC, *et al.* Efficacy and safety of epoetin alfa in critically ill patients. *N Engl J Med* 2007;357:965-976.
36. Locatelli F, Aljama P, Canaud B, *et al.* Target haemoglobin to aim for with erythropoiesis-stimulating agents: a position statement by ERBP following publication of the Trial to reduce cardiovascular events with Aranesp therapy (TREAT) study. *Nephrol Dial Transplant* 2010;25:2846-2850.
37. KDOQI Clinical Practice Guideline and Clinical Practice Recommendations for anemia in chronic kidney disease: 2007 update of hemoglobin target. *Am J Kidney Dis* 2007;50:471-530.
38. Ebben JP, Gilbertson DT, Foley RN, *et al.* Hemoglobin level variability: associations with comorbidity, intercurrent events, and hospitalizations. *Clin J Am Soc Nephrol* 2006;1:1205-1210.
39. Boudville NC, Djurdjev O, Macdougall IC, *et al.* Hemoglobin variability in nondialysis chronic kidney disease: examining the association with mortality. *Clin J Am Soc Nephrol* 2009;4:1176-1182.
40. Fishbane S, Berns JS. Hemoglobin cycling in hemodialysis patients treated with recombinant human erythropoietin. *Kidney Int* 2005;68:1337-1343.
41. Walker R, Pussell BA. Fluctuations in haemoglobin levels in haemodialysis patients receiving intravenous epoetin alfa or intravenous darbepoetin alfa. *Nephrology (Carlton)* 2007;12:448-451.
42. Minutolo R, Chiodini P, Cianciaruso B, *et al.* Epoetin therapy and hemoglobin level variability in nondialysis patients with chronic kidney disease. *Clin J Am Soc Nephrol* 2009;4:552-559.
43. Macdougall IC, Carrera F, Braun J, *et al.* Intercurrent events and comorbid conditions influence hemoglobin level variability in dialysis patients. *Clin Nephrol* 2009;Volume 71:397-404.
44. Yang W, Israni RK, Brunelli SM, *et al.* Hemoglobin variability and mortality in ESRD. *J Am Soc Nephrol* 2007;18:3164-3170.
45. Brunelli SM, Joffe MM, Israni RK, *et al.* History-adjusted marginal structural analysis of the association between hemoglobin variability and mortality among chronic hemodialysis patients. *Clin J Am Soc Nephrol* 2008;3:777-782.
46. Eckardt KU, Kim J, Kronenberg F, *et al.* Hemoglobin Variability Does Not Predict Mortality in European Hemodialysis Patients. *J Am Soc Nephrol* 2010;21:1765-1775.
47. Scheer FA, Hilton MF, Mantzoros CS, *et al.* Adverse metabolic and cardiovascular consequences of circadian misalignment. *Proc Natl Acad Sci U S A* 2009;106:4453-4458.

48. Phillips ML. Circadian rhythms: Of owls, larks and alarm clocks. *Nature* 2009;458:142-144.
49. Elung-Jensen T, Strandgaard S, Kamper AL. Longitudinal observations on circadian blood pressure variation in chronic kidney disease stages 3-5. *Nephrol Dial Transplant* 2008;23:2873-2878.
50. Clement DL, De Buyzere ML, De Bacquer DA, *et al.* Prognostic value of ambulatory blood-pressure recordings in patients with treated hypertension. *N Engl J Med* 2003;348:2407-2415.
51. Koch BC, Nagtegaal JE, Hagen EC, *et al.* The effects of melatonin on sleep-wake rhythm of daytime haemodialysis patients: a randomized, placebo-controlled, cross-over study (EMSCAP study). *Br J Clin Pharmacol* 2009;67:68-75.
52. Karasek M, Szuflet A, Chrzanowski W, *et al.* Decreased melatonin nocturnal concentrations in hemodialyzed patients. *Neuro Endocrinol Lett* 2005;26:653-656.
53. Geron R, Shurtz-Swirski R, Sela S, *et al.* Polymorphonuclear leucocyte priming in long intermittent nocturnal haemodialysis patients--is melatonin a player? *Nephrol Dial Transplant* 2006;21:3196-3201.
54. Poeggeler B, Reiter RJ, Tan DX, *et al.* Melatonin, hydroxyl radical-mediated oxidative damage, and aging: a hypothesis. *J Pineal Res* 1993;14:151-168.
55. Quiroz Y, Ferrebuz A, Romero F, *et al.* Melatonin ameliorates oxidative stress, inflammation, proteinuria, and progression of renal damage in rats with renal mass reduction. *Am J Physiol Renal Physiol* 2008;294:F336-344.
56. Markus RP, Ferreira ZS, Fernandes PA, *et al.* The immune-pineal axis: a shuttle between endocrine and paracrine melatonin sources. *Neuroimmunomodulation* 2007;14:126-133.
57. Macdougall IC, Cooper AC. Hyporesponsiveness to erythropoietic therapy due to chronic inflammation. *Eur J Clin Invest* 2005;35 Suppl 3:32-35.
58. Vgontzas AN, Zoumakis M, Papanicolaou DA, *et al.* Chronic insomnia is associated with a shift of interleukin-6 and tumor necrosis factor secretion from nighttime to daytime. *Metabolism* 2002;51:887-892.
59. Buemi M, Allegra A, Lagana A, *et al.* Effects of the evening i.v. administration of erythropoietin in haemodialyzed patients. *Riv Eur Sci Med Farmacol* 1993;15:195-197.
60. Vaziri ND, Oveisi F, Reyes GA, *et al.* Dysregulation of melatonin metabolism in chronic renal insufficiency: role of erythropoietin-deficiency anemia. *Kidney Int* 1996;50:653-656.
61. Benz RL, Pressman MR, Hovick ET, *et al.* A preliminary study of the effects of correction of anemia with recombinant human erythropoietin therapy on sleep, sleep disorders, and daytime sleepiness in hemodialysis patients (The SLEEPO study). *Am J Kidney Dis* 1999;34:1089-1095.
62. Locatelli F, Pisoni RL, Combe C, *et al.* Anaemia in haemodialysis patients of five European countries: association with morbidity and mortality in the Dialysis Outcomes and Practice Patterns Study (DOPPS). *Nephrol Dial Transplant* 2004;19:121-132.
63. Go AS, Yang J, Ackerson LM, *et al.* Hemoglobin level, chronic kidney disease, and the risks of death and hospitalization in adults with chronic heart failure: the Anemia in Chronic Heart Failure: Outcomes and Resource Utilization (ANCHOR) Study. *Circulation* 2006;113:2713-2723.
64. Macdougall IC. Poor response to erythropoietin: practical guidelines on investigation and management. *Nephrol Dial Transplant* 1995;10:607-614.
65. Zhang Y, Thamer M, Stefanik K, *et al.* Epoetin requirements predict mortality in hemodialysis patients. *Am J Kidney Dis* 2004;44:866-876.
66. Swinkels DW, Wetzels JF. Hepcidin: a new tool in the management of anaemia in patients with chronic kidney disease? *Nephrol Dial Transplant* 2008;23:2450-2453.
67. Felker GM, Allen LA, Pocock SJ, *et al.* Red cell distribution width as a novel prognostic marker in heart failure: data from the CHARM Program and the Duke Databank. *J Am Coll Cardiol* 2007;50:40-47.
68. Pascual-Figal DA, Bonaque JC, Redondo B, *et al.* Red blood cell distribution width predicts long-term outcome regardless of anaemia status in acute heart failure patients. *Eur J Heart Fail* 2009;11:840-846.
69. Allen LA, Felker GM, Mehra MR, *et al.* Validation and potential mechanisms of red cell distribution width as a prognostic marker in heart failure. *J Card Fail* 2009;16:230-238.
70. Forhecz Z, Gombos T, Borgulya G, *et al.* Red cell distribution width in heart failure: prediction of clinical events and relationship with markers of ineffective erythropoiesis, inflammation, renal function, and nutritional state. *Am Heart J* 2009;158:659-666.

71. Hardee ME, Arcasoy MO, Blackwell KL, *et al.* Erythropoietin biology in cancer. *Clin Cancer Res* 2006;12:332-339.
72. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;420:860-867.
73. De Nicola L, Conte G, Chiodini P, *et al.* Stability of target hemoglobin levels during the first year of epoetin treatment in patients with chronic kidney disease. *Clin J Am Soc Nephrol* 2007;2:938-946.
74. Claustrat B, Brun J, Chazot G. The basic physiology and pathophysiology of melatonin. *Sleep Med Rev* 2005;9:11-24.

PART I

The role of erythropoietin in the cardiorenal syndrome

CHAPTER 2

Mechanisms of Disease: erythropoietin resistance in patients with both heart and kidney failure

Karien van der Putten[1]
Branko Braam[2]
Kim E Jie[3]
Carlo AJM Gaillard[1]

[1]Department of Internal Medicine, Meander Medical Center, Amersfoort, The Netherlands
[2]Division of Nephrology and Immunology, University of Alberta, Edmonton, Canada
[3]Department of Nephrology and Hypertension, University Medical Center Utrecht, Utrecht, The Netherlands

Nat Clin Pract Nephrol 2008;4:47-57

SUMMARY

Anemia is common in patients who have both heart failure and chronic kidney disease, and there is an association between anemia and progression of both diseases. The main causes of anemia are deficient production of erythropoietin (EPO), iron deficiency and chronic disease with endogenous EPO resistance. EPO has been successfully used for over a decade to treat anemia in patients with chronic kidney disease. Less obvious are the safety and efficacy of EPO treatment in patients with both heart failure and renal disease. Up to 10% of patients receiving EPO are hyporesponsive to therapy and require large doses of the agent. Several mechanisms could explain resistance to endogenous and exogenous EPO. Proinflammatory cytokines antagonize the action of EPO by exerting an inhibitory effect on erythroid progenitor cells and by disrupting iron metabolism (a process in which hepcidin has a central role). EPO resistance might also be caused by inflammation, which has a negative effect on EPO receptors. Furthermore, neocytolysis could have a role. As resistance to exogenous EPO is associated with an increased risk of death, it is important to understand how cardiorenal failure affects EPO production and function.

INTRODUCTION

Anemia is common in patients with both heart failure and chronic kidney disease (CKD) and is associated with a negative outcome. Whether treatment with erythropoietin (EPO) is of benefit is the subject of several ongoing studies. Treatment with EPO does not increase hemoglobin levels in a considerable proportion of patients. This fact is clinically important because resistance to EPO is associated with an increased risk of death in people with CKD. In this review, we address these issues with a focus on mechanisms that modulate the response to EPO. First, we briefly discuss the pathogenesis of the cardiorenal syndrome, and explore the causes and consequences of anemia in CKD, heart failure and the combination of the two. Thereafter, we elucidate the physiological role of EPO and discuss the mechanisms that might underlie the variability in sensitivity to endogenous, as well as exogenous, EPO.

ERYTHROPOIETIN AND THE CARDIORENAL SYNDROME

We recently proposed a model of the pathogenesis of the cardiorenal syndrome, in which cardiac and renal dysfunction mutually amplify progressive failure of both systems.[1] Inflammation, the balance between nitric oxide and reactive oxygen species, the sympathetic nervous system and the renin-angiotensin system (RAS), are the 'cardiorenal connectors', cornerstones in the pathophysiology of the cardiorenal syndrome (Figure 1).[1] More recently, we have expanded the model and explored the hypothesis that EPO could dampen the cardiorenal connectors.[2] In this review, we address the means by which the cardiorenal connectors influence the function of EPO. In acknowledgement of its pivotal role, we will specifically focus on the mechanism by which inflammation affects the physiological function of EPO. The other cardiorenal connectors are only discussed when relevant to this mechanism.

ANEMIA IN CKD, HEART FAILURE AND THE CARDIORENAL SYNDROME

Causes

Anemia associated with CKD has several causes. The main factor is inappropriate synthesis of EPO, with serum levels of EPO being disproportionately low for the degree of anemia (i.e. 'renal anemia').[3] The 'anemia of chronic disease' also has an important role in CKD.[4] Several pathophysiological mechanisms underlie this condition, including limited availability of iron for erythropoiesis, impaired proliferation of erythroid precursor cells, reduced expression of EPO and EPO receptors, and, possibly, perturbed EPO signal transduction.[5] These mechanisms will be discussed in detail later in this article. Other causes of anemia in patients with CKD are infection and absolute iron deficiency.[6] Blood loss is a common cause of anemia in patients with renal failure. This blood loss includes occult gastrointestinal blood loss, blood being retained in extracorporeal circuits during dialysis, and withdrawal of blood for laboratory tests. Hemolysis, vitamin B_{12} or folate

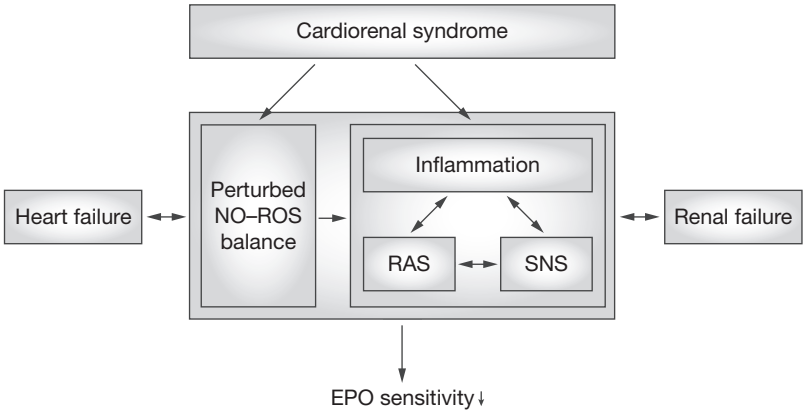

Figure 1 The pathophysiology of the cardiorenal syndrome and its effect on erythropoietin. The cardiorenal syndrome is characterized by an imbalance between nitric oxide and reactive oxygen species, by increased inflammation, by increased activity of the renin-angiotensin system, and by increased activity of the sympathetic nervous system. Together, these 'cardiorenal connectors' decrease sensitivity to EPO.
Abbreviations: EPO, erythropoietin; NO, nitric oxide; RAS, renin-angiotensin system; ROS, reactive oxygen species; SNS, sympathetic nervous system.

deficiency, hyperparathyroidism, hemoglobinopathies and malignancies can also cause anemia in patients with CKD.[6-8] The consequences of concomitant angiotensin-converting-enzyme (ACE) inhibitor therapy are complex and might contribute to suppression of erythropoiesis.[9,10]

Anemia is a common problem in chronic heart failure (CHF). Ezekowitz et al. reported that 17% of 12.065 patients with CHF were anemic.[11] Anemia in CHF develops in response to several factors. The most important form is anemia of chronic disease.[12] In addition, the creatinine clearance rates are less than 60 ml/min in approximately 25% to 50% of patients with CHF,[13] which could contribute to the development of anemia in these individuals. Other causes of anemia in patients with CHF are iron deficiency, and vitamin B_{12} or folate deficiency.[8,12]

ACE-inhibitors are part of the standard treatment regimens for CHF and, as mentioned above, whether their use contributes to the development of anemia is uncertain. Angiotensin II enhances EPO secretion and might also directly stimulate erythroid precursor cells.[14] ACE catalyzes the breakdown of N-acetyl-seryl-aspartyl-lysyl-proline, an inhibitor of erythropoiesis.[15,16] Accordingly, inhibition of the RAS would reduce hemoglobin levels. This effect has been confirmed in clinical trials in which pharmacological inhibition of the RAS was associated with a small but statistically significant reduction in hemoglobin levels.[17-19] By contrast, Androne et al. assert that activation of the RAS reduces hemoglobin concentration in patients with CHF, by hemodilution.[20] It seems, therefore, that the effects of ACE inhibition on hematocrit are complex, and available data are conflicting.

It is not surprising that anemia is a common feature of patients with both CKD and CHF. de Silva et al. found that, of 955 patients with systolic heart failure, 32% had anemia and 54% had renal insufficiency[8]. Furthermore, 41% of patients with kidney disease and 22% of patients without kidney disease had anemia. Silverberg et al. called the triad of anemia, CKD and CHF the 'cardiorenal-anemia syndrome'.[21] In this syndrome, the three

components form a vicious circle, with each constituent capable of causing or worsening the other two.

Consequences

Renal anemia has serious clinical consequences. In addition to reducing patient physical capacity and quality of life, anemia induces adaptive cardiovascular mechanisms that increase the risk of cardiovascular disease and death.[22-25] Observational studies have shown that a decreased level of hemoglobin in patients with CKD is associated with an increased risk of hospitalization and of death.[26-29] Small interventional trials have shown that treatment of anemia with EPO in CKD patients improves quality of life.[30] Some studies,[31,32] but not all,[25] have detected an association between treatment with EPO and regression of left ventricular hypertrophy. Thus, treating of anemia is important in patients with CKD, but much controversy exists about the optimum hemoglobin target concentration. Several studies have been performed to address this issue. In 1998, Besarab et al. suggested that treating patients with end-stage renal disease to a target hematocrit of 42% could be harmful.[33] The Cardiovascular Risk Reduction by Early Anemia Treatment with Epoetin Beta (CREATE) study detected no significant effect on cardiovascular events of achieving a target hemoglobin level of 13-15 g/dl in patients with CKD.[34] The Correction of Hemoglobin and Outcomes in Renal Insufficiency (CHOIR) study was terminated when an increased risk of death and cardiovascular hospitalization was detected in patients with CKD treated to achieve a target hemoglobin level of 13.5 g/dl.[35] In conclusion, no data are currently available to support the normalization of hemoglobin concentrations in patients with CKD, and large prospective trials are needed to determine an optimum target level. Until such evidence becomes available, the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF K/DOQI) guidelines recommend a target hemoglobin level of 11-12 g/dL.[36] The European best practice guidelines recommend a target hemoglobin level of above 11 g/dL, with a maximum of 12 g/dL for patients with concomitant cardiovascular disease or diabetes.[37]

A low hemoglobin concentration in patients with CHF is associated with more-severe disease, greater left ventricular mass index, and higher hospitalization and mortality rates.[11,38,39] The Anemia in Chronic Heart Failure: Outcomes and Resource Utilization (ANCHOR) study, however, showed that a very high hemoglobin level (>17 g/dL) is also predictive of an increased risk of death and hospitalization in patients with CHF.[39] Several small controlled trials have been performed to assess the effect of treatment with EPO on anemic patients with CHF. EPO treatment improved exercise capacity and left ventricular function, and reduced hospitalization rate.[40-43] In the Studies of Anemia in Heart Failure Trial (STAMINA-HeFT), 319 patients with CHF were randomly assigned to receive either darbepoietin alfa or placebo[44]. No significant differences were observed between the two groups in exercise duration, NYHA class or hospitalization, after 27 weeks. Available evidence is, therefore, contradictory, and further studies are needed to determine whether, and to what extent, correction of anemia with EPO is beneficial in patients with CHF.

ERYTHROPOIETIN, ERYTHROPOIESIS AND BEYOND

EPO acts as an erythropoietic protein; it is produced in the kidneys by peritubular cells and can also be produced in the liver.[45] The primary stimulus for production of EPO is hypoxia. A deficiency in tissue oxygen levels increases the activity of hypoxia-inducible factor 2α, which binds to hypoxia responsive elements located in the enhancer region of the EPO gene in order to activate transcription.[46,47] Other transcription factors, such as GATA2 and nuclear factor kappa B (NFκB), can modulate EPO expression; evidence indicates that both factors inhibit transcription of the EPO gene.[48,49] The transcription factor Sox6 has been shown in mice to be an important enhancer of erythropoiesis at multiple levels.[50] In the bone marrow, EPO acts synergistically with stem cell factor, granulocyte-macrophage colony-stimulating factor, interleukin (IL)-3, IL-4, IL-9 and insulin-like growth factor 1 on erythroid progenitor cells to prevent their programmed cell death, thereby stimulating proliferation and maturation of erythroid progenitors through the normoblast stage into reticulocytes and mature erythrocytes.[51] The EPO receptor is expressed primarily on erythroid cells that are between the colony-forming-unit erythroid stage and the pronormoblast stage of development. The number of EPO receptors per cell gradually decreases during erythroid differentiation.[52]

In addition to acting as an erythropoietic factor, EPO has been shown to have an important role in tissues outside of the erythropoietic system. Expression of EPO receptors and a biological response to EPO have been observed in endothelial, neural, cardiac, and other cell types.[53-55] One non-hematopoietic effect of EPO is its stimulation of dose-dependent proliferation and chemotaxis of endothelial progenitor cells *in vitro*, which promotes vascular reparative processes and neoangiogenesis.[56] This process has also been shown to occur in patients with CKD [57] and in patients with CHF.[58] EPO can also induce nitric oxide production by endothelial cells, which might exert anti-apoptotic effects.[2,59] EPO protects cardiomyocytes against ischemic injury by inhibiting apoptosis.[60] Administration of EPO to ischemic renal cells reduced apoptosis and enhanced regeneration in a study by Vesey and colleagues.[61] Thus, local binding of EPO to endothelial cells, cardiac cells and renal cells exerts cytoprotective and proliferative effects.

Erythropoietic agents were introduced in about 1990 for the treatment of anemia associated with CKD. Nowadays, EPO is also registered for use in patients with nonmyeloid malignancies treated with chemotherapy, in AIDS patients with anemia due to treatment with zidovudine, and in the perioperative stage for surgical patients. Several studies that aim to define the role of EPO treatment in CHF, the combination of CHF and CKD, and in acute myocardial infarction, are underway.

ERYTHROPOIETIN RESISTANCE

In 1979, Caro et al. measured endogenous EPO levels in healthy people with or without anemia and compared these with levels in CKD patients with anemia.[62] It seemed that EPO levels were higher in patients with CKD than in those without CKD, but were inappropriately low for the degree of anemia. Serum levels of EPO in normal individuals range between 1 and 27 mU/ml (mean 6.2 ± 4.3 mU/ml), whereas those in patients

with CKD are between 4.2 and 102 mU/ml (mean 29.5 $\pm$ 4.0 mU/ml); [63] anemia persists in patients with CKD despite average EPO levels being approximately five times higher than those in healthy individuals. This disparity indicates that, in addition to relative EPO deficiency, bone marrow response to endogenous EPO is suppressed in people with CKD. Erythropoietic agents have been used since the 1990s to treat patients with CKD, in an effort to overcome the relative EPO deficiency. Up to 10% of patients, however, have an inadequate response to therapy.[64] This finding is clinically important, because resistance to EPO is associated with an increased risk of death in patients with CKD. This effect persists after adjustment for the generally decreased hematocrit in these patients.[29]

EPO levels have also been measured in people with CHF, and have been found to be high.[65-67] Analogous to the situation in patients with CKD, the high EPO levels in patients with CHF are inappropriately low for the degree of anemia.[68] These data indicate that in CHF, as well as in CKD, there is a relative EPO deficiency as well as resistance of bone marrow to endogenous EPO.

Several mechanisms for resistance to endogenous, as well as exogenous, EPO have been proposed. The processes that cause anemia of chronic disease have a role. The primary hypothesis is that proinflammatory cytokines antagonize the action of endogenous and exogenous EPO by directly inhibiting erythroid progenitor cells and by disrupting iron metabolism. Interaction between EPO and its receptor, and intracellular EPO signaling, could also have a role. Little is known about the putative contribution of neocytolysis (selective hemolysis of young circulating red blood cells) to EPO resistance. These possible causes of EPO resistance are discussed in the following section.

MECHANISMS OF ERYTHROPOIETIN RESISTANCE

Cytokines and erythropoiesis

CKD involves a chronic inflammatory state. Patients with CKD have increased levels of markers of inflammation, such as C-reactive protein and the cytokines IL-1, IL-6, interferon (IFN)-γ and tumor necrosis factor (TNF).[69,70] Increased levels of TNF and IL-6 have also been measured in patients with CHF.[71-73] These cytokines inhibit the growth of erythroid precursor cells *in vitro*, an effect that is probably brought about through cytokine-mediated induction of apoptosis.[74,75] Taniguchi et al. showed that IFN-γ downregulates messenger RNA for EPO receptor expression, indicating that the number of EPO receptors can influence apoptosis of erythroid precursor cells.[76] In addition, IL-1 and TNF have been shown to inhibit EPO production, another mechanism by which cytokines induce apoptosis of precursor cells.[77] Moreover, cytokines have a direct toxic effect on progenitor cells, which is generated at least in part by inducing the labile free radical nitric oxide, produced by inducible nitric oxide synthase.[78]

In addition to their direct inhibitory effect on erythroid progenitor cells, cytokines can cause EPO resistance by disrupting iron metabolism (Figure 2). Iron is essential for the production of hemoglobin. Iron homeostasis involves three sources that supply the plasma compartment: iron absorption from the diet; recycled iron from red blood cells; and iron moved from storage sites in the liver. As most of the body's iron is contained in

red blood cells, nearly all of the iron for erythropoiesis is supplied by the recycling of iron from senescent red cells. Macrophages phagocytize erythrocytes, and iron is released from hemoglobin in the phagolysosome. From the macrophage, iron is transferred to the circulation by the carrier protein ferroportin.[79] Iron is transported in the plasma by transferrin, which donates iron to cells through its interaction with a specific membrane receptor, the transferrin receptor.[80]

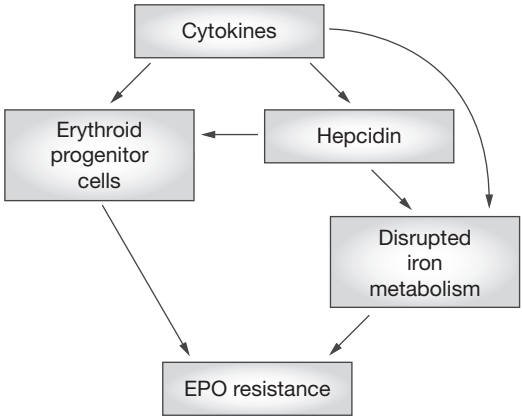

Figure 2 The role of cytokines in erythropoietin resistance. Proinflammatory cytokines induce EPO resistance by exerting both direct inhibitory effects on erythroid progenitor cells, and indirect inhibitory effects by upregulating hepcidin. Cytokines also induce EPO resistance by disrupting iron metabolism. This effect occurs via upregulation of hepcidin and via a direct effect of proinflammatory cytokines on iron homeostasis.
Abbreviation: EPO, erythropoietin.

Pro-inflammatory cytokines, mainly IL-6, affect iron metabolism by stimulating the synthesis of hepcidin. Hepcidin is a type II acute-phase protein produced in the liver that has been proposed to be the central regulator of iron metabolism (Figure 3).[81] Hepcidin is thought to control the efflux of iron into plasma transferrin by downregulating ferroportin, the efflux channel for iron in macrophages and enterocytes. It has also been suggested that hepcidin negatively regulates the expression of the apical divalent metal transporter 1 in the enterocyte.[82] These actions lead to a decreased iron concentration in the circulation. In addition to its effect on iron metabolism, hepcidin may contribute to EPO resistance by directly inhibiting erythroid-progenitor proliferation and survival (Figure 2).[83] In accordance with these findings, it has been shown that levels of hepcidin, as well as pro-hepcidin (the precursor of hepcidin), are negatively correlated with hematocrit in hemodialysis patients.[84,85]

Proinflammatory cytokines also have a direct effect on iron homeostasis (Figure 2). TNF, IL-1 and IL-6 upregulate the expression of divalent metal transporter 1 in macrophages, induce ferritin expression, and downregulate ferroportin. In addition, cytokines stimulate an increase in transferrin-receptor-mediated uptake of transferrin-bound iron into macrophages. TNF and IL-1 damage erythrocyte membranes and stimulate erythrophagocytosis. Together, these processes promote intracellular iron storage and decrease the plasma concentration of iron.[5]

To sum up, the chronic inflammatory state of CKD plus CHF limits the availability of iron for erythropoiesis, thereby leading to EPO resistance via upregulation of hepcidin and the direct effect of cytokines on iron homeostasis.

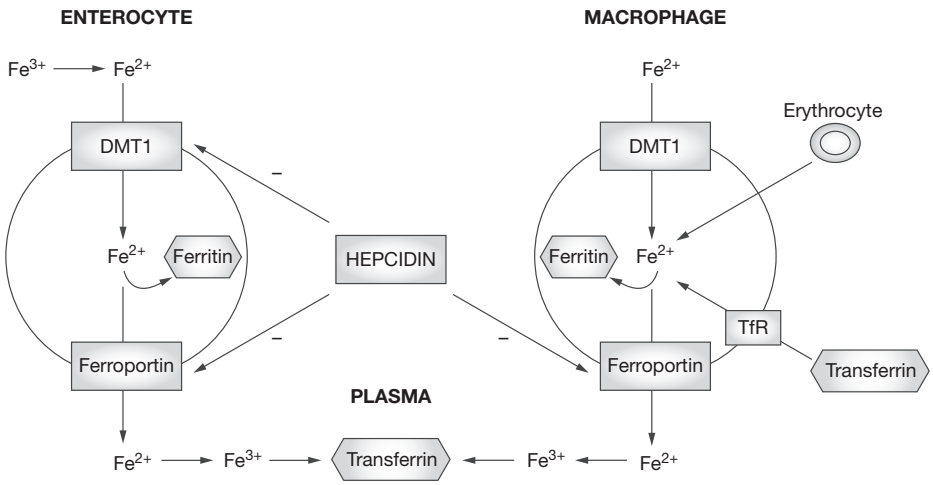

Figure 3 Iron homeostasis and the role of hepcidin. Fe^{3+} is reduced to Fe^{2+} and moves into the enterocyte via DMT1. In the enterocyte, iron can either be stored as ferritin or can be transported out by ferroportin. In plasma, $Fe^{2+}$is oxidized to Fe^{3+}, which can bind to transferrin. Macrophages take up iron by phagocytosis of erythrocytes, as Fe^{2+} via DMT1, and from transferrin via the transferrin receptor. In the macrophage, iron is stored as ferritin or is transported to the circulation via ferroportin. Hepcidin negatively regulates ferroportin in the enterocyte and macrophage, and the expression of DMT1 in the enterocyte, leading to decreased plasma iron availability.
Abbreviation: DMT1, divalent metal transporter 1.

Receptor interactions

Binding of EPO to the EPO receptor is essential for the production of mature red blood cells. The EPO receptor is a type 1 transmembrane protein that belongs to the hematopoietic cytokine receptor superfamily. The EPO-bound receptor is a dimer; however, there has been debate about whether, in the absence of ligand, the EPO receptor is a monomer or oligomer. One model proposes that two monomeric receptors become a dimer after the binding of EPO, resulting in signal transduction.[86] Another model describes a process whereby the EPO receptor appears at the cell surface as a preformed dimer. In this now generally accepted scheme, binding of ligand shifts the receptor from an inactive to an active conformation (Figure 4).[87,88] Activation of the EPO receptor is transient; it is rapidly deactivated by downregulating mechanisms, including receptor internalization and degradation.

Mechanisms that might underlie EPO resistance are defective dimerization or defective activation of the EPO receptor. It has been shown that the transmembrane domain of the EPO receptor has a powerful ligand-independent dimerizing ability.[87] Although the role of preformed dimers is still unclear, it could be that the dimerizing activity of the EPO receptor transmembrane domain sensitizes the EPO receptor such that it functions at low EPO concentrations. According to this model, absence of spontaneous dimerization of the EPO receptor could lead to EPO resistance.

Naranda et al. described how the EPO receptor can be activated in the absence of its natural ligand by a peptide that binds to a domain that differs from the EPO-binding site.[89] Mimetic peptides that bind to the EPO-binding site also exist, thereby activating the same signaling pathways as EPO itself.[90,91] EPO resistance could be induced by the presence of antagonistic peptides that bind to the EPO receptor. For example, it has been shown that cytokine-inducible SH2-containing protein (CIS) binds directly to the cytoplasmatic domain of the EPO receptor and inhibits EPO-dependent proliferation.[92] CIS production is induced by cytokines such as IFN-γ, IL-2 and IL-6. As such, cytokines might cause EPO resistance in cardiorenal failure.

Another possible cause of blunted EPO response is a decreased number of EPO receptors. IFN-γ inhibits messenger RNA for EPO receptor expression. The number of EPO receptors can also drop in response to receptor internalization and degradation, which are poorly understood mechanisms of downregulation. Beckman et al. studied the contribution of EPO-induced receptor internalization to modulation of EPO intracellular signals.[93] They showed that neither EPO activation of Janus kinase 2 (JAK2), nor tyrosine phosphorylation of the EPO receptor, was required for EPO-induced receptor downregulation. So, in contrast to other classes of growth factor receptors,[94] internalization of the EPO receptor seems to be regulated independently to EPO signaling. An increase in the rate of internalization could decrease the number of EPO receptors and lead to EPO resistance. The means by which ligand-occupied EPO receptor internalization is regulated, and whether cytokines have a role in this process, is unknown.

After internalization, proteosomes and lysosomes degrade both EPO and the EPO receptor, thereby regulating the degree of EPO signaling. In contrast to internalization, EPO and EPO receptor degradation has been shown to be dependent on JAK2 activity; blocking of JAK2 induced recycling of internalized EPO-EPO receptor-complexes to the cell surface, indicating perturbation of degradative processes.[95] Enhanced degradation of the EPO receptor could be important to the development of EPO resistance as it would diminish the rate of EPO-EPO receptor-complex recycling; however, no data are available to support this hypothesis.

Intracellular signaling

After binding to its receptor, EPO promotes activation of receptor-associated JAK2 tyrosine kinase and subsequent tyrosine phosphorylation of the EPO receptor[96]. This phosphorylation initiates intracellular signaling. Hemopoietic cell phosphatase (HCP; also known as protein tyrosine phosphatase-1 [SHP-1]) is a cytoplasmic protein that negatively regulates intracellular signal transduction.[97] HCP dephosphorylates JAK2, preventing tyrosine phosphorylation of the EPO receptor (Figure 4). Upregulation of HCP could, therefore, attenuate the EPO signaling cascade and contribute to EPO hyporesponsiveness. Indeed, levels of HCP are increased in hemodialysis patients who are resistant to EPO therapy.[98] Mechanisms that upregulate the expression of HCP have not been elucidated.

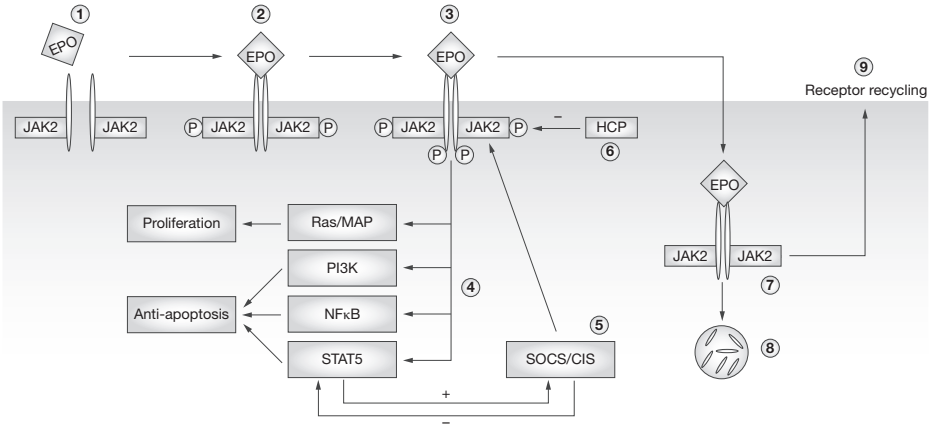

Figure 4 Overview of erythropoietin receptor activation and intracellular pathways. The EPO receptor is a preformed dimer (1). Binding of EPO leads to activation of JAK2 by transphosphorylation (2) and subsequent tyrosine phosphorylation of the cytoplasmic domain of the EPO receptor (3). This phosphorylation leads to the initiation of intracellular signaling with activation of STAT5, Ras/MAP, PI3K and NFκB (4). STAT5 induces SOCS/CIS, which attenuates EPO signaling by binding to JAK2 and by inhibiting phosphorylation of STAT5 (5). EPO signaling is terminated by HCP, which dephosphorylates JAK2 (6). The EPO-EPO receptor-complex is internalized following dephosphorylation of the receptor (7). The receptor is then degraded in the proteosome (8) or recycled to the cell surface (9).

Abbreviations: CIS, cytokine-inducible SH2-containing protein; EPO, erythropoietin; HCP, hematopoietic cell phosphatase; JAK2, Janus kinase 2; MAP, mitogen-activated protein; NFκB, nuclear factor kappa B; PI3K, phosphatidylinositol 3 kinase; SOCS, suppressor of cytokine signaling; STAT5, signal transducer and activator of transcription 5.

Several signaling pathways are activated in response to tyrosine phosphorylation of the EPO receptor, including those that involve signal transducer and activator of transcription 5 (STAT5), Ras/mitogen-activated protein (MAP), phosphatidylinositol 3 kinase (PI3-K) and NF-κB (Figure 4). Binding of EPO to its receptor induces phosphorylation of STAT5, which has a crucial role in cytokine-induced survival of hematopoietic cells. Phosphorylation of STAT5 leads to expression of anti-apoptotic proteins, including Bcl-x_l and Bcl-2.[99] Furthermore, STAT5 induces suppressors of cytokine signaling (SOCS), including CIS. SOCS and CIS are components of a negative feedback mechanism that attenuates EPO signaling by binding to JAK2 or by inhibiting tyrosine phosphorylation of STAT5.[92,100,101] SOCS and CIS are induced by proinflammatory cytokines. Thus, the presence of inflammatory cytokines could cause EPO resistance by affecting intracellular signaling.

The Ras/MAP kinase pathway is activated by EPO and is involved in cell proliferation.[102,103] EPO also activates the PI3-K pathway via phosphorylation, which leads to phosphorylation and activation of protein Kinase B (Akt).[104,105] Akt can then phosphorylate and inactivate proapoptotic molecules. Prevention of Akt phosphorylation would block the cellular protection afforded by EPO and could lead to EPO resistance; however, no studies have been performed to determine whether Akt is inhibited in cardiorenal failure.

EPO can induce the phosphorylation of inhibitor of NF-κB (I-κB), thereby activating NF-κB, which in turn enhances the transcriptional activity of target genes that encode anti-apoptotic molecules.[105] NFκB also inhibits transcription of the EPO gene. NFκB activity is

enhanced by IL-1 and TNF, another mechanism by which inflammatory cytokines affect intracellular signaling and lead to EPO resistance in cardiorenal failure.[49]

Neocytolysis

Red blood cell mass is maintained within a narrow range to optimize tissue oxygenation. Among the processes that control this parameter is neocytolysis. This physiological process is initiated by a drop in EPO levels, which leads to selective hemolysis of young circulating red blood cells (neocytes), and subsequent downregulation of red cell mass when it is excessive.[106] Two mechanisms could explain neocytolysis. EPO might regulate the secretion from endothelial cells of transforming growth factor-beta, which influences macrophage-mediated phagocytosis. Alternatively, EPO withdrawal could induce secretion of inflammatory mediators from endothelial cells and macrophages, which would expose phosphatidylserine on young red cells. Phosphatidylserine is an aminophospholipid that is normally localized to the inner leaflet of the red cell membrane; once it is exposed on the red cell surface, it marks these non-nucleated cells for destruction.[107] Neocytolysis could have a role in resistance to exogenous EPO. A small trial found that neocytolysis occurred in patients with CKD and anemia from whom EPO therapy was withheld.[108] Although the current information about the relevance of neocytolysis is limited, available data indicate that further exploration of this issue is warranted.

CONCLUSIONS

The pathophysiological basis of the frequently occurring phenomenon of cardiorenal anemia is complex, and includes inadequate EPO production as well as EPO resistance. We propose that inflammation has a key role in EPO resistance. Cardiorenal failure is a low-grade inflammatory condition in which pro-inflammatory cytokines antagonize the action of EPO by directly inhibiting erythroid progenitor cells and by disrupting iron metabolism, in which hepcidin has a central role. EPO resistance could also be caused by inflammation-induced changes in EPO receptor properties, assembly and recycling, and by interference with the post-receptor signaling routes. This latter subject is largely unexplored. Neocytolysis could also have a role in Epo resistance.

Recombinant human EPO has been registered for more than a decade for the treatment of renal anemia in patients with CKD, and has proven to be a safe and beneficial therapy. Less clear is whether treatment with EPO in patients with heart failure and kidney disease is effective and safe. Resistance to exogenous EPO is associated with an increased risk of death in hemodialysis patients. It is, therefore, important to understand more in detail how cardiorenal failure affects EPO production and function.

REFERENCES

1. Bongartz LG, Cramer MJ, Doevendans PA, *et al.* The severe cardiorenal syndrome: 'Guyton revisited'. *Eur Heart J* 2005;26:11-17.
2. Jie KE, Verhaar MC, Cramer MJ, *et al.* Erythropoietin and the cardiorenal syndrome: cellular mechanisms on the cardiorenal connectors. *Am J Physiol Renal Physiol* 2006;291:F932-944.
3. Fehr T, Ammann P, Garzoni D, *et al.* Interpretation of erythropoietin levels in patients with various degrees of renal insufficiency and anemia. *Kidney Int* 2004;66:1206-1211.
4. Jacobs C, Frei D, Perkins AC. Results of the European Survey on Anaemia Management 2003 (ESAM 2003): current status of anaemia management in dialysis patients, factors affecting epoetin dosage and changes in anaemia management over the last 5 years. *Nephrol Dial Transplant* 2005;20 Suppl 3:iii3-24.
5. Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med* 2005;352:1011-1023.
6. Eckhardt KU. Pathophysiology of renal anemia. *Clin Nephrol* 2000;53:S2-8.
7. Drueke TB, Eckardt KU. Role of secondary hyperparathyroidism in erythropoietin resistance of chronic renal failure patients. *Nephrol Dial Transplant* 2002;17 Suppl 5:28-31.
8. de Silva R, Rigby AS, Witte KK, *et al.* Anemia, renal dysfunction, and their interaction in patients with chronic heart failure. *Am J Cardiol* 2006;98:391-398.
9. Macdougall IC. The role of ACE inhibitors and angiotensin II receptor blockers in the response to epoetin. *Nephrol Dial Transplant* 1999;14:1836-1841.
10. Saudan P, Halabi G, Perneger T, *et al.* ACE inhibitors or angiotensin II receptor blockers in dialysed patients and erythropoietin resistance. *J Nephrol* 2006;19:91-96.
11. Ezekowitz JA, McAlister FA, Armstrong PW. Anemia is common in heart failure and is associated with poor outcomes: insights from a cohort of 12 065 patients with new-onset heart failure. *Circulation* 2003;107:223-225.
12. Opasich C, Cazzola M, Scelsi L, *et al.* Blunted erythropoietin production and defective iron supply for erythropoiesis as major causes of anaemia in patients with chronic heart failure. *Eur Heart J* 2005;26:2232-2237.
13. Anand IS. Pathogenesis of anemia in cardiorenal disease. *Rev Cardiovasc Med* 2005;6 Suppl 3:S13-21.
14. Mrug M, Stopka T, Julian BA, *et al.* Angiotensin II stimulates proliferation of normal early erythroid progenitors. *J Clin Invest* 1997;100:2310-2314.
15. Azizi M, Rousseau A, Ezan E, *et al.* Acute angiotensin-converting enzyme inhibition increases the plasma level of the natural stem cell regulator N-acetyl-seryl-aspartyl-lysyl-proline. *J Clin Invest* 1996;97:839-844.
16. van der Meer P, Lipsic E, Westenbrink BD, *et al.* Levels of hematopoiesis inhibitor N-acetyl-seryl-aspartyl-lysyl-proline partially explain the occurrence of anemia in heart failure. *Circulation* 2005;112:1743-1747.
17. Albitar S, Genin R, Fen-Chong M, *et al.* High dose enalapril impairs the response to erythropoietin treatment in haemodialysis patients. *Nephrol Dial Transplant* 1998;13:1206-1210.
18. Erturk S, Nergizoglu G, Ates K, *et al.* The impact of withdrawing ACE inhibitors on erythropoietin responsiveness and left ventricular hypertrophy in haemodialysis patients. *Nephrol Dial Transplant* 1999;14:1912-1916.
19. Ishani A, Weinhandl E, Zhao Z, *et al.* Angiotensin-converting enzyme inhibitor as a risk factor for the development of anemia, and the impact of incident anemia on mortality in patients with left ventricular dysfunction. *J Am Coll Cardiol* 2005;45:391-399.
20. Androne AS, Katz SD, Lund L, *et al.* Hemodilution is common in patients with advanced heart failure. *Circulation* 2003;107:226-229.
21. Silverberg DS, Wexler D, Blum M, *et al.* The correction of anemia in severe resistant heart failure with erythropoietin and intravenous iron prevents the progression of both the heart and the renal failure and markedly reduces hospitalization. *Clin Nephrol* 2002;58 Suppl 1:S37-45.
22. Levin A, Thompson CR, Ethier J, *et al.* Left ventricular mass index increase in early renal disease: impact of decline in hemoglobin. *Am J Kidney Dis* 1999;34:125-134.
23. Foley RN, Parfrey PS, Harnett JD, *et al.* The impact of anemia on cardiomyopathy, morbidity, and and mortality in end-stage renal disease. *Am J Kidney Dis* 1996;28:53-61.

24. Ma JZ, Ebben J, Xia H, *et al.* Hematocrit level and associated mortality in hemodialysis patients. *J Am Soc Nephrol* 1999;10:610-619.
25. Parfrey PS, Foley RN, Wittreich BH, *et al.* Double-blind comparison of full and partial anemia correction in incident hemodialysis patients without symptomatic heart disease. *J Am Soc Nephrol* 2005;16:2180-2189.
26. Collins AJ, Li S, St Peter W, *et al.* Death, hospitalization, and economic associations among incident hemodialysis patients with hematocrit values of 36 to 39%. *J Am Soc Nephrol* 2001;12:2465-2473.
27. Xue JL, St Peter WL, Ebben JP, *et al.* Anemia treatment in the pre-ESRD period and associated mortality in elderly patients. *Am J Kidney Dis* 2002;40:1153-1161.
28. Locatelli F, Pisoni RL, Combe C, *et al.* Anaemia in haemodialysis patients of five European countries: association with morbidity and mortality in the Dialysis Outcomes and Practice Patterns Study (DOPPS). *Nephrol Dial Transplant* 2004;19:121-132.
29. Regidor DL, Kopple JD, Kovesdy CP, *et al.* Associations between changes in hemoglobin and administered erythropoiesis-stimulating agent and survival in hemodialysis patients. *J Am Soc Nephrol* 2006;17:1181-1191.
30. Jones M, Ibels L, Schenkel B, *et al.* Impact of epoetin alfa on clinical end points in patients with chronic renal failure: a meta-analysis. *Kidney Int* 2004;65:757-767.
31. Goldberg N, Lundin AP, Delano B, *et al.* Changes in left ventricular size, wall thickness, and function in anemic patients treated with recombinant human erythropoietin. *Am Heart J* 1992;124:424-427.
32. Frank H, Heusser K, Hoffken B, *et al.* Effect of erythropoietin on cardiovascular prognosis parameters in hemodialysis patients. *Kidney Int* 2004;66:832-840.
33. Besarab A, Bolton WK, Browne JK, *et al.* The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. *N Engl J Med* 1998;339:584-590.
34. Drueke TB, Locatelli F, Clyne N, *et al.* Normalization of hemoglobin level in patients with chronic kidney disease and anemia. *N Engl J Med* 2006;355:2071-2084.
35. Singh AK, Szczech L, Tang KL, *et al.* Correction of anemia with epoetin alfa in chronic kidney disease. *N Engl J Med* 2006;355:2085-2098.
36. KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for Anemia in Chronic Kidney Disease. *Am J Kidney Dis* 2006;47:S11-145.
37. Locatelli F, Aljama P, Barany P, *et al.* Revised European best practice guidelines for the management of anaemia in patients with chronic renal failure. *Nephrol Dial Transplant* 2004;19 Suppl 2:ii1-47.
38. Anand I, McMurray JJ, Whitmore J, *et al.* Anemia and its relationship to clinical outcome in heart failure. *Circulation* 2004;110:149-154.
39. Go AS, Yang J, Ackerson LM, *et al.* Hemoglobin level, chronic kidney disease, and the risks of death and hospitalization in adults with chronic heart failure: the Anemia in Chronic Heart Failure: Outcomes and Resource Utilization (ANCHOR) Study. *Circulation* 2006;113:2713-2723.
40. Mancini DM, Katz SD, Lang CC, *et al.* Effect of erythropoietin on exercise capacity in patients with moderate to severe chronic heart failure. *Circulation* 2003;107:294-299.
41. Silverberg DS, Wexler D, Sheps D, *et al.* The effect of correction of mild anemia in severe, resistant congestive heart failure using subcutaneous erythropoietin and intravenous iron: a randomized controlled study. *J Am Coll Cardiol* 2001;37:1775-1780.
42. Ponikowski P, Anker SD, Szachniewicz J, *et al.* Effect of darbepoetin alfa on exercise tolerance in anemic patients with symptomatic chronic heart failure: a randomized, double-blind, placebo-controlled trial. *J Am Coll Cardiol* 2007;49:753-762.
43. Palazzuoli A, Silverberg D, Iovine F, *et al.* Erythropoietin improves anemia exercise tolerance and renal function and reduces B-type natriuretic peptide and hospitalization in patients with heart failure and anemia. *Am Heart J* 2006;152:1096 e1099-1015.
44. Ghali J, Anand I, Abraham W: Randomized, double-blind, placebo-controlled trial to assess the impact of darbepoetin alfa treatment on exercise tolerance in anemic patients with symptomatic heart failure: results from STAMINA-HeFT, in, Eur Soc of Heart Fail Congress 2006 (abstr 549, suppl 1)

45. Fisher JW, Koury S, Ducey T, *et al.* Erythropoietin production by interstitial cells of hypoxic monkey kidneys. *Br J Haematol* 1996;95:27-32.
46. Warnecke C, Zaborowska Z, Kurreck J, *et al.* Differentiating the functional role of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2alpha target gene in Hep3B and Kelly cells. *Faseb J* 2004;18:1462-1464.
47. Scortegagna M, Ding K, Zhang Q, *et al.* HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner. *Blood* 2005;105:3133-3140.
48. Imagawa S, Yamamoto M, Miura Y. Negative regulation of the erythropoietin gene expression by the GATA transcription factors. *Blood* 1997;89:1430-1439.
49. La Ferla K, Reimann C, Jelkmann W, *et al.* Inhibition of erythropoietin gene expression signaling involves the transcription factors GATA-2 and NF-kappaB. *Faseb J* 2002;16:1811-1813.
50. Dumitriu B, Patrick MR, Petschek JP, *et al.* Sox6 cell-autonomously stimulates erythroid cell survival, proliferation, and terminal maturation and is thereby an important enhancer of definitive erythropoiesis during mouse development. *Blood* 2006;108:1198-1207.
51. Fisher JW. Erythropoietin: physiology and pharmacology update. *Exp Biol Med (Maywood)* 2003;228:1-14.
52. Wickrema A, Krantz SB, Winkelmann JC, *et al.* Differentiation and erythropoietin receptor gene expression in human erythroid progenitor cells. *Blood* 1992;80:1940-1949.
53. Anagnostou A, Liu Z, Steiner M, *et al.* Erythropoietin receptor mRNA expression in human endothelial cells. *Proc Natl Acad Sci U S A* 1994;91:3974-3978.
54. Chong ZZ, Kang JQ, Maiese K. Hematopoietic factor erythropoietin fosters neuroprotection through novel signal transduction cascades. *J Cereb Blood Flow Metab* 2002;22:503-514.
55. Henry DH, Bowers P, Romano MT, *et al.* Epoetin alfa. Clinical evolution of a pleiotropic cytokine. *Arch Intern Med* 2004;164:262-276.
56. Bahlmann FH, De Groot K, Spandau JM, *et al.* Erythropoietin regulates endothelial progenitor cells. *Blood* 2004;103:921-926.
57. Bahlmann FH, DeGroot K, Duckert T, *et al.* Endothelial progenitor cell proliferation and differentiation is regulated by erythropoietin. *Kidney Int* 2003;64:1648-1652.
58. George J, Goldstein E, Abashidze A, *et al.* Erythropoietin promotes endothelial progenitor cell proliferative and adhesive properties in a PI 3-kinase-dependent manner. *Cardiovasc Res* 2005;68:299-306.
59. Beleslin-Cokic BB, Cokic VP, Yu X, *et al.* Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells. *Blood* 2004;104:2073-2080.
60. Parsa CJ, Matsumoto A, Kim J, *et al.* A novel protective effect of erythropoietin in the infarcted heart. *J Clin Invest* 2003;112:999-1007.
61. Vesey DA, Cheung C, Pat B, *et al.* Erythropoietin protects against ischaemic acute renal injury. *Nephrol Dial Transplant* 2004;19:348-355.
62. Caro J, Brown S, Miller O, *et al.* Erythropoietin levels in uremic nephric and anephric patients. *J Lab Clin Med* 1979;93:449-458.
63. Mason-Garcia M, Beckman BS, Brookins JW, *et al.* Development of a new radioimmunoassay for erythropoietin using recombinant erythropoietin. *Kidney Int* 1990;38:969-975.
64. Macdougall IC. Poor response to erythropoietin: practical guidelines on investigation and management. *Nephrol Dial Transplant* 1995;10:607-614.
65. Volpe M, Tritto C, Testa U, *et al.* Blood levels of erythropoietin in congestive heart failure and correlation with clinical, hemodynamic, and hormonal profiles. *Am J Cardiol* 1994;74:468-473.
66. Jensen JD, Eiskjaer H, Bagger JP, *et al.* Elevated level of erythropoietin in congestive heart failure relationship to renal perfusion and plasma renin. *J Intern Med* 1993;233:125-130.
67. George J, Patal S, Wexler D, *et al.* Circulating erythropoietin levels and prognosis in patients with congestive heart failure: comparison with neurohormonal and inflammatory markers. *Arch Intern Med* 2005;165:1304-1309.
68. van der Meer P, Voors AA, Lipsic E, *et al.* Prognostic value of plasma erythropoietin on mortality in patients with chronic heart failure. *J Am Coll Cardiol* 2004;44:63-67.

69. Stenvinkel P. Inflammatory and atherosclerotic interactions in the depleted uremic patient. *Blood Purif* 2001;19:53-61.
70. Bergstrom J, Lindholm B, Lacson E, Jr., *et al.* What are the causes and consequences of the chronic inflammatory state in chronic dialysis patients? *Semin Dial* 2000;13:163-175.
71. Deswal A, Petersen NJ, Feldman AM, *et al.* Cytokines and cytokine receptors in advanced heart failure: an analysis of the cytokine database from the Vesnarinone trial (VEST). *Circulation* 2001;103:2055-2059.
72. Levine B, Kalman J, Mayer L, *et al.* Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N Engl J Med* 1990;323:236-241.
73. Dutka DP, Elborn JS, Delamere F, *et al.* Tumour necrosis factor alpha in severe congestive cardiac failure. *Br Heart J* 1993;70:141-143.
74. Macdougall IC, Cooper AC. Erythropoietin resistance: the role of inflammation and pro-inflammatory cytokines. *Nephrol Dial Transplant* 2002;17 Suppl 11:39-43.
75. Means RT, Jr. Recent developments in the anemia of chronic disease. *Curr Hematol Rep* 2003;2:116-121.
76. Taniguchi S, Dai CH, Price JO, *et al.* Interferon gamma downregulates stem cell factor and erythropoietin receptors but not insulin-like growth factor-I receptors in human erythroid colony-forming cells. *Blood* 1997;90:2244-2252.
77. Jelkmann W. Proinflammatory cytokines lowering erythropoietin production. *J Interferon Cytokine Res* 1998;18:555-559.
78. Maciejewski JP, Selleri C, Sato T, *et al.* Nitric oxide suppression of human hematopoiesis in vitro. Contribution to inhibitory action of interferon-gamma and tumor necrosis factor-alpha. *J Clin Invest* 1995;96:1085-1092.
79. Donovan A, Lima CA, Pinkus JL, *et al.* The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell Metab* 2005;1:191-200.
80. Lawrence CM, Ray S, Babyonyshev M, *et al.* Crystal structure of the ectodomain of human transferrin receptor. *Science* 1999;286:779-782.
81. Nemeth E, Rivera S, Gabayan V, *et al.* IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 2004;113:1271-1276.
82. Mena NP, Esparza AL, Nunez MT. Regulation of transepithelial transport of iron by hepcidin. *Biol Res* 2006;39:191-193.
83. Dallalio G, Law E, Means RT, Jr. Hepcidin inhibits in vitro erythroid colony formation at reduced erythropoietin concentrations. *Blood* 2006;107:2702-2704.
84. Malyszko J, Malyszko JS, Hryszko T, *et al.* Is hepcidin a link between anemia, inflammation and liver function in hemodialyzed patients? *Am J Nephrol* 2005;25:586-590.
85. Eleftheriadis T, Kartsios C, Liakopoulos V, *et al.* Does hepcidin affect erythropoiesis in hemodialysis patients? *Acta Haematol* 2006;116:238-244.
86. Philo JS, Aoki KH, Arakawa T, *et al.* Dimerization of the extracellular domain of the erythropoietin (EPO) receptor by EPO: one high-affinity and one low-affinity interaction. *Biochemistry* 1996;35:1681-1691.
87. Constantinescu SN, Keren T, Socolovsky M, *et al.* Ligand-independent oligomerization of cell-surface erythropoietin receptor is mediated by the transmembrane domain. *Proc Natl Acad Sci U S A* 2001;98:4379-4384.
88. Rossert J, Eckardt KU. Erythropoietin receptors: their role beyond erythropoiesis. *Nephrol Dial Transplant* 2005;20:1025-1028.
89. Naranda T, Wong K, Kaufman RI, *et al.* Activation of erythropoietin receptor in the absence of hormone by a peptide that binds to a domain different from the hormone binding site. *Proc Natl Acad Sci U S A* 1999;96:7569-7574.
90. Wrighton NC, Farrell FX, Chang R, *et al.* Small peptides as potent mimetics of the protein hormone erythropoietin. *Science* 1996;273:458-464.
91. Livnah O, Stura EA, Johnson DL, *et al.* Functional mimicry of a protein hormone by a peptide agonist: the EPO receptor complex at 2.8 A. *Science* 1996;273:464-471.
92. Sasaki A, Yasukawa H, Shouda T, *et al.* CIS3/SOCS-3 suppresses erythropoietin (EPO) signaling by binding the EPO receptor and JAK2. *J Biol Chem* 2000;275:29338-29347.

93. Beckman DL, Lin LL, Quinones ME, *et al.* Activation of the erythropoietin receptor is not required for internalization of bound erythropoietin. *Blood* 1999;94:2667-2675.
94. Sorkin A, Waters CM. Endocytosis of growth factor receptors. *Bioessays* 1993;15:375-382.
95. Walrafen P, Verdier F, Kadri Z, *et al.* Both proteasomes and lysosomes degrade the activated erythropoietin receptor. *Blood* 2005;105:600-608.
96. Witthuhn BA, Quelle FW, Silvennoinen O, *et al.* JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell* 1993;74:227-236.
97. Klingmuller U, Lorenz U, Cantley LC, *et al.* Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell* 1995;80:729-738.
98. Akagi S, Ichikawa H, Okada T, *et al.* The critical role of SRC homology domain 2-containing tyrosine phosphatase-1 in recombinant human erythropoietin hyporesponsive anemia in chronic hemodialysis patients. *J Am Soc Nephrol* 2004;15:3215-3224.
99. Silva M, Grillot D, Benito A, *et al.* Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. *Blood* 1996;88:1576-1582.
100. Matsumoto A, Masuhara M, Mitsui K, *et al.* CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. *Blood* 1997;89:3148-3154.
101. Peltola KJ, Paukku K, Aho TL, *et al.* Pim-1 kinase inhibits STAT5-dependent transcription via its interactions with SOCS1 and SOCS3. *Blood* 2004;103:3744-3750.
102. Miura Y, Miura O, Ihle JN, *et al.* Activation of the mitogen-activated protein kinase pathway by the erythropoietin receptor. *J Biol Chem* 1994;269:29962-29969.
103. Carroll MP, Spivak JL, McMahon M, *et al.* Erythropoietin induces Raf-1 activation and Raf-1 is required for erythropoietin-mediated proliferation. *J Biol Chem* 1991;266:14964-14969.
104. Miura O, Nakamura N, Ihle JN, *et al.* Erythropoietin-dependent association of phosphatidylinositol 3-kinase with tyrosine-phosphorylated erythropoietin receptor. *J Biol Chem* 1994;269:614-620.
105. Kashii Y, Uchida M, Kirito K, *et al.* A member of Forkhead family transcription factor, FKHRL1, is one of the downstream molecules of phosphatidylinositol 3-kinase-Akt activation pathway in erythropoietin signal transduction. *Blood* 2000;96:941-949.
106. Rice L, Ruiz W, Driscoll T, *et al.* Neocytolysis on descent from altitude: a newly recognized mechanism for the control of red cell mass. *Ann Intern Med* 2001;134:652-656.
107. Trial J, Rice L. Erythropoietin withdrawal leads to the destruction of young red cells at the endothelial-macrophage interface. *Curr Pharm Des* 2004;10:183-190.
108. Rice L, Alfrey CP, Driscoll T, *et al.* Neocytolysis contributes to the anemia of renal disease. *Am J Kidney Dis* 1999;33:59-62.

CHAPTER 3

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

Karien van der Putten[1]
Kim E Jie[2]
Mireille E Emans[3]
Marianne C Verhaar[4]
Jaap A Joles[2]
Maarten Jan M Cramer[3]
Birgitta K Velthuis[5]
Louis Meiss[6]
Rob J Kraaijenhagen[7]
Pieter AFM Doevendans[3,8]
Branko Braam[2,9]
Carlo AJM Gaillard[1,10]

[1]Department of Internal Medicine, Meander MC, Amersfoort, The Netherlands
[2]Department of Nephrology and Hypertension, UMC Utrecht, Utrecht ,The Netherlands
[3]Department of Cardiology, UMC Utrecht, Utrecht, The Netherlands
[4]Department of Vascular Medicine, UMC Utrecht, Utrecht, The Netherlands
[5]Department of Radiology, UMC Utrecht, Utrecht ,The Netherlands
[6]Department of Radiology, Meander MC , Amersfoort, The Netherlands
[7]Department of Clinical Chemistry, Meander MC, Amersfoort, The Netherlands
[8]Interuniversity Cardiology Institute Netherlands, Utrecht, The Netherlands
[9]Division of Nephrology and Immunology, University of Alberta, Edmonton, Canada
[10]Department of Nephrology, VU University Medical Center, Amsterdam, The Netherlands

J Nephrol 2010;23:363-368

ABSTRACT

Background

Anemia 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 hematopoietic or nonhematopoietic 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 anemia (hemoglobin 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 hemoglobin 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 hemoglobin 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 hematopoietic from nonhematopoietic 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 (Trial registration: ClinicalTrials.gov identifier NCT00356733).

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 hemoglobin (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 anemia may improve outcome. In addition to well-documented hematopoietic 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 nonhematopoietic 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 hematopoietic as well as nonhematopoietic 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 anemia. Complete inclusion and exclusion criteria are outlined in Table 1. The etiology 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 suppletion, 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.

Table 1 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)
Hemoglobinopathies, bleeding or hemolysis as a cause of anemia
Deficiency of iron, folate and/or vitamin B12
Chronic inflammatory diasease or clinically significant infection
Hematological 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, hemoglobin; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycosylated hemoglobin; PTH, parathyroid hormone

Differentiation between hematopoietic effects and nonhematopoietic 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, nonhematopoietic 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 hematopoietic and nonhematopoietic effects of EPO.

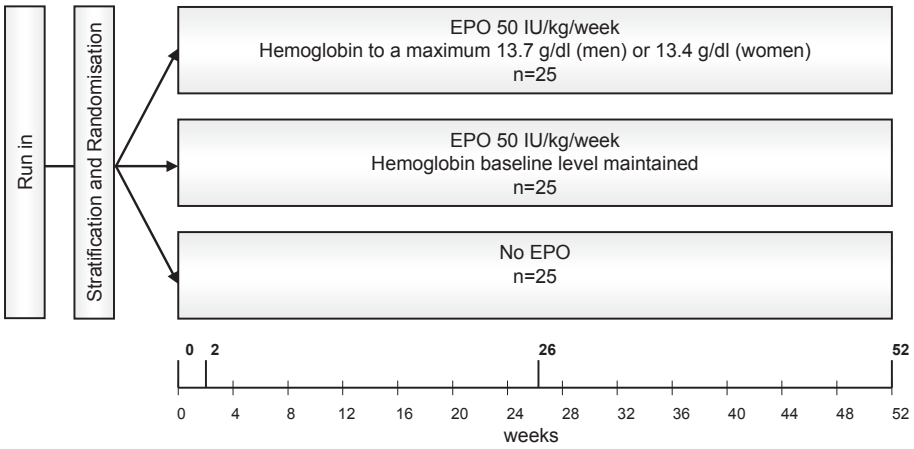

Figure 1 Study design and main time points of measurements during the study period.
Abbreviation: EPO, recombinant human erythropoietin

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 reduced left ventricular ejection fraction (LVEF) (HFREF) or heart failure with normal LVEF (HFNEF) will be included. HFNEF 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 (VO^2max). CKD is defined as estimated creatinine clearance (Cockcroft-Gault formula) 20-70 ml/min. Albuminuria is assessed by 24-hour urinary collection.

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+ hematopoietic 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 anemia treatment with EPO in patients with CKD and/or CHF is under debate. It is uncertain to what extent the hematopoietic or nonhematopoietic effects of EPO are responsible for the unexpected outcomes of recent studies.[6,7] The EPOCARES study is specifically designed to assess both the hematopoietic and nonhematopoietic 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 anemia 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 design that justifies discussion of potential. 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 anemia 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,[9] 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 anemia. 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 etiology 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 hematopoietic and nonhematopoietic 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.

REFERENCES

1. Bongartz LG, Cramer MJ, Doevendans PA, *et al.* The severe cardiorenal syndrome: 'Guyton revisited'. *Eur Heart J* 2005;26:11-17.
2. Foley RN, Parfrey PS, Harnett JD, *et al.* The impact of anemia on cardiomyopathy, morbidity, and and mortality in end-stage renal disease. *Am J Kidney Dis* 1996;28:53-61.
3. Regidor DL, Kopple JD, Kovesdy CP, *et al.* Associations between changes in hemoglobin and administered erythropoiesis-stimulating agent and survival in hemodialysis patients. *J Am Soc Nephrol* 2006;17:1181-1191.
4. de Groot K, Bahlmann FH, Sowa J, *et al.* Uremia causes endothelial progenitor cell deficiency. *Kidney Int* 2004;66:641-646.
5. Valgimigli M, Rigolin GM, Fucili A, *et al.* CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. *Circulation* 2004;110:1209-1212.
6. Drueke TB, Locatelli F, Clyne N, *et al.* Normalization of hemoglobin level in patients with chronic kidney disease and anemia. *N Engl J Med* 2006;355:2071-2084.
7. Singh AK, Szczech L, Tang KL, *et al.* Correction of anemia with epoetin alfa in chronic kidney disease. *N Engl J Med* 2006;355:2085-2098.
8. van der Putten K, Braam B, Jie KE, *et al.* Mechanisms of Disease: erythropoietin resistance in patients with both heart and kidney failure. *Nat Clin Pract Nephrol* 2008;4:47-57.
9. Szczech LA, Barnhart HX, Inrig JK, *et al.* Secondary analysis of the CHOIR trial epoetin-alpha dose and achieved hemoglobin outcomes. *Kidney Int* 2008;74:791-798.

CHAPTER 4

Hepcidin-25 is a marker of the response rather than resistance to exogenous erythropoietin in chronic kidney disease/chronic heart failure patients

Karien van der Putten[1,2]
Kim E Jie[3]
Daan van den Broek[4]
Rob J Kraaijenhagen[5]
Coby Laarakkers[6]
Dorine W Swinkels[6]
Branko Braam[7]
Carlo A Gaillard[1,8]

[1]Department of Internal Medicine, Meander MC, Amersfoort, The Netherlands
[2]Department of Cardiology, UMC Utrecht, Utrecht, The Netherlands
[3]Department of Nephrology and Hypertension, UMC Utrecht, Utrecht, The Netherlands
[4]Department of Clinical Chemistry, Lange Land Hospital, Zoetermeer, The Netherlands
[5]Department of Clinical Chemistry, Meander MC, Amersfoort, The Netherlands
[6]Department of Clinical Chemistry, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[7]Division of Nephrology and Immunology, Department of Medicine, University of Alberta, Edmonton, Canada
[8]Department of Nephrology, VU University Medical Center, Amsterdam, The Netherlands

Eur J Heart Fail 2010;12:943-950

ABSTRACT

Aims

Erythropoietin (EPO) resistance, an important cause of anemia in patients with heart and renal failure, is associated with increased mortality. The hypothesis of the present study was that exogenous EPO decreases hepcidin levels and that the decrease in hepcidin levels upon EPO treatment is related to the bone marrow response.

Methods

In the EPOCARES trial, patients with renal failure (glomerular filtration rate 20-70 mL/min), heart failure, and anemia were randomized to receive 50 IU/kg/week EPO (n=20) or not (n=13). Hemoglobin (Hb), hepcidin-25, ferritin, reticulocytes, serum transferrin receptor (sTfR), IL-6, and high-sensitivity C-reactive protein were measured at baseline and during treatment. Hepcidin-25 was measured by weak cation exchange chromatography/matrix assisted laser desorption ionization time-of-flight mass spectrometry.

Results

Baseline hepcidin levels were increased compared with a healthy reference population and were inversely correlated with Hb (r^2=0.18, P=0.02), and positively with ferritin (r^2=0.51, P< 0.001), but not with renal function, high-sensitivity C-reactive protein or IL-6. Erythropoietin treatment increased reticulocytes (P<0.001) and sTfR (P<0.001), and decreased hepcidin (P<0.001). Baseline hepcidin levels and the magnitude of the decrease in hepcidin correlated with the increase in reticulocytes (r^2=0.23, P=0.03) and sTfR (r^2=0.23, P=0.03) and also with the Hb response after 6 months (r^2=0.49, P=0.001).

Conclusion

In this group of patients with combined heart and renal failure and anemia, increased hepcidin levels were associated with markers of iron load and not with markers of inflammation. The (change in) hepcidin levels predicted early and long-term bone marrow response to exogenous EPO. In our group hepcidin seems to reflect iron load and response to EPO rather than inflammation and EPO resistance.

INTRODUCTION

Erythropoietin (EPO) resistance, defined as an inadequate bone marrow response to exogenous or endogenous EPO, contributes importantly to anemia[1] and is associated with increased mortality, particularly in patients with heart and/or kidney failure.[2-4] Hepcidin is a protein closely related to iron homeostasis, and is an acute-phase protein mainly produced in the liver. It has been suggested to play a central role in the development of EPO resistance.[5]

Enhancement of erythropoiesis by EPO requires intact EPO signaling (EPO receptor, downstream JAK/STAT signaling and transcriptional response) and effective mobilization of iron stores.[1] Hepcidin inhibits the efflux of iron into plasma transferrin by downregulating ferroportin, the efflux channel for iron in macrophages and in enterocytes.[6] Enhanced synthesis of hepcidin thus leads to inhibition of iron absorption in the small intestine and sequestering of iron in macrophages, resulting in limited iron availability for erythropoiesis. In addition to its effect on iron metabolism, hepcidin may contribute to EPO resistance through a direct inhibitory effect on erythroid progenitor proliferation and survival.[7]

In patients with chronic kidney disease (CKD) and in hemodialysis patients, it has been shown that hepcidin levels are higher than in healthy controls.[8-10] No information on hepcidin levels in patients with chronic heart failure (CHF) is available, with the exception of one study measuring levels of prohepcidin.[11] However, prohepcidin levels do not correlate with urinary and serum hepcidin, nor do they respond to relevant physiological stimuli.[5]

Increased iron stores and inflammation induce hepcidin synthesis in the liver, whereas suppression occurs during hypoxia, iron deficiency, and increased erythropoiesis.[12] Hepcidin thus integrates the input from both inflammatory and erythropoietic pathways.

In vitro, it has been demonstrated that EPO decreases hepcidin expression in hepatocytes.[13] In addition, Ashby et al.[14] showed that exogenous EPO decreases hepcidin levels in hemodialysis patients, using an immunoassay which measures total hepcidin. In the present study, we measured hepcidin-25 levels in CKD patients, using a mass spectrometry based assay. Unlike immunoassays, this assay does not measure total hepcidin, but quantifies hepcidin-25 and hepcidin-20 separately.[15]

We hypothesized that EPO, which is the primary signal that triggers erythropoiesis, decreases hepcidin-25 levels in patients with combined heart and renal failure. We tested whether, as in CKD patients, hepcidin levels are elevated in iron-replete, EPO naïve patients with heart and renal failure and if EPO treatment modulates hepcidin levels. Furthermore, we studied whether baseline hepcidin levels determine the bone marrow response to EPO and if a change in hepcidin levels upon EPO treatment predicts EPO responsiveness.

METHODS

Patients

In an open-label, randomized trial, 33 patients with the combination of CHF, CKD, and anemia were included. Recently a classification of patients with both heart and renal failure was proposed by Ronco et al.[16] Using this classification, we studied patient with cardiorenal syndrome (CRS) Type 2 and 4. The current study is part of the EPOCARES trial of which a detailed protocol description is available (ClinicalTrials.gov NCT 00356733).[17] Ethics approval was obtained and all subjects signed informed consent. All procedures were in accordance with the Helsinki Declaration. Complete inclusion and exclusion criteria are outlined in Table 1. Anemia was defined as hemoglobin (Hb) between 10.3 and 12.6 g/dL in men and between 10.3 and 11.9 g/dL in women, measured after 4 weeks of oral iron therapy. Chronic kidney disease was defined as a glomerular filtration rate (GFR) of 20–70 mL/min according to the Cockcroft–Gault equation. Chronic heart failure was defined as NYHA class II or higher, based on symptoms, signs, and objective evidence of an abnormality in cardiac structure or function according to echocardiography.[18,19] Patients with heart failure with reduced ejection fraction (HFREF) or heart failure with normal EF (HFNEF) were included. Heart failure with normal EF was defined as an EF>50%, left ventricular (LV) end-diastolic volume index <97 mL/m^2, and evidence of diastolic LV dysfunction.[20]

Table 1 Inclusion and exclusion criteria for the EPOCARES study

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 (HFNEF and/or HFREF)
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)
Hemoglobinopathies, bleeding or hemolysis as a cause of anemia
Deficiency of iron, folate and/or vitamin B12
Chronic inflammatory disease or clinically significant infection
Hematological malignancy of solid tumor < 3 years ago

Abbreviations: GFR, glomerular filtration rate; HFNEF, heart failure with normal ejection fraction; HFREF, heart failure with reduced ejection fraction; Hb, hemoglobin; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycosylated hemoglobin; PTH, parathyroid hormone

Study design

After written consent was obtained, run-in treatment was started, comprising of oral iron supplementation, aspirin when indicated, and maximal tolerated dosages of a ß-blocker, an ACE-inhibitor or an angiotensin receptor blocker. Patients were randomized once they had been clinically stable on standard treatment for at least 4 weeks. At this point, patients were randomized on a 2:1 basis to receive 50 IU/kg/week Epoetin beta (Neorecormon, Roche Pharmaceuticals) (n=20) or not (n=13). Measurements were performed at baseline and after 2 weeks of EPO therapy. This interval of 2 weeks was specifically chosen, as the Hb level does not increase until about 4 weeks after starting erythropoiesis-stimulating agent treatment. In addition, Hb measurements were repeated monthly for a period of 6 months.

Measurements

All blood samples were drawn between 8 and 9 a.m. Serum urea nitrogen, creatinine, and Hb were assessed using routine methods. High sensitivity C-reactive protein was determined by particle-enhanced immunonephelometry using a standard Cardio-Phase high-sensitivity C-reactive protein for BNII (Dade Behring Holding GmbH, Liederbach, Germany). Plasma IL-6 levels (pg/mL) were measured in duplicate using a commercially available high-sensitive ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Ferritin was determined using a sandwich immunoassay on an Acces 2 immunoanalyser within a Dx automated system from Beckman Coulter (Brea, CA, USA). Transferrin saturation (TSAT) was calculated from serum iron and transferrin estimates obtained with standard methods on a Beckman Coulter Dx. Serum Transferrin Receptor (sTfR) assay was performed with an immunoassay on a BNProSpec nephelometer from Siemens (Marburg, Germany). Reticulocyte count and RetHe (reticulocyte Hb equivalent) were performed using flow cytometric analysis with Ret-Search (II) dye on a Sysmex XE-2100 haematology analyser (Toa Medical, Kobe, Japan). NT-proBNP was measured using an electrochemiluminescence immunoassay on a Cobas CA6000 from Roche (Mannheim, Germany). Levels of Cystatin-C were measured by means of the N-Latex Cystatin-C assay and a PROspec nephelometer (Siemens Healthcare Diagnostics, Breda, the Netherlands). Serum EPO levels were measured by a two-site sandwich chemiluminescent immunoassay on an IMMULITE 2000 platform (Siemens Healthcare Diagnostics, Breda, the Netherlands). To define EPO levels as appropriate or inappropriate for a given degree of anemia, we calculated the observed/ predicted log (EPO) ratio (O/P ratio). Erythropoietin levels were defined as inappropriate at an O/P ratio<0.80. The O/P ratio was 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.[21]: log(Epo)=4.746-(0.275×Hb).

Serum hepcidin-25 measurements were performed by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (TOF MS). An internal standard (synthetic hepcidin-24; Peptide International Inc.) was used for quantification.[15] 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 of this method was 0.5 nM; average

coefficients of variation (CV) were 2.7% (intra-run) and 6.5% (inter-run). The median reference level of serum hepcidin-25 as measured at 08.30 a.m. is 2.9 nM, range 0.5–8.2 nM.[22]

Definition of hemoglobin response

In the EPOCARES trial the EPO patients were divided into two groups.[17] Of the 20 patients in the current study who received EPO, 11 patients received EPO to a target Hb level of 13.7 g/dL for men and 13.4 g/dL for women, while the remaining nine patients received EPO to maintain baseline Hb for the first 6 months by sequential blood withdrawal of 250 mL, with a maximum frequency of once every 2 weeks. For assessment of Hb response, the difference in Hb level after 6 months was assessed and subsequently increased by adding 0.24 g/dL for each phlebotomy.

Statistical analysis

The study is part of the EPOCARES study. The EPOCARES study is not powered for hard endpoints, but for intermediate endpoints as measured by biomarkers, imaging data, and cell studies. Power analysis of several biomarkers 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. Data are expressed as means ± SD or median with interquartile range as appropriate. Unpaired data were compared using the Fishers' Exact test (for categorical variables), and the unpaired t-test or Mann-Whitney U test (for continuous variables). Paired data were compared with the use of the paired t-test or Wilcoxon rank sum test. Correlations were calculated using Pearson's correlation test. Skewed data were log-transformed before correlations were calculated. P-values<0.05 were considered to represent statistical significance. The Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) version 17 was employed for all statistical analysis.

RESULTS

Overall characteristics of the patients

Baseline characteristics of the EPO treated and non-EPO treated patients are displayed in Table 2. The two groups were not different for age, renal function, left ventricular ejection fraction (LVEF), and all other measured parameters. Chronic kidney disease is evident from the decreased GFR, heart failure from elevated brain natriuretic peptide levels and decreased LVEF. The average O/P (observed/predicted, see methods section) ratio was just below 0.80 in both groups, indicating that endogenous EPO production was partly preserved. As patients with overt inflammation were excluded, high-sensitivity C-reactive protein levels are only slightly increased.

Table 2 Baseline characteristics

Characteristic	EPO (n=20)	No EPO (n=13)	P-value
Age (years)			
Median	77	72	0.34
Interquartile range	70-81	64-67	
Male sex, no. (%)	12 (60)	9 (69)	0.72
Diabetes, no. (%)	7 (35)	4 (31)	1.00
Hemoglobin (g/dl)	11.6 ± 1.0	11.9 ± 0.7	0.32
GFR (ml/min)	35 ± 12	39 ± 22	0.44
Plasma creatinine (mg/dl)	2.14 ± 0.90	2.30 ± 0.99	0.64
Urea nitrogen (mg/dl)	45 ± 21	38 ± 17	0.34
Cystatin C (mg/l)	2.22 ± 0.96	2.10 ± 0.69	0.70
NT-proBNP (pg/ml)			
Median	1832	1239	0.25
Interquartile range	684-3959	501-2207	
LVEF (%)	43 ± 14	46 ± 13	0.52
hs-CRP (mg/l)			
Median	5	4	0.69
Interquartile range	1-19	1-10	
O/P ratio	0.72 ± 0.19	0.79 ± 0.24	0.42

Means ± SD are shown, unless otherwise stated. P-values were calculated with the use of the Fishers' Exact test (for categorical variables), and the unpaired t-test or Mann-Whitney U test (for continuous variables). To convert the values for urea nitrogen to micromoles per liter, multiply by 0.357. To convert the values for creatinine to micromoles per liter, multiply by 88.4.

Abbreviations: GFR, glomerular filtration rate; NT-ProBNP, N-terminal prohormone brain natriuretic peptide; LVEF, left ventricular ejection fraction; hs-CRP, high-sensitivity C-reactive protein; O/P ratio, observed/ predicted ratio.

Baseline hepcidin levels

With a median level of 6.3 nM (interquartile range 3.9-8.6), hepcidin levels of the total study population at baseline were increased when compared with levels in a healthy reference population (median level of 2.9 nM).[22] At baseline, hepcidin levels were inversely correlated with Hb (r^2=0.18, P=0.02; Figure 1A). Furthermore, hepcidin levels at baseline were positively correlated with ferritin (r^2=0.51, P<0.001; Figure 1B) and TSAT (r^2=0.14, P=0.03; Figure 1C). There was no significant association between hepcidin and GFR (r^2=0.01, P=0.64), Cystatin-C (r^2=0.03, P=0.25), high-sensitivity C-reactive protein (r^2=0.00, P=0.90), IL-6 (r^2=0.01, P=0.65), O/P ratio (r^2=0.03, P=0.38), reticulocytes (r^2=0.03, P=0.38), sTfR (r^2=0.12, P=0.05), or RetHe (r^2=0.00, P=0.84).

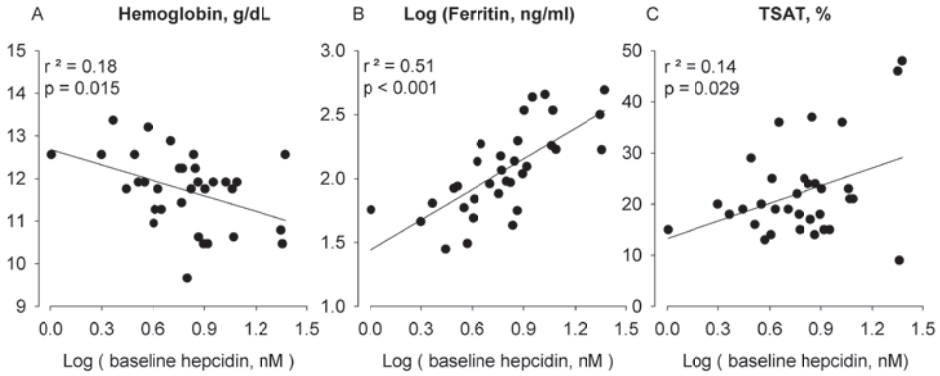

Figure 1 The correlation between log-transformed baseline hepcidin levels and baseline levels of hemoglobin, log-transformed ferritin, and TSAT in 33 cardiorenal patients. Baseline levels of hepcidin are correlated to baseline levels of hemoglobin, ferritin, and TSAT (A, B, and C).

Abbreviations: Hb, hemoglobin; TSAT, transferrin saturation.

Response to erythropoietin treatment

In the EPOCARES study patients were stratified on the basis of erythropoietin sensitivity as measured by the O/P ratio.[21] The actual erythropoietic response in the EPO treated patients was measured in two ways. The increase in reticulocyte level at 2 weeks was designated 'early response of the bone marrow' to EPO whereas Hb levels after 6 months were used to estimate the 'long-term effect' of EPO on the bone marrow. Although factors other than the response of the bone marrow to EPO may play a role in the Hb response at 6 months, none were identified. For instance, no clinical evidence of blood loss was observed in our patients. Furthermore it should be pointed out that in the EPOCARES study the EPO treated patients were divided in two groups.[17] One group was targeted to maintain their baseline Hb level, despite EPO treatment, and underwent periodic withdrawal of 250 mL of blood at a maximal frequency of 2 weeks. In these patients, the Hb response to EPO was imputed by adding 0.24 g/dL to each phlebotomy.

Acute response to erythropoietin treatment

Table 3 shows the effect of 2 weeks of EPO treatment vs. no EPO treatment on all variables. Two weeks of EPO treatment increased reticulocytes ($P<0.001$; Figure 2B) and sTfR ($P<0.001$), but decreased hepcidin levels ($P<0.001$; Figure 2A). In addition, TSAT ($P=0.01$) and ferritin levels ($P=0.02$) decreased in response to EPO treatment. In the EPO treated patients, baseline levels of hepcidin correlated with the increase in reticulocytes ($r^2=0.35$, $P=0.006$; Figure 3A). The magnitude of the decrease in hepcidin correlated with the increase in reticulocytes ($r^2=0.23$, $P=0.03$; Figure 3B) and the increase in sTfR ($r^2=0.23$, $P=0.03$). The magnitude of decrease in hepcidin was not correlated to baseline levels of IL-6 ($r^2<0.01$, $P=0.82$), high-sensitivity C-reactive protein ($r^2<0.01$, $P=0.86$), or TSAT ($r^2=0.02$, $P=0.54$). The magnitude of decrease in hepcidin did correlate to baseline ferritin levels ($r^2= 0.34$, $P=0.008$). There was no correlation between O/P ratio at baseline and increase in reticulocyte count ($r^2<0.01$, $P=0.99$) or sTfR ($r^2=0.07$, $P=0.25$).

Table 3 Effects of 2 weeks erythropoietin vs. no erythropoietin treatment

Variable	EPO (n=20)			No EPO (n=13)		
	t=0	t=2 weeks	P-value	t=0	t=2 weeks	P-value
Hb(g/dl)	11.6±1.0	11.9±1.2	0.07	11.9±0.7	11.7±0.9	0.28
Reticulocytes ($x10^{12}$/l)	0.045±0.015	0.069±0.019	<0.001	0.043±0.014	0.044±0.016	0.78
sTfR (mg/l)	1.4±0.5	1.8±0.5	<0.001	1.3±0.4	1.4±0.4	0.13
RetHe (fmol)	1.84±0.13	1.76±0.26	0.22	1.87±0.11	1.82±0.14	0.28
Hepcidin (nM)	9.2±6.6	4.7±4.2	<0.001	5.1±2.3	7.0±6.3	0.35
Erythropoietin (IU/l)	12±7	34±20	<0.001	10±6	12±6	0.30
Ferritin (ng/ml)	166±133	141±125	0.02	136±122	157±154	0.12
TSAT (%)	22±10	18±6	0.01	22±8	22±5	0.86
IL-6 (pg/ml)	4.5±3.6	4.5±3.2	0.14	5.0±3.5	4.4±3.1	0.92

Means±SD are shown. P-values were calculated with the use of the paired t-test or Wilcoxon rank sum test. Abbreviations: Hb, hemoglobin; sTfR, serum transferrin receptor; RetHe, reticulocyte Hb equivalent; TSAT, transferrin saturation; IL-6, interleukin-6.

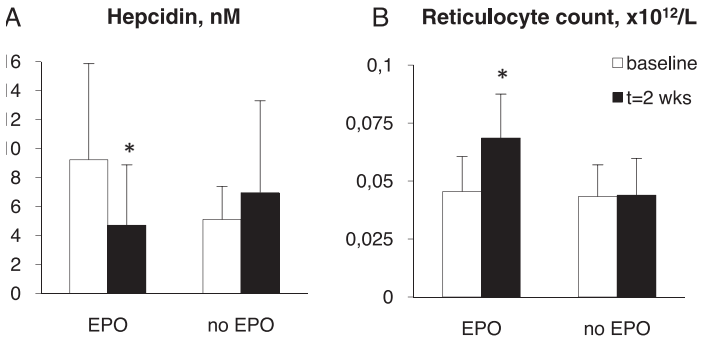

Figure 2 Hepcidin levels and reticulocytes after two weeks of EPO treatment. (A) Two weeks of erythropoietin treatment decreased log transformed hepcidin levels in cardiorenal patients. (B) The effect of 2 weeks of erythropoietin treatment on reticulocyte count. Error bars represent SD; * denotes P< 0.001.

Hemoglobin response after 6 months

Age, renal function, baseline Hb, and baseline ferritin were not associated with response to EPO. Also, baseline levels of hepcidin did not correlate with Hb response after 6 months of EPO treatment (r^2=0.07, P=0.27). However, the magnitude of the decrease in hepcidin after 2 weeks of EPO correlated with Hb response after 6 months (r^2=0.49, P=0.001; Figure 4). As expected, after exclusion of the five patients who underwent an EPO dose decrease (as necessitated by the safety procedures described in the EPOCARES protocol) during the first 6 months, this correlation remained highly significant (r^2=0.45, P=0.012). The magnitude of the increase in reticulocytes after 2 weeks of EPO also correlated with Hb response after 6 months (r^2=0.21, P=0.046). There was no correlation between O/P ratio at baseline and Hb response after 6 months (r^2=0.12, P=0.15).

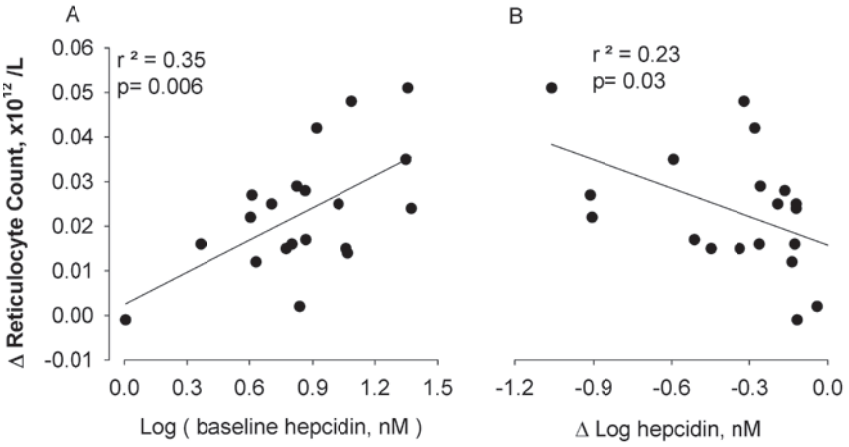

Figure 3 Correlations between hepcidin and reticulocyte count. (A) The correlation between log-transformed baseline hepcidin levels and reticulocyte increase after 2 weeks of erythropoietin treatment in 20 cardiorenal patients. (B) The correlation between log-transformed hepcidin decrease and reticulocyte increase in response to 2 weeks of erythropoietin treatment.

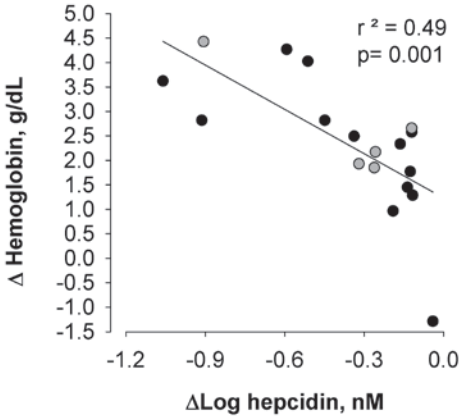

Figure 4 The correlation between the log-transformed hepcidin decrease after 2 weeks of erythropoietin and hemoglobin response after 6 months of erythropoietin treatment. In five patients the erythropoietin dose was decreased during the 6-month period. These patients are marked separately in grey.

DISCUSSION

In the present study, hepcidin levels were increased in patients with cardiorenal failure and higher baseline hepcidin levels were associated with an increased rather than decreased early bone marrow response, as determined by the increase in reticulocyte level, to EPO. A stronger decrease in hepcidin after 2 weeks of EPO treatment was correlated with an increased early bone marrow response to EPO and with Hb response after 6 months. In our study involving patients with combined heart and renal failure, hepcidin levels appeared to reflect systemic iron load rather than inflammation. Importantly, when EPO is administered, hepcidin levels predict short- and long-term bone marrow responses in patients with cardiorenal failure at an early stage of treatment.

Several putative mechanisms may lead to increased hepcidin levels, such as decreased renal clearance of hepcidin.[9] In addition, the inflammatory state that accompanies CRS may increase hepcidin. However, we were not able to demonstrate a correlation between hepcidin levels and high-sensitivity C-reactive protein or IL-6, which suggests that in our patient group increased hepcidin levels were attributable to other factors. Hepcidin levels correlated with Hb, ferritin, and TSAT, which is in accordance with hepcidin being regulated by erythropoietic activity, iron stores, and iron availability. This finding is in keeping with the contention that hepcidin levels reflect systemic iron load rather than inflammatory state or resistance to erythropoietin.[23,24] Indeed our, iron-replete, patient group had relatively low levels of inflammatory biomarkers, compatible with a relatively low inflammatory state. In contrast to our original hypothesis, low levels of hepcidin were associated with a decreased response to EPO in our patient group. The question remains whether, in accordance with the results of the DRIVE study group, additional (i.v.) iron therapy would have improved the response to EPO specifically in this low-hepcidin group, or whether another abnormality in the EPO-iron homeostasis axis explains the relative absence of a bone marrow response after exogenous EPO administration.[25] This may be of importance since a recent study reported favourable results of i.v. iron therapy in iron deplete heart failure patients.[26]

The finding that EPO treatment decreases serum hepcidin levels in patients with CRS is in accordance with the fact that increased erythropoiesis leads to suppression of hepcidin.[12] Pathways via which EPO treatment modulates hepcidin levels are largely unknown. Pinto et al.[13] suggested that it concerns a direct effect, mediated via a decrease of CEBPA binding to the hepcidin promoter after EPO supplementation. Alternatively, EPO administration may indirectly lead to suppression of hepcidin via increased levels of growth differentiation factor-15, which is secreted by erythroblasts[27] or via the molecule named twisted gastrulation (TWSG1). TWSG1 is expressed during erythropoiesis and acts by inhibiting bone morphogenic protein-induced expression of hepcidin.[28] Although we studied a relatively small and heterogeneous patient population, our results with respect to the hepcidin decrease in response to EPO are unambiguous. It seems that the signaling pathway that connects EPO with hepcidin is not markedly influenced by the complex systemic disturbances seen in patients with combined heart and kidney failure.

Our most important observation was that higher baseline hepcidin levels as well as a greater decrease in hepcidin upon EPO treatment are associated with an increased early bone marrow response to EPO. Moreover, a more pronounced decrease in hepcidin after 2 weeks of EPO treatment was associated with increased Hb response after 6 months. A large proportion of the patients who received EPO underwent periodical withdrawal of 250 mL of blood at a maximal frequency of every 2 weeks. The Hb response to EPO was imputed in this phlebotomy group using a conservative correction factor. The correction was necessary due to the design of the EPOCARES study.[17] It is important to note that the erythropoietic response to EPO was not only measured as the Hb response after 6 months but also as the increase in reticulocytes after 2 weeks of EPO treatment and that the magnitude of the decrease in hepcidin correlated both with the increase in reticulocytes (r^2=0.23, P=0.03; Figure 3B) as well as with Hb response after 6 months (r^2=0.49, P=0.001).

The observation that exogenous EPO can depress hepcidin and induce the bone marrow response to EPO supports the idea that EPO signaling pathways are intact in the CRS. The association between higher baseline hepcidin levels and a better response to EPO may seem paradoxical; however, it probably reflects an appropriate response to exogenous EPO in the face of an adequate systemic iron load. It is possible that patients without increased hepcidin levels who are less responsive to EPO form a group that may benefit from additional (i.v.) iron therapy.[25] At this point it is important to note that our group was stable, carefully selected and in a low inflammatory state. The question remains whether under different, high inflammatory conditions, hepcidin levels still reflect iron load rather than inflammatory state. Further studies should be directed towards discriminating the EPO response in patients with low-hepcidin levels vs. patients with high levels, the latter with and without elevated markers of systemic inflammation.

Our study has several potential clinical implications. First, our study suggests that hepcidin levels together with inflammatory markers can help to predict EPO response in patients with cardiorenal failure at an early stage of treatment. This is of importance, as high doses of EPO can have several non-hematopoietic adverse effects, for example increased thrombogenicity[29] and possibly also increased carcinogenicity.[30] Secondly, as iron availability is difficult to assess, hepcidin may have advantages beyond measurement of conventional measures of iron load. For this purpose, cheaper and high-throughput methods to reliably measure hepcidin are needed. A recently developed quantitative RadioImmonoAssay (RIA) might prove useful.[8,31]

In conclusion, our study shows that in EPO-naive, iron-replete patients with cardiorenal failure, EPO decreased hepcidin levels. Hepcidin levels were associated with markers of iron load and not with inflammatory markers, which suggests that in our patient group hepcidin reflects iron load. A greater decrease in hepcidin was associated with an increased early and long-term bone marrow response to EPO which suggests that the signaling pathway that connects EPO with hepcidin is operative and that hepcidin may be a marker of response rather than resistance to exogenous EPO.

REFERENCES

1. van der Putten K, Braam B, Jie KE, *et al.* Mechanisms of Disease: erythropoietin resistance in patients with both heart and kidney failure. *Nat Clin Pract Nephrol* 2008;4:47-57.
2. Zhang Y, Thamer M, Stefanik K, *et al.* Epoetin requirements predict mortality in hemodialysis patients. *Am J Kidney Dis* 2004;44:866-876.
3. Regidor DL, Kopple JD, Kovesdy CP, *et al.* Associations between changes in hemoglobin and administered erythropoiesis-stimulating agent and survival in hemodialysis patients. *J Am Soc Nephrol* 2006;17:1181-1191.
4. Szczech LA, Barnhart HX, Inrig JK, *et al.* Secondary analysis of the CHOIR trial epoetin-alpha dose and achieved hemoglobin outcomes. *Kidney Int* 2008;74:791-798.
5. Swinkels DW, Wetzels JF. Hepcidin: a new tool in the management of anaemia in patients with chronic kidney disease? *Nephrol Dial Transplant* 2008;23:2450-2453.
6. Nemeth E, Rivera S, Gabayan V, *et al.* IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 2004;113:1271-1276.
7. Dallalio G, Law E, Means RT, Jr. Hepcidin inhibits in vitro erythroid colony formation at reduced erythropoietin concentrations. *Blood* 2006;107:2702-2704.
8. Ganz T, Olbina G, Girelli D, *et al.* Immunoassay for human serum hepcidin. *Blood* 2008;112:4292-4297.
9. Tomosugi N, Kawabata H, Wakatabe R, *et al.* Detection of serum hepcidin in renal failure and inflammation by using ProteinChip System. *Blood* 2006;108:1381-1387.
10. Zaritsky J, Young B, Wang HJ, *et al.* Hepcidin--A Potential Novel Biomarker for Iron Status in Chronic Kidney Disease. *Clin J Am Soc Nephrol* 2009.
11. Adlbrecht C, Kommata S, Hulsmann M, *et al.* Chronic heart failure leads to an expanded plasma volume and pseudoanaemia, but does not lead to a reduction in the body's red cell volume. *Eur Heart J* 2008;29:2343-2350.
12. Kemna EH, Tjalsma H, Willems HL, *et al.* Hepcidin: from discovery to differential diagnosis. *Haematologica* 2008;93:90-97.
13. Pinto JP, Ribeiro S, Pontes H, *et al.* Erythropoietin mediates hepcidin expression in hepatocytes through EPOR signaling and regulation of C/EBPalpha. *Blood* 2008;111:5727-5733.
14. Ashby DR, Gale DP, Busbridge M, *et al.* Plasma hepcidin levels are elevated but responsive to erythropoietin therapy in renal disease. *Kidney Int* 2009;75:976-981.
15. Swinkels DW, Girelli D, Laarakkers C, *et al.* Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. *PLoS ONE* 2008;3:e2706.
16. Ronco C, Haapio M, House AA, *et al.* Cardiorenal syndrome. *J Am Coll Cardiol* 2008;52:1527-1539.
17. van der Putten K, Jie KE, Emans ME, *et al.* Erythropoietin treatment in patients with combined heart and renal failure: objectives and design of the EPOCARES study. *J Nephrol* 23:363-368.
18. Dickstein K, Cohen-Solal A, Filippatos G, *et al.* ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the diagnosis and treatment of acute and chronic heart failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). *Eur J Heart Fail* 2008;10:933-989.
19. Hunt SA, Abraham WT, Chin MH, *et al.* ACC/AHA 2005 Guideline Update for the Diagnosis and Management of Chronic Heart Failure in the Adult: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure): developed in collaboration with the American College of Chest Physicians and the International Society for Heart and Lung Transplantation: endorsed by the Heart Rhythm Society. *Circulation* 2005;112:e154-235.
20. Paulus WJ, Tschope C, Sanderson JE, *et al.* How to diagnose diastolic heart failure: a consensus statement on the diagnosis of heart failure with normal left ventricular ejection fraction by the Heart Failure and Echocardiography Associations of the European Society of Cardiology. *Eur Heart J* 2007;28:2539-2550.
21. Opasich C, Cazzola M, Scelsi L, *et al.* Blunted erythropoietin production and defective iron supply for erythropoiesis as major causes of anaemia in patients with chronic heart failure. *Eur Heart J* 2005;26:2232-2237.

22. Kroot JJ, Hendriks JC, Laarakkers CM, *et al.* (Pre)analytical imprecision, between-subject variability, and daily variations in serum and urine hepcidin: implications for clinical studies. *Anal Biochem* 2009;389:124-129.
23. Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med* 2005;352:1011-1023.
24. Kato A, Tsuji T, Luo J, *et al.* Association of prohepcidin and hepcidin-25 with erythropoietin response and ferritin in hemodialysis patients. *Am J Nephrol* 2008;28:115-121.
25. Singh AK, Coyne DW, Shapiro W, *et al.* Predictors of the response to treatment in anemic hemodialysis patients with high serum ferritin and low transferrin saturation. *Kidney Int* 2007;71:1163-1171.
26. Anker SD, Comin Colet J, Filippatos G, *et al.* Ferric carboxymaltose in patients with heart failure and iron deficiency. *N Engl J Med* 2009;361:2436-2448.
27. Tanno T, Bhanu NV, Oneal PA, *et al.* High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. *Nat Med* 2007;13:1096-1101.
28. Tanno T, Porayette P, Sripichai O, *et al.* Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells. *Blood* 2009.
29. Fuste B, Serradell M, Escolar G, *et al.* Erythropoietin triggers a signaling pathway in endothelial cells and increases the thrombogenicity of their extracellular matrices in vitro. *Thromb Haemost* 2002;88:678-685.
30. Fenner MH, Ganser A. Erythropoietin in cancer-related anemia. *Curr Opin Oncol* 2008;20:685-689.
31. Grebenchtchikov N, Geurts-Moespot AJ, Kroot JJ, *et al.* High-sensitive radioimmunoassay for human serum hepcidin. *Br J Haematol* 2009:: *In press*.

CHAPTER 5

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

Mireille E Emans[1,2]
Karien van der Putten[2,8]
Karlijn L van Rooijen[2,5]
Rob J Kraaijenhagen[3]
Dorine Swinkels[4]
Wouter W van Solinge[5]
Maarten J Cramer[1]
Pieter AFM Doevendans[1]
Branko Braam[6]
Carlo AJM Gaillard[2,7]

[1]Department of Cardiology, University Medical Center Utrecht, the Netherlands
[2]Department of Internal Medicine, Meander Medical Center Amersfoort, the Netherlands
[3]Department of Clinical Chemistry, Meander Medical Center Amersfoort, the Netherlands
[4]Department of Clinical Chemistry, Radboud University Nijmegen Medical Center, the Netherlands
[5]Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, the Netherlands
[6]Division of Nephrology and Immunology, University of Alberta, Edmonton, Canada
[7]Department of Nephrology, VU University Medical Center, Amsterdam, the Netherlands
[8]Department of Nephrology, Leiden University Medical Center, the Netherlands

Submitted

ABSTRACT

Background

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

Methods and Results

In the EPOCARES study, which investigates the role of EPO in 54 iron supplemented anemic patients with CHF and chronic kidney disease (CKD) (n=35 treated with 50 IU/kg/wk Epoetin 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 EPO treatment ($r=-0.18$, $p=0.31$) and the increase of hemoglobin after 6 months EPO treatment ($r=0.26$, $p=0.35$). However, RDW was negatively correlated with functional iron availability (reticulocyte hemoglobin 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 anemic 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] The pathophysiological mechanism responsible for the association between RDW and adverse outcomes, as reported in these retrospective, cross-sectional cohort studies, is open to question. Anemia is highly prevalent in chronic heart failure (CHF) and is associated with morbidity and mortality.[7] Most factors that cause anemia 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 hemoglobin 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 anemia of CHF and chronic kidney disease (CKD).[8] Resistance to EPO is associated with morbidity and mortality.[9] Several authors hypothesize that EPO resistance could explain the association between RDW and outcome.[2,4,10] 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 EPOCARES study created an opportunity to investigate the association between RDW and EPO resistance in iron-supplemented, EPO naive patients with CHF and CKD.[9] Since 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 anemia[11], 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 hemoglobin 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.[12] In short, the EPOCARES study is an open-label, prospective, randomized trial, in which patients with CHF, CKD (GFR by Cockroft-Gault equation of 20-70 ml/min) and mild anemia (hemoglobin 10.3-12.6 g/dL for men and 10.3-11.9 g/dL for women) are included to test the hematopoietic and non-hematopoietic responses to EPO treatment. Exclusion criteria, amongst others, were erythropoietic therapy within 6 months, bleeding, hemolysis, hemoglobinopathies, chronic inflammatory disease

or malignancy. Hemoglobin (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.[13] Patients with heart failure with reduced left ventricular ejection fraction (HFREF) as well as patients with preserved left ventricular ejection fraction (HFPEF) were included.[14] 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 ACE-inhibitor or an angiotensin receptor blocker for at least four weeks. One group received 50 IU/kg/wk Epoetin 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 Epoetin 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

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,[15] 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 reticulocyte hemoglobin 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 hematology 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.[16,17] EPO resistance was measured in multiple ways. Endogenous EPO resistance was determined by defining the EPO levels

for the degree of anemia, 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).[11] 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 (TOF MS).[18] Serum hepcidin-25 concentrations were expressed as nmol/l. The lower limit of detection (LLOD) of this method was 0.5 nM; average coefficients of variation (CV) 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.[19,20]

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 paired 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 one patient was excluded due to malignancy (diagnosed on routine X-ray). Of two patients, RDW data at baseline could not be ascertained. Baseline characteristics of the 54 patients for this study are presented in table 1, divided according to tertiles. Univariate linear correlations are listed in table 2. In table 3, the multivariable regression analysis is shown. All patients had CKD, CHF and anemia, as shown by the decreased MDRD, the higher NT-proBNP, the lower LVEF and the lower Hb-levels. Vitamin B12 and folate levels were normal and hemolysis 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.

Table 1 Baseline characteristics, stratified by RDW values

Characteristic	All patients n=54	1st tertile RDW < 13.4%	2nd tertile RDW 13.4-14.7%	3rd tertile RDW ≥ 14.8 %	P-value
Age (years)	74 [69-80]	72 [68-75]	77 [71-82]	77 [66-81]	0.27
Male sex, no. (%)	35 (65%)	12 (67%)	12 (67%)	11 (61%)	0.49
Hemoglobin (g/dL)	11.8 ± 0.9	11.6 ± 0.9	12.1 ± 0.9	11.8 ± 0.9	0.36
Hematocrit (%)	0.35 [0.33-0.37]	0.35 ± 0.03	0.32 ± 0.12	0.34 ± 0.09	0.71
MCV (/μm³)	90 ± 4	90 ± 3	91 ± 5	89 ± 4	0.60
eGFR (ml/min/1.73m²)	35 ± 14	30 ± 14	39 ± 15	35 ± 11	0.18
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
NTproBNP (pg/mL)	1453 [718-2655]	1128 [482-1887]	1306 [718-2162]	2352 [926-6688]	0.08
LVEF (%)	44 ± 12	43 ± 9	44 ± 12	35 ± 13	0.97
Iron (μmol/L)	10 [9-14]	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 ± 0.2	2.3 ± 0.3	2.4 ± 0.5	0.19
TSAT (%)	20 [16-25]	24 [19-29]	20 [16-25]	17 [14-20]	**0.020**
Ret-He (fmol)	1.90 ± 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 (x10¹²/L)	0.046 ± 0.015	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**
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)	37 ± 3	37 ± 2	38 ± 2	35 ± 4	**0.045**
Cholesterol (mmol/L)	4.1 ± 1.1	4.6 ± 1.3	4.1 ± 1.0	3.6 ± 1.0	**0.044**

Mean ± standard deviation or median [interquartile range] are shown.

Abbreviations: MCV, mean corpuscular volume; eGFR, estimated glomerular filtration rate; NTproBNP, N-terminal pro-brain natriuretic peptide; LVEF, left ventricular ejection fraction; TSAT, transferrin saturation; Ret-He, reticulocyte hemoglobin content; sTfR, serum transferrin receptor; O/P ratio, log observed/predicted ratio; IRF, immature reticulocyte fraction; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; LDH, lactate dehydrogenase

Table 2 Univariate correlation coefficients of clinical and biochemistry variables with RDW, n=54

Variable	r	P-value
Age	0.25	0.08
Hemoglobin	-0.05	0.72
Hematocrit	0.09	0.52
MCV	-0.08	0.58
eGFR	0.13	0.37
Cystatin C	-0.26	0.85
NTproBNP	0.24	0.10
LVEF	-0.17	0.22
Serum iron	-0.33	0.02
Ferritin	-0.21	0.16
Transferrin	0.34	0.012
TSAT	-0.39	0.005
Ret-He	-0.48	<0.001
Hepcidin	-0.25	0.07
sTfR	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	0.07
Folate	0.07	0.61
LDH	0.21	0.13
Albumin	-0.17	0.23
Total cholesterol	-0.27	0.05

All non-parametric variables were considered for analysis after logarithmic transformation.
Abbreviations: MCV, mean corpuscular volume; eGFR, estimated glomerular filtration rate; NTproBNP, N-terminal pro-brain natriuretic peptide; LVEF, left ventricular ejection fraction; TSAT, transferrin saturation; Ret-He, reticulocyte hemoglobin content; sTfR, serum transferrin receptor; O/P ratio, log observed/predicted ratio; IRF, immature reticulocyte fraction; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; LDH, lactate dehydrogenase

RDW

The median RDW value was 14.0% (IQR 13.3-15.1), and 26% of the patients had a level above the upper limit (>15%) which corresponds with data from other studies.[1-5,21] In our normocytic anemic population, a higher RDW was not associated with Hb levels, hematocrit or mean corpuscular volume (MCV). RDW showed no correlation with renal function, as measured by creatinine, MDRD or cystatin C, nor with cardiac function as measured by left ventricular ejection fraction or NTproBNP levels.

Iron metabolism

In these patients on oral iron supplementation 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 suppletion. Indeed, also 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), indicating that there was no iron-restricted erythropoiesis. Therefore no direct evidence of decreased functional iron availability in the patients that received EPO was observed.[22] Total iron store parameters did not correlate with RDW. However, functional iron availability was negatively associated with RDW; patients in the highest RDW-tertile had both significant lower TSAT levels and RET-He levels (figure 1A). Hepcidin-25 showed no significant correlation with RDW.

EPO levels, erythropoietic activity and EPO resistance

There was a positive correlation between RDW and erythropoietic activity as measured by sTfR and IRF (figure 1B and 1C), but this was apparently not related to higher endogenous EPO levels.

The EPO level was defined for the degree of anemia by the 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.

Of the 54 patients, 35 patients received EPO treatment. After two weeks of EPO, 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). The magnitude of increase in reticulocyte count however did not correlate with the baseline RDW values (figure 2A). Neither did the extent in increase of sTfR and IRF after two weeks EPO treatment correlate with baseline RDW (figure 2B and 2C).

In 17 of the 35 patients who received EPO treatment, the Hb was increased. 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 RDW and response to exogenous EPO.

Inflammation

A positive correlation was observed between RDW and IL6, but this was not the case with CRP, nor with hs-CRP. There was a negative correlation between IL6 with RET-He (resp p=0.03, r= -0.30). As previously described, there was no correlation between both IL6 and hs-CRP with hepcidine-25 (resp. p=0.93, r=-0.12 and p=0.27, r=0.20) in our population.[23]

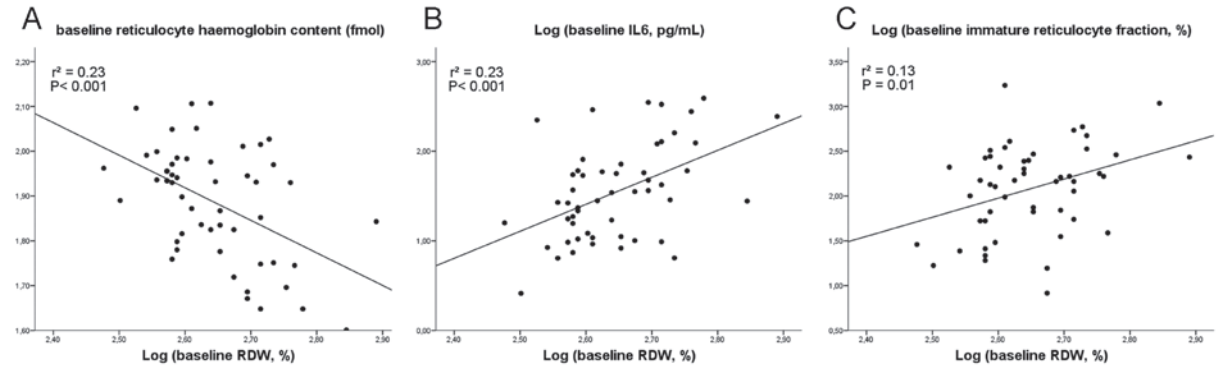

Figure 1 The correlation between log-transformed baseline RDW values and baseline levels of (A) reticulocyte hemoglobin content, (B) log transformed interleukin-6 and (C) log transformed immature reticulocyte fraction in 54 cardiorenal patients.

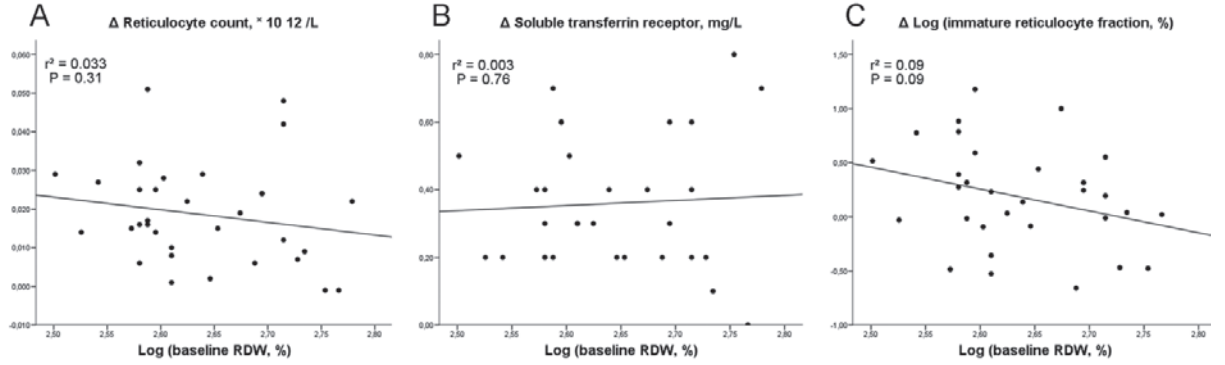

Figure 2 The correlation between log-transformed baseline RDW values and (A) the reticulocyte increase, (B) the increase in soluble transferrin receptor and (C) the increase in immature reticulocyte fraction, after two weeks of erythropoietin treatment in 35 cardiorenal patients.

Multivariable regression model

After entering all biomarkers with a significant univariate correlation with 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).

Table 3 Stepwise multivariate linear regression for RDW, n=54

Variable	Step no.	Multiple r^2	β coefficient	P-value
sTfR	1	0.218	0.483	<0.001
IL-6	2	0.356	0.394	<0.001
Ret-He	3	0.434	-0.309	<0.001
IRF	4	0.529	0.278	<0.001

All non-parametric variables were considered for analysis after logarithmic transformation.
Abbreviations: sTfR, serum transferrin receptor; IL-6, interleukin-6; Ret-He, reticulocyte hemoglobin content; IRF, immature reticulocyte fraction

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 outcomes 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 IL6 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.[23,24]

EPO resistance, defined as an inadequate bone marrow response to exogenous or endogenous EPO, contributes to anemia[9] and is associated with increased mortality in patients with heart and/or kidney failure.[25,26] Indeed, EPO levels in patients with CKD and/or CHF are higher as compared to healthy controls, but inappropriately low for the degree of anemia.[8,11] This observation indicates a relative EPO deficiency as well as a reduced bone marrow response to endogenous EPO.[9] 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 Hemoglobin and Outcomes in Renal Insufficiency (CHOIR) study, the use of high-dose EPO in EPO-resistant patients was associated with increased morbidity and mortality.[25] 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.[2,4,10] 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 (IRF and sTfR). At this point it is important to note that increased erythropoietic activity not necessarily results in increased hemoglobin levels since the level of hemoglobin 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.[16] 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.[27] Förhécz et al. also reported a positive correlation between sTfR and RDW in a nonanemic 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 anemic patients with heart and kidney failure.

RDW is most commonly used in the differential diagnosis of iron deficiency anemia, in which MCV is decreased and RDW is increased. Decreased functional iron availability plays a role in anemia of CHF and CKD. We demonstrate a negative correlation between TSAT, RET-He, sTfR and RDW in our iron-supplemented patients. Reticulocyte hemoglobin content (RET-He) is considered to be a very sensitive indicator reflecting iron availability for erythropoiesis[28] and is an indicator for iron-restricted erythropoiesis in patients receiving EPO.[22] Compared with the use of ferritin or TSAT, it showed higher sensitivity and specificity.[15] Therefore, although 41% of our orally iron-suppelemented patients had a ferritin level <100ng/ml, indicating possible lower iron stores, the lack of decrease in RET-He after EPO therapy suggests that there was no overtly iron-restricted erythropoiesis. Since 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 IL6. 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. anemia and inflammation (IL6). Hepcidin thus integrates input from erythropoietic and inflammatory pathways.[29] In patients with CKD[30] it has been shown that hepcidin levels are higher compared to healthy controls, but in patients with CHF and anemia this was not confirmed.[24,31] In our patients with the combination of heart- and kidney failure and anemia, 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 cannot 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.[23,24] Thus, in our study RDW was associated with IL6 but not with other markers of inflammation. Although data exist that IL6 can influence iron absorption during the hypoxic exposure, via a mechanism independent of hepcidin[32], at this point it is unclear how IL6 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 anemic 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 anemic 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 anemic patients with CKD and CHF. However, RDW was associated with erythropoietic activity, decreased functional iron availability and IL6. 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.

REFERENCES

1. Felker GM, Allen LA, Pocock SJ, *et al.* Red cell distribution width as a novel prognostic marker in heart failure: data from the CHARM Program and the Duke Databank. *J Am Coll Cardiol* 2007;50:40-47.
2. Forhecz Z, Gombos T, Borgulya G, *et al.* Red cell distribution width in heart failure: prediction of clinical events and relationship with markers of ineffective erythropoiesis, inflammation, renal function, and nutritional state. *Am Heart J* 2009;158:659-666.
3. Pascual-Figal DA, Bonaque JC, Redondo B, *et al.* Red blood cell distribution width predicts long-term outcome regardless of anaemia status in acute heart failure patients. *Eur J Heart Fail* 2009;11:840-846.
4. Allen LA, Felker GM, Mehra MR, *et al.* Validation and potential mechanisms of red cell distribution width as a prognostic marker in heart failure. *J Card Fail* 2010;16:230-238.
5. Tonelli M, Sacks F, Arnold M, *et al.* Relation Between Red Blood Cell Distribution Width and Cardiovascular Event Rate in People With Coronary Disease. *Circulation* 2008;117:163-168.
6. Perlstein TS, Weuve J, Pfeffer MA, *et al.* Red blood cell distribution width and mortality risk in a community-based prospective cohort. *Arch Intern Med* 2009;169:588-594.
7. Groenveld HF, Januzzi JL, Damman K, *et al.* Anemia and mortality in heart failure patients a systematic review and meta-analysis. *J Am Coll Cardiol* 2008;52:818-827.
8. Belonje AM, Voors AA, van der Meer P, *et al.* Endogenous erythropoietin and outcome in heart failure. *Circulation* 2010;121:245-251.
9. van der Putten K, Braam B, Jie KE, *et al.* Mechanisms of Disease: erythropoietin resistance in patients with both heart and kidney failure. *Nat Clin Pract Nephrol* 2008;4:47-57.
10. van Kimmenade RR, Mohammed AA, Uthamalingam S, *et al.* Red blood cell distribution width and 1-year mortality in acute heart failure. *Eur J Heart Fail* 2010;12:129-136.
11. Opasich C, Cazzola M, Scelsi L, *et al.* Blunted erythropoietin production and defective iron supply for erythropoiesis as major causes of anaemia in patients with chronic heart failure. *Eur Heart J* 2005;26:2232-2237.
12. van der Putten K, Jie KE, Emans ME, *et al.* Erythropoietin treatment in patients with combined heart and renal failure: objectives and design of the EPOCARES study. *J Nephrol* 2010;23:363-368.
13. Dickstein K, Cohen-Solal A, Filippatos G, *et al.* ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the diagnosis and treatment of acute and chronic heart failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). *Eur J Heart Fail* 2008;10:933-989.
14. Paulus WJ, Tschope C, Sanderson JE, *et al.* How to diagnose diastolic heart failure: a consensus statement on the diagnosis of heart failure with normal left ventricular ejection fraction by the Heart Failure and Echocardiography Associations of the European Society of Cardiology. *Eur Heart J* 2007;28:2539-2550.
15. Wish JB. Assessing iron status: beyond serum ferritin and transferrin saturation. *Clin J Am Soc Nephrol* 2006;1 Suppl 1:S4-8.
16. Briggs C. Quality counts: new parameters in blood cell counting. *Int J Lab Hematol* 2009;31:277-297.
17. Davis BH, Ornvold K, Bigelow NC. Flow cytometric reticulocyte maturity index: a useful laboratory parameter of erythropoietic activity in anemia. *Cytometry* 1995;22:35-39.
18. Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, *et al.* Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. *Clin Chem* 2010;56:1570-1579.
19. Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, *et al.* Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. *Clin Chem* 2009;56:1570-1579.
20. Peters HP, Laarakkers CM, Swinkels DW, *et al.* Serum hepcidin-25 levels in patients with chronic kidney disease are independent of glomerular filtration rate. *Nephrol Dial Transplant* 2010;25:848-853.
21. Al-Najjar Y, Goode KM, Zhang J, *et al.* Red cell distribution width: an inexpensive and powerful prognostic marker in heart failure. *Eur J Heart Fail* 2009;11:1155-1162.
22. Schoorl M, Schoorl M, Nube MJ, *et al.* Erythropoiesis activity, iron availability and reticulocyte hemoglobinization during treatment with hemodialysis and in subjects with uremia. *Clin Lab* 2006;52:621-629.

23. van der Putten K, Jie KE, van den Broek D, *et al.* Hepcidin-25 is a marker of the response rather than resistance to exogenous erythropoietin in chronic kidney disease/chronic heart failure patients. *Eur J Heart Fail* 2010;12:943-950.
24. Matsumoto M, Tsujino T, Lee-Kawabata M, *et al.* Iron regulatory hormone hepcidin decreases in chronic heart failure patients with anemia. *Circ J* 2010;74:301-306.
25. Szczech LA, Barnhart HX, Inrig JK, *et al.* Secondary analysis of the CHOIR trial epoetin-alpha dose and achieved hemoglobin outcomes. *Kidney Int* 2008;74:791-798.
26. Zhang Y, Thamer M, Stefanik K, *et al.* Epoetin requirements predict mortality in hemodialysis patients. *Am J Kidney Dis* 2004;44:866-876.
27. Choi JW. Serum transferrin receptor concentrations correlate more strongly with red cell indices than with iron parameters in iron-deficient adolescents. *Acta Haematol* 2003;110:213-216.
28. Bartels PC, Schoorl M, Schoorl M. Hemoglobinization and functional availability of iron for erythropoiesis in case of thalassemia and iron deficiency anemia. *Clin Lab* 2006;52:107-114.
29. Swinkels DW, Wetzels JF. Hepcidin: a new tool in the management of anaemia in patients with chronic kidney disease? *Nephrol Dial Transplant* 2008;23:2450-2453.
30. Zaritsky J, Young B, Wang HJ, *et al.* Hepcidin--a potential novel biomarker for iron status in chronic kidney disease. *Clin J Am Soc Nephrol* 2009;4:1051-1056.
31. Divakaran V, Mehta S, Yao D, *et al.* Hepcidin in anemia of chronic heart failure. *Am J Hematol* 2010;86:107-109.
32. Raja KB, G OL-D, Peters TJ, *et al.* Role of interleukin-6 in hypoxic regulation of intestinal iron absorption. *Br J Haematol* 2005;131:656-662.

CHAPTER 6

High cumulative incidence of cancer in patients with cardio-renal-anemia syndrome

Nicolas F Schroten[1*]
Karien van der Putten[2*]
Frans H Rutten[1]
Adry Diepenbroek[2]
Arend Mosterd[3,4]
Carlo AJM Gaillard[2,5]

[1]Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands
[2]Department of Internal Medicine, Meander Medical Center, Amersfoort, The Netherlands
[3]Department of Cardiology, Meander Medical Center, Amersfoort, The Netherlands
[4]Heart and Lung Center, University Medical Center Utrecht, Utrecht, The Netherlands
[5]Department of Nephrology, VU University Medical Center, Amsterdam, The Netherlands

*The first two authors contributed equally

Eur J Heart Fail 2010;12:855-860

ABSTRACT

Aims

The combination of chronic kidney disease (CKD), chronic heart failure (HF), and anemia, the so-called cardio-renal anemia syndrome (CRA) is associated with dysregulation of erythropoietin levels and inflammation. Both have been associated with the development of cancer. This study aimed to determine the cumulative incidence of cancer in patients with CRA, as compared with anemic CKD and control patients.

Methods

Patients aged <80 years who attended the nephrology or cardiology outpatient clinics between March 2006 and November 2007 were eligible for inclusion in this retrospective case-control study if hemoglobin <8.1 mmol/L (13 g/dL) and serum creatinine >80 mmol/L (0.90 mg/dL). Medical records dating back to 1996 were reviewed. The relationship between cancer and CRA, chronic HF, CKD, and anemia was analyzed using logistic regression analysis.

Results

Data from 1087 patients were reviewed. We identified 348 patients with both CKD and anemia, of whom 132 (38.3%) had CRA. The control group included 264 patients attending the hypertension outpatient clinic. Patients with CRA had a 19% cumulative incidence of cancer compared with 11% for patients with anemia, CKD and no chronic HF, and 11% in the control group. The odds ratio (OR) for cancer was 1.8 (95% CI 1.0-3.2) for the CRA group compared with the control group. Chronic HF was an independent risk factor for cancer after correction for age and gender (adjusted OR 2.0; 95% CI 1.2-3.3, P=0.007).

Conclusion

The cumulative incidence of cancer among patients with CRA is high compared with controls and to anemic CKD patients without chronic HF. Chronic HF was an independent risk factor for cancer. These results stress the importance of clarifying the mechanisms involved in the development of cancer in CRA.

INTRODUCTION

Chronic kidney disease (CKD) and anemia of chronic disease (ACD) are interrelated and common in patients with chronic heart failure (HF). The combination of chronic HF, CKD, and ACD is known as the cardio-renal-anemia syndrome (CRA).[1]

Several mechanisms have been proposed to explain the strong association between chronic HF and CKD. Previously, we described a model in which the renin–angiotensin system (RAS), the balance between the nitric oxide (NO) and reactive oxygen species (ROS), the sympathetic nervous system and inflammatory mediators, interact. A disturbance in one of these connectors may lead to a vicious circle, where failure of the heart or kidney eventually leads to progressive failure of both organs.[1]

In addition, there is an increasing interest in the effects of erythropoietin (EPO) in heart and kidney failure. EPO is a glycoprotein hormone that is well known for its erythropoietic effects. EPO also has important non-hematopoietic effects, including protection of the heart and kidney in hypoxic conditions.[2-4] Observational studies have shown that EPO levels are elevated in patients with moderate kidney disease as compared with patients with preserved renal function as well as compared with patients with severely perturbed renal function.[5,6] This observation suggests a compensatory response of EPO production in mild CKD, but exhaustion of the response in advanced CKD. In chronic HF patients EPO levels tend to be elevated.[7]

The most important stimulus for the production of EPO is renal hypoxia via hypoxia-inducible factor (HIF)-2α.[8,9] Other important factors that influence the EPO levels are cytokines (IL-1,TNF-α)[10] and the RAS.[11-13] Despite high levels of EPO, patients with chronic HF and CKD are often anemic. This contra-intuitive phenomenon is explained by an insufficient elevation of EPO levels in relation to the degree of anemia, as well as the so-called 'EPO resistance' phenomenon. EPO resistance is probably caused by inflammatory mediators (i.e. IL-1, IL-6, interferon-γ, TNF-α) which act directly on erythropoiesis by down-regulating messenger RNA for EPO receptor expression in erythroid progenitor cells and indirectly by disruption of iron metabolism, mainly through stimulation of the production of hepcidin.[14]

The EPO receptor is also expressed in several non-erythroid tissues, including neural tissues, the breast and the gut.[3,15] EPO triggers several important signaling cascades that inhibit apoptosis, and stimulate cell proliferation and angiogenesis. EPO has been associated with cancers of the breast, bladder, kidney, and bowel, as well as hematological cancers.[3,16,17] In the condition of 'EPO resistance', the bone marrow response to EPO is decreased. However, since different types of EPO receptors exist in different tissues,[18] these other tissues may not be resistant to EPO, and thus may have a preserved response to elevated levels of EPO. Prolonged exposure to high levels of endogenous EPO and inflammation, as is the case in CRA, could therefore lead to an increased risk of developing cancer.[19] Several studies, in support of this hypothesis, have shown a higher incidence of cancer in patients with end-stage CKD compared with the general population.[20-26] Wong et al. found in a population-based cohort an excess risk for malignancies starting at an estimated glomerular filtration rate (eGFR)<55 mL/min/1.73 m^2 and increasing linearly as eGFR declined further.[20] We therefore hypothesized that patients with CRA will have a higher incidence rate of cancer than patients without CRA. To our knowledge, this is the first study of the relationship between CRA and cancer.

METHODS

Study design

This retrospective case-control study was conducted at the Meander Medical Centre in Amersfoort, a large regional teaching hospital in the Netherlands, affiliated with the University Medical Centre Utrecht. The investigation conforms to the principles outlined in the Declaration of Helsinki. All patients who attended the cardiology and nephrology outpatient clinics between March 2006 and November 2007 were screened for hemoglobin (Hb) and creatinine levels. Patients aged<80 years, with a Hb <8.1 mmol/L (13 g/dL) and creatinine <80 mmol/L (0.9 mg/dL) were eligible for this study and their medical history dating back 10 years was reviewed. The diagnoses of chronic HF, CKD, ACD, and cancer were established according to the following criteria.

Chronic kidney disease

Renal function was calculated using the short 'Modification of Diet in Renal Disease (MDRD) formula' for the eGFR.[27] CKD was defined as a stable eGFR <60 mL/min/1.73 m^2 for at least three consecutive months. Thus, patients with a temporary decrease in eGFR (caused by an acute illness) were excluded.

Anemia of chronic disease

ACD was defined as a decreased Hb level for more than six consecutive months [women <7.8 mmol/L (12 g/dL) and men <8.1 mmol/L (13 g/dL)], without evidence of a deficiency of iron, vitamin B12 or folic acid. Patients with anemia due to surgery or an acute event or illness were excluded, as were patients who used erythropoiesis stimulating agents.

Chronic heart failure

The diagnosis of chronic HF was established by two cardiologists according to the criteria of the European Society of Cardiology.[28] That is, symptoms suggestive of HF (such as dyspnoea on exertion, fatigue) with (systolic and/or diastolic) ventricular dysfunction at rest, preferably assessed by echocardiography.[28] Systolic ventricular dysfunction was defined as a left ventricular ejection fraction <45% and diastolic function was assessed using standard methods including the E/A ratio, deceleration time, indexed left atrial volume, tricuspid flow parameters (S/D), and isovolumetric relaxation time. Since tissue Doppler imaging (TDI) measurements were only available in a minority of patients, e' and E/e' could not be used as a measure of diastolic function. Finally, patients who had been diagnosed with chronic HF by their own cardiologist were also classified as such.

Cardio-renal-anemia syndrome

Using the classifications described above, patients with CKD, ACD, and chronic HF were classified as CRA.

Cancer

Patients with cancer in the 10 years prior to the investigation date were classified as such, except for those with basal cell carcinoma and premalignant lesions. All cancers were coded according to the organ-system in which they primarily had developed.

Control group

A sample of patients from the hypertension outpatient clinic with an eGFR ≥ 60 mL/min/1.73 m^2 and Hb > 8.0 mmol/L (13 g/dL), were used as controls. The control group was matched for age and gender with the CRA patients. Age stratified cumulative incidence rates of cancer from the general population in the Netherlands were obtained from the comprehensive cancer centre (IKC), as an additional external control group.

Data analysis

Differences between groups were compared with the $\chi 2$ test (categorical variables) and Mann-Whitney U test (continuous variables, non-normal distribution). For each group, the cumulative incidence of cancer in the prior 10 years was calculated. The period of 10 years was chosen in concordance with data from the IKCs in the Netherlands, which also served as an external validation of our control group. Confidence intervals of the cumulative incidence of cancer were calculated using the usual methods for dichotomous outcome.[29] We applied multivariable logistic regression analysis to calculate the odds ratios (OR) of ACD, CKD, and chronic HF, corrected for each other and for age and gender differences. Data were analyzed using SPSS 14.0®.

RESULTS

Patient characteristics

We identified 1087 patients with anemia and eGFR <60 mL/min/1.73 m^2. Of these, 29 patients (1%) were excluded because data on renal function (n=14), or Hb levels (n=15) were missing. Another 233 patients (21%) were excluded because conclusive information about the presence of HF was missing (Figure 1). Overall 825 patients had reduced Hb and creatinine levels; however, only 348 patients met the criteria for both CKD plus ACD. Of these, 132 (38.3%) patients had concomitant chronic HF, thus meeting the criteria for CRA (Figure 1).

General characteristics of the study population are shown in Table 1. There was a statistically significant difference in age and gender in the CKD/ACD group as compared with the CRA and control groups, whereas diabetes was slightly more prevalent in patients with CRA as compared with the CKD/ACD and control groups. In the CRA group the mean (SD) left ventricular ejection fraction was 37 (±13%) and median (Q1–Q3) BNP level was 1670 (767–4016 pg/mL).

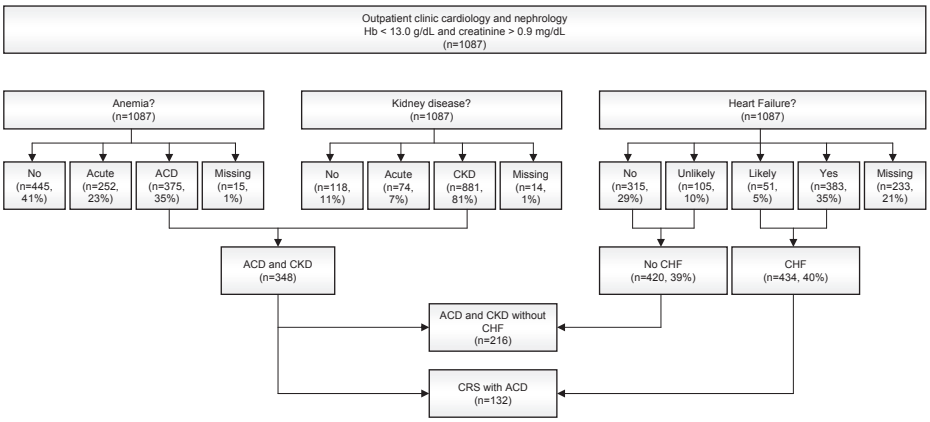

Figure 1 Selection of patients. In total 1087 patients were selected from the outpatient clinics because of hemoglobin <8.1 mmol/l (13.0 g/dL) and creatinine >80 mmol/l (0.9 mg/dL). They were all scored for chronic heart failure, anemia of chronic disease, and chronic kidney disease. Patients with both chronic kidney disease and anemia of chronic disease were subdivided into a group with chronic heart failure, chronic kidney disease, and anemia of chronic disease (cardio-renal-anemia syndrome) and a group with chronic kidney disease plus anemia of chronic disease, without chronic heart failure.

Abbreviations: ACD, anemia of chronic disease; CKD, chronic kidney disease; CHF, chronic heart failure.

Table 1 Characteristics of 348 patients with chronic kidney disease plus anemia of chronic disease, divided into those with and without concurrent chronic heart failure compared with controls (n=264)

	CRA	CKD/ACD No CHF	Control group
Number of patients	132	216	264
Men	87 (66%)	116 (54%)**	174 (66%)
Age (years)	73.5 (68–77)	71 (62–76)**	74 (68–77)
Hemoglobin (mmol/L)	7.2 (±0.6)**	7.3 (±0.6)**	9.0 (±0.8)
eGFR-MDRD (mL/min/1.73 m^2)	39 (±14)**	37 (±14)**	76 (±12)
Diabetes			
Yes	70 (53%)*	98 (45%)	111 (42%)
No	61 (47%)	116 (54%)	153 (58%)
Unknown	1 (1%)	2 (1%)	0 (0%)
Smoking			
Yes	24 (18%)	34 (16%)	42 (16%)
No	81 (51%)	145 (71%)	222 (84%)
Unknown	27 (21%)	37 (17%)	0 (0%)
On dialysis	6 (4.5%)**	9 (4.5%)**	0 (0%)
Renal transplantation	2 (1.5%)*	6 (2.8%)**	0 (0%)

*P< 0.05 compared with control group.

**P <0.01 compared with control group.

Data are presented as mean (±SD), median (Q1–Q3), or absolute (%).

Abbreviations: CRA, cardio-renal-anemia syndrome; CKD/ACD No CHF, chronic kidney disease and anemia of chronic disease, without heart failure.

Association of chronic heart failure with cancer

Univariate analysis of the 825 patients with reduced Hb and creatinine levels showed a significant relation between chronic HF and cancer [OR 1.9 (95% CI 1.3-2.6)]. After multivariable logistic regression analysis including renal function, anemia, age, and gender, chronic HF remained a significant risk factor with an OR 2.0 (95% CI 1.2-3.3, P=0.007; Table 2).

Table 2 Multivariable logistic regression analysis of the correlation with malignancies in 1087 screened patients

	Odds ratio	95.0% CI for odds ratio		Significance
		Lower	Upper	
CHF	2.01	1.21	3.33	0.007
CKD	0.48	0.25	0.95	0.03
ACD	1.12	0.70	1.7	0.65
Age	1.04	1.01	1.07	0.03
Gender	1.10	0.67	1.78	0.71
Constant	0.02			0.001

Abbreviations: CHF, chronic heart failure; CKD, chronic kidney disease; ACD, anemia of chronic disease.

The cumulative incidence of malignancies in the CRA group was significantly higher than in the hypertensive control group [OR 1.8 (95% CI 1.0-3.2)] and than in the CKD/ACD group [OR 1.9 (95% CI 1.0-3.6)]. Patients with CRA had a 19% cumulative incidence of cancer during 10 years of follow-up. In the CKD/ACD group, as well as in the hypertensive control patients, the cumulative incidence was 11%. There was no significant difference in cancer incidence between the CKD/ACD group and the hypertensive control group [OR 0.9 (95% CI 0.5-1.7)].

The cumulative incidence of cancer for different age strata is listed in Table 3. The median (Q1–Q2) year malignancy was diagnosed was 2003 (1998–2006). Age stratified cancer incidence data provided by the IKCs in the Netherlands were comparable with data from our control group of patients with hypertension.

Cancer of the digestive tract was the most often detected cancer in patients with CRA, accounting for 17-30% of all cancers (Table 4), whereas urinary tract cancer accounted for 12-17% of all cancers, cancer of the male reproductive tract for 8–23%, breast for <5%, and skin for 0-13%.

Table 3 Cumulative incidence of cancer during 10 years follow-up in 348 patients with chronic kidney disease plus anemia of chronic disease, divided into those with and without concurrent chronic heart failure compared with controls (n=5264)

	CRA		CKD/ACD No CHF		Controls	
Age	n	Cumulative incidence malignancies	n	Cumulative incidence malignancies	n	Cumulative incidence malignancies
<60	6	0 (0%)	45	3 (7%)	12	0 (0%)
60-64	12	2 (17%)	16	2 (13%)	22	2 (9%)
65-69	22	2 (9%)	34	2 (6%)	44	4 (9%)
70-74	34	7 (21%)	51	9 (18%)	67	10 (15%)
75+	58	14 (24%)	70	7 (10%)	119	14 (12%)
Total	132	25 (19%)	216	23 (11%)	264	30 (11%)

Abbreviatons: CRA, cardio-renal-anemia syndrome; CKD/ACD No CHF, chronic kidney disease and anemia of chronic disease, without heart failure.

Table 4 Distribution of cancer in 348 patients with chronic kidney disease plus anemia of chronic disease, divided into those with and without concurrent chronic heart failure compared with controls (n=5264)

Type of cancer	CRA	CKD plus ACD	Controls
Gastrointestinal	5 (20%)	4 (17%)	9 (30%)
Male reproductive	2 (8%)	3 (13%)	7 (23%)
Female reproductive	0 (0%)	1 (4%)	0 (0%)
Breast	0 (0%)	1 (4%)	1 (3%)
Urinary tract	3 (12%)	4 (17%)	4 (13%)
Skin	3 (12%)	0 (0%)	4 (13%)
Kidney	2 (8%)	3 (13%)	0 (0%)
Hematological	1 (4%)	0 (0%)	3 (10%)
Head and neck	0 (0%)	1 (4%)	1 (3%)
Lower airways	4 (16%)	0 (0%)	0 (0%)
Endocrine glands	1(4%)	0 (0%)	0 (0%)
Central nervous system	0 (0%)	1 (4%)	0 (0%)
Multiple	4 (16%)	5 (22%)	1 (3%)
Total	25 (100%)	23 (100%)	30 (100%)

Abbreviations: CRA, cardio-renal-anemia syndrome; CKD/ACD No CHF: chronic kidney disease and anemia of chronic disease, without heart failure.

DISCUSSION

The main finding of our study is that in patients with kidney disease and anemia, HF is associated with a higher incidence of cancer (adjusted OR 2.0). Patients with CRA had higher cumulative incidence rates of cancer than patients with CKD and ACD without HF and control patients from the hypertension outpatient clinic with normal renal function and Hb levels.

To our best knowledge this is the first study that reports the cumulative incidence rates of cancer in patients with CRA. Previous studies have shown a higher incidence of cancer in patients on dialysis or with end-stage kidney disease compared with the general population.[20-26] Recently Wong et al. reported in a population-based cohort an increased risk for malignancies starting at an eGFR <55 mL/min/1.73 m^2.[20] We were not able to confirm this finding in our study since we failed to show a difference in the incidence of cancer in patients with CKD/ACD (without chronic HF) as compared with the hypertensive control population. However, the increase in incidence of cancer in patients with mild CKD as reported by Wong et al. is relatively small and our study may have lacked the power to detect differences in the incidence of malignancies as reported in patients with mild CKD.

Our study does not provide data about the causative relation between cancer and CRA nor to putative mechanisms involved in the unexpected increased cancer incidence in the CRA group compared with the CKD/ACD (without chronic HF) group. In our view there are, however, several plausible pathophysiological mechanisms that may be related to a higher risk of cancer in patients with CRA. Putative mechanisms involved are prolonged exposure to hypoxia resulting in increased levels of HIF-1α and/or long-term elevation of endogenous EPO levels leading to activation of EPO-R on non-erythroid tissues. In addition several authors hypothesize that changes commonly reported in CKD and chronic HF such as elevated levels of inflammatory mediators and/or ROS may be involved. Regardless of the mechanisms involved, our (hypothesis generating) study stresses the importance of further research into the relationship between CRA and malignancies, as well as into the mechanisms involved. Basic research and prognostic follow-up studies in patients with early stages of CRA are urgently needed. If indeed inflammation or EPO play a role in the pathophysiological mechanism, intervention studies evaluating the therapeutic effect of exogenous EPO may provide important new data and randomized clinical trials that are underway, need to be closely monitored for differences in the incidence of malignancies. The TREAT study, for example, showed an increased cancer related mortality in patients treated with erythropoiesis stimulating agents.[30]

Since our study had a retrospective design, it is important to point out and discuss the limitations of the study in more detail. To minimize the risk of 'reverse causality', which is present in all retrospective studies, we excluded patients who developed chronic HF, CKD, or ACD within 3 months after the diagnosis of cancer and patients in whom chronic HF or CKD could be attributed to medication (e.g. adriamycin). Secondly, in the analysis of the data we corrected for several confounders, however, unfortunately, we were not able to correct for all possible confounders because of incomplete patient records. We did not find a significant difference in the prevalence of smoking between the groups. Small

differences between groups may exist, due to inaccurate reporting in the electronic patient files, but this does not appear sufficient to entirely explain our observations. Although the control population was selected from the same hospital, referral, and work-up bias between patients with CRA and controls, and thus residual confounding, cannot be fully ruled out. To minimize the risk of work-up bias we selected a control group with a similar diagnostic work-up (laboratory analysis, chest X-rays etc) as HF and CKD patients. Finally, survival bias has to be taken into account. Any retrospective study may underestimate the true cumulative incidence of cancer because only survivors are included, especially in populations with a short life expectancy. However, survival bias could both weaken and strengthen the association between HF and malignancies.

CONCLUSIONS

The high cumulative incidence of malignancies in patients with CRA as compared with patients with CKD and anemia is remarkable and stresses the importance of diagnosing chronic HF in patients with CKD and ACD. Although causality cannot be proven, these patients appear to be at a greater risk of developing malignancies, in addition to the increased risk of cardiovascular morbidity and mortality. Moreover, since clinical studies are now examining the beneficial effects of EPO treatment in patients with chronic HF and CKD, the effects of EPO treatment in patients with chronic HF need to be closely monitored.

REFERENCES

1. Bongartz LG, Cramer MJ, Doevendans PA, *et al.* The severe cardiorenal syndrome: 'Guyton revisited'. *Eur Heart J* 2005;26:11-17.
2. Jie KE, Verhaar MC, Cramer MJ, *et al.* Erythropoietin and the cardiorenal syndrome: cellular mechanisms on the cardiorenal connectors. *Am J Physiol Renal Physiol* 2006;291:F932-944.
3. Hardee ME, Arcasoy MO, Blackwell KL, *et al.* Erythropoietin biology in cancer. *Clin Cancer Res* 2006;12:332-339.
4. Lipsic E, Westenbrink BD, van der Meer P, *et al.* Low-dose erythropoietin improves cardiac function in experimental heart failure without increasing haematocrit. *Eur J Heart Fail* 2008;10:22-29.
5. Fehr T, Ammann P, Garzoni D, *et al.* Interpretation of erythropoietin levels in patients with various degrees of renal insufficiency and anemia. *Kidney Int* 2004;66:1206-1211.
6. Radtke HW, Claussner A, Erbes PM, *et al.* Serum erythropoietin concentration in chronic renal failure: relationship to degree of anemia and excretory renal function. *Blood* 1979;54:877-884.
7. Westenbrink BD, Visser FW, Voors AA, *et al.* Anaemia in chronic heart failure is not only related to impaired renal perfusion and blunted erythropoietin production, but to fluid retention as well. *Eur Heart J* 2007;28:166-171.
8. Nangaku M, Inagi R, Miyata T, *et al.* Hypoxia and hypoxia-inducible factor in renal disease. *Nephron Exp Nephrol* 2008;110:e1-7.
9. Rankin EB, Biju MP, Liu Q, *et al.* Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. *J Clin Invest* 2007;117:1068-1077.
10. Jelkmann W. Molecular biology of erythropoietin. *Intern Med* 2004;43:649-659.
11. Donnelly SM, Miller JA. Losartan may modulate erythropoietin production. *J Renin Angiotensin Aldosterone Syst* 2001;2:255-260.
12. Wang AY, Yu AW, Lam CW, *et al.* Effects of losartan or enalapril on hemoglobin, circulating erythropoietin, and insulin-like growth factor-1 in patients with and without posttransplant erythrocytosis. *Am J Kidney Dis* 2002;39:600-608.
13. Freudenthaler SM, Schreeb K, Korner T, *et al.* Angiotensin II increases erythropoietin production in healthy human volunteers. *Eur J Clin Invest* 1999;29:816-823.
14. van der Putten K, Braam B, Jie KE, *et al.* Mechanisms of Disease: erythropoietin resistance in patients with both heart and kidney failure. *Nat Clin Pract Nephrol* 2008;4:47-57.
15. Spano JP, Milano G, Rixe C, *et al.* JAK/STAT signalling pathway in colorectal cancer: a new biological target with therapeutic implications. *Eur J Cancer* 2006;42:2668-2670.
16. Mohyeldin A, Lu H, Dalgard C, *et al.* Erythropoietin signaling promotes invasiveness of human head and neck squamous cell carcinoma. *Neoplasia* 2005;7:537-543.
17. Acs G, Zhang PJ, McGrath CM, *et al.* Hypoxia-inducible erythropoietin signaling in squamous dysplasia and squamous cell carcinoma of the uterine cervix and its potential role in cervical carcinogenesis and tumor progression. *Am J Pathol* 2003;162:1789-1806.
18. Mannello F, Tonti GA. Erythropoietin and its receptor in breast cancer: putting together the pieces of the puzzle. *Oncologist* 2008;13:761-768.
19. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;420:860-867.
20. Wong G, Hayen A, Chapman JR, *et al.* Association of CKD and cancer risk in older people. *J Am Soc Nephrol* 2009;20:1341-1350.
21. Holley JL. Screening, diagnosis, and treatment of cancer in long-term dialysis patients. *Clin J Am Soc Nephrol* 2007;2:604-610.
22. Mandayam S, Shahinian VB. Are chronic dialysis patients at increased risk for cancer? *J Nephrol* 2008;21:166-174.
23. Iseki K, Osawa A, Fukiyama K. Evidence for increased cancer deaths in chronic dialysis patients. *Am J Kidney Dis* 1993;22:308-313.
24. Stewart JH, Buccianti G, Agodoa L, *et al.* Cancers of the kidney and urinary tract in patients on dialysis for end-stage renal disease: analysis of data from the United States, Europe, and Australia and New Zealand. *J Am Soc Nephrol* 2003;14:197-207.

25. Port FK, Ragheb NE, Schwartz AG, *et al.* Neoplasms in dialysis patients: a population-based study. *Am J Kidney Dis* 1989;14:119-123.
26. Cengiz K. Increased incidence of neoplasia in chronic renal failure (20-year experience). *Int Urol Nephrol* 2002;33:121-126.
27. Smilde TD, van Veldhuisen DJ, Navis G, *et al.* Drawbacks and prognostic value of formulas estimating renal function in patients with chronic heart failure and systolic dysfunction. *Circulation* 2006;114:1572-1580.
28. Dickstein K, Cohen-Solal A, Filippatos G, *et al.* ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the diagnosis and treatment of acute and chronic heart failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). *Eur J Heart Fail* 2008;10:933-989.
29. Newcombe RG. Improved confidence intervals for the difference between binomial proportions based on paired data. *Stat Med* 1998;17:2635-2650.
30. Pfeffer MA, Burdmann EA, Chen CY, *et al.* A trial of darbepoetin alfa in type 2 diabetes and chronic kidney disease. *N Engl J Med* 2009;361:2019-2032.

PART II

Hemoglobin variability in CKD patients

CHAPTER 7

Hemoglobin variability in patients with chronic kidney disease in the Netherlands

Karien van der Putten[1]
Frederieke H van der Baan[2]
Huub Schellekens[2]
Carlo AJM Gaillard [1,3]

[1]Department of Internal Medicine, Meander MC, Amersfoort, the Netherlands
[2]Department of Innovation Studies, Utrecht University, Utrecht, the Netherlands
[3]Department of Nephrology, VU University Medical Center, Amsterdam, the Netherlands

Int J Artif Organs 2009;32:787-793

ABSTRACT

Aim

Hemoglobin cycling has been reported in hemodialysis patients treated with erythropoietic-stimulating agents (ESA) and is associated with increased mortality. Information on hemoglobin cycling in Europe is limited. We investigated hemoglobin variability in the Netherlands. Hemodialysis and peritoneal dialysis patients were studied and predialysis patients were enrolled.

Methods

This observational retrospective study was executed in a Dutch dialysis centre. We studied 157 patients from 2005 to 2007: 56 hemodialysis, 12 peritoneal dialysis and 29 predialysis patients, all treated with ESA; and 60 predialysis patients without ESA. Patients were divided on the basis of their pattern of hemoglobin fluctuation around a target range of 11-12 g/dL. In dialysis patients, the amount of time that hemoglobin remained within the target range was calculated. For all patients, the magnitude of hemoglobin fluctuations was assessed (i.e. the difference between hemoglobin maximum and minimum) and data on ESA dose changes and hospitalizations were collected.

Results

None of the ESA treated patients had stable hemoglobin levels within the target range over a one-year period. Predialysis patients without ESA also showed variable hemoglobin levels. A stepwise decrease in the magnitudes of hemoglobin fluctuation was observed in the hemodialysis patients, peritoneal dialysis patients, predialysis patients using ESA, to the predialysis patients without ESA, respectively.

Conclusion

In the Netherlands, hemoglobin variability is common in hemodialysis and peritoneal dialysis patients, but also in predialysis patients. The results of this study warrant further research into the relationship between hemoglobin variability and clinical outcomes.

INTRODUCTION

Anemia is common in patients with chronic kidney disease (CKD) and is associated with poor outcome.[1-4] Observational data indicate that hemoglobin (Hb) levels between 11.5 and 13 g/dL are associated with the greatest survival. Hemoglobin values >13.5 g/dL are associated with a trend toward increased death risk, as are Hb levels lower than 11.5 g/dL.[1] In keeping with the upper limit found in this observational study, a recent randomized controlled trial in CKD patients showed that targeting to achieve a hemoglobin level of 13.5 g/dL was associated with increased risk.[5]

The guidelines for managing anemia in patients with CKD do not agree on the target level of Hb. The revised European Best Practice Guideline (EBPG) recommends a target Hb concentration of at least 11 g/dL and advices against Hb levels above 12 g/dL for patients with severe cardiovascular disease.[6] The National Kidney Foundation's Kidney Disease Outcomes Quality Initiative (NKF-K/DOQI) guideline recommends initiation of ESA at an Hb level of 10 g/dL and a Hb target of 11-12 g/dL, up to a maximum of 13 g/dL.[7] The EBPG for the Netherlands is currently being revised, but up until now, a similar Hb target range of 11-12 g/dL was recommended[8], in agreement with previous K/DOQI guidelines.

Maintaining a patient's Hb at such a narrow target window is challenging. It has been observed that hemodialysis patients receiving ESA frequently show fluctuations in their Hb level over time.[9-13] Fishbane et al. call this phenomenon cycling, defined as repeated, cyclical, up and down movements of absolute Hb levels during ESA treatment.[14] Yang et al. define Hb variability by measuring the residual standard deviation of Hb.[15] Many factors are associated with the variability of Hb levels, including ESA dose adjustments, intravenous iron dose adjustments, hospitalizations, infections[11], route of ESA administration[16], variation of hydration status[17], blood loss, and dialysis adequacy.[18] The exact mechanism is not fully understood, however, and is likely to be multifactorial. Variability of Hb over time is associated with increased mortality in dialysis patients.[15,19,20] Yang et al. demonstrated that for each 1 g/dL increase in Hb variability, the adjusted rate of death was increased by 33%.[15] However, the observational nature of these studies does not allow the conclusion of a causal relationship between Hb cycling and increased mortality risk.

Up until now, most studies on Hb cycling focused on US dialysis patients[9-13]. In view of the difference between the US and Europe regarding the reimbursement and organizational structure of dialysis centers, the question whether cycling also occurs in Western Europe is of importance. Furthermore, since changes in ESA dose were found to be associated with Hb cycling[11], predialysis patients receiving ESA are expected to experience Hb cycling as well. Therefore, information about Hb variability in predialysis patients may aid in determining whether Hb variability depends on individual responsiveness to ESA, intensity of the therapeutic intervention or events related to the dialysis treatment.[21-23] The aim of this retrospective study is to investigate the phenomenon of Hb cycling in CKD patients (dialysis, PD and predialysis) in the Netherlands and to study factors associated with Hb cycling.

METHODS

Study population

This single-centre, retrospective study in patients with CKD, was conducted in the Meander Medical Dialysis Centre in Amersfoort, the Netherlands. Four different patient groups were studied: (1) chronic intermittent hemodialysis (HD) patients treated with ESA; (2) continuous ambulatory and cycler assisted peritoneal dialysis (CAPD and CCPD) patients treated with ESA; (3) predialysis patients treated with ESA; and (4) predialysis patients not treated with ESA. All patients who started treatment with HD, CAPD or CCPD and were treated with ESA were included. Predialysis patients were included if estimated GFR was 40 ml/min/1.73m^2 or less, using the MDRD (Modified Diet in Renal Disease) formula. The first four months of ESA treatment were not included, since this period is generally required to reach target Hb concentrations.[8]

Data collection

Data were collected during the arbitrarily selected period between January 1, 2005 to January 1, 2007. Hb levels were obtained from the Labosys® laboratory system (Philips Healthcare, Best, Netherlands). For dialysis patients, records of ESA doses were obtained from hospital pharmacy records. For the predialysis patients, the required data on ESA doses were obtained from the patient files. Information about hospitalizations was obtained from electronic patient files, as were the patient characteristics, including gender, age, weight, co-morbidity, medication use, MDRD and cause of CKD.

Assessment of hemoglobin cycling and time in target

We determined the number of patients maintaining Hb levels below, within and above a target range of 11-12 g/dL over a period of 6,12 and 24 months. It must be noted that at the time of the study, this range of 11-12 g/dL was not perceived as the predefined target range in all the patient groups.

According to the classification described by Ebben et al[13], patients were divided on the basis of their pattern of Hb fluctuation: SL = stable low (Hb levels below target range); SI = stable within the target range (Hb levels within target range); SH = stable high (Hb levels above target range); LAL = low amplitude swing with low Hb levels (Hb levels both within and below target range); LAH = low amplitude swing with high Hb levels (Hb levels both within and above target range); HA = high amplitude swing (Hb levels below, within and above target range).

To divide the patients in one of the 6 cycle groups, all available Hb levels over a period of 6, 12 or 24 months were used. To calculate the amount of time that Hb levels remained within the target range, moving averages of the Hb levels were used. The moving average was calculated over a 6-week period, moving in 3-week steps:

$$MA_1 = \frac{\sum_{i=wk1}^{wk6}(Hb.measurements)_i}{\sum_{i=wk1}^{wk6} n_i},\ MA_2 = \frac{\sum_{i=wk4}^{wk9}(Hb.measurements)_i}{\sum_{i=wk4}^{i=wk9} n_i},\ \text{etc.}$$

(MA = moving average, n = number of Hb measurements over given period)

Applying this method, small fluctuations are removed. The time in target could not be assessed in predialysis patients, due to a shortage of Hb measurements per year in this group. For each patient, the maximum and minimum Hb values, based on all available Hb measurements, were determined to calculate the Hb variability.

Data analysis

Data are presented as means ± standard deviation. Within the cycle groups, the distribution of the variables 'time in target', 'ESA dose changes' and 'hospitalizations' is non-parametric, therefore the cycle groups were compared using the Kruskal Wallis test.

RESULTS

Data were calculated over a period of 6, 12 and 24 months. For clarity reasons, mainly the results over a 12-month period are shown. The characteristics of the study population are displayed in Table 1.

Table 1 Patient characteristics

Characteristic	HD	PD	Predialysis with ESA	Predialysis no ESA
Total (nr)	56	12	29	60
Age (yr)	74 [32 – 89]	53 [34 – 86]	75 [24 – 89]	73 [33 – 92]
Gender (male: female) (%)	61 : 39	33 : 67	48 : 52	73 : 27
Weight (kg)	74.3 ± 16.1	73.3 ± 22.0	69.6 ± 11.4	83.4 ± 16.6
Diabetes mellitus (%)	28.6	16.7	41.3	40.0
Hypertension (%)	71.4	83.3	65.5	73.3
ESA treatment (%)	100.0 (i.v.)	100.0 (s.c.)	100.0 (s.c.)	0.0
Iron treatment (%)	100.0 (i.v.)	66.7 (oral)	72.4 (oral)	28.3 (oral)
Cause of CKD (%)				
Vascular	60.7	33.3	75.9	70.0
Glomerular	7.1	25.0	3.5	6.7
Interstitial	3.6	0.0	3.5	6.7
Hereditary	7.1	0.0	6.9	3.3
Urological	7.1	8.3	3.5	3.3
Other	0.0	0.0	3.5	3.3
Unknown	14.3	33.3	3.5	6.7

The variable 'age' is presented as median with the total range, the variable 'weight' is presented as mean with standard deviation. The categorical variables are presented as percentages of the total.
Abbreviations: i.v., intravenous; s.c., subcutaneous.

Hemodialysis patients

In Figure 1, the effect of time on the distribution of HD patients among the cycling groups is shown. When analyzed over a 6-month period, 39.1% of the HD patients show a high amplitude swing, whereas analysis over a 2-year period resulted in 93.1% of the patients in the high amplitude group. Over a 1-year period, on average 29±16 Hb measurements were available per patient. Over this period, 78.6% of the HD patients experienced a high amplitude swing and no patients remained stable, either inside or outside target range (Figure 2). The average amplitude of the Hb variability of the total HD population was 4.0±1.0 g/dL. Among the cycle groups, Hb levels were in target a comparable amount of time. In the LAH cycle group, Hb levels were within the target range 29±10% of time. The LAL group remained within target 20±12% of time and the HA group 28±15% of time. The same was the case for the number of ESA dose changes. In the HD population, 2.0±1.7 ESA dose changes per year per patient were observed. ESA dose was changed 2.1±1.6 times per year in the HA cycle group, 1.3±1.3 times in the LAH cycle group, and the LAL cycle group had 1.5±1.8 ESA dose changes per year. The number of hospitalizations was significantly different amongst the cycle groups (p=0.02). The HD patients in the LAL cycle group experienced most hospitalizations per patient year: 2.8±3.6. The LAH group experienced 0.3±0.5 hospitalizations and the HA group 1.8±1.8 hospitalizations per patient per year.

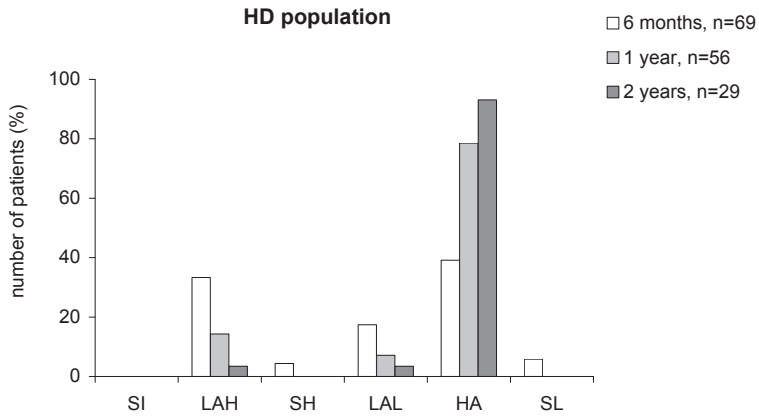

Figure 1 Hemodialysis population, classified according to Ebben's principle[13], analyzed over a period of 6 months, 1 year and 2 years.
Abbreviations: HD, hemodialysis; SI, stable within the target range; LAH, low amplitude swing with high Hb levels; SH, stable high; LAL, low amplitude swing with low Hb levels; HA, high amplitude swing; SL, stable low

Peritoneal dialysis patients

On average, 14±6 Hb measurements were available per patient over the 1-yr period. All cycle groups were represented, except the SI group, and 33.3% of the PD patients (n=4) experienced a high amplitude swing (Figure 2). The average amplitude of the Hb variability of the PD population was 3.1±0.3 g/dL. In the LAH cycle group, Hb levels were within the target range 43±35% of time. The LAH patients remained within target 15±9% of time and the HA patients 27±6% of time. The 3 patients experiencing stable

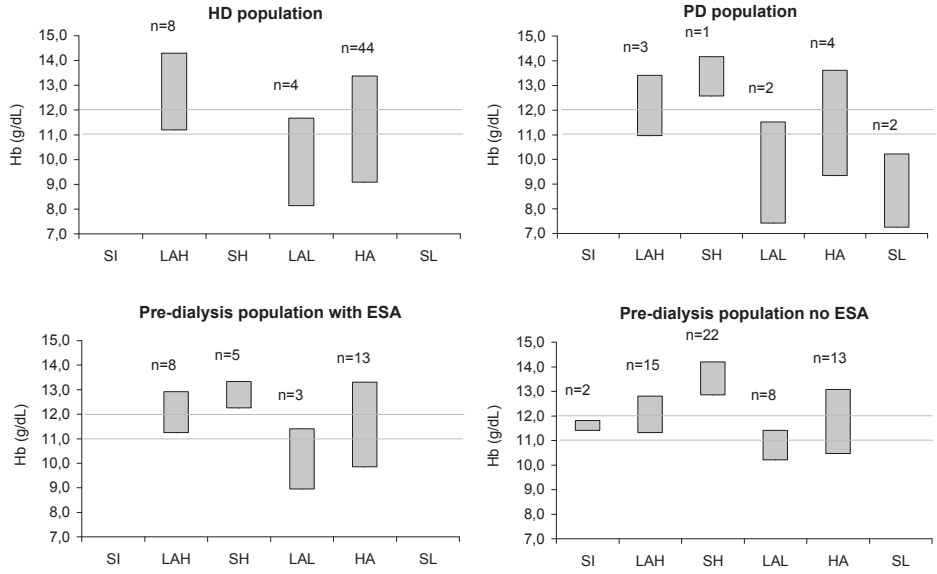

Figure 2 Average Hb variability over a 1-year period, classified according to Ebben's principle.[13]
Abbreviations: HD, hemodialysis; PD, peritoneal dialysis; ESA, erythropoietic-stimulating agent; SI, stable within the target range; LAH, low amplitude swing with high Hb levels; SH, stable high; LAL, low amplitude swing with low Hb levels; HA, high amplitude swing; SL, stable low

Hb levels outside the target range (SH and SL) did not remain between 11 and 12 g/dL at any time. In the PD population, on average 1.0±1.0 ESA dose changes per patient per year were observed. The HA cycle group experienced 1.8±1.0 ESA dose changes per patient per year, the LAH group had 0.3±0.6 dose changes, the LAL patients had 1.0±1.4 dose changes and the SL patients had 1.0±1.4 ESA dose changes per patient per year. The ESA dose for the patient with stable high Hb levels was not changed over the 1 yr period. The small number of patients in this group does not allow statistical testing with respect to hospitalization according to cycle groups. As an alternative, the number of hospitalizations per cycle group is reported. The two patients in the LAL cycle group experienced most hospitalizations per patient year: 2.0±2.8. The LAH patients experienced on average 0.3±0.6 hospitalizations per year, the single SH patient was not admitted in the hospital during the year of analysis, the HA patients experienced 1.8±0.9 hospitalizations and the two SL patients had 0.5±0.7 hospitalizations per year.

Predialysis patients with ESA treatment

On average, 11±8 Hb measurements were available per patient over the 1-yr period. No patients remained stable in or below target range and 44.8% of the patients experienced a high amplitude swing (Figure 2). The average amplitude of the Hb variability of the predialysis population with ESA treatment was 2.4±0.5 g/dL. In this population, on average 1.1±1.1 ESA dose changes per year per patient were observed. The LAH cycle group had 0.7±0.6 ESA dose changes per year, the LAL cycle group had 1.0±1.0 ESA dose changes and the HA cycle group experienced 1.8±1.3 ESA dose changes. Interestingly, the

SH cycle group had very few ESA dose changes per patient per year: 0.2±0.3 (p=0.03). However, it should be pointed out that the most recent KDOQI guidelines were updated in September 2007 based on studies published in late 2006.[7] The Dutch guideline during this retrospective study was based on the revised EBPG 2004.[6,8] In this guideline the upper limit received less emphasis than in the most recent KDOQI guidelines. The patients in the LAL cycle group experienced on average 1.3±1.5 hospitalizations per year, the HA group 0.7±0.9 hospitalizations, and the SH group 0.6±1.3. No patients in the LAH cycle group were admitted to the hospital over the 1 yr period. These values show no significant difference (p=0.20).

Predialysis patients without ESA treatment

On average, 8±3 Hb measurements were available per patient over the 1-yr period. In this group, 36.7% experienced Hb levels that were stable above 12 g/dL and no patients remained stable below 11 g/dL (Figure 2). The average amplitude of the Hb variability of the predialysis patients without ESA treatment was 1.6±0.3g/dL. The patients in the HA cycle group experienced on average 0.6±0.8 hospitalizations per year, the LAL group 0.3±0.7 hospitalizations, the LAH group 0.3±0.6 and the SH group 0.2±0.4. The two patients in the SI cycle group were not admitted in the hospital over the 1- year period. These values show no significant difference (p=0.28).

DISCUSSION

The main finding of this study is that Hb cycling occurs in the Netherlands in dialysis patients as well as in predialysis patients. Over a 1-year period, no CKD patients using ESA had Hb levels stable within a target range of 11-12 g/dL. The HD population showed overall Hb variability over the largest range: Hb values varied on average 4.0±1.1g/dL. Comparing the four patient groups, there was a stepwise decrease in the magnitude of Hb fluctuation starting, respectively, from the HD patients, the PD patients, the predialysis patients using ESA, to the predialysis patients not using ESA.

The finding that patients receiving ESA had greater variability than patients not receiving ESA is in keeping with recent studies and points to the current practice of prescribing ESA as one of the causes of Hb variability.[21,22,24]

Several measures exist to define magnitude, frequency and duration of variability.[25] The within patient standard deviation (SD) and residual variation from least-squares regression lines (residual SD) are accurate measures of variability, but do not reflect patterns of Hb variability.[15] The classification described by Ebben does provide patterns of variability but statistical analysis is limited since it does not provide a quantitative measure of Hb variability.[13] For the purpose of the analysis of our small pilot study, the classification described by Ebben was used.

With regard to the concept of hemoglobin variability, there are two other issues of great importance: the identification of factors causing variation; and the clinical consequences of variation. For instance, most[19,20,24] but not all studies[26] report that variability is associated with mortality, however, there are not data to support the concept that variability is the

cause of increased mortality.

There are several possible factors that could play a role in the development of Hb cycling. One of those factors is adjustment of ESA dose. Fishbane et al. found that in hemodialysis patients, 84% of up-excursions were associated with an ESA dose increase and 62% of down-excursions were related to dose reduction.[11] We could not confirm this in the HD patients in our study, as the number of ESA dose changes was comparable between the different cycle groups. In the predialysis patients treated with ESA, the HA cycle group had significantly more ESA dose changes than the other cycle groups.

Another key factor that is associated with cycling is hospitalization. In the current study, among the ESA-treated patient populations, patients in the LAL cycle group experienced most hospitalizations and patients in the LAH cycle group experienced least hospitalizations. This is in accordance with the results found by Ebben et al, who showed that patients in the SL, HA and LAL groups had a higher percentage of hospital admission than patients in the LAH, SI and SL groups.[13]

This study has limitations that should be considered. The data were collected retrospectively in one dialysis centre. The obtained database therefore reflects only a small sample of the entire Dutch dialysis and predialysis population. Nonetheless, the findings of this study are consistent with those in other publications.[9,11,13]

In conclusion, this study shows that in the Netherlands, Hb variability is common in HD and PD patients, but also in predialysis patients. The magnitude of Hb fluctuation seems to decrease stepwise starting, respectively, from the HD patients, PD patients, the predialysis patients treated with ESA, to the predialysis patients not treated with ESA. It was observed that over a 1-year period, no CKD patients using ESA had Hb levels stable within the target range as recommended by the most recent guidelines. This reflects the difficulty of maintaining Hb levels within a narrow range as recommended by the most recent guidelines. This is probably caused by provider practices, such as frequent ESA adjustments, in combination with patient factors, such as co-morbidity and hospitalization. The question is whether adaptation of treatment policies can contribute to decrease cycling, and whether this could influence outcome. To answer this question, further studies are needed to clarify the relationship between provider practices and Hb variability and mortality.

REFERENCES

1. Regidor DL, Kopple JD, Kovesdy CP, *et al.* Associations between changes in hemoglobin and administered erythropoiesis-stimulating agent and survival in hemodialysis patients. *J Am Soc Nephrol* 2006;17:1181-1191.
2. Rao M, Pereira BJ. Optimal anemia management reduces cardiovascular morbidity, mortality, and costs in chronic kidney disease. *Kidney Int* 2005;68:1432-1438.
3. Foley RN, Parfrey PS, Harnett JD, *et al.* The impact of anemia on cardiomyopathy, morbidity, and and mortality in end-stage renal disease. *Am J Kidney Dis* 1996;28:53-61.
4. Collins AJ, Ma JZ, Ebben J. Impact of hematocrit on morbidity and mortality. *Semin Nephrol* 2000;20:345-349.
5. Singh AK, Szczech L, Tang KL, *et al.* Correction of anemia with epoetin alfa in chronic kidney disease. *N Engl J Med* 2006;355:2085-2098.
6. Locatelli F, Aljama P, Barany P, *et al.* Revised European best practice guidelines for the management of anaemia in patients with chronic renal failure. *Nephrol Dial Transplant* 2004;19 Suppl 2:ii1-47.
7. KDOQI Clinical Practice Guideline and Clinical Practice Recommendations for anemia in chronic kidney disease: 2007 update of hemoglobin target. *Am J Kidney Dis* 2007;50:471-530.
8. Anemia directive based on the Revised European best practice guidelines for the management of anaemia in patients with chronic renal failure. *http://www.nefro.nl* 2004.
9. Berns JS, Elzein H, Lynn RI, *et al.* Hemoglobin variability in epoetin-treated hemodialysis patients. *Kidney Int* 2003;64:1514-1521.
10. Lacson E, Jr., Ofsthun N, Lazarus JM. Effect of variability in anemia management on hemoglobin outcomes in ESRD. *Am J Kidney Dis* 2003;41:111-124.
11. Fishbane S, Berns JS. Hemoglobin cycling in hemodialysis patients treated with recombinant human erythropoietin. *Kidney Int* 2005;68:1337-1343.
12. Collins AJ, Brenner RM, Ofman JJ, *et al.* Epoetin alfa use in patients with ESRD: an analysis of recent US prescribing patterns and hemoglobin outcomes. *Am J Kidney Dis* 2005;46:481-488.
13. Ebben JP, Gilbertson DT, Foley RN, *et al.* Hemoglobin level variability: associations with comorbidity, intercurrent events, and hospitalizations. *Clin J Am Soc Nephrol* 2006;1:1205-1210.
14. Fishbane S, Berns JS. Evidence and implications of haemoglobin cycling in anaemia management. *Nephrol Dial Transplant* 2007;22:2129-2132.
15. Yang W, Israni RK, Brunelli SM, *et al.* Hemoglobin variability and mortality in ESRD. *J Am Soc Nephrol* 2007;18:3164-3170.
16. Goodkin DA, Gimenez LF, Graber SE, *et al.* Hematocrit stability following intravenous versus subcutaneous administration of epoetin alfa to dialysis patients: a post hoc analysis. *Clin Nephrol* 1999;51:367-372.
17. Bellizzi V, Minutolo R, Terracciano V, *et al.* Influence of the cyclic variation of hydration status on hemoglobin levels in hemodialysis patients. *Am J Kidney Dis* 2002;40:549-555.
18. Ifudu O, Feldman J, Friedman EA. The intensity of hemodialysis and the response to erythropoietin in patients with end-stage renal disease. *N Engl J Med* 1996;334:420-425.
19. Gilbertson DT, Ebben JP, Foley RN, *et al.* Hemoglobin level variability: associations with mortality. *Clin J Am Soc Nephrol* 2008;3:133-138.
20. Brunelli SM, Joffe MM, Israni RK, *et al.* History-adjusted marginal structural analysis of the association between hemoglobin variability and mortality among chronic hemodialysis patients. *Clin J Am Soc Nephrol* 2008;3:777-782.
21. Spiegel DM. Hemoglobin Variability in Chronic Kidney Disease: A Cross-Sectional Study. *Am J Med Sci* 2009.
22. Minutolo R, Chiodini P, Cianciaruso B, *et al.* Epoetin therapy and hemoglobin level variability in nondialysis patients with chronic kidney disease. *Clin J Am Soc Nephrol* 2009;4:552-559.
23. DeFrancisco AL, Macdougall IC, Carrera F, *et al.* Intercurrent events and comorbid conditions influence hemoglobin level variability in dialysis patients. *Clin Nephrol* 2009;71:397-404.

24. Boudville NC, Djurdjev O, Macdougall IC, *et al.* Hemoglobin variability in nondialysis chronic kidney disease: examining the association with mortality. *Clin J Am Soc Nephrol* 2009;4:1176-1182.
25. Kalantar-Zadeh K, Aronoff GR. Hemoglobin variability in anemia of chronic kidney disease. *J Am Soc Nephrol* 2009;20:479-487.
26. Eckardt KU, Fouqueray B, Macdougal IC, *et al.* Hemoglobin variability is not an independent predictor of mortality in a European haemodialysis populaton: results of the ARO study. *World congress of Nephrology, Milan* 2009.

CHAPTER 8

Determinants of hemoglobin variability in CKD patients with and without renal replacement therapy

Frederieke H van der Baan[1*]
Karien van der Putten[2,3*]
Willem Bax[4]
Carlo AJM Gaillard[2,5]

[1]Julius Center for Health Sciences and Primary Care, Utrecht University, Utrecht, The Netherlands
[2]Department of Internal Medicine, Meander MC, Amersfoort, the Netherlands
[3]Department of Nephrology, Leiden University Medical Center, Leiden, the Netherlands
[4]Department of Internal Medicine, Medical Center Alkmaar, Alkmaar, the Netherlands
[5]Department of Nephrology, VU University Medical Center, Amsterdam, the Netherlands

*The first two authors contributed equally

Submitted

ABSTRACT

Background and objectives

Patients with CKD exhibit a significant variation in intra-individual hemoglobin (Hb) levels, which are often outside the target range. In this study we investigated to what degree CKD patients maintain Hb levels within the recommended target range, which factors are associated with Hb variability and which differences exist between CKD treatment modalities.

Design, setting, participants, & measurements

This retrospective study was executed in 20 Dutch dialysis centres. We studied 898 hemodialysis (HD), 184 peritoneal dialysis (PD) and 115 predialysis patients, all treated with erythropoiesis stimulating agents (ESA), and 198 predialysis patients without ESA. Hb amplitude, residual SD and the pattern of Hb fluctuation (the classification as described by Ebben) were used as measures of Hb variability.

Results

Over a 1-year period, 1.7% of ESA-treated patients had stable Hb levels within the target range of 11-12 g/dl. In HD and PD patients, Hb levels remained within the target range for respectively 25 and 22% of time. A stepwise decrease in Hb fluctuation was observed from respectively the HD patients, PD patients, predialysis patients using ESA, to the predialysis patients without ESA. Frequent Hb sampling and ESA dose adjustments, average ESA dose and hospitalizations were the main predictors of Hb variability.

Conclusions

Maintaining Hb within targets according to the guidelines is rarely achieved in daily practice, which can be attributed to the narrow target range and to provider practices. It may be hypothesized that adaptation of the guideline as well as ESA prescribing protocols contribute to decrease Hb variability.

INTRODUCTION

In CKD patients anemia is common and associated with increased morbidity and mortality.[1-4] No trials that accurately define the optimal and safe goal for treatment of anemia with erythopoiesis stimulating agents (ESA) are available. Following recent studies that failed to show a beneficial effect of (targeting for) normalization of hemoglobin (Hb) levels[5,6], the Anaemia Working Group of ERBP maintains its view that "Hemoglobin values of 11–12 g/dL should be generally sought in the CKD population without intentionally exceeding 13 g/dL" and that the doses of ESA therapy to achieve the target hemoglobin should also be considered.[7,8]

How these guidelines should be interpreted and implemented is matter of debate.[9] There are no data that show that implementing treatment strategies to actively reach and maintain the Hb target is beneficial. Furthermore the target Hb levels are in a narrow range. Hemodialysis patients treated with ESA show variable Hb levels that are often not maintained within the target.[10-14] Also in CKD patients not treated with ESA, a 'background' Hb variability exists.[15,16] Moreover, in healthy subjects, an intra-individual physiological day-to-day variation in Hb levels is seen with a coefficient of variation of 4.5%.[17] Therefore, the feasibility of maintaining a patient's Hb levels in the recommended range can be questioned.

The aim of this retrospective multicentre study was to investigate to what degree CKD patients maintain Hb levels within the recommended target range, which factors predict Hb variability and which differences exist between CKD treatment modalities. We studied patients on HD, PD, and not on renal replacement therapy (RRT). In these treatment groups we aimed to identify patient-related, treatment-related as well as dialysis center-related factors associated with Hb variability using several different measures of Hb variability. Within patient standard deviation (SD) and residual variation from least-squares regression lines (residual SD) are accurate measures of variability but do not reflect patterns of Hb variability.[18] The classification described by Ebben does provide patterns of variability but statistical analysis is limited since it does not provide a quantitative measure of Hb variability.[13] Therefore, for the purpose of this study, we compared Hb amplitude, residual SD and the classification as described by Ebben.

MATERIALS AND METHODS

Study population

This multicentre retrospective study in patients with CKD was conducted in 20 dialysis centers (3 university medical centers and 17 regional hospitals) in the Netherlands. Four patient groups were studied: (1) hemodialysis (HD) patients treated with ESA, (2) peritoneal dialysis (PD) patients treated with ESA, (3) predialysis patients treated with ESA and (4) predialysis patients not treated with ESA. All ESA treated patients who received HD or PD for at least 6 successive months were included. Predialysis patients were included when treated at an outpatient clinic designated for predialysis patients for a period of at least 12 successive months. The first four months of ESA treatment were not included in the analysis, since this period is generally required to reach target Hb concentrations.

Data collection

Data were collected from January 1st 2005 to January 1st 2007. For all predialysis patients the estimated Glomerular Filtration Rate (eGFR) according to the modification of diet in renal disease (MDRD) formula was calculated. Furthermore, we collected Hb, ferritin, parathyroid hormone (PTH) and C-reactive protein (CRP) levels. For dialysis patients, records of ESA and iron doses were obtained from hospital pharmacy records. For the predialysis patients, the required data on ESA and iron doses were obtained from the patient files. Information about hospitalizations, surgeries and blood transfusions was obtained from electronic patient files, as were the patient characteristics.

Assessment of hemoglobin variability and time in target

The degree of Hb variability was assessed using four different methods. First, we used Hb amplitude as a measure of Hb variability. For each patient, the maximum and minimum Hb value, based on all available Hb measurements over a 1-year period, was determined to calculate the Hb amplitude. Secondly, in HD and PD patients, we determined residual variation from least-squares regression lines (residual SD), an accurate measure of variability although it does not reflect patterns of Hb variability.[18] Therefore, we also determined the number of patients maintaining Hb levels below, within and above a target range of 11-12 g/dl over a period of 12 months. According to the classification described by Ebben et al[13], patients were divided on the basis of their pattern of Hb fluctuation: stable low (Hb levels below target range), stable within the target range (Hb levels within target range), stable high (Hb levels above target range), low amplitude with low Hb levels (Hb levels both within and below target range), low amplitude with high Hb levels (Hb levels both within and above target range) and high amplitude (Hb levels below, within and above target range). To allocate the patients to one of the 6 groups, all available Hb levels over a period of 12 months were used. Only ESA-treated patients were classified according to this method, since the target range of 11–12 g/dl does apply to these patients only.

Finally, we calculated the amount of time that Hb levels remained within the target range. For these calculations, moving averages of Hb levels were used.[10] The moving average was calculated over a 6-week period, moving in 3-week steps. In predialysis patients the number of Hb measurements did not allow assessment of time in target. Average Hb levels were determined for each patient, based on all available Hb measurements.

Definition of variables

Renal function, ferritin and PTH were calculated by averaging respectively all eGFR values and ferritin- and PTH-levels over 1 year. The number of blood transfusions per patient was low; therefore the dichotomised variable 'transfusion' was used. 'Infection' was defined as the number of 2-week episodes with a mean CRP level above 15 mg/l over a 1-year period. 'Hospitalization' was defined as a stay at the hospital of minimally one night. ESA doses were calculated only for the patients who did not switch ESA's over the 1-year period. 'ESA dose' was defined as the mean dose per week (Darbepoetin alfa doses (μg/week) were converted to Epoetin alpha or beta doses in IU/week using a factor 200).

Data analysis

Data are presented as means ± standard deviation or medians with interquartile range as appropriate. The 4 patient groups were compared using ANOVA or Kruskal Wallis test for continuous variables, depending on the distribution of the data. Categorical variables were compared using the Chi Square test. To determine univariate associations between continuous variables and Hb amplitude, Pearson's correlations were used after evaluation of scatterplots. Univariate associations between categorical variables and Hb amplitude were examined using ANOVA. To characterize the independent contribution of the variables to Hb amplitude, multivariate analysis was performed using forward linear regression analysis. P-values <0.05 were considered to represent statistical significance. Conditional imputation was used for the missing data in BMI (12.4% missing), since data were expected to be missing completely at random. In other cases of missing data, complete case analysis was performed. The Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) version 17 was employed for all statistical analysis. Validity of the statistical analysis was monitored by the Center of Biostatistics of the University of Utrecht.

RESULTS

A total of 898 HD patients, 184 PD patients, 115 predialysis patients using ESA and of 198 predialysis patients not using ESA were included in the analysis. In table 1, the characteristics of the study population are shown. The PD patients had a lower median age than the other 3 patient groups whereas the HD patients had the lowest BMI of the four groups. The subdivision of the types of ESA that were used is different across the 4 patient groups, as are the ESA doses. Also, as expected, iron treatment differed between the groups.

Hb variability characteristics

Table 2 shows the Hb variability characteristics of the 4 patient groups. The number of Hb measurements differed significantly among the groups, with exception of the 2 predialysis patient groups, who had a comparable number of Hb measurements. Average Hb levels were significantly lower in both dialysis patient groups when compared with the patients without RRT ($p<0.01$). Hb amplitude was significantly different in the 4 groups ($p<0.01$). In both HD patients and PD patients, a strong correlation was found between Hb amplitude and residual SD ($r=0.63$, $p<0.001$ and $r=0.82$, $p<0.001$ respectively). The Hb levels of HD and PD patients were found to be within the target range for a comparable amount of time (respectively 25 and 22%).

Table 1 General patient characteristics

Characteristic	HD (n=897)	PD (n=183)	Predialysis with ESA (n=115)	Predialysis no ESA (n=198)
Age (yr)	70 [57-78]	60 [48 -71]	72 [60 - 78]	71 [60 - 77]
Gender (% male)	60.1	59.6	60.9	65.7
BMI (kg/m^2)	25.8 ± 4.3	26.2 ± 4.3	26.6 ± 4.4	27.2 ± 4.5
Diabetes mellitus (%)	26.2	20.2	27.0	33.8
Hypertension (%)	64.9	69.9	69.6	72.7
eGFR (ml/min)	-	-	14.7 [10.7 - 21]	18.9 [13.1 - 29.7]
ESA treatment (%)	100.0	100.0	100.0	0.0
Epoetin beta	48.4	50.8	30.4	-
Epoetin alpha	9.8	0.0	1.7	-
Darbepoetin alpha	22.2	31.1	50.4	-
Switch	19.6	18.1	17.5	-
ESA dose (IU/week)	9326 [5945 -14375]	6000 [4000 - 9805]	3449 [2000 - 6000]	-
Iron treatment (%)	94.5	78.1	73.9	47.5
Ferritin (µg/L)	380.7 [213.1-635.7]	181.6 [87.2 - 329.0]	*	*

Data are expressed as mean ± SD, or median [IQR]. *, not reported due to high number of missing values. HD, hemodialysis; PD, peritoneal dialysis; ESA, erythropoiesis-stimulating agent; BMI, Body Mass Index; eGFR, estimated glomerular filtration rate

Table 2 Hb variability over a 12-month period

Variable	HD (n=897)	PD (n=183)	Predialysis with ESA (n=115)	Predialysis no ESA (n=198)
Hb measurements (no.)	16 [13-24]	10 [8 - 14]	7 [5 - 10]	5 [4 - 8]
Patients per cycle group (%)				
Stable within target	0.1	0.5	1.7	-
Low ampl. high Hb	21.0	20.2	29.6	-
Stable high	8.0	16.4	29.6	-
Low ampl. low Hb	4.7	7.7	6.1	-
High ampl.	65.8	51.4	29.6	-
Stable low	0.4	3.8	3.4	-
Average Hb (g/dl)	11.9 ± 0.9	11.9 ± 1.2	12.2 ± 1.2	12.4 ± 1.3
Hb amplitude (g/dl)	3.8 ± 1.6	3.0 ± 1.6	2.3 ± 1.5	1.7 ± 1.3
Residual SD (g/dl)	0.8 ± 0.4	0.7 ± 0.4	-	-
Time in target range (%)	25.0 [12.5-40.6]	21.9 [7.1-50.0]	-	-

Data are expressed as mean ± SD, or median [IQR]. HD, hemodialysis; PD, peritoneal dialysis; ESA, erythropoiesis-stimulating agent; Hb ,hemoglobin

Univariate analysis

To determine a possible association between various (patient and treatment) factors and the degree of Hb variability, correlations were calculated between these factors and Hb amplitude. The results for continuous variables are displayed in table 3. Considering the categorical variables, HD patients receiving 1 or more blood transfusions over a 1-year period had a significantly higher Hb amplitude than HD patients not receiving a blood transfusion (5.2±1.6 vs 3.5±1.5 g/dl, $p<0.001$). This was also the case for PD patients (4.3±1.5 vs 2.9±1.6 g/dl, $p=0.001$) and the predialysis patients using ESA (4.0±1.8 vs 2.2±1.3 g/dl, $p<0.001$). In the predialysis patients not using ESA, the incidence of blood transfusions was too low for calculations. Gender, the presence of diabetes mellitus and hypertension were not associated with Hb amplitude in any of the 4 patient groups. The type of ESA was associated with Hb amplitude only in the HD patients. Patients using Epoetin alpha had the lowest Hb amplitude (mean 3.3 g/dl, $p=0.011$), while patients using Epoetin beta and Darbepoetin alpha had comparable Hb amplitudes (mean 3.8 vs 3.9 g/dl respectively).

Multivariate analysis

To further characterize the independent as well as cumulative association of the variables with Hb variability, we constructed multiple linear regression models for each of the four patient groups. For the two dialysis patient groups, regression models were constructed for both Hb amplitude and residual SD. The results of these analyses are displayed in tables 4a, b, c and d. Combinations involving the significant factors associated with Hb amplitude predicted 38% of the variance in Hb amplitude in HD patients, 48% in PD patients, 58% in predialysis patients using ESA and 31% in predialysis patients not using ESA. The regression models for residual SD predicted 15% of residual SD in HD patients and 32% in PD patients.

Table 3 Univariate analysis: associations with Hb amplitude

	HD (n=897)		PD (n=183)		Predialysis with ESA (n=115)		Predialysis no ESA (n=198)	
Variable	**r**	**p-value**	**r**	**p-value**	**r**	**p-value**	**r**	**p-value**
Age (yr)	-0.08	0.02	-0.19	0.01	-0.04	0.64	-0.03	0.67
BMI (kg/m^2)	-0.16	<0.001	-0.09	0.20	-0.19	0.05	-0.01	0.89
eGFR (ml/min)	-	-	-	-	-0.06	0.51	-0.04	0.58
PTH (pmol/L)	0.01	0.78	-0.03	0.70	0.14	0.17	0.02	0.78
Ferritin (µg/L)	0.05	0.13	0.21	0.004	*		*	
Hb meas. (no.)	0.43	<0.001	0.62	<0.001	0.62	<0.001	0.60	<0.001
Average Hb (g/dL)	-0.25	<0.001	-0.15	0.04	-0.33	<0.001	-0.24	0.001
ESA dose (IU/week)	0.17	<0.001	0.36	<0.001	0.28	0.006	-	-
ESA dose change (no.)	0.32	<0.001	0.34	<0.001	0.51	<0.001	-	-
Iron dose change (no.)	0.07	0.04	0.16	0.04	0.45	<0.001	0.13	0.07
Infection (no.)	0.34	<0.001	0.46	<0.001	0.32	<0.001	0.36	<0.001
Hospitalization (no.)	0.39	<0.001	0.48	<0.001	0.40	<0.001	0.47	<0.001
Surgery (no.)	0.28	<0.001	0.36	<0.001	0.23	0.01	0.17	0.01

*, not reported due to high number of missing values. HD, hemodialysis; PD, peritoneal dialysis; ESA, erythropoiesis-stimulating agent; Hb, hemoglobin; BMI, Body Mass Index. eGFR, estimated glomerular filtration rate. PTH, parathyroid hormone.

Table 4a Final multiple linear regression model for Hb amplitude and Residual SD in hemodialysis patients

	Hb amplitude (g/dL)		Residual SD (g/dL)	
Variable	**Standardized β**	**p-value**	**Standardized β**	**p-value**
Age (yr)	-0.10	0.001		
BMI (kg/m^2)	-0.10	0.003		
Ferritin (μg/L)	0.11	<0.001	0.16	<0.001
Hb meas. (no.)	0.17	<0.001		
ESA dose change (no.)	0.30	<0.001	0.36	<0.001
Iron dose change (no.)	-0.08	0.01	-0.09	0.02
Transfusion (y/n)	0.20	<0.001		
Infection (no.)	0.09	0.02		
Hospitalization (no.)	0.25	<0.001	0.13	<0.001
Intercept	3.70		0.31	
R^2	0.38		0.15	

Abbrevations: BMI, Body Mass Index; ESA, erythropoiesis-stimulating agent; Hb, hemoglobin

Table 4b Final multiple linear regression model for Hb amplitude and Residual SD in peritoneal dialysis patients

	Hb amplitude (g/dL)		Residual SD (g/dL)	
Variable	**Standardized β**	**p-value**	**Standardized β**	**p-value**
Age (yr)	-0.13	0.04	-0.16	0.03
Ferritin (μg/L)	0.13	0.04		
Average Hb (g/dL)			0.17	0.02
ESA dose (IU/wk)	0.25	<0.001	0.30	<0.001
ESA dose change (no.)	0.29	<0.001	0.40	<0.001
Infection (no.)	0.33	<0.001		
Surgery (no.)	0.23	<0.001	0.20	0.005
Intercept	2.15		-0.02	
R^2	0.48		0.32	

The number of Hb measurements was not entered into the model to prevent multicollinearity.
Abbrevations: ESA, erythropoiesis-stimulating agent; Hb, hemoglobin

Table 4c Final multiple linear regression model for Hb amplitude in predialysis patients with ESA

Variable	Standardized β	p-value
BMI (kg/m^2)	-0.30	<0.001
ESA dose (IU/wk)	0.30	<0.001
ESA dose change (no.)	0.27	0.004
Iron dose change (no.)	0.31	0.001
Hospitalization (no.)	0.37	0.001
Intercept	3.77	
R^2	0.58	

The number of Hb measurements was not entered into the model to prevent multicollinearity.
Abbrevations: BMI, Body Mass Index; ESA, erythropoiesis-stimulating agent; Hb, hemoglobin

Table 4d Final multiple linear regression model for Hb amplitude in predialysis patients without ESA

Variable	Standardized β	p-value
Hb meas. (no.)	0.38	<0.001
Average Hb (g/dL)	-0.19	0.01
Hospitalization (no.)	0.20	0.02
Intercept	3.52	
R^2	0.31	

Abbrevation: Hb, hemoglobin

Comparison of dialysis centers

A total of 19 dialysis centers was compared to determine whether center-related factors could predict Hb amplitude in HD patients. There was no relation between Hb amplitude and the size of the dialysis centers. HD patients treated in university medical centers showed a higher Hb amplitude when compared to patients treated in regional hospitals (4.1 vs 3.7 g/dL, p=0.02). The number of Hb measurements was comparable between university vs. regional clinics. However, HD patients in university medical centers had more ESA dose adjustments (p<0.001) and blood transfusions (p=0.002) than patients in regional hospitals.

A substantial variation was found in the number of Hb measurements in HD patients between all 19 centers (figure 1). When divided in tertiles according to Hb amplitude, the centers in the lowest tertile had less Hb measurements than the centers in the upper tertile (p=0.04). No relation was found between prescribed ESA dose or the number of ESA dose changes and Hb amplitude across the centers.

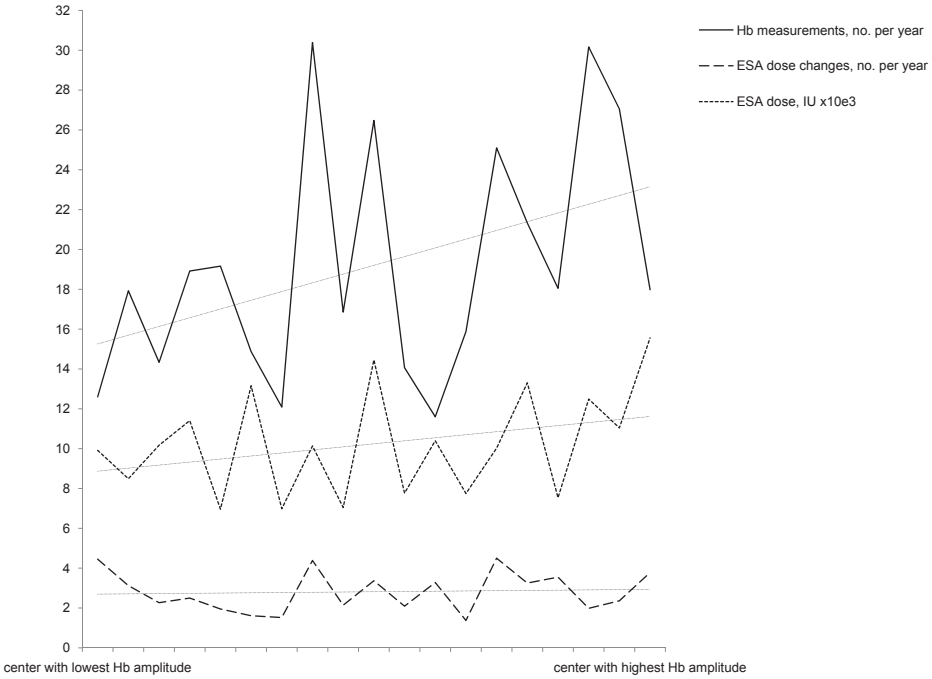

Figure 1 Variation in the number of Hb measurements, the number of ESA dose changes and ESA dose between the centers.

DISCUSSION

In this study, which was performed in the Netherlands, Hb variability was a common phenomenon. There was a significant difference in magnitude of Hb variability between the HD patients, the PD patients, the predialysis patients with ESA, and the predialysis patients not using ESA ($p<0.01$). In the predialysis patients not using ESA, the mean Hb amplitude over a 1-year period was 1.7 g/dl. Thus, in a patient group not treated with ESA, and, generally speaking, with less co-morbidity, the level of 'background' Hb variability is considerable. The residual SD values of 0.77 and 0.73 g/dL for respectively HD and PD patients are comparable with levels reported from U.S. as well as European HD databases.[19,20] Furthermore our data confirmed that over a 1-year period, according to the Ebben classification, only a very small percentage of patients had stable Hb levels within the target range of 11-12 g/dl. Together with the fact that the HD and PD patients in our study had Hb levels within the target range for respectively 25 and 22% of time, this illustrates the difficulty of maintaining Hb levels within the narrow range as recommended by the most recent guidelines.[7,8,21]

We found a stepwise decrease in the magnitude of hemoglobin variation from respectively the HD patients, the PD patients, the predialysis patients with ESA, to the predialysis patients not using ESA (table 2). This is in agreement with our earlier work.[22] Although our study was designed to study the predictive value of several variables,

we hypothesize that (some of) these variables may have a causal role, explaining the difference in Hb variability between the CKD treatment modalities. First, the number of Hb measurements could play a role. In both HD and PD patient groups, Hb levels were measured more often than in predialysis patients. Also, in multivariate analysis for all patients, the number of Hb measurements was an independent predictor for Hb amplitude (data not shown). This appears to be in contrast with data suggesting that frequent Hb monitoring would prevent unnecessary ESA dose adjustments, thereby reducing Hb variability.[23] Frequent Hb sampling directly leads to increased detection of changes in Hb amplitude. Therefore, an association between number of Hb measurements and variability may seem self-explanatory. On the other hand, increased Hb sampling may lead to an increased number of ESA dose adjustments. Indeed, there was a significant correlation between the number of Hb measurements and the number of ESA dose changes in all patients using ESA ($p<0.001$). Also the number of ESA dose changes was a main independent predictor of Hb amplitude in all EPO treated patient groups, as determined by multivariate testing (table 4a, b, c). ESA dosing could also be a factor that could contribute to the stepwise decrease in Hb amplitude between the three ESA-treated patient groups, as the average ESA dose was different among the groups ($P<0.001$) and was correlated with Hb amplitude ($p<0.001$). Again however, we can only speculate about causality. Patient factors -in particular hospitalizations- are also clearly associated with increased Hb variability. This was shown in univariate as well as multivariate analysis.

When analyzing the association between dialysis center-related factors and Hb amplitude, we found that Hb amplitude was significantly higher in university hospitals as compared to regional clinics ($p=0.02$). One may hypothesize that a combination of treatment factors (frequent ESA dose adjustments) and patient factors (blood transfusions) have a role here.

Combining all significant factors associated with Hb amplitude, we were able to predict 38% of Hb amplitude in the HD patients, 48% in the PD patients, 58% in the predialysis patients using ESA and 31% in the predialysis patients not using ESA. The percentage in the last group is the lowest, possibly caused by the contribution of ESA treatment on Hb amplitude in the other 3 groups. When comparing the multivariate models for Hb amplitude and residual SD, the predictive value differs remarkably. An earlier study reported similar low predictive values in multivariate analysis when using residual SD.[15]

All together, the current practice of blood sampling and ESA prescribing, in combination with patient factors, were the main predictors for Hb variability. This is in accordance with several recent studies.[12-14,24-26] The question is whether adaptation of treatment policies can contribute to decrease Hb variability, and whether this could influence outcome. Several[15,18,27] but not all[20] studies report that variability is associated with mortality. However, it is unknown whether Hb variability is the *cause* of increased mortality. Recently it has been put forward that the number of months with hemoglobin values below the target range rather than hemoglobin variability itself is the primary driving force of the increased risk[20,28], fueling not only the discussion whether cycling has a causative relation with mortality but also questioning whether any association exists. Indeed studies using Ebben's classification invariably reported an increase in mortality in the stable low hemoglobin groups.[15,20,28] However, even if Hb variability is not associated

with mortality, it is of clinical importance since the phenomenon has a marked influence on the ability of the care provider to reach and maintain treatment guidelines.

In summary, this study shows that in CKD patients in the Netherlands, Hb variability is common and is mainly associated with an increased number of Hb measurements, frequent ESA dose adjustments, and patient factors. The magnitude of Hb fluctuation decreased stepwise from the HD patients, to the PD patients, to the predialysis patients using ESA, with the lowest -but considerable- degree of Hb fluctuation in the predialysis patients not using ESA. The time that Hb levels remained within the target range as recommend by the guidelines was low in both the HD and PD patients. Although it is unknown whether hemoglobin variability is causally related to mortality, the results of this study raise two important questions. First, what are optimal anemia treatment (and target) strategies? A large body of evidence suggests that reaching *and* maintaining the hemoglobin targets in the current guidelines is not feasible. Should we continue to define hemoglobin targets? Secondly, would adaptation of ESA prescribing as well as blood sampling protocols decrease Hb variability?

REFERENCES

1. Astor BC, Muntner P, Levin A, *et al.* Association of kidney function with anemia: the Third National Health and Nutrition Examination Survey (1988-1994). *Arch Intern Med* 2002;162:1401-1408.
2. Foley RN, Parfrey PS, Harnett JD, *et al.* The impact of anemia on cardiomyopathy, morbidity, and and mortality in end-stage renal disease. *Am J Kidney Dis* 1996;28:53-61.
3. Locatelli F, Pisoni RL, Combe C, *et al.* Anaemia in haemodialysis patients of five European countries: association with morbidity and mortality in the Dialysis Outcomes and Practice Patterns Study (DOPPS). *Nephrol Dial Transplant* 2004;19:121-132.
4. Collins AJ, Ma JZ, Ebben J. Impact of hematocrit on morbidity and mortality. *Semin Nephrol* 2000;20:345-349.
5. Singh AK, Szczech L, Tang KL, *et al.* Correction of anemia with epoetin alfa in chronic kidney disease. *N Engl J Med* 2006;355:2085-2098.
6. Drueke TB, Locatelli F, Clyne N, *et al.* Normalization of hemoglobin level in patients with chronic kidney disease and anemia. *N Engl J Med* 2006;355:2071-2084.
7. Locatelli F, Aljama P, Barany P, *et al.* Revised European best practice guidelines for the management of anaemia in patients with chronic renal failure. *Nephrol Dial Transplant* 2004;19 Suppl 2:ii1-47.
8. Locatelli F, Aljama P, Canaud B, *et al.* Target haemoglobin to aim for with erythropoiesis-stimulating agents: a position statement by ERBP following publication of the Trial to reduce cardiovascular events with Aranesp therapy (TREAT) study. *Nephrol Dial Transplant* 2010;25:2846-2850.
9. Collins AJ, Ebben JP, Gilbertson DT. EPO adjustments in patients with elevated hemoglobin levels: provider practice patterns compared with recommended practice guidelines. *Am J Kidney Dis* 2007;49:135-142.
10. Berns JS, Elzein H, Lynn RI, *et al.* Hemoglobin variability in epoetin-treated hemodialysis patients. *Kidney Int* 2003;64:1514-1521.
11. Collins AJ, Brenner RM, Ofman JJ, *et al.* Epoetin alfa use in patients with ESRD: an analysis of recent US prescribing patterns and hemoglobin outcomes. *Am J Kidney Dis* 2005;46:481-488.
12. Fishbane S, Berns JS. Hemoglobin cycling in hemodialysis patients treated with recombinant human erythropoietin. *Kidney Int* 2005;68:1337-1343.
13. Ebben JP, Gilbertson DT, Foley RN, *et al.* Hemoglobin level variability: associations with comorbidity, intercurrent events, and hospitalizations. *Clin J Am Soc Nephrol* 2006;1:1205-1210.
14. Lacson E, Jr., Ofsthun N, Lazarus JM. Effect of variability in anemia management on hemoglobin outcomes in ESRD. *Am J Kidney Dis* 2003;41:111-124.
15. Boudville NC, Djurdjev O, Macdougall IC, *et al.* Hemoglobin variability in nondialysis chronic kidney disease: examining the association with mortality. *Clin J Am Soc Nephrol* 2009;4:1176-1182.
16. Spiegel DM. Hemoglobin variability in chronic kidney disease: a cross-sectional study. *Am J Med Sci* 2009;337:340-343.
17. Borel MJ, Smith SM, Derr J, *et al.* Day-to-day variation in iron-status indices in healthy men and women. *Am J Clin Nutr* 1991;54:729-735.
18. Yang W, Israni RK, Brunelli SM, *et al.* Hemoglobin variability and mortality in ESRD. *J Am Soc Nephrol* 2007;18:3164-3170.
19. Arneson TJ, Zaun D, Peng Y, *et al.* Comparison of methodologies to characterize haemoglobin variability in the US Medicare haemodialysis population. *Nephrol Dial Transplant* 2009;24:1378-1383.
20. Eckardt KU, Kim J, Kronenberg F, *et al.* Hemoglobin variability does not predict mortality in European hemodialysis patients. *J Am Soc Nephrol* 2010;21:1765-1775.
21. KDOQI Clinical Practice Guideline and Clinical Practice Recommendations for anemia in chronic kidney disease: 2007 update of hemoglobin target. *Am J Kidney Dis* 2007;50:471-530.
22. van der Putten K, van der Baan FH, Schellekens H, *et al.* Hemoglobin variability in patients with chronic kidney disease in the Netherlands. *Int J Artif Organs* 2009;32:787-793.
23. Gaweda AE, Nathanson BH, Jacobs AA, *et al.* Determining optimum hemoglobin sampling for anemia management from every-treatment data. *Clin J Am Soc Nephrol* 2010;5:1939-1945.

24. Minutolo R, Chiodini P, Cianciaruso B, *et al.* Epoetin therapy and hemoglobin level variability in nondialysis patients with chronic kidney disease. *Clin J Am Soc Nephrol* 2009;4:552-559.
25. Bradbury BD, Danese MD, Gleeson M, *et al.* Effect of Epoetin alfa dose changes on hemoglobin and mortality in hemodialysis patients with hemoglobin levels persistently below 11 g/dL. *Clin J Am Soc Nephrol* 2009;4:630-637.
26. Macdougall IC, Carrera F, Braun J, *et al.* Intercurrent events and comorbid conditions influence hemoglobin level variability in dialysis patients. *Clin Nephrol* 2009;Volume 71:397-404.
27. Brunelli SM, Joffe MM, Israni RK, *et al.* History-adjusted marginal structural analysis of the association between hemoglobin variability and mortality among chronic hemodialysis patients. *Clin J Am Soc Nephrol* 2008;3:777-782.
28. Gilbertson DT, Ebben JP, Foley RN, *et al.* Hemoglobin level variability: associations with mortality. *Clin J Am Soc Nephrol* 2008;3:133-138.

PART III

Anemia and circadian body functions in CKD patients

CHAPTER 9

Impairment of endogenous melatonin rhythm is related to the degree of chronic kidney disease (CREAM study)

Birgit CP Koch[1]
Karien van der Putten[2]
Eus JW Van Someren[3,4]
Jos PM Wielders[5]
Piet M ter Wee[6]
J Elsbeth Nagtegaal[1]
Carlo AJM Gaillard[2]

[1]Department of Clinical Pharmacy, Meander MC, Amersfoort, The Netherlands
[2]Department of Internal Medicine, Meander MC, Amersfoort, The Netherlands
[3]Netherlands Institute for Neuroscience
[4]Departments of Neurology, Clinical Neurophysiology and Medical Psychology, VU University Medical Center, Amsterdam, the Netherlands
[5]Department of Clinical Chemistry, Meander MC, Amersfoort, The Netherlands
[6]Department of Nephrology, VU University Medical Center, Amsterdam, The Netherlands

Nephrol Dial Transplant 2010;25:513–519

ABSTRACT

Background

The nocturnal endogenous melatonin rise, which is associated with the onset of sleep propensity, is absent in hemodialysis patients. Information on melatonin rhythms in chronic kidney disease (CKD) is limited. Clear relationships exist between melatonin, core body temperature and cortisol in healthy subjects. In CKD, no data are available on these relationships. The objective of the study was to characterize the rhythms of melatonin, cortisol and temperature in relation to renal function in patients with CKD.

Methods

From 28 patients (mean age 71 years) with various degrees of renal function, over a 24-h period, blood samples were collected every 2 h. An intestinal telemetric sensor was used to measure core temperature. The presence of diurnal rhythms was examined for melatonin, temperature and cortisol. Correlation analysis was performed between Cockcroft–Gault GFR (GFR), melatonin, cortisol and temperature parameters.

Results

The mean GFR was 57±30 ml/min. The subjects exhibited melatonin ($n=24$) and cortisol ($n=2$) rhythms. GFR was significantly correlated to melatonin amplitude ($r=0.59$, $P=0.003$) and total melatonin production ($r=0.51$, $P=0.01$), but not to temperature or cortisol rhythms. Interestingly, no associations were found between the rhythms of temperature, melatonin and cortisol.

Conclusions

As melatonin amplitude and melatonin rhythm decreased with advancing renal dysfunction, follow-up research into circadian rhythms in patients with CKD is warranted.

INTRODUCTION

Chronic kidney disease (CKD) is associated with circadian rhythm disturbances, such as sleep-wake problems, that have a major influence on quality of life and morbidity.[1,2] Circadian rhythms are fluctuations in body functions within a period of around 24 h. They are driven by the biological 'clock' located in the hypothalamic suprachiasmatic nucleus. Circadian rhythms are described as having a 'mean', a 'period', an 'amplitude' (the difference between the maximum and the mean of the curve of the rhythm), an 'acrophase' (time of the peak level) and a 'bathyphase' (time of the trough level).[3] Individual peripheral tissue specific oscillators, which are under the influence of the master circadian pacemaker in the suprachiasmatic nucleus, might be disturbed in CKD due to impaired partial oxygen pressure and blood flow in the kidney.[3-5] Indeed, CKD patients often exhibit a deregulated circadian blood pressure rhythm, such as nocturnal non-dipping profile,[6] which is a risk factor for cardiovascular disease.

The pineal hormone melatonin, which is normally only secreted during the night, is an important marker of the circadian timing system.[7] Melatonin levels are usually nearly undetectable during daytime. The onset of the evening rise of endogenous melatonin is called the dim light melatonin onset (DLMO) and can be calculated as the first interpolated point >10 pg/ml after which the serum concentration continues to rise.[8] This rise is associated with the onset of sleep propensity in healthy subjects.[9] In addition, endogenous melatonin reinforces the nocturnal decrease of central temperature, facilitated by increases in skin blood flow,[10] an event that facilitates sleep propensity.[11] Furthermore, it has been suggested that melatonin can affect the production of cortisol in primates.[12] The nocturnal endogenous melatonin rise is absent in hemodialysis (HD) patients.[13,14] Information on melatonin rhythm in CKD patients is limited.

The main objective of this study was to investigate the relationship between melatonin rhythm and renal function in CKD patients. If indeed melatonin rhythm is impaired in these patients, follow-up research on exogenous melatonin might be warranted to restore the melatonin rhythm and improve sleep, as was demonstrated in HD patients.[15] The secondary objective was to investigate the circadian rhythms of cortisol and core body temperature and their synchronization with melatonin rhythm in CKD patients.

SUBJECTS AND METHODS

The study population consisted of patients with various degrees of renal function ($n = 32$) admitted to our hospital. The patients were stable, not acutely ill and were awaiting a procedure, such as an elective surgical procedure. The inclusion and exclusion criteria are outlined in Table 1. The medical ethical committee approved the protocol of the study (ClinicalTrials. gov: NCT00698360), and informed consent was obtained from all subjects.

Table 1 Inclusion and exclusion criteria

Inclusion Criteria
Age >18 years, <85 years
GFR-Cockcroft-Gault >10 ml/min
Exclusion Criteria
Acute renal failure (Δ GFR-Cockroft >10 ml/min in 2 proceeding weeks)
Instable Angina Pectoris
Heart failure NYHA class IV
Hypoxia (SO_2 < 95%)
Treatment with erythropoietin, melatonin or hypnotics
Deficiency of iron, folate and/or vitamin B12
Hemoglobinopathies, bleeding or haemolysis as a cause of anaemia
Chronic inflammatory disease or clinically significant infection
Alcohol and/or drug abuse

Abbreviations: GFR, glomerular filtration rate; NYHA, New York heart association; SO2, oxygen saturation.

Study protocol

All subjects followed a semi-constant routine protocol and stayed in a dimly lit room. Normal sleep and wake times were observed. The patients were admitted to our hospital and were free to move during the study. From 6 pm till 8 am the intensity of the ambient light was <20 lux and from 8 am till 6 pm the intensity of the ambient light was <200 lux.[16] Over a 24-h period, blood samples were collected every 2–3 h (access via a permanent peripheral intravenous cannula) in 6-ml serum tubes and allowed to clot for 10 min at room temperature. Thereafter, samples were immediately centrifuged and separated in 1-ml aliquots and stored at −70°C until assay. Core body temperature was continuously measured using telemetry.

Analysis of laboratory parameters

Melatonin levels in serum were measured by the commercially available RIA kit (Bühlmann Laboratories, Allschwill, Switzerland). Aliquots of 400 µl of a serum sample were added directly in assay tubes having an inter-assay CV of 17.8% at 20.1 ng/l. The detection limit was 0.5 pg/ml. All samples originating from one subject were analyzed in the same run. Cortisol levels were measured by a competitive chemiluminescent enzyme immunoassay on an IMMULITE 2000 platform (Siemens Healthcare Diagnostics, Breda, the Netherlands) having an inter-assay CV of 9.6% at 0.43 µmol/l. Aliquots of 200 µl of a serum sample were added directly in assay tubes. The detection limit was 0.03 µmol/l. Furthermore, standard laboratory parameters were measured. Because we included patients with a glomerular filtration rate (GFR) >60 ml/min, we estimated GFR by means of the Cockcroft–Gault method, as MDRD estimated GFR is not validated for GFR > 60 ml/min.

Core body temperature

Core body temperature was continuously measured by means of a Jonah capsule® (Respironics, Bend, OR, USA). This capsule is a disposable ingestible core body temperature sensor that telemetrically transmits information to a monitor, which has to stay <0.5 m from the ingested capsule. Transmissions begin around 1 min after activation and are repeated approximately every 15 s thereafter. Due to individual variation of gastrointestinal motility, the first 2 h of data collection were deleted, as only intestinal positioning of the capsule provides valid data.[17]

Statistical analysis

The presence of a 24-h rhythm time series was set to be examined by fitting of a dual harmonic cosine function for temperature, a baseline cosine function for melatonin and a skewed cosine function for cortisol. [18-21] The functions that parsimoniously and robustly capture the specific 24-h profiles of melatonin and cortisol cannot be linearized[18] and therefore we applied non-linearizable functions. The characteristics of the individual curves were obtained from individual fits for each subject, resulting in a number of parameters for each subject. Group differences on these parameters were evaluated with ordinary statistical methods. In the case of non-symmetrically distributed parameters, the data were log-transformed before correlations were calculated. This was the case for total melatonin production. Pearson's correlation analysis was performed to quantify the associations between the descriptive parameters for the whole range of renal function and the rhythms in melatonin, cortisol and core body temperature. *P*-values <0.05 were considered to represent statistical significance. The Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) version 17 was employed for all statistical analyses.

RESULTS

The 24-h study period was completed by only 28 patients, as 4 patients were excluded due to hospital discharge (n=3) and problems with blood flow (n=1) through the cannula. The general characteristics of the patients are displayed in Table 2. The mean age was 71, and 8 female patients were included. The causes of CKD are described in Table 2. Diabetes mellitus was reported in 29% of the population, which is comparable to the prevalence of diabetes mellitus in the general Dutch CKD population. Furthermore, Table 2 reveals that antihypertensive medication was frequently used.

Melatonin

Figure 1 shows the discrete mean melatonin concentrations and the standard deviations of the raw data at different time points in different renal function groups of all patients (n=28). The patients with the worst renal function (GFR<30 ml/min) had the lowest mean melatonin concentrations and patients with the best renal function (GFR>80 ml/min) had the highest melatonin concentrations. In the group with the worst renal function, the DLMO concentration of 10 pg/ml was reached. However, the normal rise above this value was not observed in this patient group, as can be seen in Figure 1. The melatonin rhythm

parameters are presented in Table 3. The goodness of fit for the melatonin curve (R^2) was 0.95±0.02 (mean ± SEM). In our patient group, 24 patients expressed a melatonin rhythm. The individual eGFRs of the patients without a melatonin rhythm varied considerably (eGFR range 17-79 ml/min). In the patients with a melatonin rhythm (n=24), the amplitude of the melatonin rhythm was correlated with GFR (r=0.59, P=0.003), as shown in Figure 2. Correction for age and gender did not change the outcomes. The log-transformed total 24-h melatonin production (area under the curve, AUC) was also correlated with GFR (r=0.51, P=0.01). As the use of beta blockers was equally divided across the different levels of renal function, it is unlikely that the association of renal dysfunction with melatonin levels results from beta-blocker use (F=4.23, P=0.18).

Table 2 Baseline characteristics (n = 28)

Characteristic	
Age (years)	71.3 ± 6.8
Gender (M:F)	20:8
BMI (kg/m^2)	
Median	25.8
Interquartile range	24.3-28.1
Diabetes (%)	29
Smoking (%)	14
Antihypertensive treatment (%)	86
Cause of CKD in GFR<60 (14 patients)	
Vascular	3
Diabetes Mellitus	4
Nephrosclerosis	6
Hypertension	1
Hemoglobin (g/dl)	13.3 ± 1.4
GFR-Cockcroft (ml/min/1.73m^2)	61 ± 34.9
Systolic blood pressure (mmHg)	125 ± 20
Diastolic blood pressure (mmHg)	73 ± 14

Plus-minus values are means ± SD.
Abbreviations: BMI, body mass index; GFR, glomerular filtration rate.

Cortisol

The cortisol rhythm parameters are shown in Table 3. The goodness of fit for the cortisol curve (R^2) was 0.72±0.02 (mean±SEM). Most patients (n=22), as expected, exhibited a cortisol rhythm. The patients with an absent cortisol rhythm were different than the patients with an absent melatonin rhythm. No correlations between cortisol amplitude and GFR (P=0.52) and between melatonin amplitude and cortisol amplitude (P=0.53) were found in the patients with a cortisol rhythm. In addition, no associations between melatonin acrophase and cortisol acrophase (P=0.11) and between melatonin acrophase and cortisol bathyphase (P=0.24) were found.

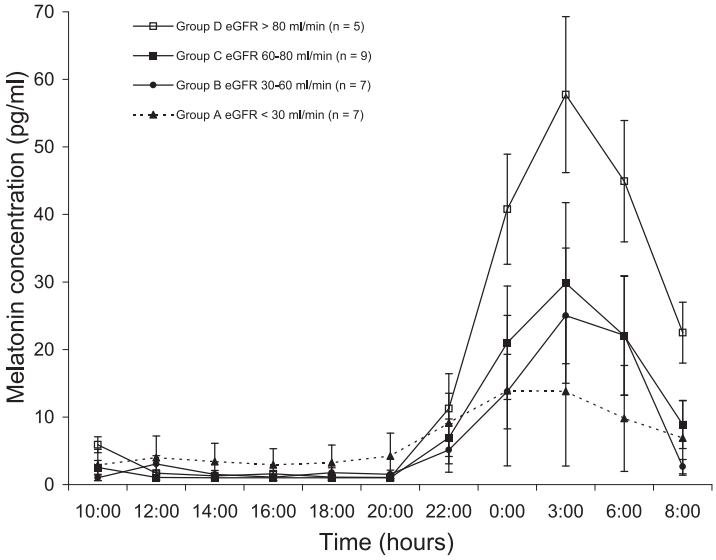

Figure 1 Discrete mean melatonin concentrations (of the raw data) in four groups with increasingly affected renal functions (n = 28). The error bars reflect the standard deviations of the raw data. The horizontal axis reflects the time of day (hours). The vertical axis reflects the mean melatonin concentration (in pg/ml).
Abbrevation: eGFR, estimated glomerular filtration rate

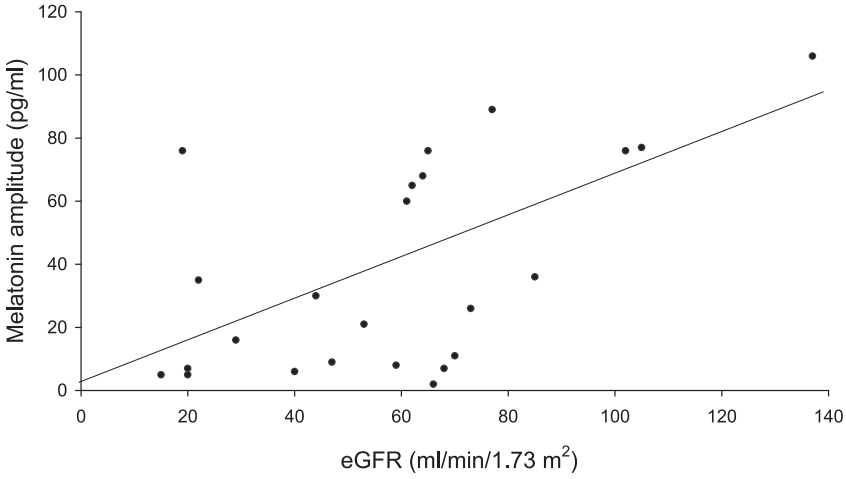

Figure 2 Scatter plot of melatonin amplitude versus GFR in the patients with a melatonin rhythm (n = 24). The horizontal axis reflects the estimated GFR (ml/min/1.73 m2). The vertical axis reflects the melatonin amplitude (pg/ml).
Abbrevation: eGFR, estimated glomerular filtration rate

Table 3 Rhythm characteristics of melatonin, cortisol and core body temperature

Melatonin (n=24, no detectable rhythm in 4 patients)	**Median (IQR)**
Acrophase (hours)	03:16 (2:49–3:56)
Time above baseline (min)	294 (18–393)
Amplitude (pg/ml)	28 (17-74)
Production (area under the curve -pg/ml*minute)	646 (536-756)
Correlation between amplitude and mean	R=0.978 (P<0.001)
Cortisol (n=22, no detectable rhythm in 6 patients)	
Acrophase (hours)	07:22 (6:10–8:46)
Maximum (µmol/L)	0.43 (0.38–0.52)
Bathyphase (hours)	0:30 (22:59–1:44)
Minimum (µmol/L)	0.09 (0.0–0.16)
Amplitude (µmol/L)	0.31 (0.24–0.42)
Core body temperature (n=28)	
Minimum (°C)	36.9 (36.4–37.0)
Bathyphase (hours)	04:42 (03:11–05:49)

Values are expressed as median and interquartile range (IQR).

Core body temperature

Due to instabilities of the temperature curves and missing data during daytime (unfortunately often observed in these temperature studies[16]), the temperature amplitude could not be determined and only the timing and the level of the nocturnal minimum were established after smoothing (centered rectangular 15 min moving average). The temperature data are shown in Table 3. Correlations between temperature minimum (P=0.26) and GFR or temperature bathyphase (P=0.07) and GFR were not to be established. Furthermore, no correlations, in contrast to normal observations, were found between the temperature bathyphase and the cortisol bathyphase (P=0.201) or between the temperature bathyphase and melatonin acrophase (P=0.24).

DISCUSSION

The main finding of our study is that renal function is associated with melatonin amplitude as well as total melatonin production. Renal function was not associated with cortisol or body temperature rhythm parameters. Interestingly, no association between the phases of the rhythms of melatonin, cortisol and core body temperature could be detected.

Melatonin

To our knowledge, this is the first study that shows a direct association between a decrease in renal function and decreases in melatonin rhythm and melatonin production. Patients with the worst renal function, shown in Figure 1, still expressed a DLMO concentration

of 10 pg/ml. However, a normal rise above this value was not to be established. Recently, we have found an abolished nocturnal melatonin rise in daytime HD patients.[15] After administration of exogenous melatonin in HD patients, the absent endogenous melatonin rise was recovered and an improvement in objective as well as subjective sleep was measured. In the present study, we found the presence of a melatonin rhythm in the majority of patients (n=24). We demonstrated, however, a decrease in melatonin amplitude and total melatonin production with advancing renal dysfunction.

Sleep measurements were not performed in our study, which need to be incorporated in follow-up research on circadian rhythm and CKD. In addition to a role in sleep-wake rhythm, other effects of endogenous melatonin in CKD need further investigation. For example, endogenous melatonin was found to have a stimulating and protective effect on activity and quantity of anti-oxidative stress enzymes.[22,23] This property can be of interesting value in this patient group as oxidative stress is often seen in CKD patients. A primed state of polymorphonuclear cells, responsible for oxidative stress in HD patients, was associated with lower nocturnal plasma melatonin levels.[24] In addition, melatonin has also been associated with the immune response, impairing the mounting of an inflammatory response, while melatonin produced at the site of the injury by immunocompetent cells exerts anti-inflammatory effects.[25] Exogenous melatonin has also been useful in restoring the dipping profile in male patients with essential hypertension.[26] More research on endogenous en exogenous melatonin is needed to further establish the role of melatonin in CKD patients.

Earlier studies in HD patients found an absence of nocturnal melatonin rise.[13,14] When comparing CKD stage 4 patients and HD patients, the concentration at night was found to be even lower in HD patients, which suggests an additional effect of the dialysis process on the melatonin rhythm,[14] which is—as we here demonstrate—already compromised in CKD patients.

Several factors affect the production of melatonin in CKD. Firstly, a decline in melatonin levels has been reported to be the result of impairment in beta-adrenoreceptor mediated responsiveness in renal insufficiency.[27] The adrenergic system plays an important role in the synthesis of serotonin N-acetyltransferase (NAT), the key enzyme in melatonin biosynthesis.[28] Although suppression of NAT was observed in rats rendered uremic by partial nephrectomy,[29] research on NAT concentrations has not been performed in humans with CKD. Secondly, metabolic acidosis and reduction of airway muscle tone due to accumulation of uremic toxins result in an increased prevalence of sleep apnea in CKD, which is associated with increased melatonin levels during the afternoon.[30] However, as we did not investigate the prevalence of sleep apnea in our population, we cannot confirm this. Considering the characteristics, medical history and BMI of the included patients, it seems unlikely that the prevalence of sleep apnea would be increased in these patients. Thirdly, uremia has been associated with daytime sleepiness.[31] Daytime sleepiness may impair the regulation of the sleep-wake rhythm. This dysregulation might negatively affect the melatonin rhythm. Melatonin can also be deregulated due to erythropoietin deficiency anemia as often found in CKD patients.[5] The fact that exogenous erythropoietin treatment can restore the circadian rhythm of melatonin in CKD[5,32] could suggest that a relationship exists between melatonin rhythm and endogenous erythropoietin levels. We

investigated this relationship in the other part of the submitted CREAM study.[33] Finally, medication can impair the melatonin production. Beta blockers and benzodiazepines, often used in the CKD population, can flatten the normal nocturnal rise.[28] Benzodiazepines were not used during our study, but beta blockers were still taken (n=23). As the use of beta blockers was equally divided across the different levels of renal function, it is unlikely that the association of renal dysfunction with melatonin levels results from beta blocker use. Overall, antihypertensive treatment was frequently used, which could be the reason for the adequate blood pressure control.

The duration of our study period was 24 h. As we know from other studies diurnal melatonin measurements are a fingerprint for the melatonin rhythm of a subject, and therefore, longer study periods are not needed.[28] The mathematical model for the description of melatonin rhythms was developed by means of 24-h measurements.[18] As we used the same constant routine measurements in all patients, and excluded other influences, 24 h is a normal observation period. Other possible influences on melatonin production are corrected (beta blockers), excluded (bright light, hypnotics) or minimized using a standard protocol. As all female patients were post menopausal, a difference in gender was not suspected. Correction for gender was performed to exclude a possible gender effect. The distribution of male and female patients was not equal (Table 2). Research with more patients might be needed to confirm the absence of a gender effect.

Core body temperature

In healthy persons, core body temperature variations over time follow an asymmetric sinusoidal rhythm with the temperature minimum normally occurring between 4 am and 6 am. Sleep propensity is closely linked to core body temperature, probably most specifically to the temperature of the skin.[34] Abnormalities in the rhythms in core and skin temperature have been reported in some patients with insomnia.[35] Dialysis patients show impaired nerve conduction, possibly due to uremia.[36] Patients with proteinuria exhibit impaired vascular endothelial function, which can result in impairment in blood flow response to heating in the skin,[37] which leads to less thermal conductivity.[38] These observations led us to investigate whether CKD patients have an abnormal core body temperature. We failed to find an association between CKD severity and core body temperature, however. Additional studies are needed to confirm the absence of such a relationship, as well as to investigate possible alterations in skin temperature regulation, which are associated with sleep problems in dialysis patients.[39]

Melatonin has been proposed to be an endogenous synchronizer, able to stabilize circadian rhythms under normal circumstances.[28] The effect of melatonin on the temperature rhythms, under normal circumstances, meets this hypothesis.[10,11] In our patient group, the timing of the core body temperature minimum was not associated with the timing of the melatonin peak. Autonomic deregulation, which might dissociate circadian rhythms, may be involved.[40] It is also possible that the normal synchronization aspects of endogenous melatonin disappear when melatonin rhythm is impaired, which might result in a dissociation of melatonin and temperature rhythm. Further research on this theory is needed.

Cortisol

Cortisol is synthesized in the adrenal gland under influence of ACTH. Under normal circumstances it exhibits a circadian rhythm. Normal levels of cortisol can be found in the range of 0.15-0.70 μmol/l during daytime and <0.20 μmol/l at nighttime. As the kidney contributes to the excretion of cortisol, the serum half-life of cortisol becomes prolonged in advanced CKD.[41] Both normal and elevated levels of serum cortisol have been reported in CKD.[42,43] We found cortisol levels in CKD not to be markedly different to reported normal values. The cortisol levels were also not related to renal function.

Furthermore, we did not find any association between cortisol rhythm parameters and renal function. Most patients exhibited a normal cortisol rhythm, which has also been shown in children with CKD.[44] Besides this paper from 1975, there is a paucity of research on cortisol and circadian rhythm in CKD. Due to frequently observed sleep-wake disturbances in CKD patients, affected cortisol rhythms might have been expected.[45] For example, Knutson et al. reported that partial sleep deprivation resulted in changes in cortisol.[46] As patients in our study were admitted to the hospital, it should be noted that the environment in which cortisol is assessed affects its diurnal profile; for example, humans measured in a hospital setting showed elevated levels respective to their home-assessed levels, especially in the evening.[47]

Experimental studies have suggested melatonin to affect cortisol and sleep-wake rhythms.[7,13] However, in patients with abnormal melatonin profiles, ACTH and cortisol levels were not changed.[48] Normal nocturnal melatonin levels have also been found in patients with steroid production disorders.[49] In our study, we also did not find a relationship between melatonin and cortisol.

In conclusion, we have demonstrated that melatonin production is increasingly compromised with advancing renal dysfunction and that CKD patients may have compromised coupling of melatonin, cortisol and temperature rhythms. Our findings suggest the need for placebo-controlled studies into the application of exogenous melatonin to mimic the endogenous melatonin rhythm aiming to reinforce the coherence between rhythms and improving sleep disturbances in patients with CKD.

REFERENCES

1. De Santo RM, Bartiromo M, Cesare MC, *et al.* Sleeping disorders in early chronic kidney disease. *Semin Nephrol* 2006;26:64-67.
2. Parker KP. Sleep disturbances in dialysis patients. *Sleep Med Rev* 2003;7:131-143.
3. Minors DS, Waterhouse JM. Circadian rhythms and their mechanisms. *Experientia* 1986;42:1-13.
4. Koopman MG, Koomen GC, Krediet RT, *et al.* Circadian rhythm of glomerular filtration rate in normal individuals. *Clin Sci (Lond)* 1989;77:105-111.
5. Vaziri ND, Oveisi F, Reyes GA, *et al.* Dysregulation of melatonin metabolism in chronic renal insufficiency: role of erythropoietin-deficiency anemia. *Kidney Int* 1996;50:653-656.
6. Farmer CK, Goldsmith DJ, Cox J, *et al.* An investigation of the effect of advancing uraemia, renal replacement therapy and renal transplantation on blood pressure diurnal variability. *Nephrol Dial Transplant* 1997;12:2301-2307.
7. Claustrat B, Brun J, Chazot G. The basic physiology and pathophysiology of melatonin. *Sleep Med Rev* 2005;9:11-24.
8. Lewy AJ, Sack RL. The dim light melatonin onset as a marker for circadian phase position. *Chronobiol Int* 1989;6:93-102.
9. Tzischinsky O, Shlitner A, Lavie P. The association between the nocturnal sleep gate and nocturnal onset of urinary 6-sulfatoxymelatonin. *J Biol Rhythms* 1993;8:199-209.
10. van der Helm-van Mil AH, van Someren EJ, van den Boom R, *et al.* No influence of melatonin on cerebral blood flow in humans. *J Clin Endocrinol Metab* 2003;88:5989-5994.
11. Van Someren EJW. Sleep propensity is modulated by circadian and behavior-induced changes in cutaneous temperature. *J Therm Biol* 2004;29:437-444.
12. Torres-Farfan C, Richter HG, Rojas-Garcia P, *et al.* mt1 Melatonin receptor in the primate adrenal gland: inhibition of adrenocorticotropin-stimulated cortisol production by melatonin. *J Clin Endocrinol Metab* 2003;88:450-458.
13. Koch BC, Hagen EC, Nagtegaal JE, *et al.* Effects of nocturnal hemodialysis on melatonin rhythm and sleep-wake behavior: an uncontrolled trial. *Am J Kidney Dis* 2009;53:658-664.
14. Karasek M, Szuflet A, Chrzanowski W, *et al.* Decreased melatonin nocturnal concentrations in hemodialyzed patients. *Neuro Endocrinol Lett* 2005;26:653-656.
15. Koch BC, Nagtegaal JE, Hagen EC, *et al.* The effects of melatonin on sleep-wake rhythm of daytime haemodialysis patients: a randomized, placebo-controlled, cross-over study (EMSCAP study). *Br J Clin Pharmacol* 2009;67:68-75.
16. Nagtegaal JE, Kerkhof GA, Smits MG, *et al.* Delayed sleep phase syndrome: A placebo-controlled cross-over study on the effects of melatonin administered five hours before the individual dim light melatonin onset. *J Sleep Res* 1998;7:135-143.
17. McKenzie JE, Osgood DW. Validation of a new telemetric core temperature monitor. *J Therm Biol* 2004;29:605-611.
18. Van Someren EJ, Nagtegaal E. Improving melatonin circadian phase estimates. *Sleep Med* 2007;8:590-601.
19. Ruf T. The baseline cosinus function: a periodic regression model forn biological rhythms. *Biol Rhythm Res* 1996;27:153-165.
20. Batschelet E. *Circular Statistics in Biology*: London: Academic Press, 1981.
21. Bardsley WG, Bukhari NAJ, Ferguson MWJ. Evaluation of model discrimination, parameter estimation and goodness of fit in nonlinear regression problems by test statistics distributions. *Comput Chem* 1995;19:75-84.
22. Poeggeler B, Reiter RJ, Tan DX, *et al.* Melatonin, hydroxyl radical-mediated oxidative damage, and aging: a hypothesis. *J Pineal Res* 1993;14:151-168.
23. Reiter RJ. Melatonin: clinical relevance. *Best Pract Res Clin Endocrinol Metab* 2003;17:273-285.
24. Geron R, Shurtz-Swirski R, Sela S, *et al.* Polymorphonuclear leucocyte priming in long intermittent nocturnal haemodialysis patients—is melatonin a player? *Nephrol Dial Transplant* 2006;21:3196-3201.
25. Markus RP, Ferreira ZS, Fernandes PA, *et al.* The immune-pineal axis: a shuttle between endocrine and paracrine melatonin sources. *Neuroimmunomodulation* 2007;14:126-133.

26. Scheer FA, Van Montfrans GA, van Someren EJ, *et al.* Daily nighttime melatonin reduces blood pressure in male patients with essential hypertension. *Hypertension* 2004;43:192-197.
27. Souchet T, Bree F, Baatard R, *et al.* Impaired regulation of beta 2-adrenergic receptor density in mononuclear cells during chronic renal failure. *Biochem Pharmacol* 1986;35:2513-2519.
28. Nagtegaal JE, Smits MG, Kerkhof GA. Chronobiological, clinical and pharmacological aspects of melatonin in human circadian rhythm dysfunction. In: Haldar C, Singaravel M, Kumar Maitra S (eds). *Treatise on Pineal Gland and Melatonin.* Enfield, UK: Science Publishers, 2002:461-489.
29. Holmes EW, Hojvat SA, Kahn SE, *et al.* Testicular dysfunction in experimental chronic renal insufficiency: a deficiency of nocturnal pineal N-acetyltransferase activity. *Br J Exp Pathol* 1989;70:349-356.
30. Ulfberg J, Micic S, Strom J. Afternoon serum-melatonin in sleep disordered breathing. *J Intern Med* 1998;244:163-168.
31. Hanly PJ, Gabor JY, Chan C, *et al.* Daytime sleepiness in patients with CRF: impact of nocturnal hemodialysis. *Am J Kidney Dis* 2003;41:403-410.
32. Benz RL, Pressman MR, Hovick ET, *et al.* A preliminary study of the effects of correction of anemia with recombinant human erythropoietin therapy on sleep, sleep disorders, and daytime sleepiness in hemodialysis patients (The SLEEPO study). *Am J Kidney Dis* 1999;34:1089-1095.
33. van der Putten K, Koch BC, van Someren EJ, et al. The role of renal function loss on circadian misalignment of cytokines EPO, IGF-1, IL-6 and TNF-α in chronic renal disease. Submitted.
34. Van Someren EJ. Mechanisms and functions of coupling between sleep and temperature rhythms. *Prog Brain Res* 2006;153:309-324.
35. Lack LC, Gradisar M, Van Someren EJ, *et al.* The relationship between insomnia and body temperatures. *Sleep Med Rev* 2008;12:307-317.
36. Robles NR, Alvarez-Lobato VC, Caravaca F, *et al.* Sympathetic skin response in peritoneal dialysis patients. *Asaio J* 2003;49:88-90.
37. van Ree RM, Oterdoom LH, de Vries AP, *et al.* Circulating markers of endothelial dysfunction interact with proteinuria in predicting mortality in renal transplant recipients. *Transplantation* 2008;86:1713-1719.
38. Petrofsky JS, McLellan K, Bains GS, *et al.* Skin heat dissipation: the influence of diabetes, skin thickness, and subcutaneous fat thickness. *Diabetes Technol Ther* 2008;10:487-493.
39. Parker KP, Bailey JL, Rye DB, *et al.* Lowering dialysate temperature improves sleep and alters nocturnal skin temperature in patients on chronic hemodialysis. *J Sleep Res* 2007;16:42-50.
40. Hathaway DK, Cashion AK, Milstead EJ, *et al.* Autonomic dysregulation in patients awaiting kidney transplantation. *Am J Kidney Dis* 1998;32:221-229.
41. Palmer BF. Metabolic disturbances in chronic renal failure. *Saudi J Kidney Dis Transpl* 2002;13:273-280.
42. Nolan GE, Smith JB, Chavre VJ, *et al.* Spurious overestimation of plasma cortisol in patients with chronic renal failure. *J Clin Endocrinol Metab* 1981;52:1242-1245.
43. Rosman PM, Benn R, Kay M, *et al.* Cortisol binding in uremic plasma. I. Absence of abnormal cortisol binding to cortisol binding to corticosteroid-binding globulin. *Nephron* 1984;37:160-165.
44. Betts PR, Howse PM, Morris R, *et al.* Serum cortisol concentrations in children with chronic renal insufficiency. *Arch Dis Child* 1975;50:245-247.
45. Taheri S. Sleep and metabolism: bringing pieces of the jigsaw together. *Sleep Med Rev* 2007;11:159-162.
46. Knutson KL, Spiegel K, Penev P, *et al.* The metabolic consequences of sleep deprivation. *Sleep Med Rev* 2007;11:163-178.
47. Scheer FA, Van Paassen B, Van Montfrans GA, *et al.* Human basal cortisol levels are increased in hospital compared to home setting. *Neurosci Lett* 2002;333:79-82.
48. Vaughan GM. Melatonin in humans. *Pineal Res Rev* 1984;2:141-202.
49. Werner S, Brismar K, Wetterberg L. Circadian rhythms of melatonin, prolactin, growth hormone, and cortisol in patients with pituitary adenomas, empty cell syndrome and Cushing syndrome due to adrenal tumors. *Adv Biosci* 1981;29:357-363.

CHAPTER 10

The role of renal function loss on circadian misalignment of cytokines EPO, IGF-1, IL-6 and TNF-α in chronic renal disease

Karien van der Putten[1]
Birgit CP Koch[2]
Eus JW van Someren[3]
Jos PM Wielders[4]
Piet ter Wee[5]
J Elsbeth Nagtegaal[6]
Carlo AJM Gaillard[1,5]

[1]Department of Internal Medicine, Meander MC, Amersfoort, The Netherlands
[2]Department of Hospital Pharmacy, Erasmus MC, Rotterdam, The Netherlands
[3]Netherlands Institute for Neuroscience, Amsterdam; Department of Integrative Neurophysiology, VU University, Amsterdam, and Departments of Neurology and Radiology, Sleep Center Leiden, Leiden University Medical Center, Leiden, the Netherlands
[4]Department of Clinical Chemistry, Meander MC, Amersfoort, the Netherlands
[5]Department of Nephrology, VU University Medical Centre, Amsterdam, The Netherlands
[6]Department of Clinical Pharmacy, Meander MC, Amersfoort, The Netherlands

Accepted in Neuroendocrinol Lett

ABSTRACT

Objective

Chronic inflammation plays a pivotal role in the development of renal disease. Circadian sleep-wake rhythm is disturbed in renal disease. Awareness of other disturbed rhythms, such as inflammation processes, can affect the treatment of patients with renal disease. Knowledge of possibly related circadian misalignment of the cytokines erythropoietin (EPO), Insulin Growth Factor-1 (IGF-1) and interleukins (IL) however is limited. We therefore performed an observational study. The objective of this study was to characterize levels of EPO, IGF-1 and inflammation markers IL-6 and TNF-α, related to renal function.

Methods

The study population consisted of patients with various degrees of renal function, admitted to our hospital. During 24 hours, blood of 28 subjects with various degrees of renal function was collected every 2 hours. The patients were stable, not acutely ill and they were waiting for a procedure, such as elective surgery. Circadian parameters of EPO, IGF-1, IL-6 and TNF-α were measured in serum and were correlated with glomerular filtration rate (GFR) and Hb, using Pearson correlations.

Results

Although diurnal variations in EPO level were found in 15 out of 28 patients, the curves did not show a consistent phase. The presence of an EPO rhythm was not related to GFR. No diurnal rhythm could be detected for IGF-1, IL-6 and TNF-α. Mean levels of IGF-1 were correlated inversely to mean levels of EPO ($p=0.03$). When divided based on GFR and Hb subjects with GFR 0-30 ml/min and lower Hb had the highest IGF-1 levels ($p=0.02$). A relationship between IL-6, TNF-α, EPO and GFR was not found.

Conclusion

The existence of a circadian (mis)alignment of EPO, IGF-1, IL-6 and TNF-α was not found. The association between high IGF-1 and low Hb suggests that EPO and IGF-1 have an alternating role, dependent on GFR, in stimulating erythropoiesis. These results could have consequences for the treatment of anemia.

INTRODUCTION

Patients with chronic kidney disease exhibit markedly disrupted circadian body functions. For example, the diurnal blood pressure rhythm is disturbed in patients with chronic kidney disease (CKD), showing a nocturnal non-dipping profile[1], which is associated with increased cardiovascular mortality.[2] The nocturnal endogenous melatonin rise, which is associated with the onset of nocturnal sleep propensity, is decreased in patients with reduced GFR and completely absent in hemodialysis patients.[3-5] Melatonin secretion is governed by the suprachiasmatic nucleus (SCN) in the hypothalamus. Importantly, in addition to the central endogenous timing system, in peripheral cells oscillators share a similar core clock based on transcriptional activators (such as Clock), and of feedback repressors (such as Cry1, Cry2). Thus, circadian oscillations of the core clock entrain circadian rhythms in expression of output genes in peripheral cells, which are, in turn, translating these transcriptional oscillations into tissue-specific functional rhythms.[6] Recently it was shown that Clock deficient mice and (Cry1,Cry2) double knockout mice had marked, "clinically important", disturbances in water or sodium homeostasis.

Renal disease is, in addition to loss of melatonin rhythm, associated with anemia, erythropoietin (EPO) deficiency, and with inflammation, endothelial dysfunction and EPO resistance. The endothelium is an important non-hematological target of EPO[7] and both anemia and EPO resistance are important predictors of cardiovascular complications and mortality in CKD. It is unknown whether changes in circadian rhythms and loss of melatonin secretion in CKD are associated with changes in EPO effect and/or rhythm. Associations between melatonin and erythropoietin rhythm have been published[8] and EPO response in the treatment of anemia in CKD is influenced by the time of administration.[9] There are several additional reasons that support the existence of a circadian rhythm of EPO and other cytokines. The primary stimulus for EPO production is (lack of) oxygen availability. The fact that during the night the metabolic requirement for oxygen is lower, could lead to decreased levels of EPO during nighttime.

To our knowledge, no information is available on whether IGF-1 exhibits a circadian rhythm. IGF-1 is produced mainly in the liver under the influence of growth hormone (GH), which is, however, secreted pulsatile in a circadian fashion.[10] An increase in IL-6 and TNF-α has been associated with an increased need in exogenous EPO.[11] In healthy volunteers the concentration of Il-6 is regulated in a circadian fashion with peak levels in the early morning and early evening and through levels later in the morning and at night. TNF-α has shown to have peak levels at night.[12]

We therefore asked the question whether changes in circadian rhythms and loss of melatonin secretion in CKD are associated with changes related to EPO and modulators of its erythropoietic effect. In the present study we examined the circadian (mis) alignment of the cytokines EPO, IGF-1, co-factor in erythropoiesis[13] and inflammatory markers (related to EPO resistance[14]), in comparison to changes in melatonin rhythm, in CKD.

MATERIAL AND METHODS

Subjects

The study population consisted of patients with various degrees of renal function (n=32, age 71±7, 29% female), admitted to our hospital. The patients were stable, not acutely ill and they were waiting for a procedure, such as elective surgery. The inclusion and exclusion criteria are outlined in Table I. The Medical-Ethical Committee approved the protocol of the study (ClinicalTrials.gov: NCT00698360), and informed consent was obtained from all subjects.

Table I Inclusion and exclusion criteria

Inclusion Criteria
Age >18 years, <85 years
GFR-Cockcroft-Gault >10 ml/min
Exclusion Criteria
Acute renal failure (Δ GFR-Cockroft >10 ml/min in 2 proceeding weeks)
Instable Angina Pectoris
Heart failure NYHA class IV
Hypoxia (SO_2 < 95%)
Treatment with erythropoietin, melatonin or hypnotics
Deficiency of iron, folate and/or vitamin B12
Hemoglobinopathies, bleeding or hemolysis as a cause of anemia
Chronic inflammatory disease or clinically significant infection
Alcohol and/or drug abuse

Abbreviations: GFR, glomerular filtration rate; NYHA, New York heart association; SO2, oxygen saturation.

Study protocol

Over a 24-hour period, blood samples for measurement of serum EPO, total IGF-1, TNF-α and IL-6 were collected every 2-3 hours (access via a permanent peripheral intravenous cannula) in 6-ml serum tubes and allowed to clot for 10 minutes at room temperature. Thereafter, samples were immediately centrifuged and separated in 1 ml-aliquots and stored at -70°C until assay. In addition, blood was withdrawn in a 3-ml EDTA tube for measurement of hemoglobin (Hb) in supine position after 0, 12 and 24 hours.

Measurements

Medical history and medication use were recorded and blood pressure was measured in all participants. Serum EPO levels were measured by a two-site sandwich chemiluminescent immunoassay on an IMMULITE 2000 platform (Siemens Healthcare Diagnostics, Breda, the Netherlands) having an inter-assay CV of 7.2% at 16 IU/l. Total IGF-1 levels were measured using an enzyme-labeled chemiluminescent immunometric assay on an IMMULITE 2000 platform (Siemens Healthcare Diagnostics, Breda, the Netherlands) with

an inter-assay CV of 6.9 % at 128 ng/ml. Levels of Cystatin-C were measured once at the start of the study, by means of the N-Latex Cystatin-C assay and a PROspec nephelometer (Siemens Healthcare Diagnostics, Breda, the Netherlands) having an inter-assay CV of 2.2 % at 0.90 mg/l. All samples originating from one subject were analyzed in the same run. IL-6 and TNF-α are measured by means of an ELISA kit (enzyme immunoassay, Pelikine ™. The sensitivity for IL-6 is 0,3 pg/ml, and no cross-reactivity is observed. The sensitivity for TNF-α is 1-3 pg/ml, and no cross-reactivity is observed. Furthermore, standard laboratory testing was performed. Glomerular Filtration Rate (GFR) was calculated according to the Cockcroft-Gault method.

Statistical analysis

The sample size was based on an analysis of statistical power using data of a pilot study on the relationship between circadian EPO rhythm and renal disease in pre-terminal uremia.[9] Given the prior variances and mean difference in EPO amplitude between healthy subjects and predialysis patients, a sample of n=32 was chosen to obtain a power of 0.90 at an alpha of 0.05). For IGF-1 rhythm detection, previous studies that demonstrated circadian rhythms used a sample size of n=6.[10,15] The existence of a circadian rhythm was examined by fitting of a cosine function to time series.[16] In case of non-symmetrically distributed parameters, the data were log-transformed before correlation calculations were performed. This was the case for Cystatin-C, mean EPO levels, EPO amplitude and mean IGF-1, IL-6 and TNF-α levels. Continuous variables were compared using unpaired t-tests and categorical variables were compared using Fishers' Exact Test. Data regarding EPO and IGF-1, IL-6 and TNF-α rhythm/production were correlated with GFR and Hb using Pearson correlations. P-values <0.05 were considered to represent statistical significance. The Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) version 17 was employed for all statistical analysis.

RESULTS

The 24-hour study period was completed by 28 subjects, as hospital discharge and problems with blood flow through the cannula resulted in missing a significant amount of data in 4 patients. The general characteristics of the subjects are displayed in Table 2.

EPO

In 15 out of the 28 subjects, a significant cosine rhythm was present for EPO (Table 3). However, the acrophase (time of peak concentration) varied widely among these subjects. No determinants for the existence of an EPO rhythm could be identified. The presence of an EPO rhythm was not related to GFR (p=0.46) and no correlation was found between amplitude of EPO rhythm and GFR (p=0.10). Furthermore, no relation was found between the presence of an EPO rhythm and several general characteristics of the study population (CKD cause, gender (p=0.69), age (p=0.54), BMI (p=0.85), diabetes mellitus (DM) (p=1.00), smoking (p=0.60)). Also, no relation was found between EPO rhythm and medication use (beta-blocking agents (p=1.00), ACE-inhibitors (p=0.70),

acetylsalicylic acids (p=0.71)). As expected, the mean EPO levels for all 28 subjects were correlated to GFR (r=0.52, p=0.005) and to levels of Cystatin-C (r=-0.52, p=0.005). Mean levels of EPO for all subjects were not correlated to Hb (p=0.29).

Table 2 Baseline characteristics (n = 28)

Characteristic	
Age (years)	71 ± 7
Gender (M:F)	71 : 29
BMI (kg/m^2)	
Median	25.8
Interquartile range	24-28
Diabetes (%)	29
Smoking (%)	14
Systolic blood pressure (mmHg)	125 ± 20
Diastolic blood pressure (mmHg)	73 ± 14
Hemoglobin (g/dL)	13.3 ± 1.4
GFR-Cockcroft (ml/min)	57 ± 30
Cystatin C (mg/l)	
Median	1.01
Interquartile range	0.79-1.98

Plus-minus values are means ± SD. Conversion factors for units: serum hemoglobin in g/dl to g/L x10; serum GFR in mL/min/1.73 m^2 to mL/s/1.73 m^2 x0.01667.
Abbreviations: BMI, body mass index; GFR, glomerular filtration rate.

Table 3 EPO rhythm characteristics (n=15)

Variable	Median [interquartile range]
EPO level (IU/l)	8.9 [7.8-15.7]
Peak EPO level (IU/l)	12.0 [9.4-20.4]
Acrophase (hours after midnight)	14.2 [10.3-22.1]
Amplitude (IU/l)	2.7 [1.5-3.3]

Abbreviation: EPO, erythropoietin

IGF-1

Cosine curve fitting of the IGF-1 time series did not reveal a clear rhythm for IGF-1 in any of the subjects. On an individual basis, there was some time-of-day variation in levels of IGF-1. However, no systematic peak time range was found. Mean levels of IGF-1 did not correlate to GFR (p=0.26) or to Cystatin-C (p=0.08), but interestingly, mean levels of IGF-1 did correlate inversely to mean levels of EPO (r=-0.41, p=0.03) (Figure 1). Levels of IGF-1 did not correlate to Hb (p=0.68). To determine the relation between IGF-1, Hb and GFR, participants were divided into six groups based on the degree of CKD (GFR 0-30, 30-60, >60) and Hb lower/higher than 12.6 g/dL. Subjects with GFR 0-30 ml/min and a low Hb had the highest IGF-1 levels (p=0.02, ANOVA) (Figure 2).

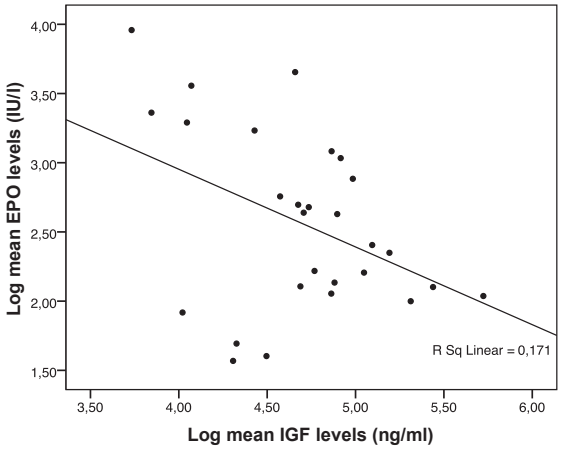

Figure 1 The correlation between log transformed IGF-levels and log transformed EPO levels (n=28, p=0.03).
Abbreviation: IGF-1, Insulin-like Growth Factor-1; EPO, erythropoietin

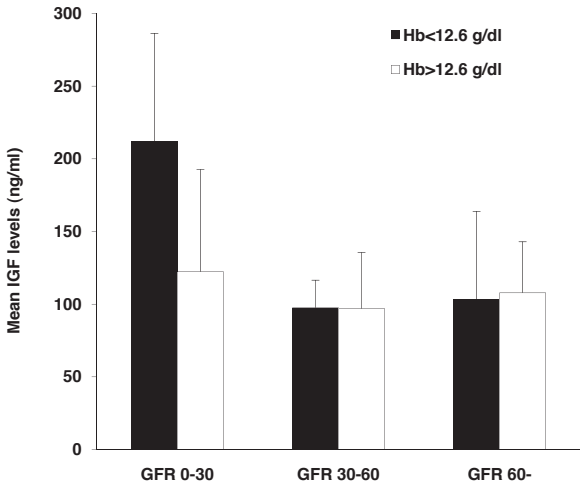

Figure 2 The relation between IGF-1, Hb and GFR. Subjects with GFR 0-30 ml/min and a low Hb had the highest IGF-1 levels (p=0.02).
Abbrevations: IGF-1, Insulin-like Growth Factor-1; Hb, hemoglobin; GFR, glomerular filtration rate

IL-6 and TNF-α

Cosine curve fitting of the Il-6 and TNF-α time series did not reveal a clear circadian rhythm in any of the subjects. The mean level of IL-6 was 9.6 pg/ml (normal < 9,7 pg/ml) and the mean level of TNF-α was 0,8 pg/ml (normal < 2 pg/ml). On an individual basis, there was some time-of-day variation in levels of IL-6. However, no systematic peak time range was found. Mean levels of Il-6 and TNF-α did not correlate with EPO, IGF-1 and GFR (all $p > 0.1$). There seemed to be an association between mean levels of IL-6 and Hb ($p = 0.1$, $r = 0.49$). No other relationships were found.

DISCUSSION

The main finding of our study is that circadian (mis)alignment for cytokines EPO, IGF-1 and inflammation is not a consequence of the degree of CKD, in contrast to melatonin rhythm.[3] Secondly, circadian rhythms of these parameters or associations between these parameters amongst themselves could not be identified. Interestingly, mean levels of IGF-1 correlated inversely to mean levels of EPO. Subjects with the greatest decrease in renal function and the lowest Hb levels had the highest IGF-1 levels.

EPO

In the present study we found no clinical significant circadian EPO rhythm. EPO is a glycoprotein that acts to prevent the programmed cell death of erythroid progenitor cells in the bone marrow, thereby stimulating these cells to proliferate and mature.[17] EPO is mainly produced in the kidney by peritubular cells[18] and the primary stimulus for its production is hypoxia.[19] In CKD, production of EPO is disrupted, which is one of the main reasons for anemia in chronic renal disease patients.[14]

It is unclear whether EPO is regulated in a circadian manner in healthy persons.[20,21] With exception of one small series of 5 uremic patients[22], no study has been performed on a circadian rhythm of EPO in CKD. In our study, we found diurnal variations for EPO in 15 of the 28 subjects. The peak times however varied, and therefore it is unlikely that EPO levels are regulated intrinsically in a circadian fashion. No relation was found between the presence of an EPO rhythm and the degree of renal disease. Our search for additional factors that could influence EPO levels did not reveal any other determinants for the existence of a rhythm for EPO. It thus remains unclear why the 15 subjects in our study did demonstrate an EPO rhythm, while the other 13 subjects did not. A possible explanation could be that in the subjects with an EPO rhythm, more fluctuations in blood pressure were present, as blood pressure is related to EPO levels.[23] Unfortunately, the frequency of blood pressure measurements that we used was too low to detect such fluctuations. Another possible explanation could be the presence of unrecognized sleep apnea. In sleep apnea, the diurnal variation in EPO levels is higher than in healthy persons due to nocturnal hypoxemia.[24] In our study, we did not perform nocturnal oxygen measurements.

IGF-1

In the present study we have found no significant IGF-1 rhythm. IGF-1 has been shown to exhibit EPO-like erythropoietic activity.[25] IGF-1 is produced in the liver under the influence of GH. In CKD, the release of GH is increased and the metabolic clearance rate is decreased, leading to increased circulating levels of GH.[26] This would lead to higher levels of IGF-1, however in CKD, IGF-1 synthesis in the liver is impaired. Despite ensuing normal IGF-1 levels, the effectiveness of IGF-1 is reduced. This is due to decreased levels of free bioactive IGF-1, as levels of circulating inhibitory binding proteins are higher.[27] To our knowledge, no information is available on whether IGF-1 exhibits a circadian rhythm in healthy adults and in patients with CKD. In our study, we were unable to demonstrate a circadian rhythm for IGF-1 in subjects with a decreased GFR. We also found no rhythm for IGF-1 in subjects with a normal GFR. Possibly the relatively old age of our study population (mean age 71.3 yrs) has influenced our results. At the age of 65 years, daily spontaneous GH secretion is reduced by 50–70%, leading to a decline in IGF-1 levels.[28] Theoretically, this could lead to an increased difficulty in detecting a circadian rhythm. Another explanation for the absence of an IGF-1 rhythm in our study could be the relatively high prevalence (29%) of DM amongst the subjects, as DM is associated with lower levels of IGF-1.[29]

EPO and IGF-1

Mean levels of IGF-1 were correlated inversely to mean levels of EPO. When divided based on GFR and Hb, subjects with the lowest GFR (0-30 ml/min) and lowest Hb levels had the highest IGF-1 levels.

Brox et al. showed that EPO and IGF-1 act synergistically on erythropoiesis in a mouse model of CKD[13] in inducing a substantial rise in Hb. In addition, several possible feedback mechanisms between EPO production and IGF-1 secretion have been suggested.[30,31] In our study, the fact that mean levels of IGF-1 correlated inversely to mean levels of EPO and that subjects with the greatest decrease in CKD and a relatively low Hb had the highest IGF-1 levels, suggests that EPO and IGF-1 both have a role on erythropoiesis in CKD. Taken together, it is possible to speculate that IGF-1constitutes a synergistic role with EPO in erythropoiesis and that when EPO falls a compensatory increase of IGF-1 occurs. However, until now the therapeutic application of IGF-1 is limited by the difficulty of assessing pituitary functional status in CKD patients, and by the interaction with IGF-binding proteins that determine its bioavailability.

IL-6 and TNF-α

In the present study we found no circadian IL-6 and TNF-α rhythm, Inflammation, in this study characterized by IL-6 and TNF-α, has been linked to EPO-resistance and renal disease. An increase in IL-6 and TNF- α has been associated with an increased need for exogenous EPO.[11] In healthy volunteers the concentration of Il-6 is regulated in a circadian fashion with peak levels in the early morning and early evening and through levels later in the morning and at night.[12] TNF-α has shown to have peak levels at night. In a study with 60 patients with CKD IL-6 was significantly increased in comparison to the healthy control

group. Information on times of blood collection was not available.[32] Knowledge on TNF-α levels in renal disease is absent. In our patient group IL-6 levels were raised in contrast to TNF-α levels. No circadian rhythm in IL-6 was found, and furthermore no relationship was found between inflammation and EPO levels, IGF-1 levels, or GFR. This finding is in keeping with another study that showed no association between GFR and IL-6.[32]

In conclusion, we could not demonstrate a GFR-dependent circadian regulation for EPO, IGF-1 (a co-stimulator for EPO) and/or in the levels of inflammatory markers (inhibitors of the action of EPO), which is in contrast to melatonin secretion in CKD. . Furthermore we could not identify the main driving factor for rhythmic fluctuations in endogenous EPO levels on the basis of the results of this study. Due to the varying peak times, it is unlikely that it concerns a circadian (mis)alignment, therefore providing no reason for specific timing of administration of exogenous EPO. Also for IGF-1, TNF-α and IL-6 levels, we were unable to identify a rhythm in any of the patients. Finally, the correlation between IGF-1 and EPO as well as the association between IGF-1, Hb and renal function warrants future research on the role of IGF-1 in CKD patients with persistent anemia.

REFERENCES

1. Elung-Jensen T, Strandgaard S, Kamper AL. Longitudinal observations on circadian blood pressure variation in chronic kidney disease stages 3-5. *Nephrol Dial Transplant* 2008;23:2873-2878.
2. Clement DL, De Buyzere ML, De Bacquer DA, *et al.* Prognostic value of ambulatory blood-pressure recordings in patients with treated hypertension. *N Engl J Med* 2003;348:2407-2415.
3. Koch BC, van der Putten K, Van Someren EJ, *et al.* Impairment of endogenous melatonin rhythm is related to the degree of chronic kidney disease (CREAM study). *Nephrol Dial Transplant* 2010;25:513-519.
4. Karasek M, Szuflet A, Chrzanowski W, *et al.* Decreased melatonin nocturnal concentrations in hemodialyzed patients. *Neuro Endocrinol Lett* 2005;26:653-656.
5. Koch BC, Nagtegaal JE, Hagen EC, *et al.* The effects of melatonin on sleep-wake rhythm of daytime haemodialysis patients: a randomized, placebo-controlled, cross-over study (EMSCAP study). *Br J Clin Pharmacol* 2009;67:68-75.
6. Firsov D, Bonny O. Circadian regulation of renal function. *Kidney Int* 2010;78:640-645.
7. Congote LF, Sadvakassova G, Dobocan MC, *et al.* Erythropoietin-dependent endothelial proteins: potential use against erythropoietin resistance. *Cytokine* 2010;51:113-118.
8. Vaziri ND, Oveisi F, Reyes GA, *et al.* Dysregulation of melatonin metabolism in chronic renal insufficiency: role of erythropoietin-deficiency anemia. *Kidney Int* 1996;50:653-656.
9. Buemi M, Allegra A, Lagana A, *et al.* Effects of the evening i.v. administration of erythropoietin in haemodialyzed patients. *Riv Eur Sci Med Farmacol* 1993;15:195-197.
10. Stratakis CA, Mastorakos G, Magiakou MA, *et al.* 24-hour secretion of growth hormone (GH), insulin-like growth factors-I and -II (IGF-I, -II), prolactin (PRL) and thyrotropin (TSH) in young adults of normal and tall stature. *Endocr Res* 1996;22:261-276.
11. Macdougall IC, Cooper AC. Hyporesponsiveness to erythropoietic therapy due to chronic inflammation. *Eur J Clin Invest* 2005;35 Suppl 3:32-35.
12. Vgontzas AN, Zoumakis M, Papanicolaou DA, *et al.* Chronic insomnia is associated with a shift of interleukin-6 and tumor necrosis factor secretion from nighttime to daytime. *Metabolism* 2002;51:887-892.
13. Brox AG, Zhang F, Guyda H, *et al.* Subtherapeutic erythropoietin and insulin-like growth factor-1 correct the anemia of chronic renal failure in the mouse. *Kidney Int* 1996;50:937-943.
14. van der Putten K, Braam B, Jie KE, *et al.* Mechanisms of Disease: erythropoietin resistance in patients with both heart and kidney failure. *Nat Clin Pract Nephrol* 2008;4:47-57.
15. Heuck C, Skjaerbaek C, Orskov H, *et al.* Circadian variation in serum free ultrafiltrable insulin-like growth factor I concentrations in healthy children. *Pediatr Res* 1999;45:733-736.
16. Van Someren EJ, Nagtegaal E. Improving melatonin circadian phase estimates. *Sleep Med* 2007;8:590-601.
17. Fisher JW, Koury S, Ducey T, *et al.* Erythropoietin production by interstitial cells of hypoxic monkey kidneys. *Br J Haematol* 1996;95:27-32.
18. Fisher JW. Erythropoietin: physiology and pharmacology update. *Exp Biol Med (Maywood)* 2003;228:1-14.
19. Scortegagna M, Ding K, Zhang Q, *et al.* HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner. *Blood* 2005;105:3133-3140.
20. Roberts D, Smith DJ. Erythropoietin does not demonstrate circadian rhythm in healthy men. *J Appl Physiol* 1996;80:847-851.
21. Klausen T, Dela F, Hippe E, *et al.* Diurnal variations of serum erythropoietin in trained and untrained subjects. *Eur J Appl Physiol Occup Physiol* 1993;67:545-548.
22. Buemi M, Allegra A, Aloisi C, *et al.* The circadian rhythm of erythropoietin in subjects with pre-terminal uremia. *Clin Nephrol* 1992;37:159-160.
23. Schmieder RE, Langenfeld MR, Hilgers KF. Endogenous erythropoietin correlates with blood pressure in essential hypertension. *Am J Kidney Dis* 1997;29:376-382.
24. Cahan C, Decker MJ, Arnold JL, *et al.* Diurnal variations in serum erythropoietin levels in healthy subjects and sleep apnea patients. *J Appl Physiol* 1992;72:2112-2117.

25. Correa PN, Axelrad AA. Production of erythropoietic bursts by progenitor cells from adult human peripheral blood in an improved serum-free medium: role of insulinlike growth factor 1. *Blood* 1991;78:2823-2833.
26. Haffner D, Schaefer F, Girard J, *et al.* Metabolic clearance of recombinant human growth hormone in health and chronic renal failure. *J Clin Invest* 1994;93:1163-1171.
27. Tonshoff B, Powell DR, Zhao D, *et al.* Decreased hepatic insulin-like growth factor (IGF)-I and increased IGF binding protein-1 and -2 gene expression in experimental uremia. *Endocrinology* 1997;138:938-946.
28. Lombardi G, Di Somma C, Rota F, *et al.* Associated hormonal decline in aging: is there a role for GH therapy in aging men? *J Endocrinol Invest* 2005;28:99-108.
29. Colao A, Di Somma C, Cascella T, *et al.* Relationships between serum IGF1 levels, blood pressure, and glucose tolerance: an observational, exploratory study in 404 subjects. *Eur J Endocrinol* 2008;159:389-397.
30. Lissoni P, Perego M, Veronese E, *et al.* Endocrine effects of erythropoietin in cancer patients. *Hematology* 2004;9:363-367.
31. Sohmiya M, Kato Y. Human growth hormone and insulin-like growth factor-I inhibit erythropoietin secretion from the kidneys of adult rats. *J Endocrinol* 2005;184:199-207.
32. Oberg BP, McMenamin E, Lucas FL, *et al.* Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. *Kidney Int* 2004;65:1009-1016.

CHAPTER 11

Summary

The prevalence of chronic kidney disease (CKD) as well as chronic heart failure (CHF) is increasing worldwide.[1,2] The combination of CKD and CHF, i.e. the cardiorenal syndrome (CRS), is also an emerging problem, which is accompanied by high morbidity and mortality.[3,4] This high risk is amplified in the presence of anemia, which frequently occurs in CKD and in CHF.[5,6] Treatment of anemia with human recombinant erythropoietin (EPO) has shown beneficial effects in several small studies[7-9], but this could not be confirmed in larger randomized trials.[10-12] Mechanisms underlying these paradoxical effects of EPO are not clear. In this thesis, we aimed to study the role of endogenous as well as exogenous EPO in patients with heart and/or renal failure.

ANEMIA AND ERYTHROPOIETIN TREATMENT IN THE CARDIORENAL SYNDROME

The presence of anemia in cardiorenal failure is mainly caused by defective endogenous EPO production, in combination with a decreased bone marrow response to exogenous and endogenous EPO (i.e. EPO resistance). Clinically, EPO resistance is an important phenomenon, as the need for high dosages of EPO is associated with an increased mortality risk.[13,14] In **part I**, we focused on several aspects of EPO resistance in cardiorenal patients. In **chapter 2**, we reviewed mechanisms underlying endogenous as well as exogenous EPO resistance in patients with CRS. Inflammation appears to have a central role in EPO resistance. In CRS, pro-inflammatory cytokines antagonize the action of EPO by directly inhibiting erythroid progenitor cells[15-17] and by disrupting iron metabolism, in which hepcidin has a central role.[18-20]

In addition to its well known hematopoietic function, EPO has been shown to exert several non-hematopoietic effects. For example, EPO promotes vascular reparative processes in both CKD[21] and CHF[22], and exerts anti-apoptotic effects[23,24]. Importantly, it could also be that EPO intervenes in the pathophysiology of the CRS by dampening the cardiorenal connectors, i.e. inflammation, the balance between nitric oxide (NO) and reactive oxygen species (ROS), the sympathetic nervous system and the renin-angiotensin system.[25,26]

In **chapter 3**, the rationale and design of the EPOCARES study (Erythropoietin in the CardioRenal Syndrome) were described. In this translational study, we aimed to identify pathophysiologic mechanisms of CRS and to elucidate whether EPO indeed affects the cardiorenal connectors. Furthermore, the EPOCARES study was uniquely designed to distinguish hematopoietic from non-hematopoietic effects of EPO treatment. After a 4-week run-in period on standard treatment, patients with CRS and anemia were assigned to receive a fixed dose of (50 IU/kg/week) EPO, but were maintained at baseline hemoglobin (Hb) levels for the first six months by sequential phlebotomy; a fixed dose of EPO to increase Hb levels to a maximum of 13.7 g/dL in men and 13.4 g/dL in women; or to receive standard care without EPO. Follow-up was 12 months. With this design, acute effects of EPO could be discerned from chronic effects, and Hb effects were separated from non-Hb effects of EPO. In this way, this study attempts to explain the fragile balance between desirable and undesirable effects of EPO. For this purpose, cardiac

and renal function as well as a panel of biomarkers and iron parameters were assessed. Furthermore, the effects of EPO on monocyte gene expression profiles and on endothelial progenitor cells were evaluated. In this thesis, results of the iron studies in CRS were discussed.

As mentioned above, hepcidin has a central role in the pathogenesis of EPO resistance. In **chapter 4**, we show that in CRS patients in the EPOCARES study, hepcidin levels are increased compared with a healthy reference population. The levels of hepcidin were correlated with markers or iron load (Hb and ferritin), but not with markers of inflammation (C-reactive protein and IL-6). Thus, in these CRS patients, hepcidin levels reflect iron load rather than inflammatory status. Higher baseline levels of hepcidin were associated with an increased rather than decreased early bone marrow response (as determined by the increase in reticulocyte level) to EPO. A stronger decrease in hepcidin after 2 weeks of EPO treatment was correlated with an increased early bone marrow response to EPO and with Hb response after 6 months. This suggests that hepcidin levels together with inflammatory markers can help to predict EPO response in patients with cardiorenal failure at an early stage of treatment. This is of importance, as high doses of EPO can have several non-hematopoietic adverse effects. The results of this study also raise the question whether hepcidin could be a target in the treatment of anemia in CKD. Indeed, recently, in a mouse model of anemia of inflammation, it has been demonstrated that while administration of EPO by itself could not ameliorate anemia, a combination of EPO and hepcidin-neutralizing antibody was effective in preventing anemia.[27] Furthermore, it has been shown that hepcidin can be antagonized by blocking pathways that regulate its production.[28,29] However, the risks and clinical benefits of these therapies remain to be evaluated.

Red cell distribution width (RDW), a measure of anisocytosis, is associated with adverse outcomes in patients with CHF.[30,31] It has been hypothesized that a disturbed bone marrow response to EPO could explain the association between morphological changes in the red blood cell (RDW) and outcome.[32] In **chapter 5**, we tested this hypothesis in CRS patients from the EPOCARES study. EPO resistance was assessed using 3 measures; 1. the log observed/predicted ratio (O/P), which reflects the EPO level for the degree of anemia, 2. the extent in increase of reticulocyte count, serum transferrin receptor or immature reticulocyte fraction after two weeks of exogenous EPO treatment, and 3. the hemoglobin increase after 6 months of EPO treatment. We found that in our CRS population, RDW was not correlated with EPO resistance. Furthermore, treatment with EPO induced an increase in RDW, which also contradicts an association between RDW and EPO resistance. RDW was correlated with IL-6, but not with hepcidin. In accordance with other studies, there was an independent association between RDW and markers of functional iron availability and erythropoietic activity. Thus, it seems that the association between RDW and outcome is mainly due to the fact that the erythrocyte may be viewed as a 'barometer' for overall cardiovascular health. 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.

In both CKD and CHF, levels of endogenous EPO are elevated when compared to healthy controls.[33-35] However, these patients often remain anemic, indicating a disturbed

bone marrow response to EPO. Prolonged exposure to high levels of endogenous EPO and inflammation, as is the case in CRS, could lead to an increased risk of developing cancer.[36] **Chapter 6** showed the results of a retrospective case-control study in which we examined the incidence of cancer in CRS patients. We found that in patients with CKD and anemia, the presence of heart failure is associated with a higher incidence of cancer (adjusted odds ratio 2.0). Patients with CRS and anemia had higher cumulative incidence rates of cancer than patients with CKD and anemia without CHF and control patients. Although our (hypothesis generating) study does not provide data about the causative relation between CRS and cancer, it stresses the importance of further research into the relationship between CRS and malignancies, as well as into the mechanisms involved. If indeed inflammation or EPO play a role in the pathophysiological mechanism, this could have important therapeutical consequences. Anti-inflammatory agents might prove useful in preventing progression of CRS, but also as anti-cancer therapy.[37] Furthermore, in patients with CRS and/or cancer, exogenous EPO should be used with caution, as the risk will possibly outweigh the benefits.[12]

HEMOGLOBIN VARIABILITY IN CKD PATIENTS

Until more evidence is available with regard to optimal and safe Hb levels in CKD patients, the Anemia Working Group of ERBP as well as the National Kidney Foundation's Kidney Disease Outcomes Quality Initiative (NKF-K/DOQI) guideline recommend a target Hb level of 11-12 g/dL and advise against Hb levels above 13 g/dL.[38,39] However, several studies have shown that in both hemodialysis and predialysis patients, Hb levels are often not maintained within this target range.[40-42]

The oscillations or fluctuations of an individual patient's Hb over time are referred to as 'Hb variability'. This phenomenon is clinically relevant, as many,[41,43,44], but not all[45] studies show that Hb variability is associated with increased mortality. In **Part II**, the focus was on various aspects of the concept of hemoglobin variability in CKD patients.

In **chapter 7,** Hb variability was assessed using a pilot study in four patient groups in a single Dutch dialysis center: hemodialysis (HD) and peritoneal dialysis (PD) patients treated with EPO, predialysis patients using EPO, and predialysis patients not using EPO. We showed that Hb variability was very common, with none of the studied CKD patients using EPO having Hb levels stable within a target range of 11-12 g/dL over a 1-year period. Comparing the four patients groups, there was a stepwise decrease in the magnitude of Hb fluctuation starting, respectively, from the HD patients, the PD patients, the predialysis patients using EPO, to the predialysis patients not using EPO. In chapter 8, we tried to explain this finding.

Chapter 8 showed the results of a retrospective study that was executed in 20 Dutch dialysis centers. In 1393 CKD patients, we demonstrated that Hb variability is a common phenomenon. We confirmed the stepwise decrease in Hb variability when comparing the same four patient groups as in chapter 7. Using multivariate regression models, we found that the frequency of Hb testing, EPO prescribing patterns and hospitalization were the main predictors for Hb variability. Due to the retrospective design of the study, we could

not prove causative relations, however it can be hypothesized that adaptation of treatment policies can contribute to decrease Hb variability. A prospective study using EPO dosing protocols could answer this question. Furthermore, the HD and PD patients in our study had Hb levels within the target range for respectively 28 and 29% of time, which illustrates the difficulty of maintaining Hb levels within the narrow range as recommended by the most recent guidelines. It seems that Hb targets should be defined on an individual basis, and adjusted depending on the clinical context of the patient. In this way, unnecessary and possibly hazardous EPO dosing regimens can be prevented.

ANEMIA AND CIRCADIAN BODY FUNCTIONS IN CKD PATIENTS

In **part III**, we presented the results of the CREAM (Circadian Rhythm of Erythropoietin and Melatonin in CKD)-study. Circadian rhythms are fluctuations in body functions within a period of around 24 h. Circadian misalignment can have important consequences on the pathology of cardiovascular disease and sleep-wake disturbances.[46,47] The pineal hormone melatonin, which is normally only secreted during the night, is an important marker of the circadian timing system. In HD patients, this nocturnal melatonin rise is absent.[48] In **chapter 9**, we showed that in 28 CKD patients with eGFR 30-80 ml/min, melatonin production is increasingly compromised with advancing renal dysfunction. This warrants further research into the causes of dysregulated melatonin secretion and into the effect of exogenous melatonin suppletion on melatonin rhythm in order to improve sleeping disorders in early stages of CKD. In addition to a role in sleep-wake rhythm, other effects of endogenous melatonin have been shown. For example, endogenous melatonin has anti-oxidative effects[49,50], as well as anti-inflammatory effects.[51,52] Therefore, it can be hypothesized that dysregulation of melatonin secretion has a role in the pathophysiology of (cardio)renal failure. These effects of endogenous melatonin in CKD need further investigation. Furthermore, in chapter 9, we did not find a connection between melatonin, cortisol and temperature rhythms in CKD patients, while in healthy persons this is the case. This supports the hypothesis that melatonin acts as an endogenous synchronizer, able to stabilize circadian rhythms.[53]

In addition to inducing sleep-wake disturbances and cardiovascular disease, circadian misalignment could also be important in the pathophysiology of anemia in cardiorenal failure. Based on the findings that the therapeutic effect of exogenous EPO administration is dependent on the time of administration[54] and that exogenous administration of EPO can recover the defective melatonin rhythm in dialysis patients[55,56], we hypothesized that a relation exists between melatonin rhythm and endogenous EPO levels in patients with CKD. In **chapter 10,** we could not demonstrate a clinical significant rhythm for EPO in 28 CKD patients. We also studied whether IL-6 and TNF, pro-inflammatory cytokines that are associated with an inadequate response to EPO in CKD[57], are regulated in an circadian manner in CKD. While in healthy persons this is indeed the case[58], in CKD no rhythm could be demonstrated. IGF-1, which acts as a pro-erythropoietic cytokine, also showed no circadian rhythm. Interestingly however, is that levels of IGF-1 were inversely correlated to levels of EPO and that subjects with the greatest decrease in CKD and a relatively low

Hb had the highest IGF-1 levels. All together, we failed to substantiate our hypothesis that the GFR-related decrease in melatonin rhythm is associated with circadian changes in EPO levels, in the levels of IGF-1 (a co- stimulator of EPO) and/or in the levels of inflammatory markers (inhibitors of the action of EPO). The association between high IGF-1 and low Hb suggests that EPO and IGF-1 have an alternating role, dependent on GFR, in stimulating erythropoiesis. However, until now the therapeutic application of IGF-1 is limited by the difficulty of assessing pituitary functional status in CKD patients, and by the interaction with IGF-binding proteins that determine its bioavailability.

ANEMIA TREATMENT IN CARDIORENAL FAILURE: FUTURE PERSPECTIVES

In this thesis, we found a clear connection between hepcidin levels and response to exogenous EPO, suggesting that hepcidin could be a target of anemia treatment. Currently, this hypothesis is subject of ongoing research.[27-29] Other approaches to stimulate erythropoiesis are also under development. Peginesatide (Hematide™) is a synthetic EPO mimetic peptide with a chemical structure unrelated to EPO that is being evaluated in phase III clinical trials. These trials will reveal whether it provides advantages over the current forms of recombinant human EPO with regard to reduced immunogenicity and reduced frequency of administration. In addition, clinical trials studying the effects of hypoxia-inducible transcription factors (HIF)-stabilizers are being carried out. These agents have been shown to increase plasma levels of endogenous EPO[59], however, the safety of HIF stabilizers requires careful consideration, given the biological potential of HIF in mediating hypoxia-driven processes, such carcinogenic effects.[60]

In addition to the dose and the type drug to stimulate erythropoiesis, the Hb levels that we target for should be considered. As increasing EPO dosages to achieve a certain target is associated with an increased mortality risk, it seems more appropriate to adjust the target itself. This especially accounts for patients with a history of thrombotic events or cancer.

REFERENCES

1. U S Renal Data System, USRDS 2010 Annual Data Report. *Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD,* 2010.
2. McCullough PA, Philbin EF, Spertus JA, *et al.* Confirmation of a heart failure epidemic: findings from the Resource Utilization Among Congestive Heart Failure (REACH) study. *J Am Coll Cardiol* 2002;39:60-69.
3. Scrutinio D, Passantino A, Santoro D, *et al.* The cardiorenal anaemia syndrome in systolic heart failure: prevalence, clinical correlates, and long-term survival. *Eur J Heart Fail* 2010.
4. Smith GL, Lichtman JH, Bracken MB, *et al.* Renal impairment and outcomes in heart failure: systematic review and meta-analysis. *J Am Coll Cardiol* 2006;47:1987-1996.
5. Foley RN, Parfrey PS, Harnett JD, *et al.* The impact of anemia on cardiomyopathy, morbidity, and and mortality in end-stage renal disease. *Am J Kidney Dis* 1996;28:53-61.
6. Groenveld HF, Januzzi JL, Damman K, *et al.* Anemia and mortality in heart failure patients a systematic review and meta-analysis. *J Am Coll Cardiol* 2008;52:818-827.
7. Jones M, Ibels L, Schenkel B, *et al.* Impact of epoetin alfa on clinical end points in patients with chronic renal failure: a meta-analysis. *Kidney Int* 2004;65:757-767.
8. Mancini DM, Katz SD, Lang CC, *et al.* Effect of erythropoietin on exercise capacity in patients with moderate to severe chronic heart failure. *Circulation* 2003;107:294-299.
9. Frank H, Heusser K, Hoffken B, *et al.* Effect of erythropoietin on cardiovascular prognosis parameters in hemodialysis patients. *Kidney Int* 2004;66:832-840.
10. Drueke TB, Locatelli F, Clyne N, *et al.* Normalization of hemoglobin level in patients with chronic kidney disease and anemia. *N Engl J Med* 2006;355:2071-2084.
11. Singh AK, Szczech L, Tang KL, *et al.* Correction of anemia with epoetin alfa in chronic kidney disease. *N Engl J Med* 2006;355:2085-2098.
12. Pfeffer MA, Burdmann EA, Chen CY, *et al.* A trial of darbepoetin alfa in type 2 diabetes and chronic kidney disease. *N Engl J Med* 2009;361:2019-2032.
13. Szczech LA, Barnhart HX, Inrig JK, *et al.* Secondary analysis of the CHOIR trial epoetin-alpha dose and achieved hemoglobin outcomes. *Kidney Int* 2008;74:791-798.
14. Zhang Y, Thamer M, Stefanik K, *et al.* Epoetin requirements predict mortality in hemodialysis patients. *Am J Kidney Dis* 2004;44:866-876.
15. Taniguchi S, Dai CH, Price JO, *et al.* Interferon gamma downregulates stem cell factor and erythropoietin receptors but not insulin-like growth factor-I receptors in human erythroid colony-forming cells. *Blood* 1997;90:2244-2252.
16. Jelkmann W. Proinflammatory cytokines lowering erythropoietin production. *J Interferon Cytokine Res* 1998;18:555-559.
17. Maciejewski JP, Selleri C, Sato T, *et al.* Nitric oxide suppression of human hematopoiesis in vitro. Contribution to inhibitory action of interferon-gamma and tumor necrosis factor-alpha. *J Clin Invest* 1995;96:1085-1092.
18. Nemeth E, Rivera S, Gabayan V, *et al.* IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 2004;113:1271-1276.
19. Mena NP, Esparza AL, Nunez MT. Regulation of transepithelial transport of iron by hepcidin. *Biol Res* 2006;39:191-193.
20. Dallalio G, Law E, Means RT, Jr. Hepcidin inhibits in vitro erythroid colony formation at reduced erythropoietin concentrations. *Blood* 2006;107:2702-2704.
21. Bahlmann FH, DeGroot K, Duckert T, *et al.* Endothelial progenitor cell proliferation and differentiation is regulated by erythropoietin. *Kidney Int* 2003;64:1648-1652.
22. George J, Goldstein E, Abashidze A, *et al.* Erythropoietin promotes endothelial progenitor cell proliferative and adhesive properties in a PI 3-kinase-dependent manner. *Cardiovasc Res* 2005;68:299-306.

23. Calvillo L, Latini R, Kajstura J, *et al.* Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. *Proc Natl Acad Sci U S A* 2003;100:4802-4806.
24. Vesey DA, Cheung C, Pat B, *et al.* Erythropoietin protects against ischaemic acute renal injury. *Nephrol Dial Transplant* 2004;19:348-355.
25. Bongartz LG, Cramer MJ, Doevendans PA, *et al.* The severe cardiorenal syndrome: 'Guyton revisited'. *Eur Heart J* 2005;26:11-17.
26. Jie KE, Verhaar MC, Cramer MJ, *et al.* Erythropoietin and the cardiorenal syndrome: cellular mechanisms on the cardiorenal connectors. *Am J Physiol Renal Physiol* 2006;291:F932-944.
27. Sasu BJ, Cooke KS, Arvedson TL, *et al.* Antihepcidin antibody treatment modulates iron metabolism and is effective in a mouse model of inflammation-induced anemia. *Blood* 2010;115:3616-3624.
28. Yu PB, Hong CC, Sachidanandan C, *et al.* Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. *Nat Chem Biol* 2008;4:33-41.
29. Babitt JL, Huang FW, Xia Y, *et al.* Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance. *J Clin Invest* 2007;117:1933-1939.
30. Felker GM, Allen LA, Pocock SJ, *et al.* Red cell distribution width as a novel prognostic marker in heart failure: data from the CHARM Program and the Duke Databank. *J Am Coll Cardiol* 2007;50:40-47.
31. Pascual-Figal DA, Bonaque JC, Redondo B, *et al.* Red blood cell distribution width predicts long-term outcome regardless of anaemia status in acute heart failure patients. *Eur J Heart Fail* 2009;11:840-846.
32. Allen LA, Felker GM, Mehra MR, *et al.* Validation and potential mechanisms of red cell distribution width as a prognostic marker in heart failure. *J Card Fail* 2010;16:230-238.
33. Fehr T, Ammann P, Garzoni D, *et al.* Interpretation of erythropoietin levels in patients with various degrees of renal insufficiency and anemia. *Kidney Int* 2004;66:1206-1211.
34. Radtke HW, Claussner A, Erbes PM, *et al.* Serum erythropoietin concentration in chronic renal failure: relationship to degree of anemia and excretory renal function. *Blood* 1979;54:877-884.
35. Westenbrink BD, Visser FW, Voors AA, *et al.* Anaemia in chronic heart failure is not only related to impaired renal perfusion and blunted erythropoietin production, but to fluid retention as well. *Eur Heart J* 2007;28:166-171.
36. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;420:860-867.
37. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001;357:539-545.
38. Locatelli F, Aljama P, Canaud B, *et al.* Target haemoglobin to aim for with erythropoiesis-stimulating agents: a position statement by ERBP following publication of the Trial to reduce cardiovascular events with Aranesp therapy (TREAT) study. *Nephrol Dial Transplant* 2010;25:2846-2850.
39. KDOQI Clinical Practice Guideline and Clinical Practice Recommendations for anemia in chronic kidney disease: 2007 update of hemoglobin target. *Am J Kidney Dis* 2007;50:471-530.
40. Ebben JP, Gilbertson DT, Foley RN, *et al.* Hemoglobin level variability: associations with comorbidity, intercurrent events, and hospitalizations. *Clin J Am Soc Nephrol* 2006;1:1205-1210.
41. Boudville NC, Djurdjev O, Macdougall IC, *et al.* Hemoglobin variability in nondialysis chronic kidney disease: examining the association with mortality. *Clin J Am Soc Nephrol* 2009;4:1176-1182.
42. Fishbane S, Berns JS. Hemoglobin cycling in hemodialysis patients treated with recombinant human erythropoietin. *Kidney Int* 2005;68:1337-1343.
43. Yang W, Israni RK, Brunelli SM, *et al.* Hemoglobin variability and mortality in ESRD. *J Am Soc Nephrol* 2007;18:3164-3170.
44. Brunelli SM, Joffe MM, Israni RK, *et al.* History-adjusted marginal structural analysis of the association between hemoglobin variability and mortality among chronic hemodialysis patients. *Clin J Am Soc Nephrol* 2008;3:777-782.
45. Eckardt KU, Kim J, Kronenberg F, *et al.* Hemoglobin Variability Does Not Predict Mortality in European Hemodialysis Patients. *J Am Soc Nephrol* 2010;21:1765-1775.
46. Scheer FA, Hilton MF, Mantzoros CS, *et al.* Adverse metabolic and cardiovascular consequences of circadian misalignment. *Proc Natl Acad Sci U S A* 2009;106:4453-4458.
47. Phillips ML. Circadian rhythms: Of owls, larks and alarm clocks. *Nature* 2009;458:142-144.

48. Koch BC, Nagtegaal JE, Hagen EC, *et al.* The effects of melatonin on sleep-wake rhythm of daytime haemodialysis patients: a randomized, placebo-controlled, cross-over study (EMSCAP study). *Br J Clin Pharmacol* 2009;67:68-75.
49. Geron R, Shurtz-Swirski R, Sela S, *et al.* Polymorphonuclear leucocyte priming in long intermittent nocturnal haemodialysis patients--is melatonin a player? *Nephrol Dial Transplant* 2006;21:3196-3201.
50. Poeggeler B, Reiter RJ, Tan DX, *et al.* Melatonin, hydroxyl radical-mediated oxidative damage, and aging: a hypothesis. *J Pineal Res* 1993;14:151-168.
51. Quiroz Y, Ferrebuz A, Romero F, *et al.* Melatonin ameliorates oxidative stress, inflammation, proteinuria, and progression of renal damage in rats with renal mass reduction. *Am J Physiol Renal Physiol* 2008;294:F336-344.
52. Markus RP, Ferreira ZS, Fernandes PA, *et al.* The immune-pineal axis: a shuttle between endocrine and paracrine melatonin sources. *Neuroimmunomodulation* 2007;14:126-133.
53. Nagtegaal JE, Smits MG, Kerkhof GA. Chronobiological, clinical and pharmacological aspects of melatonin in human circadian rhythm dysfunction. In: Haldar C, Singaravel M, Kumar Maitra S (eds). *Treatise on Pineal Gland and Melatonin.* Enfield, UK: Science Publishers, 2002:461-489.
54. Buemi M, Allegra A, Lagana A, *et al.* Effects of the evening i.v. administration of erythropoietin in haemodialyzed patients. *Riv Eur Sci Med Farmacol* 1993;15:195-197.
55. Vaziri ND, Oveisi F, Reyes GA, *et al.* Dysregulation of melatonin metabolism in chronic renal insufficiency: role of erythropoietin-deficiency anemia. *Kidney Int* 1996;50:653-656.
56. Benz RL, Pressman MR, Hovick ET, *et al.* A preliminary study of the effects of correction of anemia with recombinant human erythropoietin therapy on sleep, sleep disorders, and daytime sleepiness in hemodialysis patients (The SLEEPO study). *Am J Kidney Dis* 1999;34:1089-1095.
57. Macdougall IC, Cooper AC. Hyporesponsiveness to erythropoietic therapy due to chronic inflammation. *Eur J Clin Invest* 2005;35 Suppl 3:32-35.
58. Vgontzas AN, Zoumakis M, Papanicolaou DA, *et al.* Chronic insomnia is associated with a shift of interleukin-6 and tumor necrosis factor secretion from nighttime to daytime. *Metabolism* 2002;51:887-892.
59. Bernhardt WM, Wiesener MS, Scigalla P, *et al.* Inhibition of prolyl hydroxylases increases erythropoietin production in ESRD. *J Am Soc Nephrol* 2010;21:2151-2156.
60. Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* 2006;441:437-443.

APPENDIX 1

The cardiorenal syndrome: a classification into 4 groups?

Karien van der Putten[1]
Lennart G Bongartz[2]
Branko Braam[3]
Carlo A Gaillard[1]

[1]Department of Internal Medicine, Meander MC, Amersfoort, The Netherlands
[2]Department of Cardiology, UMC Utrecht, Utrecht, The Netherlands
[3]Division of Nephrology and Immunology, University of Alberta, Edmonton, Canada

J Am Coll Cardiol 2009;53:1340; author reply 1340-1341

The recent publication by Ronco et al.[1] provides a thorough description of the pathophysiology of the cardiorenal syndrome (CRS) and comes with a proposal for a new classification. The innovative classification is based on the putative causative pathophysiological mechanism that underlies failure of the heart and kidneys. The classification focuses on 2 aspects of the process: duration (acute onset vs. chronic disease), and the sequence of events (kidney failure first vs. heart failure first). For clinical purposes, the distinction between acute and chronic CRS proposed by the authors seems very useful: they propose a clinical syndrome where acute heart failure (HF) leads to acute kidney injury (AKI) (type 1) and vice versa (AKI leading to acute HF; type 3). This seems relevant, because both conditions have a different pathogenesis and require a different clinical approach. However, the distinction between CRS type 2 (chronic HF leading to progressive chronic kidney disease [CKD]) and CRS type 4 (CKD leading to chronic HF and increased risk of cardiovascular events) is based on the assumption that, also in advanced and chronic disease, 2 different pathophysiological mechanisms can be distinguished. In the paper by Bongartz et al.,[2] we postulate a model of (chronic) cardiorenal interactions, with a quite different philosophy. In that model, we have searched for the common denominators in heart and renal failure (i.e., for similar pathophysiological interactions in "heart-kidney" and "kidney-heart" failure). We have been able to identify 4 cardiorenal connectors: inflammation, nitric oxide/reactive oxygen species balance, the sympathetic nervous system, and the renin-angiotensin system. In our view these connectors are, in addition to the well-known hemodynamic interactions between heart and kidney, responsible for the strongly progressive nature of the disease process, because they evoke positive feedback mechanisms. Therefore, the sequence in which the 2 conditions arise is not important. Furthermore, we question the feasibility of the distinction between CRS type 2 and 4 in terms of diagnosis. In addition, both groups are similar in terms of management, because they both should receive optimal interventions to block the interaction between the cardiorenal connectors, in addition to the specific measures that apply to chronic HF and CKD.

REFERENCES

1. Ronco C, Haapio M, House AA, *et al.* Cardiorenal syndrome. *J Am Coll Cardiol* 2008;52:1527-1539.
2. Bongartz LG, Cramer MJ, Doevendans PA, *et al.* The severe cardiorenal syndrome: 'Guyton revisited'. *Eur Heart J* 2005;26:11-17.

APPENDIX 2

Epoetin alfa in critically ill patients

Karien van der Putten[1]
Branko Braam[2]
Carlo A Gaillard[1]

[1]Department of Internal Medicine, Meander MC, Amersfoort, The Netherlands
[2]Division of Nephrology and Immunology, University of Alberta, Edmonton, Canada

N Engl J Med 2007;357:2515-2516; author reply 2516-2517

To the Editor: Corwin et al. report both desired and unwanted effects of erythropoietin in their trial involving patients in the intensive care unit. Paradoxical effects of erythropoietin have been reported in other groups of patients and conditions, and the dose of erythropoietin may be important in this respect. The statement that erythropoietin failed to decrease red-cell transfusions between days 1 and 29, however, may be based on a false premise, since it is generally accepted that in the first 14 days of its administration, this agent does not increase hemoglobin and thus should not influence the need for transfusion.[1] We are concerned about whether iron treatment in the participating patients was adequate and similar in the two groups. Among patients with chemotherapy-related anemia who receive similar amounts of epoetin, intravenous, but not oral, iron supplementation improves the response to epoetin.[2] In addition, oral iron supplements fail to maintain adequate iron stores in epoetin treated patients undergoing hemodialysis.[3] Therefore, it is possible that in the study by Corwin et al., patients in the epoetin alfa group received more parenteral iron than patients in the placebo group. Parenteral iron, as well as the high dose of erythropoietin, might have played a role in the observed, undesired effects of erythropoietin.

REFERENCES

1. van Iperen CE, Gaillard CA, Kraaijenhagen RJ, *et al.* Response of erythropoiesis and iron metabolism to recombinant human erythropoietin in intensive care unit patients. *Crit Care Med* 2000;28:2773-2778.
2. Auerbach M, Ballard H, Trout JR, *et al.* Intravenous iron optimizes the response to recombinant human erythropoietin in cancer patients with chemotherapy-related anemia: a multicenter, open-label, randomized trial. *J Clin Oncol* 2004;22:1301-1307.
3. Macdougall IC, Tucker B, Thompson J, *et al.* A randomized controlled study of iron supplementation in patients treated with erythropoietin. *Kidney Int* 1996;50:1694-1699.

NEDERLANDSE SAMENVATTING

In dit proefschrift worden 3 verschillende aspecten van anemie (bloedarmoede) en erythropoietine (EPO) behandeling bij patiënten met (hart- en) nierfalen behandeld, te weten de respons op endogeen/exogeen EPO, het concept van hemoglobine (Hb) variabiliteit, en de rol van circadiane ritmiek. In deze samenvatting zullen deze aspecten puntsgewijs worden besproken.

HET CARDIORENAAL SYNDROOM

Chronisch nierfalen is een veel voorkomende aandoening die ieder jaar in frequentie toeneemt. Naar schatting 10% van de volwassenen in Europa lijdt in meer of mindere mate aan deze ziekte. Patiënten met nierfalen hebben een sterk verhoogd risico om vroegtijdig te overlijden. Dit wordt met name veroorzaakt doordat deze patiënten vaker hart-en vaatziekten krijgen, zoals een hartinfarct en hartfalen. Omgekeerd krijgen patiënten met hartfalen ook vaker te maken met chronisch nierfalen. De combinatie van hart- en nierfalen wordt het cardiorenaal syndroom genoemd (CRS). In dit syndroom leidt het primaire falen van één van beide organen tot progressieve schade in het andere orgaan. Twee mechanismen staan hierbij centraal. Ten eerste veranderingen in de bloeddruk en de weerstand van de bloedvaten, en ten tweede de 'cardiorenale connectoren': 4 factoren die gestoord zijn in patiënten met CRS, te weten de aanwezigheid van ontstekingseiwitten (1) en oxidatieve stress (2), en een sterk geactiveerd renine-angiotensine systeem (3) en sympathisch zenuwstelsel (4).

ANEMIE IN HET CARDIORENAAL SYNDROOM

Patiënten met CRS hebben frequent anemie, hetgeen gepaard gaat met een verdere toename van het risico op overlijden. Deze anemie wordt veroorzaakt doordat de nieren niet meer in staat zijn om voldoende EPO aan te maken, en doordat het lichaam een verminderde respons heeft op EPO. Sinds enkele decennia worden patiënten met bloedarmoede ten gevolge van chronisch nierfalen behandeld met recombinant humaan EPO; een medicijn dat de aanmaak van rode bloedcellen stimuleert. Recent is echter aangetoond dat EPO behandeling niet de verwachte gunstige effecten heeft, zoals een daling in de sterfte. De oorzaak van deze teleurstellende bevinding ligt mogelijk in het feit dat EPO zowel erythropoietische (toename van rode bloedcellen) alsook non-erythropoietische (o.a. toegenomen vaatproliferatie, verhoogde thromboseneiging en hypertensie) effecten heeft waarbij beide effecten zowel gunstig als ongunstig kunnen zijn. Het netto effect bij een individuele patiënt is met de huidige kennis moeilijk voorspelbaar en wordt wellicht bepaald door de EPO dosis en de karakteristieken van de patiënt.

DEEL I: RESPONS OP ENDOGEEN/EXOGEEN EPO IN HET CARDIORENAAL SYNDROOM

Zoals gezegd wordt anemie in CRS voor een belangrijk deel veroorzaakt doordat het lichaam verminderd reageert op EPO. Dit verschijnsel wordt ook wel 'EPO resistentie' genoemd. Klinisch is dit een relevant fenomeen, aangezien het bestaan ervan gepaard gaat met een toegenomen sterfte. **Hoofdstuk 2** geeft een overzicht van de mechanismen die leiden tot het ontstaan van resistentie voor zowel endogeen als exogeen EPO. Het blijkt dat de aanwezigheid van ontstekingseiwitten (inflammatie) een centrale rol speelt. Door de inflammatie wordt de ontwikkeling van de rode bloedcel geremd, en tevens wordt het ijzermetabolisme verstoord, onder andere via hepcidine.

In **hoofdstuk 3** wordt de opzet van de EPOCARES (EPO in het CardioRenaal Syndroom)-studie beschreven. Deze studie had als doel om het ontstaansmechanisme van het CRS op te helderen, en om erythropoietische van niet-erythropoietische effecten van EPO te onderscheiden. Hiertoe werden patiënten met gecombineerd hart-en nierfalen en anemie geïncludeerd en ingedeeld in drie behandelgroepen: één groep ontving een vaste dosis EPO per week, maar werd de eerste zes maanden op hetzelfde bloedgehalte gehouden door middel van aderlating; één groep werd behandeld met dezelfde vaste EPO dosis, en mocht stijgen in het bloedgehalte; één groep ontving standaard zorg zonder EPO. De patiënten werden een jaar gevolgd. Door middel van deze studieopzet kunnen de gunstige en niet-gunstige effecten van EPO worden geëvalueerd. Voor dit doel werden diverse metingen verricht: meting van hart- en nierfunctie, bepaling van een set van biomarkers en ijzermetabolisme-parameters, genexpressie analyse en meten van het EPO-effect op endotheel voorloper cellen.

In **hoofdstuk 4** hebben wij de rol van hepcidine in het ontstaan van EPO resistentie onderzocht. Hepcidine is een door de lever gevormd acute-fase eiwit dat het ijzermetabolisme reguleert. Hepcidine remt het vrijkomen van ijzer uit diverse cellen, waardoor er een tekort ontstaat aan ijzer dat gebruikt kan worden voor de aanmaak van rode bloedcellen. In de EPOCARES studie hebben we onderzocht of hepcidine verhoogd is bij CRS patiënten en of hepcidine een oorzakelijke rol speelt bij het ontstaan van EPO resistentie. Inderdaad bleek dat CRS patiënten hogere hepcidine spiegels hadden dan een gezonde controle populatie. Hepcidine was gecorreleerd met markers voor ijzervoorraad, maar niet met markers voor inflammatie. De verhoogde hepcidine waarden in deze (stabiele) CRS patiënten weerspiegelen dus de totale ijzervoorraad, maar niet de mate van inflammatie. Behandeling met EPO gedurende twee weken leidde tot een daling in hepcidine. De daling in hepcidine correleerde met de mate van stijging van het aantal jonge rode bloedcellen en tevens met de mate van stijging in het bloedgehalte na 6 maanden. Dit suggereert dat hepcidine levels voorspellend zijn voor zowel de vroege als de late reactie van het beenmerg op EPO therapie. Deze bevinding heeft mogelijk klinische consequenties, omdat deze de mogelijkheid biedt om in een vroeg stadium van behandeling de respons op EPO te voorspellen. Hiermee kunnen onnodige hoge EPO doseringen worden vermeden, wat de kans op nadelige effecten verkleint. Tevens roept deze studie de vraag op of hepcidine zèlf een target zou kunnen zijn in de behandeling van anemie. Inderdaad is dit het onderwerp van diverse studies die momenteel worden

uitgevoerd, waarbij in dierproeven inmiddels is aangetoond dat hepcidine-antagonisten effectief zijn.

Hoofdstuk 5 beschrijft of er een verband bestaat tussen red cell distribution width (RDW) en EPO resistentie. Recent is aangetoond dat RDW, hetgeen een maat is voor de variatie in grootte van rode bloedcellen, een erg sterke voorspeller is voor sterfte bij patiënten met hartfalen. Het mechanisme van deze associatie tussen morfologische veranderingen in de rode bloedcel en slechte prognose is onduidelijk. Sommige auteurs hypothetiseren dat EPO resistentie een causale rol speelt. Hierop hebben wij in de EPOCARES studie onderzocht of RDW bepaald wordt door EPO resistentie, danwel door andere factoren zoals een verstoord ijzermetabolisme. EPO resistentie werd gemeten op verschillende manieren: De endogene EPO resistentie werd uitgedrukt door de geobserveerde/voorspelde log EPO ratio, hetgeen endogene EPO levels definieert t.o.v. de mate van anemie. De exogene EPO resistentie werd gemeten door 1) de stijging in jonge rode bloedcellen en markers voor erythropoietische activiteit na twee weken EPO therapie en 2) de stijging in bloedgehalte na 6 maanden EPO therapie. Het bleek dat RDW niet gecorreleerd is met EPO resistentie in onze CRS patiënten. RDW was gecorreleerd met interleukine-6 (een ontstekingseiwit), maar niet met hepcidine. Tevens was er een associatie tussen RDW en markers voor ijzerbeschikbaarheid en erythropoietische activiteit. Een verhoogd RDW wordt dus mogelijk bepaald door zowel functioneel ijzergebrek (dit geeft kleinere rode bloedcellen) als een verhoogde erythropoietische activiteit (dit geeft grotere rode bloedcellen). Verder onderzoek is noodzakelijk om te bepalen waarom dit leidt tot een hoger risico.

In zowel hart- als nierfalen zijn endogene EPO levels verhoogd in vergelijking tot gezonde controles. Desondanks zijn CRS patiënten anemisch, wat duidt op het bestaan van EPO resistentie. Mechanistisch beschouwd zou het mogelijk kunnen zijn dat langdurige blootstelling aan hoge endogene EPO spiegels en inflammatie, zoals het geval is in CRS, een verhoogd risico geeft op kanker. Om die reden hebben wij in **hoofdstuk 6** onderzocht of kanker vaker voorkomt in patiënten met CRS. Het bleek dat in patiënten met chronisch nierfalen en anemie, de aanwezigheid van hartfalen geassocieerd was met een hogere incidentie van kanker. Bij patiënten met de trias hartfalen/nierfalen/anemie kwam kanker vaker voor dan bij patiënten met chronisch nierfalen en anemie, zonder hartfalen. Ondanks het feit dat we met deze resultaten niet kunnen aantonen dat er een oorzakelijk verband bestaat tussen CRS en kanker, laat deze studie wel zien dat er nader onderzoek dient te geschieden naar het verband tussen CRS en kanker, alsook naar de mechanismen die hierbij een rol spelen. Als het namelijk zo blijkt te zijn dat inflammatie of EPO inderdaad een rol spelen in het ontstaan van kanker, dan zou dit belangrijke therapeutische consequenties met zich meebrengen. Anti-inflammatoire medicijnen zouden kunnen bijdragen aan het voorkomen van progressie van CRS, maar ook een therapie kunnen zijn om kanker af te remmen. Daarnaast dient men bij het gebruik van exogeen EPO bij patiënten met CRS en/of kanker voorzichtigheid in acht te nemen, gezien het potentiële carcinogene effect van dit middel.

DEEL II: HEMOGLOBINE VARIABILITEIT

Zoals eerder genoemd is in diverse grote studies aangetoond dat behandeling met EPO bij patiënten met chronisch nierfalen niet altijd gunstige effecten heeft. Het lijkt zo te zijn dat zowel een te laag als een te hoog hemoglobine (Hb – een maat voor het bloedgehalte) vaak geassocieerd is met negatieve uitkomsten. Totdat duidelijk is wat precies een optimaal en veilig Hb is, wordt in de richtlijnen voor nefrologen een Hb level voorgeschreven van 11-12 g/dL, tot een maximum van 13 g/dL. Het blijkt echter dat in de dagelijkse praktijk de Hb waarde van een patiënt zeer moeizaam binnen de aanbevolen grens is te houden is. Dit geldt voor patiënten met chronisch nierfalen die dialyse ondergaan, maar ook voor nierpatiënten in het pre-dialyse stadium. De schommelingen in het Hb gehalte bij een individuele patiënt worden ook wel 'Hb variabiliteit' genoemd. Hierbij zijn twee zaken van belang, te weten de factoren die Hb variabiliteit veroorzaken, evenals de klinische consequenties. Oorzakelijke factoren zijn waarschijnlijk de wijze waarop behandeling met EPO geschiedt, en mogelijk ook het type EPO. Ook factoren binnen de patiënt zelf, zoals ziekenhuisopname en infecties, lijken een rol te spelen. Over de klinische consequenties verschillen de diverse studies, hoewel de meeste (observationele) studies laten zien dat Hb variabiliteit geassocieerd is met een verhoogde kans op overlijden.

Hoofdstuk 7 laat de resultaten zien van een pilot studie waarin we Hb variabiliteit hebben onderzocht bij 4 patiëntgroepen met chronisch nierfalen binnen 1 ziekenhuis: hemodialyse (HD) patiënten, peritoneaal dialyse (PD) patiënten, pre-dialyse patiënten mét EPO gebruik, en pre-dialyse patiënten zonder EPO gebruik. Over een studieperiode van 1 jaar had géén van de patiënten die behandeld werden met EPO, stabiele Hb levels binnen de marge van 11-12 g/dL. Ook bleek er een stapsgewijze afname te bestaan in de mate van Hb variabiliteit, met de grootste Hb variabiliteit in de HD patiënten, vervolgens in de PD patiënten, dan de pre-dialyse patiënten zonder EPO, met de minste variabiliteit in pre-dialyse patiënten zonder EPO.

In **hoofdstuk 8** hebben we dit fenomeen nader onderzocht. Dit hoofdstuk laat de resultaten zien van een retrospectieve studie die we uitvoerden in 20 Nederlandse dialyse centra. In totaal werden 1393 patiënten met chronisch nierfalen onderzocht. Dezelfde 4 patiëntgroepen als in hoofdstuk 7 werden vergeleken, en het stapsgewijze verschil in Hb variabiliteit werd bevestigd. Door middel van multivariate regressie analyse kon worden aangetoond dat de wijze van EPO prescriptie, het aantal Hb metingen en ziekenhuisopname de belangrijkste voorspellers zijn voor Hb variabiliteit. Omdat het een retrospectieve studie betreft kan geen uitspraak worden gedaan over of causaliteit hier een rol speelt. Een prospectieve studie waarin gebruik wordt gemaakt van protocollen voor de wijze van EPO prescriptie en bloedafnames, zou een antwoord kunnen geven op deze vraag en ook duidelijk kunnen maken of aanpassing van behandelingsstrategieën de mate van Hb variabiliteit kan doen afnemen. Een andere vraag is of de richtlijn voor Hb targets in de huidige vorm wel praktisch haalbaar is. Uit onze studie blijkt dat de Hb levels van HD en PD patiënten voor slechts respectievelijk 28 en 29% van de tijd binnen de gestelde marges zijn. Mogelijk verdient het de voorkeur om Hb targets te definiëren op een individuele basis. Hiermee kunnen onnodige en potentieel gevaarlijke EPO dosisaanpassingen worden voorkomen.

DEEL III: ANEMIE EN CIRCADIANE RITMIEK

Veel lichaamsfuncties worden gereguleerd volgens een circadiaan ritme, dat wil zeggen dat het een ritme betreft met een periode van 24 uur. Een verstoring in dit circadiane ritme kan het risico op hart- en vaatziekten vergroten, en ook nadelige effecten hebben voor het slaap-waak ritme. In patiënten met chronisch nierfalen is het circadiane ritme van diverse lichaamsfuncties verstoord. Zo blijkt dat in hemodialyse patiënten de normale nachtelijke stijging van melatonine afwezig is. Dit leidt tot een verstoring in het slaap-waak ritme, en waarschijnlijk ook tot een toename in oxidatieve stress en inflammatie.

In **hoofdstuk 9** hebben we onderzocht of de productie van melatonine ook gestoord is in patiënten met chronisch nierfalen die (nog) niet behandeld worden met dialyse. Hiertoe werden melatonine spiegels gemeten in 28 patiënten met chronisch nierfalen over een periode van 24 uur. Het bleek dat de productie van melatonine verslechterde bij een toegenomen nierinsufficiëntie. Dit suggereert dat exogeen melatonine toegepast zou kunnen worden om het endogene melatonine ritme te herstellen. Mogelijk leidt dit vervolgens tot een verbetering van de slaap, en ook tot verlaging van inflammatie en oxidatieve stress, hetgeen een remmend effect zou kunnen hebben op de progressie van (hart-) en nierfalen. Hiernaar moet echter nog onderzoek worden uitgevoerd.

Naast de rol van circadiane ritmiek bij slaapstoornissen en het ontstaan van hart-en vaatziekten, onderzochten wij de rol van circadiane ritmes bij het ontstaan van anemie bij chronisch nierfalen. Hiervoor hebben we in **hoofdstuk 10** gekeken naar factoren die de bloedaanmaak remmen, en naar factoren die de bloedaanmaak stimuleren. Zowel IL-6 en TNF zijn ontstekingseiwitten waarvan de aanwezigheid geassocieerd is met EPO resistentie, en dus met een remmend effect op de erythropoiese. Bij gezonde personen worden deze eiwitten gereguleerd in een circadiaan ritme. In hoofdstuk 10 wordt aangetoond dat dit bij patiënten met chronisch nierfalen niet het geval is. EPO en IGF-1 zijn de pro-erythropoietische factoren die werden onderzocht in hoofdstuk 10. Op basis van de bevindingen dat het therapeutisch effect van exogeen EPO afhankelijk is van het tijdstip van toediening, en dat exogeen EPO het melatonine ritme in dialysepatiënten kan herstellen, veronderstelden wij dat er een verband bestaat tussen melatonine ritme en endogene EPO spiegels. Deze hypothese werd niet bevestigd, aangezien er geen significant circadiaan EPO ritme werd aangetoond in 28 patiënten met een verschillende mate van nierfalen. Datzelfde gold voor IGF-1. Een opmerkelijke bevinding van dit onderzoek was dat de spiegels van IGF-1 negatief gecorreleerd waren aan waardes van EPO en dat patiënten met de slechtste nierfunctie en een relatief laag Hb de hoogste IGF-1 levels hadden. Dit suggereert dat EPO en IGF-1 een alternerende rol hebben, afhankelijk van de nierfunctie, in het stimuleren van de bloedaanmaak en dat er mogelijk een therapeutische rol weggelegd is voor IGF-1 in de behandeling van anemie.

Samenvattend laat dit proefschrift zien dat behandeling van anemie bij (hart- en) nierfalen met EPO weloverwogen moet geschieden. Toekomstig onderzoek dient gericht te zijn op het ontwikkelen van nieuwe pro-erythropoietische middelen, met een mogelijke rol voor hepcidine als target. Naast het type medicament dat wordt gebruikt voor de behandeling van anemie, is de dosering van het middel belangrijk. Omdat hoge EPO doseringen geassocieerd zijn met een verhoogde kans op overlijden, is het beter om Hb targets te definiëren op een individuele basis. Dit geldt in het bijzonder voor patiënten met trombose of kanker in het verleden.

DANKWOORD

Zoals alle promovendi ben ook ik blij dat het er op zit. Al moet ik zeggen dat ik met een goed gevoel terug kijk op de afgelopen jaren. De afwisseling tussen kliniek en onderzoek was een hele goede, omdat te lang van hetzelfde niets voor mij is. Maar toch heb ik al de goede herinneringen voornamelijk te danken aan de mensen met wie ik heb gewerkt. Een aantal van hen wil ik hieronder in het bijzonder bedanken.

Allereerst wil ik mijn promotoren en co-promotor bedanken voor de uitstekende begeleiding tijdens mijn promotieonderzoek. Pieter, ik waardeer jouw directe manier van communiceren enorm. Zowel 'live' als via de e-mail. Op de meeste vragen had ik binnen 5 minuten een antwoord, in maximaal 5 woorden. En wat fijn dat jij nooit van een mug een olifant hebt gemaakt. Carlo, ik heb enorm veel respect voor jou als dokter, als onderzoeker en als coach. De manier waarop je de EPOCARES-studie draaiende hield, en zorgde dat iedereen gelijk gestemd was, heeft mede bijgedragen aan de totstandkoming van dit boekje. Jouw creatieve manier van denken zorgde ervoor dat ik altijd stof had om over na te denken en om te analyseren. Je luisterende oor en peptalks tijdens de gebruikelijke promoveer-dipjes hebben me ook enorm geholpen, waarvoor dank! En dan nog iets waarover ik me blijf verbazen: hoe kun je op de hoogte zijn van alle medische ins en outs, van alle digitale-gadget ins en outs, van alle literaire ins en outs, en ga zo maar door. Zitten er bij jou soms 48 uur in 1 dag? Branko, jij had meestal aan een half woord genoeg om te begrijpen wat ik bedoelde. Dat maakte de communicatie - meestal via e-mail of telefoon in verband met jouw emigratie naar Canada - toch heel efficiënt. Ik wil je bedanken voor je enthousiaste instelling van de afgelopen jaren. Jij wist elke teleurstelling bij mij om te zetten in iets positiefs.

Graag wil ik de leden van de leescommissie bedanken voor hun bereidheid om dit proefschrift te beoordelen: Prof. Dr. Gerard Pasterkamp, Prof. Dr. Ton Rabelink , Prof. Dr. Wouter van Solinge, Prof. Dr. Marianne Verhaar en Prof. Dr. Adriaan Voors.

De EPOCARES studie was niet van de grond gekomen zonder een heel team aan gemotiveerde en geïnspireerde mensen. Allereerst gaat mijn dank uit naar Adry. Samen hebben we van 'niets' 'iets' gemaakt. En ook nog eens 'iets' heel succesvols! Een complexe klinische studie als de EPOCARES-studie uitvoeren is niet niks (en dat is zachtjes uitgedrukt). Dankzij jou was dit toch mogelijk, omdat je zorgde voor een logistiek plan, voor digitale ondersteuning, en ook nog voor uitstekende begeleiding van de patiënten. Je bent een nurse-practioner van onschatbare waarde! Kim, al vanaf het eerste moment wist ik dat ik aan jou een goede had. Een strakke planning, nooit iets kwijt en altijd alles op orde. Je hebt je vol toewijding gestort op het labwerk, of het nou 1e kerstdag was of niet. Je mooie proefschrift van een jaar geleden levert hiervan het bewijs. Ik wil je bedanken voor de goede samenwerking en veel succes wensen met je opleiding! Mireille, mijn 'lotgenoot'. Wat fijn dat onze ups en downs elkaar altijd zo mooi afwisselden. Daardoor hoefden we geen van beiden te lang in de put te zitten. Dank voor al je relativerende woorden. Al zitten we al een tijdje niet meer naast elkaar in een raamloos mini-hokje in de kelder, aarzel niet om te bellen voor een peptalk. Ook jij gaat uiteindelijk gewoon promoveren! En je bezit binnenkort een nieuwe titel: die van cardioloog! Karlijn, enorm

bedankt voor het tijdelijk overnemen van de klinische taken in de EPOCARES-studie. Het is bewonderenswaardig hoe weinig tekst en uitleg je nodig had om dit als een hele goede dokter te doen! Je interesse voor de rode bloedcel werd hierdoor ook weer aangewakkerd, waardoor je nu inmiddels je eigen project hebt. Heel veel succes met je promotie-traject en je opleiding tot internist! Marloes, bedankt voor alle ritjes van- en naar Amersfoort als koerier en voor al je labwerkzaamheden. En last but not least wil ik Birgitta, Lennart, Jaap, Maarten-Jan en Marianne bedanken voor de inspirerende en kritische input tijdens de 3-maandelijkse EPOCARES-meetingen.

Het EPOCARES onderzoek was niet mogelijk geweest zonder de medewerking van de nefrologen en de cardiologen in het UMCU en het Meander MC. In het bijzonder wil ik noemen Renate Bosma, Chris Hagen, Bas Hamer, Marcel Landman, Arend Mosterd, Simone Roeffel, Jeff Senden, Peter Verheggen, Evelyn de Vreij en Thierry Wildbergh. Bedankt voor het trouw reageren op de post-itjes op de statussen van jullie patiënten.

Frederieke, als niet-medicus kwam je werken aan je afstudeerproject in het Meander MC. Ongelofelijk hoe snel jij je aanpaste aan het ziekenhuiswereldje en je de medische terminologie eigen maakte. Al kon je na een half jaar de verhalen over bloed nog steeds niet echt waarderen…. Je afstudeerproject heeft geresulteerd in meerdere posters en publicaties, hetgeen genoeg zegt over jouw kwaliteiten! Dank voor de gezellige en efficiënte samenwerking en veel succes gewenst bij je promotietraject.

Nicolas, jij kwam als student naar het Meander MC voor je wetenschappelijke stage. Ik heb me vaak verbaasd over je sterke analytische vermogen en je computervaardigheden. Met welk programma kun jij niet overweg? Je hebt zonder klagen eindeloos patiëntengegevens gescreend en geanalyseerd, bedankt hiervoor! Uiteindelijk is dit terechtgekomen in een hele mooie publicatie; dat doen niet veel studenten je na. Je bent nu je wetenschappelijke talenten verder aan het ontplooien in Groningen, heel veel succes daarmee.

Birgit, jij hebt mij laten zien hoe je als onderzoeker in de periferie een (heel mooi!) proefschrift kan schrijven. Problemen oplossen in plaats van problemen maken, daar ben jij heel goed in. Ik vond het heel leuk om met je samen te werken; onze overlegmomenten waren efficiënt, en de ritjes van en naar Eus waren altijd gezellig! Elsbeth, dank je wel voor je inspirerende bijdrage aan het CREAM onderzoek. Beste Eus, ik weet niet hoe je al die modellen precies geprogrammeerd hebt, maar wat ik wel weet is dat je Birgit en mijzelf enorm hebt geholpen bij het CREAM onderzoek, bedankt hiervoor!

Het verkrijgen van een werkplek als arts-onderzoeker is vaak lastig, en dat geldt met name in een perifere kliniek. Gelukkig ben ik tijdelijk geadopteerd geweest door de afdeling Klinische Chemie, zodat ik beschikte over een kamer met raam. En een kamer met gezelschap! Daan, ik vond het super dat jij je spulletjes aan de kant schoof om een plek voor mij vrij te maken. Rob Kraaijenhagen en Jos Wielders, bedankt voor het mogelijk maken van al mijn onderzoeksprojecten en voor alle brainstormsessies. Dit is ook de plek om alle labmedewerkers te bedanken. Voor het keer op keer uitvoeren van de ingewikkelde protocollen met al die buisjes, per patiënt, per visit….

Het screenen van patiënten, de randomisatie-en controle visits, alle telefoontjes van patiënten, etc, dit alles werd afgewisseld door een heleboel gezellige momenten met alle dames van de poli nierziekten. In het bijzonder wil ik Yvonne en Rianne bedanken. Voor jullie vrolijkheid, kopjes thee, en betrokkenheid. En natuurlijk voor de goede zorg voor de patiënten en jullie ondersteunende rol bij de EPOCARES studie.

Heel erg veel dank aan de staf en de collega's van de afdeling nefrologie in het LUMC. Beste Ton, Hans, Paul, Eelco, Zeynep, Andre, Marlies, Aiko, Joris, Edwin, Hanneke en Karen: ondanks dat ik pas een korte staat van dienst heb op de afdeling, hebben jullie me enorm gesteund de afgelopen tijd. Dit waardeer ik enorm! Jullie hebben mij de mogelijkheid gegeven om mijn proefschrift af te maken, maar zorgen ook voor een hele stimulerende omgeving voor het voltooien van mijn opleiding tot nefroloog. Ook wil ik Noeleen, Ruth, Jeannet, Arnelle, Eva en Marianne bedanken voor hun steun. Het geeft een goed gevoel te weten dat jullie zo betrokken zijn!

Lieve Eli, 15 jaar geleden kwamen we elkaar tegen in de collegebanken en sindsdien hebben we al veel meegemaakt samen. Je bent niet alleen een hele lieve vriendin, maar ook is het met jou nooit saai, omdat jij je over zo'n beetje alles in het leven kunt verbazen. Over bijna elk onderwerp kunnen wij een gesprek hebben, omdat we eindeloos filosoferen over 'hoe het nou zit'. Ik vind het heel bijzonder dat je mijn paranimf wilt zijn. Ben je toch nog een keer 'getuige'!

Lieve Maris, we zijn o zo verschillend, toch zijn we erg goede vriendinnen. En er zijn toch wel een aantal eigenschappen die we delen. Bijvoorbeeld de interesse voor wetenschap. Jouw afstudeerscriptie groeide zelfs uit tot een half proefschrift. Gezellige telefoongesprekken over multivariate analyse…ik vond het leuk dat er iemand met dezelfde vragen zat als ik! Het zou me overigens niets verbazen als jij toch ook nog een keer dr. voor je naam plakt. Super dat je bij mijn promotie mijn paranimf bent!

Lieve Suus en Sjoerd, zus & broer. Heerlijk dat ik me bij jullie altijd thuis kan voelen. En fijn dat jullie de medische gesprekken aan tafel altijd hebben gedoogd. Ze waren dus toch ergens goed voor! Lieve pap en mam, het betekent enorm veel voor mij dat jullie er altijd voor mij en mijn gezin zijn. Oneindig veel dank voor alles.

En dan natuurlijk lieve Falco. Dank je wel voor het aanhoren van al mijn promotie-perikelen. Voor je 24-uurs-bereikbare-helpdesk functie. Jij bent de grappigste, sportiefste en liefste echtgenoot die ik me maar kan wensen. En de beste papa voor Max!

CURRICULUM VITAE

Karien van der Putten is geboren op 12 april 1978 te Nijmegen. Na gewoond te hebben in Malden en in Groningen, kwam zij uiteindelijk samen met haar ouders, haar zus en broertje in Zaanstad terecht. Hier behaalde zij haar VWO diploma. Vervolgens keerde zij in 1996 terug naar Groningen voor haar studie geneeskunde. Haar wetenschappelijke stage liep zij in Sydney en de co-schappen volbracht zij in Enschede en in Ghana.

Na het behalen van haar arts-examen in 2003, verhuisde zij naar Amsterdam en begon met de opleiding tot internist in het Meander MC in Amersfoort. In 2006 startte zij onder leiding van prof. Pieter Doevendans, prof. Carlo Gaillard en dr. Branko Braam met promotie-onderzoek aan de Universiteit Utrecht, met als onderwerp hart-en nierfalen. Het resultaat van dit onderzoek is beschreven in dit proefschrift. Daarnaast voltooide zij haar vooropleiding tot internist, en begon in 2010 met het aandachtsgebied nefrologie in het LUMC te Leiden.

In 2009 trouwde zij en kreeg samen met haar man Falco een zoon, genaamd Max. In ditzelfde jaar verhuisde het jonge gezin van Amsterdam naar Heemstede. Vanuit daar reist zij nu dagelijks naar het LUMC voor het voltooien van haar opleiding tot nefroloog.