CIRCULATING CELLS IN HEART AND RENAL FAILURE

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Circulating Cells in Heart and Renal Failure

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IN HART- EN NIERFALEN

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Voor mijn ouders

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GENERAL INTRODUCTION

CIRCULATING CELLS IN HEART AND RENAL FAILURE

CHRONIC KIDNEY DISEASE AND CARDIOVASCULAR RISK

Chronic kidney disease (CKD) is a worldwide public health problem. In the European Union about 360.000 patients are on renal replacement therapy. The number of patients with earlier stages of CKD exceeds 50 times that reaching end-stage renal disease[1]. The burden of CKD has grown substantially and incidence and prevalence are still increasing[2]. Besides development of end-stage renal disease, which requires renal replacement therapy and is associated with lower quality of life[3] and high mortality rates[2], CKD patients have a high risk of cardiovascular disease (CVD), including coronary heart disease, cerebrovascular disease, peripheral vascular disease and heart failure. At start of dialysis, only 15% of patients have normal left ventricular structure and function on echocardiography[4] and 40% have evidence of coronary heart disease[5]. High prevalence of CVD is not only a major problem in end-stage renal disease. Already in mild to moderate CKD, increased risk of CVD and higher mortality have been reported[6-10].

A decline in renal function is an independent risk factor for atherosclerotic CVD[11]. Patients with CKD show increased prevalence of classical risk factors such as advanced age, hypertension and diabetes mellitus. In addition, CKD related factors, i.e. highly oxidative products and inflammatory markers, may induce endothelial dysfunction[12]. Endothelial dysfunction is considered to be the first step in the development of atherosclerosis. Subsequently, an inflammatory response occurs including the influx of low density lipoprotein, leukocyte adherence and infiltration into the endothelial layer and accumulation of smooth muscle cells, contributing to formation of the atherosclerotic lesion. Maintaining and repairing the integrity of endothelium is important to prevent the development and progression of atherosclerosis.

CARDIORENAL FAILURE

Whereas CKD patients are more likely to develop chronic heart failure (CHF) than the general population[7], patients with CHF more frequently demonstrate impaired renal function . The presence of CKD in CHF is an independent prognostic factor for all-cause mortality, cardiovascular mortality and hospitalizations for heart failure[13-16]. The combination of renal dysfunction and heart failure has been described as the cardiorenal syndrome (CRS)[17], an emerging problem following the rise in prevalence of CKD as well as CHF. We recently proposed a model of the CRS in which cardiac and renal dysfunction mutually amplify progressive failure of both organs[17], resulting in strikingly high morbidity and mortality[7-10, 13-16]. In this thesis, CRS is defined as the combination of CHF and CKD (i.e. CRS type 2 and 4 as described by Ronco et al.[18]) and does not include acute heart or kidney disease.

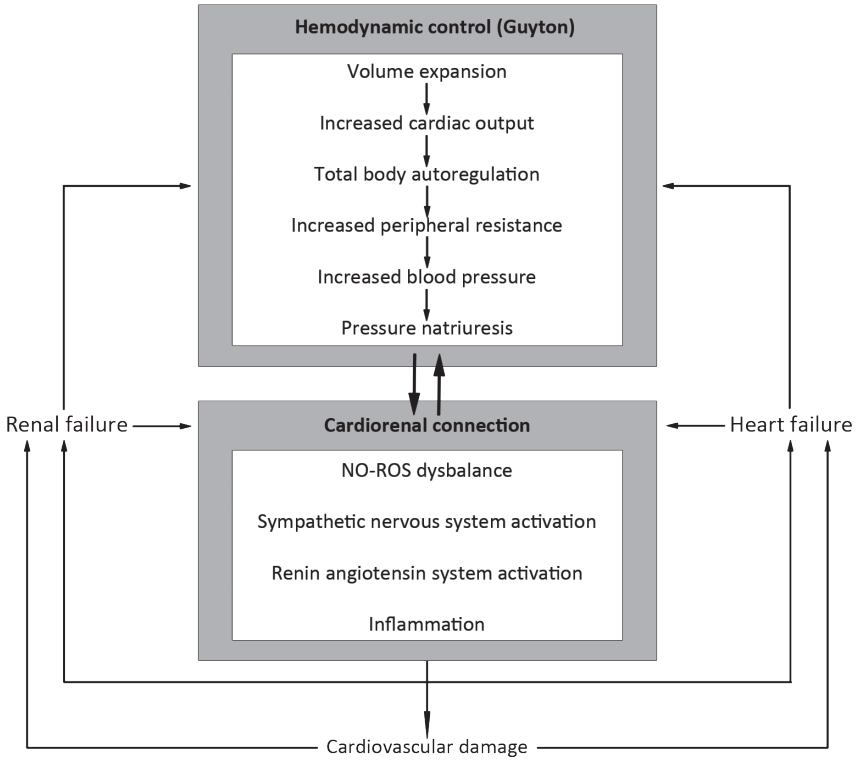
Several factors may contribute to the strong connection between heart and kidney disease. Historically, physiological heart and kidney interaction has been explained by Guyton's hemodynamic model, comprising extracellular fluid volume control, cardiac output and blood pressure[19]. In addition, a number of cardiorenal connectors specifically present in CHF and CKD have recently been proposed to play a key role in the pathophysiology of CRS[17]. Key components are enhanced inflammation and oxidative stress and increased activation of the renin angiotensin system and sympathetic nervous system (figure 01). Derangement of one connector leads to disturbance of the other connectors, resulting in a vicious circle with progressive loss of cardiac and renal function.

In CRS patients anemia frequently occurs due to a (relative) erythropoietin (EPO) deficit, in which disturbance of the cardiorenal connectors may contribute to decreased EPO sensitivity in the BM[20]. Anemia may further aggravate cardiac and renal failure and is associated with worse outcome in CHF[21] and CKD[22, 23]. It is likely that interventions at the level of all key connectors are needed to successfully interfere with the clinical course of CRS.

EPO TREATMENT IN THE CARDIORENAL SYNDROME

Since increased mortality was reported in CKD and CHF in the presence of anemia, effects of hemoglobin (Hb) normalization by EPO treatment have been investigated. Several small studies showed protective effects of EPO on cardiac and renal function in anemic CHF patients. EPO therapy was associated with improvement in functional New York Heart Association class and left ventricular ejection fraction, a reduced need for hospitalization and stabilization of serum creatinine levels[24-26]. In predialysis CKD patients with anemia, EPO treated patients showed a lower relative risk of cardiac disease and death compared to patients who received no or infrequent EPO therapy[27]. In addition, regression of left ventricular hypertrophy[28] and delayed progression of kidney disease[29] have been observed in anemic CKD patients treated with

FIGURE 01 PATHOPHYSIOLOGY OF THE CARDIORENAL SYNDROME



Published by Bongartz et al.[17]

EPO. In contrast, 3 large randomized trials did not show benefit or even demonstrated detrimental effects of anemia correction in CKD and end-stage renal disease patients[30-32]. It appears that EPO might be beneficial in some, but not all patients. It remains to be characterized which patients will encounter benefits or disadvantages. A predictive tool indicating EPO response in an early phase will provide great value in the optimization of treatment in CRS patients.

The mechanism underlying observed effects of EPO is not clear. Besides correction of anemia, several studies support non-hematopoietic capacities of EPO. EPO receptor expression is present on different cell types, including cells in the heart, kidney and vasculature[33-35]. Anti-apoptotic actions of EPO have been demonstrated in cardiomyocytes[36] and proximal tubular cells after hypoxia[37], independent of hemoglobin effects. Moreover, EPO may promote vascular repair processes by enhancing mobilization[38] and function of bone marrow (BM)-derived progenitor cells in CKD[39] and CHF[40]. EPO also demonstrated beneficial effects on the cardiorenal connectors, which will be reviewed in this thesis. It might be that EPO exerts beneficial effects in CRS by either resolving the absolute deficiency or the relative insensitivity by dampening cardiorenal connectors, thereby interrupting the vicious circle and thus intervening in the pathophysiology of CRS. To elucidate which cellular pathways EPO induces in patients, and to distinguish hematopoietic from non-hematopoietic effects of EPO, a clinical study is urgently needed.

CIRCULATING CELLS IN HEART AND RENAL FAILURE

Heart and renal failure both result in disturbances of the systemic environment. BM and circulating cells are consequently exposed to harmful factors such as uremic toxins, oxidative stress and inflammation. In this thesis we will focus on two circulating cell types that play a key role in heart and renal failure: monocytes and bone marrow derived progenitor cells. Monocytes are actively involved in the progression of the organ disease

process and in the development and progression of atherosclerosis. Bone marrow derived progenitor cells may take part in maintenance of endothelial integrity[41] or may contribute to atherogenesis[42].

MONOCYTES IN HEART AND RENAL FAILURE

Monocytes, a mononuclear cell (MNC) type characterized by the expression of cell surface marker CD14, are particularly involved in the initiation of tissue damage. Once infiltrated, monocytes differentiate towards macrophages or dendritic cells to elicit an immune response together with activated T-lymphocytes. Increased infiltrating inflammatory cells such as macrophages and T-lymphocytes have been found in failing myocardium[43] as well as in glomerular and tubulointerstitial kidney disease[44, 45]. Experimental evidence suggests that infiltration of myocardium by peripheral blood MNC contributes to left ventricular remodeling[46, 47]. In addition, increased adhesiveness of monocytes has been related to worse clinical outcome in CHF[48]. Several studies have revealed the importance of interstitial macrophage infiltration in CKD disease progression as well[49-51].

Monocytes also play a critical role in initiation and progression of atherosclerosis[52]. After endothelial cell activation, an inflammatory response occurs. Adhesion and infiltration of monocytes in the vascular wall is one of the first histological signs of atherosclerosis. Once infiltrated into the subendothelial layer, monocytes differentiate towards macrophages and interaction with oxidized low density lipoprotein results in the formation of characteristic foam cells. Secretion of cytokines by macrophages stimulates chemotaxis and attracts T-lymphocytes, which in turn further activates macrophages, endothelial cells and vascular smooth muscle cells[53]. Proliferated smooth muscle cells migrate into the intimal layer of the vessel wall, extracellular matrix synthesis results in a fibrous cap and an advanced atherosclerotic plaque has been formed.

Monocytes can thus be seen as important initiators of tissue and vascular damage. Together with activated T-lymphocytes, they participate in the progression of heart failure, renal failure and the development of atherosclerosis. Activated circulating cells could mirror changes occurring in inflammatory cells infiltrating the failing heart, kidney or vessels, and may act as a biosensor, adapting to changes in the systemic environment[54]. In cardiovascular diseases, peripheral blood MNCs have been used to examine disease-related modifications in gene expression[55-58]. Moreover, gene expression profiling can be helpful in the evaluation of treatment effects[59, 60]. It has previously been shown that leukocytes from hypertensive patients displayed altered gene expression, which could be normalized by antihypertensive therapy[59]. Taken together, we hypothesize that gene expression profiling of monocytes from CRS patients may be of great value to determine disease effects and to evaluate effects of EPO treatment. For successful implementation of gene expression profiling, several conditions should be met. Patient material should preferably be easy to obtain. Monocytes can be isolated by relatively minimal invasive methods. Blood withdrawal (time and procedure), monocyte processing and storage as well as microarray technique should be standardized to achieve optimal representative results[54].

BONE MARROW DERIVED PROGENITOR CELLS IN HEART AND RENAL FAILURE

ENDOTHELIAL PROGENITOR CELLS

BM-derived endothelial progenitor cells (EPC) play a key role in endothelial repair. In response to endothelial damage-induced growth factor and cytokine release, resident endothelial cells start to proliferate, but also EPC are mobilized from the BM to the vasculature. Circulating EPC can home to the site of vascular damage and may replace damaged or lost endothelial cells[61]. In addition, EPC may have paracrine effects on local endothelial cells by secretion of angiogenic growth factors and cytokines[62].

Impaired EPC number and function may contribute to the pathogenesis of CVD. Indeed, the presence of cardiovascular risk factors and CVD has been related to reduced EPC levels and EPC dysfunction[63, 64]. In healthy men and in CVD patients, EPC levels have been correlated with endothelial dysfunction, an early marker of atherosclerosis[65, 66]. Importantly, low EPC levels could predict the occurrence of cardiovascular events and death in patients with coronary artery disease[67] and CHF[68].

EPC availability and function can be affected through multiple mechanisms. Reduced levels of EPC may result from decreased production in the BM, decreased mobilization to the damaged vessel wall following cytokine and growth factor release, diminished survival or enhanced recruitment of EPC to the vessel wall. Differentiation towards an endothelial phenotype is necessary to be functional at the site of endothelial damage. In renal disease, the accumulation of toxins, including highly oxidative products[69], may adversely affect vascular progenitor cell availability and function

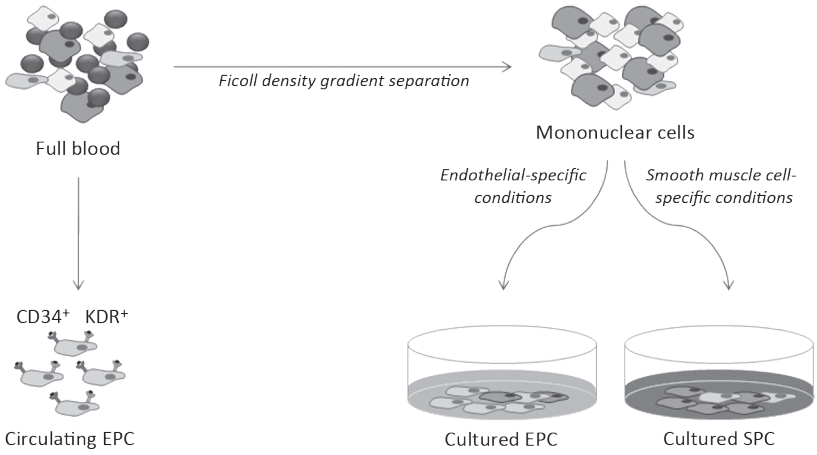
at one or more of these stages. Reduced EPC levels have been demonstrated in adult patients with end-stage renal disease[39, 70], which may add to their increased CVD risk. Children with predialysis CKD or end-stage renal disease are already at increased cardiovascular risk[71]. We hypothesize that in pediatric CKD patients reduced EPC levels may also contribute to this risk. In addition, we examine whether altered EPC number and function may also contribute to the accelerated atherosclerosis development in predialysis CKD.

In CHF, a negative correlation between EPC levels and functional New York Heart Association (NYHA) class has been reported[68]. However, others found higher levels of EPC in mild CHF stages, together with increased levels of EPC mobilizing factors, whereas EPC levels in severe disease were decreased, despite similar increases in progenitor cell mobilizing SDF-1α and VEGF[72, 73]. The latter observation suggests a protective compensatory response to the vascular risk burden in mild CHF, but exhaustion or suppression of BM progenitor cells in advanced CHF. Increased inflammation in severe CHF, known to exert a suppressive effect on hematopoiesis[74], may contribute to the impaired BM EPC response at this advanced stage[73]. We hypothesize that in CRS, the combined presence of even mild stages of both CHF and CKD is associated with impaired levels of circulating EPC, due to the accumulation of uremic toxins (e.g. reactive oxygen species), decreased nitric oxide availability and increased presence of inflammatory markers[17, 75-77].

The discovery of EPC as endogenous vascular repair system protecting against the development of atherosclerosis has gained major interest in CVD research and may lead to novel therapeutic options. One of the therapeutic drugs with potential to improve EPC number and function is EPO. Experimental models demonstrated EPC mobilization from the BM upon EPO treatment, resulting in increased neovascularization in ischemic hind limb and myocardium[38, 78]. EPO also enhanced EPC proliferation and differentiation in patients with advanced renal failure[39, 79],

PROGENITOR CELL CHARACTERIZATION IN PERIPHERAL BLOOD

Several methods have been used to determine EPC number and function (figure). Originally, EPC were described as cultured adherent spindle-shaped endothelial-like cells, resulting from in vitro outgrowth of peripheral blood MNC in specific medium favoring differentiation towards an endothelial phenotype[61]. Outgrowth EPC are mostly monocyte-derived[88, 89]. They lack the progenitor cell characteristic of clonal expansion, but are potent secretors of angiogenic factors, contributing to endothelial repair through paracrine stimulation of the resident endothelium[62, 88]. Outgrowth SPC are obtained by MNC culture in smooth muscle-cell specific medium. A different type of circulating EPC is a subfraction of $CD34^+$-hematopoietic stem cells (HSC), characterized by expression of cell surface markers of endothelial (VEGFR-2 or KDR) lineages. $CD34^+KDR^+$-cells have the ability for clonal expansion and are present in low numbers in the circulation[90, 91].



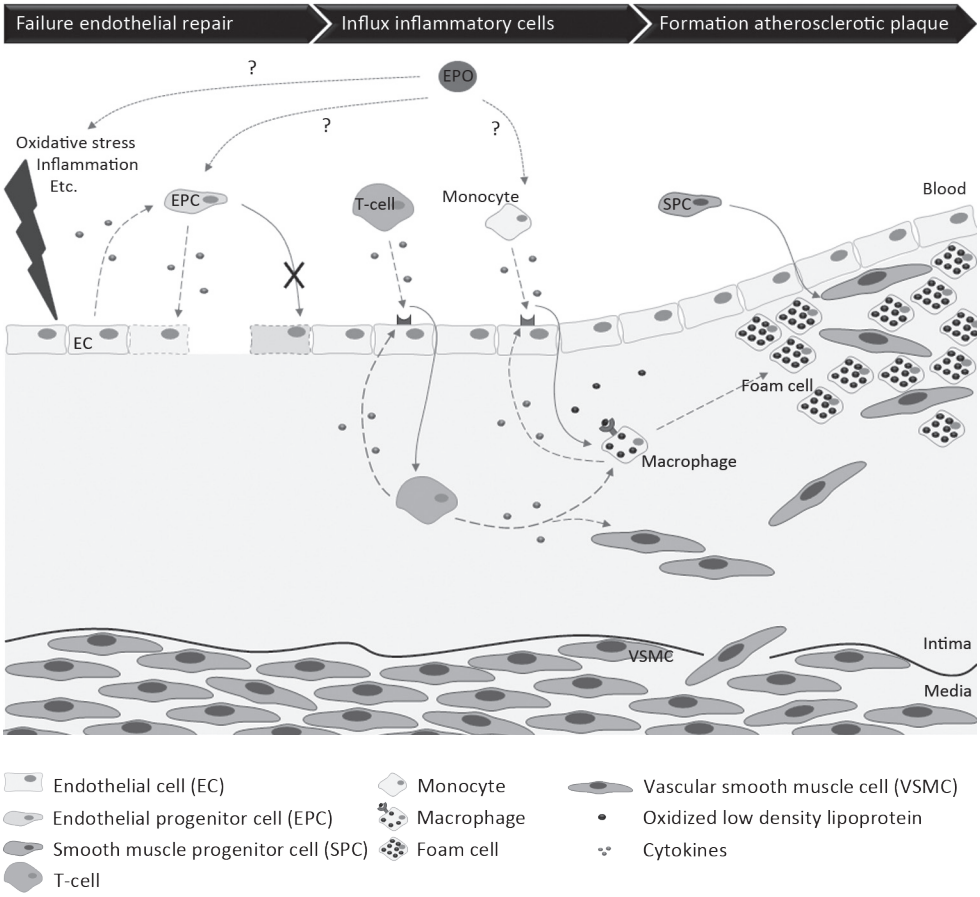
Methods of vascular progenitor cell identification in peripheral blood. Adapted from Westerweel[92]

and promoted EPC proliferative and adhesive properties in patients with CHF[40]. Moreover, EPO may improve EPC survival by anti-apoptotic effects[36, 37]. These findings suggest that EPO treatment may beneficially modulate EPC in patients with CRS.

SMOOTH MUSCLE PROGENITOR CELLS

Circulating vascular progenitor cells may differentiate towards smooth muscle cells in the neointima and myofibroblasts in the interstitium of the vessel wall[80-82]. Animal and human studies demonstrated that these smooth muscle progenitor cells (SPC) may contribute to the development of atherosclerosis[42, 83]. On the other hand, human SPC injection in atherosclerotic-prone mice resulted in plaque stabilization, suggesting a beneficial role of SPC as well[84]. Outgrowth of MNC towards cells with smooth muscle cell characteristics (i.e. alpha smooth muscle actin, calponin, collagen-1) has been demonstrated in humans[85, 86] (see box). In diabetic patients and in patients with coronary artery disease increased SPC levels have been observed, which may contribute to vascular complications[86, 87].

FIGURE 02 CIRCULATING CELLS IN ATHEROSCLEROSIS DEVELOPMENT



From left to right: upon endothelial damage, cytokines are released that trigger local EC proliferation and attract EPC. Disease circumstances may inhibit EPC to incorporate in the EC layer and to secrete cytokines that stimulate local EC proliferation. Due to loss of endothelial integrity and upregulation of adhesion molecules, T-cells and monocytes infiltrate into the vascular wall. Monocytes interact with oxLDL to form foam cells and release cytokines that increase the inflammatory process. VSMC proliferate and migrate from the media to the intima to contribute to atherosclerotic plaque formation stimulated by T-cell cytokines. Bone marrow derived SPC may also participate in the progression of plaque formation. EPO may intervene at different stages of atherosclerosis development.

In conclusion, heart and renal failure result in high cardiovascular morbidity and mortality in which circulating cells play a key role. Monocytes are actively involved in the progression of organ failure and development of atherosclerosis. BM-derived progenitor cells may differentiate towards an endothelial cell phenotype or smooth muscle cell phenotype and protect against or contribute to the development of atherosclerosis. Non-hematopoietic effects of EPO therapy may be beneficial in patients with heart and renal disease, but the exact mechanism of action is unknown (figure 02). Characterization of monocyte gene expression profiles and progenitor cell levels and function may provide further inside in the disease mechanism and treatment effects.

OUTLINE

This thesis focuses on the characterization of circulating cells in cardiac and renal failure.
The cardiorenal syndrome is an emerging problem with remarkably high cardiovascular morbidity and mortality. Cardiorenal connectors -inflammation, oxidative stress, the renin angiotensin system and sympathetic nervous system- have been proposed as cornerstones in the pathophysiology of the CRS[17]. Increased activity of these connectors forms a vicious circle, contributing to organ disease progression and development of CVD. EPO treatment has been shown to exert beneficial effects on heart and kidney function[24-26], but the underlying mechanism is unknown. **Chapter 2** reviews the possible non-hematopoietic effects of EPO on the cardiorenal connectors.

We have constructed the EPOCARES ('Erythropoietin in the CardioRenal syndrome') clinical trial to elucidate hematopoietic and non-hematopoietic effects of EPO. Patients with combined cardiac and renal failure and anemia were included and randomized to receive EPO treatment for 1 year or to be left untreated. In the first half year, EPO-treated patients were either allowed to increase in hemoglobin levels until a specified maximum or were kept on baseline hemoglobin levels by phlebotomy. The study design of this trial is described in **chapter 3**.

Monocytes circulate through the vasculature and are exposed to systemic disturbances in CRS. Moreover, monocytes play a central role in progression of heart failure, renal failure and atherosclerosis. Modulations of monocyte gene expressions may therefore reflect disease-related processes. We questioned whether monocyte transcriptomes in CRS patients from our EPOCARES study reflect the altered systemic environment when compared to age-and gender-matched healthy controls, thereby providing information on the pathophysiology underlying CRS. Furthermore, we evaluated the effect of 18 days EPO treatment on monocyte transcriptomes, when a hematopoietic response did not yet occur. The development of the bioinformatics pathway used to analyze monocyte gene expression differences is specified in **chapter 4**. Results of this transcriptome study are described in **chapter 5**.

Atherosclerosis is the main cause underlying cardiovascular morbidity and mortality. The initial step in the development of atherosclerosis is endothelial dysfunction. Maintenance of endothelial integrity thus plays a central role in the protection against CVD. BM-derived EPC form an endogenous vascular repair system. EPC are recruited from the BM upon endothelial damage. They home to the damaged vascular wall and take care of repair by incorporating in the endothelial layer or by paracrine stimulation of resident endothelial cells[61, 62]. Several factors negatively influence EPC, which may result in increased risk of CVD. In **chapter 6** we investigated EPC levels in physiologic aging, which is associated with endothelial dysfunction. Decreased EPC levels and function have been described in pathophysiological circumstances, such as coronary artery disease, diabetes mellitus and end-stage CKD[63, 70, 87, 93]. Since children with CKD have substantially increased risk of CVD, we explored whether EPC levels are altered in children with predialysis CKD and end-stage CKD on hemodialysis. Results are described in **chapter 7**. In addition to decreased EPC levels, preserved SPC levels have been demonstrated in end-stage renal disease patients, which may contribute to vascular complications. In **chapter 8** we investigated whether an imbalance between EPC and SPC is already present in patients with predialysis CKD. This imbalance may result in impaired regenerative and enhanced profibrotic tendency and contribute to the increased risk of atherosclerotic CVD. In **chapter 9** we compared EPC levels and function in the CRS patients of our EPOCARES study with healthy age-matched controls. EPO is a promising agent to improve vascular regeneration, since it enhances EPC mobilization and function[39, 40, 79]. We therefore evaluated the effect of short- and long-term EPO treatment on EPC levels and function.

The studies described in this thesis are summarized and discussed in **Chapter 10**.

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CH.02

ERYTHROPOIETIN AND THE CARDIORENAL SYNDROME: CELLULAR MECHANISMS ON THE CARDIORENAL CONNECTORS

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ABSTRACT

We have recently proposed the severe cardiorenal syndrome (SCRS), in which cardiac and renal failure mutually amplify progressive failure of both organs. This frequent pathophysiological condition has an extremely bad prognosis. Positive feedback between inflammation, the renin-angiotensin system, the balance between the nitric oxide and reactive oxygen species and the sympathetic nervous system form the cardiorenal connectors and are cornerstones in the pathophysiology of the severe cardiorenal syndrome. Absolute deficit of erythropoietin (EPO) and decreased sensitivity to EPO in this syndrome both contribute to the development of anemia, which is more pronounced than renal anemia in the absence of heart failure. Besides expression on erythroid progenitor cells, EPO receptors are present in the heart, kidney and vascular system, in which activation results in anti-apoptosis, proliferation, and possibly anti-oxidation and anti-inflammation. Interestingly, EPO can improve cardiac and renal function. We have therefore reviewed the literature with respect to EPO and the cardiorenal connectors. Indeed, there are indications that EPO can diminish inflammation, reduce renin-angiotensin system activity and shift the nitric oxide and reactive oxygen species balance towards nitric oxide. Information about EPO and the sympathetic nervous system is scarce. This analysis underscores the relevance of further understanding of clinical and cellular mechanisms underlying protective effects of EPO, since this will support a better treatment of the severe cardiorenal syndrome.

INTRODUCTION

Co-existence of renal and cardiac disease is associated with high morbidity and mortality. This pathophysiological condition, in which combined cardiac and renal dysfunction amplifies progression in failure of the individual organ, has recently been denoted as the severe cardiorenal syndrome (SCRS)[1, 2].

In patients with declined renal function measured by glomerular filtration rate (GFR), an independent increased risk for cardiovascular events has been found. The hazard ratio for cardiovascular events was 1.4 with a GFR less than 60ml/min and increased with diminished renal function to 2.8 in patients with a GFR below 30ml/min[3]. A 40% higher adjusted risk for adverse cardiovascular outcomes or death was observed in those with relatively minor degrees of renal dysfunction[4]. The prevalence of chronic renal failure (CRF) is rEPOrtedly high in patients with congestive heart failure (CHF). Furthermore, the prevalence and severity of CRF correlates with the severity of CHF[5, 6]. This data supports that combined cardiac and renal failure is a profound problem and further understanding of the underlying pathophysiological and molecular mechanisms is needed to improve therapy.

Our group proposed an interactive network of cardiorenal connectors -the renin-angiotensin system (RAS), the nitric oxide and reactive oxygen species (NO-ROS) balance, the sympathetic nervous system (SNS) and inflammation- as cornerstones of the pathophysiology of SCRS[1] . Since erythropoietin (EPO) production declines in renal failure and EPO sensitivity might decrease by the cardiorenal connectors in patients with the SCRS, it is not surprising that anemia is a commonly occurring state coinciding with CRF and CHF[7]. About half of all patients with CHF have anemia and the prevalence of anemia increases with the severity of CHF, approaching 80% in those with New York Heart Association (NYHA) class IV[8]. Declining renal function is also associated with anemia, approaching 90% of patients with a glomerular filtration rate (GFR) below 30 ml/min[9]. The coexistence of heart and renal failure and anemia has been denoted as the cardiac-renal-anemia syndrome.

In this review, we focus on how EPO could dampen the cardiorenal connectors. We propose that EPO-treatment in patients with SCRS not only acts via hematopoietic effects, but also intervenes in the vicious circle of cardiorenal connectors with subsequent deteriorating effects on cardiac, renal and vascular function. Indeed, it appears that regular EPO treatment in anemic

patients with diminished renal function improves cardiac performance[10] and delays progression of kidney disease[11]. There is also evidence that EPO treatment can be of clinical benefit for patients suffering from CHF with relatively mild anemia[8]. Despite growing evidence about EPO having positive effects on both renal and cardiac function, little is known about the underlying mechanisms of action. Although examples of how the cardiorenal connectors could diminish the production of EPO exist, EPO resistance is not central in this review.

We investigate mechanisms by which (exogenous supplementation of) EPO could dampen the cardiorenal connectors in combined cardiac and renal failure. First, we will briefly recapitulate the proposed model of cardiorenal connectors in the SCRS and supply background on EPO. Subsequently, an analysis using existing literature about the effect of EPO upon these cardiorenal connectors with its possible underlying mechanisms will be provided.

THE SEVERE CARDIORENAL SYNDROME AND THE CARDIORENAL CONNECTORS

Recently, we proposed that in the SCRS, the cardiorenal connectors – the RAS, the NO-ROS balance, the SNS and inflammation - display mutual interactions and induce positive feedback loops at many points[1]. Considering the RAS, inappropriate activation of the RAS in renal and cardiac failure causes (dys)regulation of extracellular fluid volume and vasoconstriction[12], results in formation of ROS via activation of NADPH-oxidase[13, 14], leads to vascular inflammation via the nuclear factor kappa B (NF-κB) pathway[15, 16], and increases sympathetic activity[17].

On the other hand, the imbalance between NO and ROS, by increased ROS production[18], a low anti-oxidant status[19] and lower availability of NO[20], may increase activity of pre-ganglionic sympathetic neurons[21], and stimulate the RAS directly by damaging the renal tubular or interstitial cells or by afferent vasoconstriction with chronic inhibition of NO synthesis[22]. ROS are also major initiators of inflammatory response, resulting in a shift towards production of inflammatory cytokines[23].

The chronic inflammatory state which is present in both CRF and cardiac failure, in turn, can cause ROS production by activating leucocytes to release their oxidative contents[24]. As part of the systemic stress response, renin secretion is stimulated by cytokines and pro-inflammatory cytokines can stimulate norepinephrine release from sympathetic neurons[25].

Finally, the increased SNS activity in both renal and heart failure may induce inflammation by norepinephrine-mediated cytokine production[26] and by releasing neuropeptide Y which can alter cytokine release and immune cell function[27, 28]. In this way, all four cardiorenal connectors can augment each other with their deleterious effects in SCRS as a consequence. For a more detailed description of the interaction between the cardiorenal connectors in combined cardiac and renal failure, see[1].

ERYTHROPOIETIN IN THE SEVERE CARDIORENAL SYNDROME

Several clinical studies have demonstrated protective effects of EPO on cardiac and renal function[29]. In patients with heart failure, correction of anemia with EPO was associated with an improvement of NYHA class and left ventricular ejection fraction (LVEF), a reduction in the need for hospitalization and high dose diuretics, and an amelioration of peak exercise oxygen utilization and quality of life. In addition, stabilization of serum creatinine has been shown with correction of anemia[8, 30, 31]. Similar findings were observed when treating anemia in diabetics and non-diabetics with severe CHF and mild to moderate CRF[32]. In pre-dialysis CRF patients with anemia, a retrospective study of EPO-treatment not only showed lower rates of hospitalization and treatment costs at start of dialysis, but also a lower relative risk of cardiac disease and death, compared to patients who received no or infrequent EPO therapy[10]. Regression of LVH[33] and delayed progression of kidney disease[11] in anemic CRF patients treated with EPO has also been observed. When treating CRF patients with EPO, correction of hematocrit up to 36% is desirable, since ameliorated cardiac and renal function are seen at this level, while normalization to 42% might increase cardiovascular events[34].

Since EPO-treatment effectuates erythropoiesis, supplemental iron is required. Specially when given intravenously, iron may incite free radical formation and oxidative stress, which may lead to injury of cells and enhanced atherosclerosis[35]. However, opposite results contradict this association, in that higher serum iron concentrations were associated with a decreased mortality from cardiovascular disease[36]. Furthermore, hemoglobin is a crucial anti-oxidant, thus anemia means decreased anti-oxidant capacity. In the studies described above, improved cardiac and renal function were seen upon EPO-treatment, even with supplemental iron. Thus, positive effects of EPO have been demonstrated in patients with combined cardiac and renal dysfunction. These effects could result

from increased hemoglobin levels or from non-hematopoietic actions of EPO-treatment.

REGULATION OF ERYTHROPOIETIN PRODUCTION

To understand how EPO could exert its effects on the cardiorenal connectors in the SCRS, it is important to understand how EPO production is regulated, how intracellular signaling of EPO takes place and where it can influence processes by the existence of EPO-receptors (EPO-R).

EPO is a member of the cytokine super-family, with significant homology to mediators of growth and inflammation[37]. Its expression is primarily limited to cells in the fetal liver and the adult kidney. Soon after birth, the kidneys become the main site of production of (circulating) EPO[38]. Most evidence favors that peritubular interstitial cells are the primary renal site of regulated EPO production, but a tubular origin is also possible[39]. Low levels of EPO expression have also been found in other organs, including the lung, spleen, brain and testis of rats[40, 41]. EPO is secreted and circulates in the plasma with concentrations ranging from 3 to 15 U/l[42].

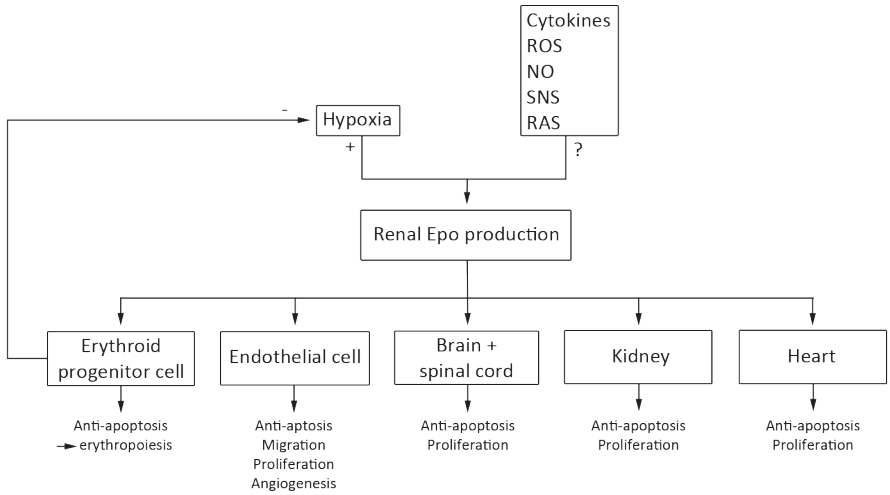
The primary stimulus of EPO production is tissue hypoxia, which activates hypoxia inducible factor-1 (HIF-1), which in turn induces transcription of the EPO-gene. The increase in EPO mRNA reaches its maximum at 4 to 8 hours after exposure to hypoxia, following the time course of HIF-1 activation. The mechanism responsible for the activation of HIF-1 proceeds via the oxygen labile subunit HIF-1α. Hypoxia blocks degradation of HIF-1α by blocking its association with von Hippel Lindau protein that targets HIF-1α for proteolysis[43].

Other stimuli can modulate EPO expression via transcription factors GATA-2 and NF-κB[44]. Cytokines such as IL-1 and tumor necrosis factor-α (TNF-α) activate GATA-2 and NF-κB, suggesting that both transcription factors are involved in the inhibition of EPO gene expression in inflammatory diseases[45, 46]. *In vitro*, pro-inflammatory cytokines such as IL-1α, IL-1β and TNF-α dose-dependently inhibit hypoxia-induced mRNA EPO gene and protein expression[47, 48]. In chronic disease, anemia is seen together with enhanced IFN-γ, IL-1 and TNF-α[49]. Contrariwise, other studies have demonstrated that IGF-1, IL-1, IL-6 and TNF-α can lead to enhanced EPO and EPO-R expression[43, 50, 51]. Moreover, NF-κB has been shown to play a key role in HIF-1-regulated EPO gene expression[44].

Several studies also describe contradictory effects of ROS on EPO gene expression and EPO production[52, 53] and similarly, the anti-oxidative extracellular superoxide dismutase can either suppress or enhance hypoxia-induced EPO gene expression[54, 55], possibly via modulation of hydrogen peroxide levels or a subsequent change in NF-κB expression[56]. NO seems to play a role in the induction of EPO production by inhibiting HIF-1α activation and destabilization of HIF-1α[57].

Finally, activity of the RAS and SNS were also shown to affect erythropoiesis. Administration of angiotensin II (Ang II) dose-dependently increases EPO production[58, 59] and angiotensin-

FIGURE 01 EPO PRODUCTION AND EFFECTS OF EPO RECEPTOR STIMULATION AT DIFFERENT TARGET ORGANS



converting enzyme inhibitors (ACEi) decrease plasma EPO concentrations, likely by inhibiting Ang II[60]. Several studies have suggested that the SNS can stimulate erythropoiesis, since reduced SNS activity is accompanied by anemia which could be corrected by administration of EPO[61, 62]. However, there is a discordance since increased SNS activity is present in both renal and cardiac failure[17, 63], whereas anemia is also present. This could be caused by predominance of factors other than SNS activity, such as inflammation, that decrease erythropoiesis.

Taken together, EPO production is primarily induced by tissue hypoxia, but inflammation, ROS, NO, the RAS and the SNS can also modulate EPO production (figure 01). However, the exact regulatory mechanisms underlying the effects of these cardiorenal connectors upon EPO production need to be clarified.

ERYTHROPOIETIN RECEPTOR EXPRESSION

The most well-known effect of EPO is activation of receptors expressed specifically on erythroid progenitor cells, thereby promoting the viability, proliferation, and terminal differentiation of erythroid precursors, and accelerating the release of reticulocytes from the bone marrow, resulting in an increase in red blood cell mass[38]. Recombinant EPO therapy has been shown to directly stimulate hematopoiesis, thereby increasing hemoglobin levels[64]. The effect of EPO on the growth of erythroid precursors is augmented by other hormones, such as androgens, thyroid hormones, somatomedins and catecholamines[65]. However, effects of EPO extend beyond hematopoiesis. In the embryo, EPO-R are found in almost every embryonic tissue; EPO acts as a major regulator of vascular formation and organ growth[66]. The expression of EPO and human EPO-binding sites in adults has been demonstrated in other tissues and organs, including human kidney, heart and vascular system (table 01).

TABLE 01 CELL PHYSIOLOGICAL EFFECTS OF THE ACTIVATED EPO RECEPTOR

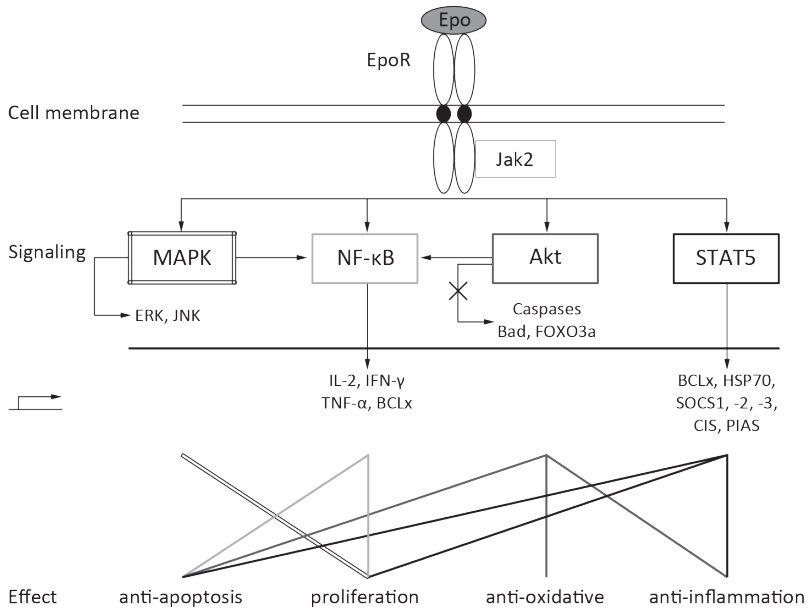
ORGAN	EPO-R	EFFECT OF ACTIVATED EPO-R
Heart	Cardiomyocyte[67, 68]	Proliferation[72], anti-apoptosis[68]
	Cardiomyoblast[69, 70]	Proliferation[69], anti-apoptosis[70]
	Cardiofibroblast[71]	
Kidney	Tubular cell[73, 74]	Anti-apoptosis[75], proliferation[74]
	Mesangial cell[74]	
	Glomerular cell[73]	
Vascular system	Endothelial cell[76, 77]	Migration, proliferation[76], anti-apoptosis[79], angiogenesis[80, 81]
	Endothelial progenitor cell	Mobilization from bone marrow[82], proliferation, differentiation[83]
	Vascular smooth muscle cell[78]	Anti-apoptosis[84]

Surprisingly, mechanisms of regulation of EPO-R expression have not been well studied[85]. It has been demonstrated that expression of the EPO-R may be enhanced in a variety of non-hematopoietic cell types by the presence of hypoxia. In rat brain, upregulation of EPO and EPO-R has been demonstrated after induction of ischemia[86]. Increased hypoxia-associated EPO and EPO-R expression has also been shown in different tumor types[87]. Whereas HIF-1α is known to mediate the expression of EPO, HIF-1α has not been identified as a regulator of EPO-R gene expression. Some studies in erythroid cell lines indicate that the transcription factors GATA-1 and Sp-1 could be involved in EPO-R gene regulation[88, 89]. Nevertheless, it seems that multiple pathways for EPO-R regulation exist and not all of them are GATA-1 dependent[89]. In the mouse brain, EPO-R transcripts decrease during development. A similar pattern is seen in erythropoiesis, in which there is also a rapid reduction in EPO-R expression as cells progress towards terminal differentiation[90]. The scant available information suggests tissue-specific EPO-R regulation, in response to different stimuli such as hypoxia or developmental aspects. However, the exact regulation of EPO-R expression in different cell types remains to be elucidated. It might even be that the cardiorenal connectors act upon EPO-R expression, thereby possibly explaining the occurrence of EPO resistance in patients with CRF.

INTRACELLULAR SIGNALING OF ERYTHROPOIETIN

The first step in EPO signaling is induction of homodimerization of EPO-R by EPO. Subsequently,

FIGURE 02 INTRACELLULAR SIGNALING ROUTES OF EPO



Positive feedback loops can be evoked in this circuitry, e.g. by NF-κB, which could be further activated by oxidative stress, caused by the inflammatory response.

one of the receptor associated Janus family of protein tyrosine kinases, JAK2, is activated, leading to tyrosine phosphorylation of the EPO-R cytosolic domain[91]. Phosphorylated tyrosines provide docking sites for proteins, such as signal transducer and activator of transcription factor 5 (Stat5), phosphoinositide-3 kinase (PI-3K), mitogen-activated protein kinase (MAPK), NF-κB and SHP1[91] (Figure 02). SHP1 can lead to JAK2 dephosphorylation and inactivation, thereby negatively regulating EPO-R signaling[92].

One well-studied non-hematopoietic action of EPO is anti-apoptosis. EPO can prevent apoptosis by consecutive activation of PI-3K and Akt[93, 94]. Akt also induces a variety of other effects, including the mediation of anti-inflammatory cellular responses. Moreover, EPO-R activation leads to increased NF-κB, followed by decreased apoptosis in erythroid progenitor cells[95]. Next to activation of the PI-3K/Akt pathway, EPO-EPO-R interaction leads to MAPK activation[96, 97], which is involved in cell proliferation[96]. The third pathway activated by EPO-R via JAK2 is Stat5 which is thought to be important for mitogenic activity[98], but also protects against apoptosis[98, 99]. Stat5 induces a variety of cellular responses, including upregulation of anti-apoptotic genes such as Bcl2, Bcl-x_L and HSP70[100-102]. Furthermore, STAT5b has shown to affect inflammation by inhibiting NF-κB mediated gene transcription, probably by competing with co-activators necessary for NF-κB signaling[103]. STAT5 induces suppressors of cytokine signaling-1 (SOCS1), SOCS2, SOCS3 and cytokine-inducible SH2-containing protein (CIS1)[104]. These SOCS family members take part in the negative feedback loop to attenuate EPO-signaling by binding to JAK2 or the activated EPO-receptor (SOCS1 and SOCS3 respectively) or by blocking STAT-binding to the EPO-receptor (CIS1)[105]. In this way, they are intrinsic modulators of JAK/STAT signaling. SOCS3 appears to be the most relevant in erythropoiesis in vivo, as SOCS3 -/- mice have severe erythrocytosis and mice overexpressing SOCS3 are anemic[106]. SOCS protein modulation could well be important in the regulation of the cardiorenal connectors, as discussed below.

KNOWN ACTIONS OF EPO ON CARDIAC, RENAL AND VASCULAR CELLS

Multiple responses of cardiac, renal and vascular cells upon binding of EPO to its receptor have

been described (figure 01). Activation of the EPO receptor has shown to stimulate proliferation in cardiomyoblasts [69] and cardiomyocytes[72]. In vitro, EPO prevents apoptosis in rat cardiomyocytes exposed to hypoxia, as well as to oxidative stress. Additionally, in vivo rodent models of coronary ischemia-reperfusion showed that administration of EPO reduces cardiomyocyte loss by ~50%, reduces infarct size, increases viable myocardium and mitigates ventricular dysfunction after myocardial infarction[68, 70, 107]. Van der Meer et al[108] performed an ischemia/reperfusion experiment in isolated rat hearts. Administration of EPO reduced the cellular damage by 56% during reperfusion, diminished apoptosis by 15% and resulted in significantly improved recovery of left ventricular pressure and coronary flow. Prevention of cardiomyocyte apoptosis in rats after administration of a derivative of EPO, lacking erythropoietic activity, indicates hemoglobin-independent cardioprotective effects[109]. This finding is supported by a rabbit study in which cardioprotective effects of EPO after infarction were seen, without an increase in hematocrit[70]. It is likely that EPO-actions on left ventricular function and coronary flow are not only mediated via anti-apoptotic effects of EPO, but also involve other actions such as increased NO-production[110].

Several studies implicated that EPO also acts upon renal cells. In vitro, human proximal tubular cells exposed to hypoxia and co-incubated with EPO showed reduced apoptosis and high levels of EPO even increased cell proliferation[111]. The same group of investigators demonstrated that EPO administration enhanced tubular regeneration and renal function recovery in rats with uni- and bilateral ischemia/reperfusion injury. Moreover, in a pig kidney endothelial cell (EC) line and in mouse mesangial cells, darbepoetin, an EPO analogue, reduced apoptosis after exposure to different toxic and hypoxic stimuli[112]. Finally, enhanced renal tubular regeneration[113], renal blood flow and GFR by EPO administration was observed in a rat model with acute renal failure induced by cisplatin[114].

The mechanism of the vascular protective effects of EPO is largely unknown. Various studies have demonstrated that EPO may affect the vascular wall upon binding to local receptors. EPO may promote vascular reparative processes[115]. It was demonstrated that EPO could enhance mobilization of bone marrow derived endothelial progenitor cells (EPC) and augmented neovascularization in animal models[82], enhanced EPC proliferation and differentiation in patients with advanced renal failure[83] and promoted EPC proliferative and adhesive properties in patients with CHF[116]. Patients with CRF have endothelial dysfunction and enhanced oxidative stress[117], whereas EPO seems to have anti-oxidative properties[118, 119] and pro-angiogenic effects on endothelial cells[81]. Furthermore, EPO stimulates human and bovine EC proliferation and migration[76, 120], and capillary outgrowth in an angiogenesis assay using adult human myocardial tissue in vitro[121]. Chronic treatment with darbepoetin prevented endothelial injury in an experimental model of progressive CRF[122]. Taken together, these studies indicate that local binding of EPO to cardiac, renal and vascular cells invokes signaling cascades, leading to a cytoprotective and proliferative response.

HOW DOES EPO INFLUENCE THE CARDIORENAL CONNECTORS?

EPO AND THE NO/ROS BALANCE: EPO AND NO

In the SCRS, balance between NO and ROS is shifted towards the latter[18-20]. Actions of EPO on NO synthesis and release, on vasodilator responses in (isolated) blood vessels and responses of vascular cells are controversial. Several lines of evidence support that EPO can regulate endothelial NO synthase (eNOS) via the PI-3K/Akt pathway[123]. Since the systemic environment reacts in a complex manner upon EPO administration, including changes in blood pressure, first the actions of EPO on cardiac, vascular and renal cells in culture will be considered.

Extended EPO exposure of EC obtained from human umbilical, coronary, dermal and pulmonary vessels induces transcription of eNOS and increases NOS activity. During hypoxia, the response of EC to EPO administration to produce NO by induction of eNOS is enhanced[77, 124]. Additionally, cardiomyocytes exposed to anoxia/reoxygenation in vitro are protected by EPO, and display increased eNOS protein expression and NO production[110]. In rats, 14 days of EPO-treatment induces eNOS protein mass in thoracic aorta. In contrast to these studies indicating enhanced eNOS expression and NO production, there was no change in eNOS expression in kidney tissue obtained from EPO treated rats[125]. Moreover, in vascular smooth muscle cells (VSMC) of rats[126], but also in EC of human coronary artery[127], EPO decreased NOS expression and NO-production. Scalera et al[42] observed an EPO-induced increase in the endogenous NO synthase inhibitor ADMA in EC, leading to decreased NO production. These differences in vitro are somewhat difficult to explain. Scalera underscores that many of the in vitro studies have applied concentrations that are only compatible with peak concentrations reached after EPO administration in vivo. In this regard, the studies summarized in the appendix

(table 02a) support the suggestion that in vitro lower EPO dosages may increase NO, while high doses may diminish NO. Besides, elevation of hematocrit and hemoglobin, and possibly blood pressure are known to independently stimulate eNOS expression and NO-production. Conversely, CRF could suppress eNOS expression[128].

Few studies evaluated the effect of EPO on endothelium-dependent vasodilatation. In rats treated with low dose of EPO for 14 days, acetylcholine caused significantly augmented concentration-dependent vasodilatation in thoracic aorta segments pre-contracted with phenylephrine[125]. Oppositely, in high dose EPO-induced hypertension in CRF rats, thoracic aorta segments showed impaired vasodilation to NO-donors, either suggesting NO scavenging by increasing ROS or downregulation of guanylate cyclase by NO[129]. Similarly, in rabbits treated with high dose EPO, the endothelium-dependent vasodilatory response was decreased. Additionally, a reduced effect of a selective NOS inhibitor (L-NAME) on acetylcholine-induced vasodilatation was observed in the EPO group, indicating that NOS activity had been inhibited or a sensitivity of NOS to L-NAME was decreased in EPO-treated rabbits[130]. Similar effects have been observed in healthy human cutaneous vessels, in which local infusion of high doses of EPO could revert endothelium-dependent vasodilatation induced by acetylcholine[131]. Taken together, there are several indications that EPO can enhance and diminish NO release, which may depend upon concentrations, and perhaps the presence of high blood pressure or uremic environment. These studies clearly indicate the urge to explore EPO actions on NO production further, to separate these factors and potentially, different effects in various cell types.

Since EPO can induce hypertension and seems to modulate NO release, several mechanisms for EPO-induced hypertension by impairment of the NO-pathway have been postulated. In a separate paragraph, these and other possible mechanisms for EPO-induced hypertension will be discussed.

EPO AND THE NO/ROS BALANCE: EPO AND ROS

Besides modulation of NO, EPO also showed the capability to modify ROS. Little information is available about the effect of EPO on ROS specifically produced by cardiac, renal or EC and most of in vivo studies have been performed among patients with end stage renal disease (ESRD) whereas our interest is pointed towards CRF (Appendix table 02b). As described below, these studies indicate that EPO indeed modulates oxidative balance. It can be hypothesized that these results may also be applicable to CRF patients, since in vitro studies show effects of EPO, apart from ESRD conditions, and the proposed mechanism of free radical capturing by EPO treatment will be applicable in CRF patients too. In rats, administration of EPO significantly reduced the increase of lipid peroxidation in cardiomyocytes after head trauma[132]. However, when treating EC with increasing amounts of EPO, Scalera[42] observed an increase in ROS production and allantoin, a marker of oxygen free radical generation. Parallel to what was observed for NO, the greatest increase in oxidative stress upon EPO treatment has been observed at high dosages (100-200 U/ml), whereas in vitro studies demonstrating diminished oxidative stress were performed with lower EPO concentrations[118].

Several studies have been conducted on oxidative stress parameters in plasma of ESRD patients receiving EPO-treatment. In this patient population, the anti-oxidative capacity of red blood cells increased[133], as supported by enhanced anti-oxidant defence enzymes such as superoxide dismutase (SOD), gluthatione peroxidase (GPx) and catalase[134-136]. SOD converts superoxide anions to H_2O_2, and H_2O_2 is subsequently detoxified by catalase or glutathione peroxidase. A decline in GPx in patients with CRF could also be prevented by EPO[137]. Besides amelioration of anti-oxidative capacity, EPO intervenes in oxidative stress by modulation of lipid peroxidation as well. It has been demonstrated that markers of lipid peroxidation, like malondialdehyde (MDA), in plasma and red blood cells of hemodialysis patients diminish following EPO treatment[135, 138]. This observation, however, is not consistent with another study indicating an increase in MDA[136]. In sum, most of the evidence indicates that EPO diminishes oxidative stress; results pointing towards an opposite effect might be explained by EPO concentration-dependency. Moreover, it must be emphasized that hypertension associated with EPO-treatment might explain elevated ROS levels and concomitant iron supplementation could also take part in free radical formation.

How EPO diminishes ROS is not clear. One option is mentioned above, namely that EPO strengthens the anti-oxidative defence systems, possibly by increasing the number of erythrocytes, which are highly effective free radical scavengers[139]. However, EPO has also been shown to protect against oxidative stress independent of erythrocyte number. In vitro, EPO scavenges hydroxyl radicals generated by the reaction between the oxidant phenylhydrazine and erythrocytes[140]. Furthermore, oxidative stress-induced cell death in cerebral ischemia

models demonstrated that neuroprotection by iron chelators is, in part, exerted by activation of a signal transduction pathway leading to increased EPO gene expression[141]. This indicates that EPO could exert anti-oxidative properties in a hemoglobin-independent manner.

EPO AND THE SYMPATHETIC NERVOUS SYSTEM

Information about the effect of EPO on this nervous system lacks for CRF patients and it has been sparsely examined in patients with ESRD. In some studies, EPO-induced hypertension in hemodialysis patients is accompanied by increased plasma norepinephrine[142-144] or enhanced vascular response to norepinephrine[145, 146], whereas other investigators could not confirm this association[33, 147, 148] or even showed a decrease in plasma norepinephrine[149, 150]. Other autonomic functions, like orthostatic blood pressure and baroreflex also did not alter in hemodialysis patients receiving EPO-treatment[151, 152]. Even in the absence of an effect on blood pressure after EPO-administration, plasma norepinephrine have been reported to increase[153, 154]. Taken together, there is little systematic study on the interaction between EPO and the SNS, and the available data is conflicting.

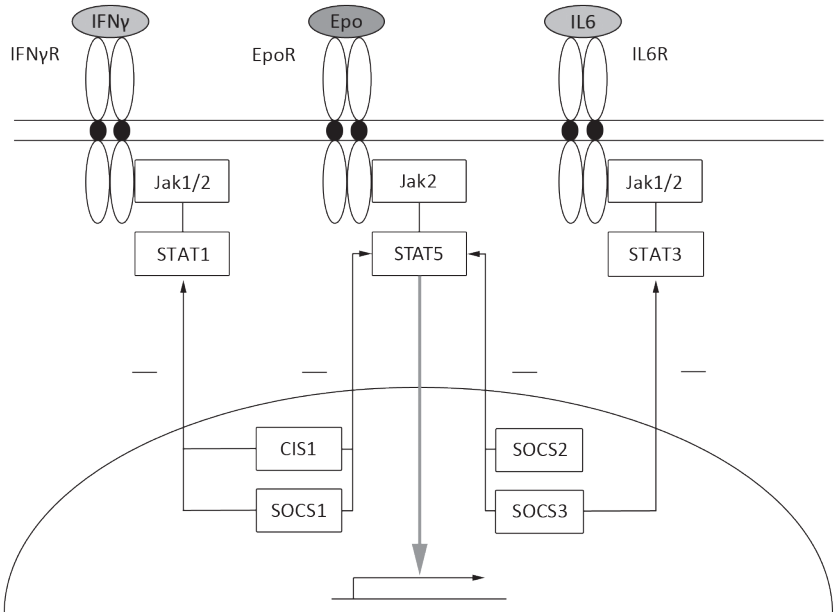
EPO AND INFLAMMATION

Several studies demonstrated protection against inflammation by EPO-administration. Animal studies showed that EPO-administration decreased the infiltration of inflammatory cells after spinal cord compression[155, 156]. Furthermore, diminished levels of IL-6, TNF-α, CRP and MCP-1 are seen in EPO-treated rodents with collagen-induced arthritis, auto-immune encephalomyelitis, cyclosporine nephropathy or cerebral ischemia[157-160]. Moreover, EPO inhibited the expression of the iNOS gene and diminished nitrite production in oligodendrocyte cultures induced by IFN- and LPS[161]. However, little is known about how EPO exactly modulates inflammation.

As mentioned, SOCS are downstream regulators of EPO signaling in the JAK/STAT pathway[105, 106], and we propose that these proteins are relevant in anti-inflammatory effects of EPO. SOCS have been demonstrated to dampen inflammation by attenuating pro-inflammatory cytokines. In vitro studies and various knockout mice studies indicate that SOCS1 negatively regulates the IFN-γ/STAT1 pathway[162, 163] and SOCS3 showed to dampen IL-6 signal transduction[164, 165]. Since EPO induces expression of SOCS1 as well as SOCS3 [104], it can be speculated that this forms the underlying mechanism of anti-inflammatory properties of EPO treatment (figure 03).

Despite the dampening effect of EPO on inflammation, EPO also induces NF-κB and activating protein-1. Activating protein-1 participates in enhanced transcription of the pro-

FIGURE 03 EPO POSSIBLY INDUCES NEGATIVE REGULATION OF THE IFN-γ AND IL-6 SIGNALING PATHWAY



inflammatory factor IL-2[166-168], whereas NF-κB has well known pro-inflammatory actions too.

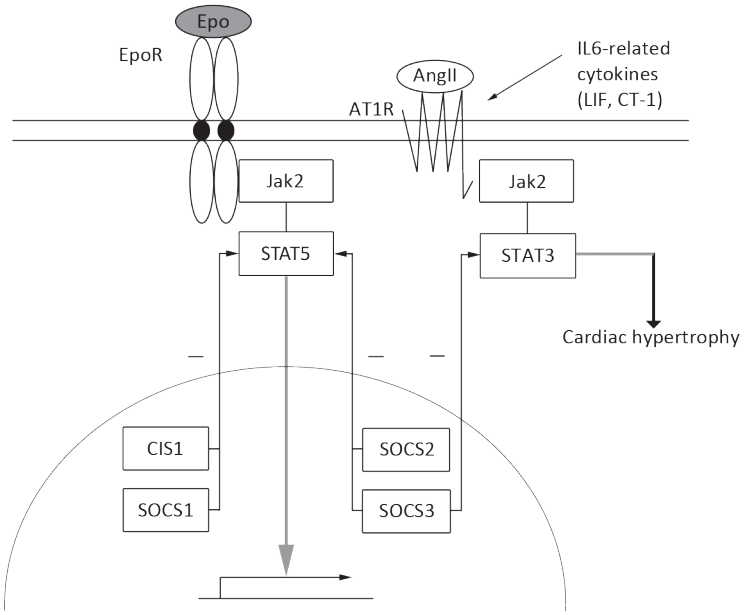
Besides the mentioned effects on erythropoiesis, inflammation also seems to affect EPO-responsiveness in patients on hemodialyses. In this field of interest, investigations among CRF patients are not available. ESRD patients with a poor response to EPO treatment express high levels of IFN-γ and TNF-α[169], both cytokines known to inhibit erythropoiesis in the bone marrow[170]. The pro-inflammatory cytokine IL-6 is also enhanced in patients who need higher doses of EPO to achieve target hematocrit[171] and C-reactive protein (CRP) is a good predictor for EPO resistance in hemodialyses patients with low hemoglobin levels[172, 173]. Finally, an improved EPO response coincided with reversal of enhanced IL-6 and CRP levels in ESRD by treatment with ultrapure dialysate[174]. As regulation of inflammatory pathways involves upregulation of SOCS, this potentially could interfere with intracellular signaling of EPO, thereby possibly explaining EPO-resistance and hampered erythropoiesis.

EPO AND THE RENIN ANGIOTENSIN SYSTEM

EPO treatment has been shown to protect against organ damage and it might be speculated that EPO diminishes RAS activation. RAS might be dampened in a direct way or in response to increased blood pressure seen after EPO-therapy. However, one of the proposed mechanisms for EPO-induced hypertension is an increased activity of the RAS.

Few in vitro studies have been performed to evaluate blood pressure-independent actions of EPO on RAS. In VSMC, EPO exposure enhances mRNA for angiotensin type 1 (AT1) and AT2 receptors and increases ligand binding[175]. Moreover, EPO has shown to increase sensitivity to Ang II by enhancing Ang II-induced intracellular calcium mobilization in VSMC[146, 176]. Furthermore, in rats treated with EPO, an increase in mRNA for renin and angiotensinogen in kidney and aorta were observed, however no change in plasma renin was shown[177]. Although most of the in vitro evidence points towards enhanced activation of RAS by EPO, this could not be confirmed by diverse in vivo studies. Despite blood pressure lowering effects of ACEi in EPO-induced hypertension, changes in plasma renin activity, Ang II or plasma aldosterone could not be demonstrated in CRF and ESRD patients treated with EPO[33, 144, 147, 148, 153, 154, 178]. In one study, plasma renin activity and aldosterone declined in ESRD patients receiving EPO-treatment for up to twelve months[179]. In in vivo studies, it is difficult to discern whether changes in RAS components result from direct effects or if they are secondary to effects of EPO on blood pressure. It might be that RAS activity decreases in response to a rise in blood pressure due to other mechanisms induced by EPO, such as increased endothelin-1 production.

FIGURE 04 EPO POSSIBLY INDUCES NEGATIVE REGULATION OF THE ANG II SIGNALING PATHWAY



To understand how EPO could modulate RAS, it seems important to know which intracellular signaling pathways are induced upon Ang II stimulation. Several studies have demonstrated activation of the JAK2/STAT pathway after interaction of Ang II with the AT1 receptor[180]. Although several STATs can be activated in different culture conditions[181-183], phosphorylated STAT3 consistently was present in Ang II-stimulated cardiac myocytes. This is interesting, since STAT3 is known for its cardiac hypertrophic effects[184, 185]. In addition to its role as blood pressure regulating hormone, Ang II also promotes inflammatory responses by facilitating the release of pro-inflammatory mediators such as IL-6, which is induced in a JAK/STAT dependent matter[186]. Studies with IL-6 and IL-6-related LIF (Leukemia Inhibiting Factor), also activators of STAT3, showed that cross talk within the JAK/STAT pathway upon different stimuli is possible, since Ang II showed inhibition of IL-6- as well as LIF-induced STAT3[187, 188]. Though the potential interaction between JAK/STAT-pathway components is intriguing, it is confusing that Ang II enhances IL-6 production, but can also dampen the IL-6 signaling pathway. EPO could intervene at different levels following Ang II stimulation; that is by upregulating SOCS with subsequent dampening of Ang II signaling, IL-6 production or IL-6 signaling. The exact mechanism remains to be elucidated.

The role of Ang II and SOCS in renal diseases has also been investigated[182]. In cultured mesangial and tubular epithelial cells, overexpression of SOCS proteins prevented Ang II-induced STAT activation. In rats infused with Ang II, SOCS1 and SOCS3 are enhanced via JAK2/STAT1 after activation of the AT1 receptor. Additionally, in SOCS3 knockout rats, JAK/STAT activation by Ang II is increased, resulting in renal damage. By enhancing negative feedback regulators, EPO might also interfere in the JAK/STAT pathway induced by Ang II (figure 04), resulting in renal and potentially also cardiac and vascular tissue protection. Although most of the mentioned studies on EPO and RAS have been performed among ESRD patients, we postulate that the proposed mechanism with intracellular signaling via JAK/STAT will also be applicable to CRF patients.

EPO EFFECTS ON BLOOD PRESSURE

Besides protective effects, EPO treatment in renal patients induces hypertension in 20-30% of cases[189] with a rise in diastolic and systolic arterial pressure of approximately 5 mmHg[190]. Postulated mechanisms for EPO-induced hypertension include increased blood viscosity, alterations in vascular smooth muscle intracellular calcium levels, direct vasopressor action, arterial remodelling through stimulation of vascular cell growth, and changes in production or sensitivity to endogenous vasopressors, such as endothelin-1, catecholamines and RAS, and vasodilatory factors, such as prostaglandins and NO[94, 191]. Several mechanisms for EPO-induced hypertension by impairment of the NO-pathway have been postulated. Interaction between EPO and its receptor induces Ca^{2+} channel activity with calcium influx, resulting in a rise in intracellular calcium levels directly followed by vasoconstriction[192]. Chronic treatment with EPO has been shown to raise cytosolic Ca^{2+} concentration, which potentially antagonizes the actions of NO and could be reversed by calcium channel blockade[127]. In addition to disturbed NO-sensitivity via increased intracellular calcium, upregulation of an inhibitor of NOS (ADMA) by EPO decreases NO production by EC in vitro[42].

Several studies have shown that increased hematocrit and erythrocyte mass do not mediate EPO-induced hypertension and conflicting results about the effects of EPO on endothelin-1, RAS, catecholamines and prostaglandins exist[191]. Moreover, it is not clear to what extent each of the proposed mechanisms contributes to the development of hypertension. Since the mechanism on EPO-induced hypertension is not well understood, it is difficult to speculate why one-fourth of patients develop hypertension upon EPO administration and others do not. Hypertension possibly develops because of rapid reversal of anemia-induced peripheral vasodilatation with a less than complete reversal of the anemia-induced rise in cardiac output. This may be explained by impaired myocardial compliance following cardiac hypertrophy, which is more or less present in individual patients. Other predisposing factors to EPO-induced hypertension have been designated, such as age, antecedent hypertension and no antiplatelet therapy[193]. Even so, increased blood pressure is obviously unwanted in patients suffering from SCRS.

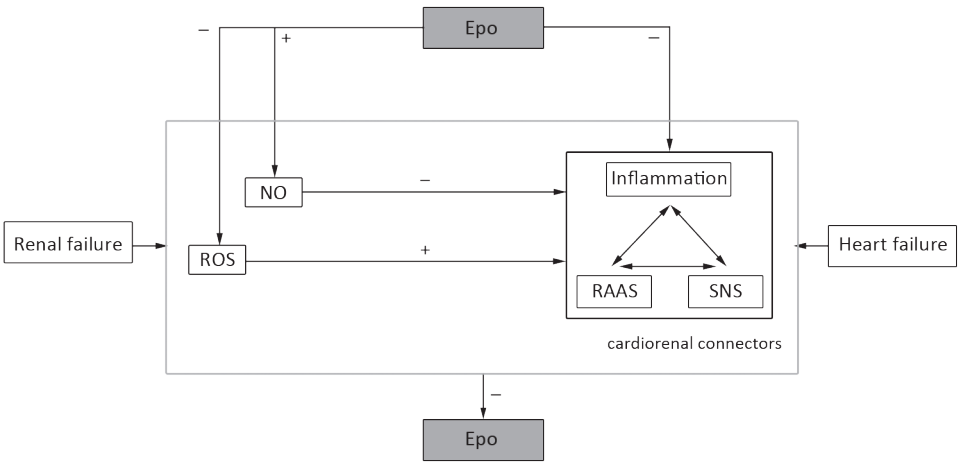
CONCLUSIONS AND PERSPECTIVES

In the SCRS, the interaction between the cardiorenal connectors leads to progressive failure of the heart and kidney. We propose that diminished EPO-production (as a result of this syndrome) and impaired responsiveness to EPO actions could further amplify this negative interaction. Administration of exogenous EPO by either resolving the absolute deficiency or the relative insensitivity could dampen the cardiorenal connectors, thereby interrupting the vicious circle and thus intervening in the pathophysiology of SCRS (figure 05). In this review, the role of EPO and its mechanism of action in the SCRS have been analyzed. Although little is known about cellular mechanisms, studies demonstrated a protective role for EPO on cardiac, renal and vascular function.

It is not yet clear to what extent each of the cardiorenal connectors mediates the development of combined cardiac and renal dysfunction and if EPO modulates inflammation, NO-ROS balance, SNS or RAS in a greater or less degree. However, it appears that disturbance of NO-ROS balance is preponderant in the pathophysiology of SCRS and therefore an important role is assigned to this component. The effect of EPO on NO seems dose-dependent, with increased NO production at EPO plasma concentrations reached during clinical application of EPO-treatment. Most evidence on inflammation indicates dampening of inflammatory cytokine production by EPO. The effect of EPO on RAS remains difficult to interpret, because of the blood pressure effects that EPO can elicit. Finally, very little is known about EPO and its effects on SNS. It should be taken into consideration that EPO-treatment comes along with iron supplementation. Iron may affect NO-ROS balance by increasing free radical production, whereas iron is required for erythropoiesis and thus helps to reverse the low anti-oxidative anemic state.

Thus far, there is also a lack of evidence about the underlying mechanism of EPO protection. As suggested in this review, intracellular signaling via the JAK/STAT pathway, with upregulation of SOCS, seems to be a feasible explanation for dampening effects of EPO on inflammation, RAS and possibly a shift in NO-ROS balance towards NO. However, to elucidate which cellular pathways EPO induces in patients, and to distinguish hematopoietic from non-hematopoietic effects of EPO, a clinical study is urgently needed.

FIGURE 05 OVERVIEW OF EPO IN THE SEVERE CARDIORENAL SYNDROME



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CH.03

ERYTHROPOIETIN TREATMENT IN PATIENTS WITH COMBINED HEART AND RENAL FAILURE; OBJECTIVE AND DESIGN OF THE EPOCARES STUDY

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ABSTRACT

Introduction: 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 erythropoietin (EPO) treatment has desirable as well as undesirable effects, related to its hematopoetic or non-hematopoioetic effects. Therefore a translational study is needed to elucidate mechanistic aspects of EPO treatment.

Methods: In this open-label randomized 12-month trial, 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 randomized into 3 groups: one group (n=25) receives a fixed dose of 50 IU/kg/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/week EPO maintaining baseline hemoglobin levels for the first six months by phlebotomy, and 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 assessed. Furthermore, the effects of EPO on monocyte gene expression profiles and on endothelial progenitor cells are evaluated .

Conclusion: This translational study is designed primarily to discern hematopoietic from non-hematopoietic effects of EPO in cardiorenal patients. The study will add insight into the mechanisms that could explain the fragile balance between desirable and undesirable effects of EPO (Trial registration: ClinicalTrials.gov identifier NCT00356733).

BACKGROUND

THE CARDIORENAL SYNDROME

Co-existence 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]. In this model, inflammation, the balance between the nitric oxide (NO) and reactive oxygen species (ROS), the sympathetic nervous system (SNS) and the renin-angiotensin system (RAS), form the so called "cardiorenal connectors" and are hypothesized to be the cornerstones in the pathophysiology of the cardiorenal syndrome[1].

A POTENTIAL ROLE FOR EPO IN THE CARDIORENAL SYNDROME

Anemia is common in the combination of CHF and CKD, leads to reduced physical capacity, and induces adaptive cardiovascular mechanisms that increase the risk of cardiovascular disease and death[2]. Observational data indicate that hemoglobin (Hb) levels are correlated with hospitalization and mortality in dialysis patients and in CHF patients[3, 4]. This led to the belief that EPO treatment of anemia may improve outcome. Small interventional trials show that EPO treatment of anemia in patients with CKD as well as in patients with CHF improves quality of life[5, 6]. Additionally, some[7, 8], though not all[9], studies have demonstrated an association between EPO treatment and regression of left ventricular (LV) hypertrophy.

EPO AND THE CARDIORENAL CONNECTORS

In addition to well documented erythropoietic effects, EPO has numerous so called non-hematopoietic (non-Hb) effects. These include neuroprotective[10], cardioprotective[11] and anti-apoptotic[12] effects. With respect to the cardiorenal connectors, there are indications that EPO can diminish inflammation, reduce RAS activity, and shift the NO/ROS balance towards NO. Very little is known about EPO and its effects on the sympathetic nervous system. One of the mechanisms underlying the effect of EPO on the cardiorenal connectors could be a modulating effect of EPO on the Janus Kinase/Signal transducer and activator of transcription (JAK/STAT) pathway[13].

EPO AND ENDOTHELIAL PROGENITOR CELLS

Bone-marrow derived endothelial progenitor cells (EPC) play an important role in vascular repair and

maintenance. In CKD, the number and function of EPC is decreased[14]. In CHF, peripheral recruitment of EPC is enhanced in early stages, whereas in advanced stages EPC levels are reduced[15]. In an experimental model of CHF, EPO induces revascularization through EPC recruitment[16]. In a pilot study in CKD patients, it is shown that EPO improves EPC levels and function[17].

PARADOXICAL EFFECTS OF EPO

Based on aforementioned 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[18, 19]. This could be related to the attained Hb levels or alternatively, to unwanted non-hematopoietic 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 outcome[4, 20].

OBJECTIVES

The objective of EPOCARES (ErythroPOietin in the CArdioREnal Syndrome) is to study hematopoietic as well as non-hematopoietic effects of EPO treatment in patients with CKD and CHF. Therefore the study design is not intended to show differences in hard endpoints, but specifically allows identification of desirable and undesirable effects of EPO as measured by biomarkers, genomics and cell studies. Importantly, the study design was specifically devised to enable differentiation of hematopoietic and non-hematopoietic effects.

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 Tables 01 and 02. The etiology of both CHF and CKD and the sequence in which the two conditions arise is not important in the selection of the patients[1, 21], but patients with active systemic disease as a cause of CHF or CKD are excluded.

The study is carried out in compliance with the Helsinki Declaration and the protocol has been approved at each participating centre by its internal review board. After an eligible patient has provided written consent, standard treatment is started in all patients, comprising of oral iron suppletion, aspirin when indicated, and maximal tolerated dosages of a beta-blocker, an 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 four weeks.

One group receives a fixed dose of 50 IU/kg/week of EPO (Neorecormon®, Roche pharmaceuticals) to increase 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/week EPO maintaining baseline Hb for the first six months by sequential blood withdrawal up to a maximum of 250 ml per 2 weeks. The third group does not receive EPO, but

TABLE 01 INCLUSION CRITERIA

Age >18 years, <85 years
eGFR by Cockcroft-Gault formula of 20-70 ml/min
Heart failure (diastolic and systolic)
Hb 10.3-12.6 g/dL in men and 10.3-11.9 g/dL in women

eGFR, estimated glomerular filtration rate; Hb, hemoglobin

TABLE 02 EXCLUSION CRITERIA

Erythropoietic therapy within 6 months before randomization
Uncontrolled hypertension (SBP>160 mmHg, DBP>100 mmHg)
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 or solid tumor <3 years ago
Enrolment in another study
Alcohol and/or drug abuse
Women with child bearing potential

SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycosylated hemoglobin; PTH, parathyroid hormone

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 six 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.

MEASUREMENTS

HB LEVEL

Hb level will be checked at least monthly in all patients. In the patient group maintained at baseline Hb, Hb level will be measured every 2 weeks during the first 6 months of the study to assess the necessity of phlebotomy.

DIFFERENTIATION BETWEEN HEMATOPOIETIC EFFECTS AND NON-HEMATOPOIETIC EFFECTS OF EPO

Figure 01 depicts the measurements that are performed throughout the study period. Since the Hb level does not increase until about four weeks after starting EPO treatment, non-hematopoietic effects of EPO treatment are assessed 18 days after initiation of treatment. Moreover, comparing the two EPO-treated groups after six months provides an additional way to discern hematopoietic and non-hematopoietic effects of EPO.

QUALITY OF LIFE (QOL)

The Minnesota Living with heart failure questionnaire will be used as a subjective index of QoL. This will be extended with the SF-36, a questionnaire containing 36 items measuring physical functioning, physical role, emotional role, vitality, general health perceptions, social functioning, body pain, and general mental health. This questionnaire has been validated for the Dutch population.

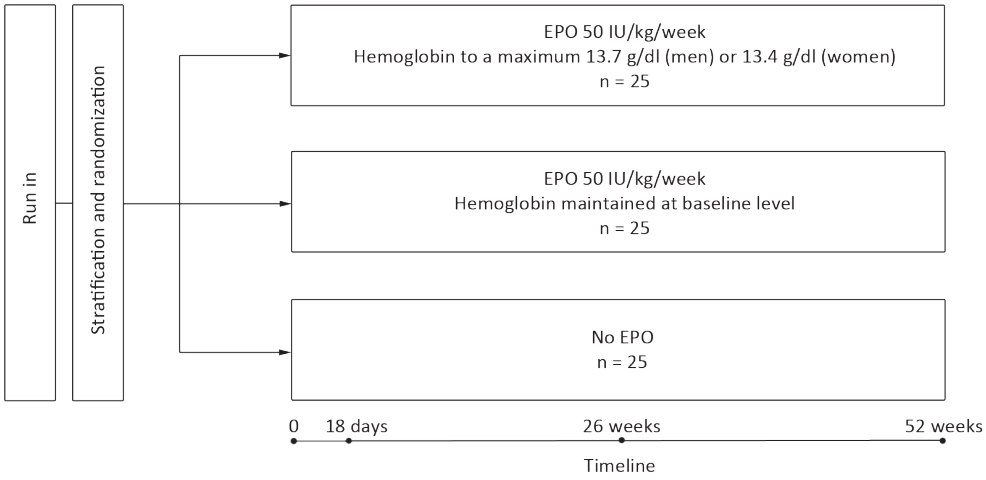
CARDIAC AND RENAL FUNCTION

CHF is defined as 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[22, 23]. Patients with heart failure with reduced LVEF (HFREF) or heart failure with normal LVEF (HFNEF) will be included. HFNEF is defined as a LVEF >50%, LV en diastolic volume index <97 ml/m^2 and evidence of diastolic LV dysfunction[24].

Echocardiography will be performed by an investigator blinded to treatment allocations. Recommendations of the American Society of Echocardiography were followed. Diastolic function will be assessed using standard methods.

Cardiovascular Magnetic Resonance Imaging (CMR) and Magnetic Resonance Angiography (MRA) of the renal arteries studies will be performed on a 1.5 Tesla Philips Intera (Philips Medical Systems)[25]. In a 45-minute protocol, both cardiac function and the renal arteries are assessed.

FIGURE 01 STUDY DESIGN AND MAIN TIME POINTS OF MEASUREMENTS DURING THE STUDY PERIOD



The cardiac function analysis will be performed by a single trained investigator using ECG-triggered multiphase, multi-slice 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 LV 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 30ml cyclic Gadolinium-based contrast (Dotarem, Geurbet, France) will be administered intravenously to obtain delayed enhancement scans of the ventricles (inversion recovery T1 pulse) in four-chamber, short axis and left two-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 two independent investigators. The LV will be divided in 17 segments according to standardized nomenclature[26]. Late enhancement will be estimated by using a 5-group classification according to the degree of LV wall involvement[27].

Cardiopulmonary exercise performance will be measured up to the symptom-limited maximum. Exercise capacity will be evaluated by peak oxygen consumption (VO_2max). The cardiac marker N-terminal pro-B-type natriuretic peptide (NT-pro-BNP) will be measured.

CKD is defined as estimated creatinine clearance 20-70 ml/min (by Cockcroft-Gault formula). Albuminuria is assessed by 24-hour urinary collection.

THE CARDIORENAL CONNECTORS

Oxidative status of the patient will be assessed measuring plasma thiobarbituric acid-reactive substances (TBARS), oxidized low density lipoprotein (Ox-LDL), asymmetric dimethylarginine (ADMA) and arylesterase. Plasma renin, pro-renin, aldosterone and catecholamines will be determined as a measure of activity of the RAS and SNS, respectively. Levels of hs-CRP, IL-6, TNF-α, high mobility group B1 (HMGB1), receptor for advanced glycation end products (RAGE) and inflammation-related gene expression in monocytes will used as indices of inflammation.

ENDOTHELIAL FUNCTION/ARTERIAL STIFFNESS

Global endothelial function will be assessed by measuring endothelial circulating markers Von Willebrand factor, soluble intercellular adhesion molecule-1, soluble vascular cell adhesion molecule-1, E-selectin, plasminogen activator inhibitor-1, vascular endothelial growth factor and monocyte chemoattractant protein. Augmentation index (the difference between early and late pressure peaks divided by the pulse pressure amplitude) will be determined from pulse waveform analysis of arterial waveforms recorded by applanation tonometry using a SphygmoCor device. The aortic pulse wave velocity will be measured using the same device by sequentially recording ECG-gated carotid and femoral artery waveforms.

CELLULAR MECHANISMS

Monocytes are both biosensors of the atherosclerotic environment and mediators of vascular damage. Monocyte gene expression profiles are determined by ILLUMINA BeadChips with real-time qPCR as confirmation. Circulating EPC, defined as $CD34^+KDR^+$-EPC, and $CD34^+$-hematopoietic stem cells are determined in peripheral blood by flow cytometry[28]. Peripheral blood-derived mononuclear cells will be isolated by Ficoll density gradient centrifugation. After 7 days culture in specific medium rich in serum and growth factors the number of EPC outgrowth will be assessed. Migratory, proliferative, adhesive and angiogenic capacity of the EPC outgrowth is determined.

IRON METABOLISM

In addition to routine iron parameters, iron metabolism will be assessed by measurement of hepcidin, using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). In addition, the Hb content of reticulocytes (CHr) will be determined, as well as the serum transferrin receptor and immature reticulocyte fractions.

STATISTICS

The study is not powered for hard endpoints, but for intermediate endpoints 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 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 mentioned variables were all around n=20. Similar trials had a comparable numbers of subjects[8, 29]. Validity of the statistical analysis will be monitored by the Center of Biostatistics of the University of Utrecht.

SAFETY CONCERNS

Hb level will be checked at least monthly in all patients. In the patients treated with EPO, blood will be withdrawn if Hb exceeds 14.0 g/dL in men or 13.8 g/dL in women, while the low dose (50 IU/kg) of EPO will be maintained. Blood will be withdrawn up to a maximum of 250 ml per session and the procedure is performed slowly. In this way, the risk of rapid volume shifts is considered minimal. If blood withdrawal is indicated more often than twice per month, EPO will be stopped for two weeks, followed by a decreased weekly dose of EPO. As the phlebotomy procedure poses a burden for the patient, patients who were maintained at baseline Hb level are allowed to increase in Hb level after 6 months.

At any time during the study, EPO will be withheld for any patient who experiences a thrombotic event or a severe or life-threatening adverse event reported by the investigator to be related to the study drug. EPO will be withheld in the case of hypertension (RR >160 mmHg systolic or >100 mmHg diastolic or symptoms due to hypertension) despite treatment with increased doses of anti-hypertensive medication. An independent data monitoring committee, consisting of 3 members, will be installed.

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 non-hematopoietic effects of EPO are responsible for the unexpected outcome of recent studies[18, 19]. The EPOCARES study is specifically designed to assess both the hematopoietic or non-hematopoietic 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 insight into mechanisms that underlie why anemia correction does not always lead to reduction of cardiovascular risk. This could help to define appropriate EPO doses, Hb targets and may help to appreciate the full range of positive and negative effects of EPO.
The study has a specifically devised design that justifies discussion of potential safety concerns in detail. One group receives a fixed low dose of EPO to a maximum Hb of approximately 13.5 g/dl. Since the study protocol was devised, two large scale studies in CKD using similar Hb targets were published that did not show beneficial effects[18] and maybe even harm[19]. 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, in order 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 a cardiovascular events[30], whereas achieving higher hemoglobin levels was not associated with worse outcomes. The intervention in the other EPO-treated group keeps the patients at their baseline level of anemia. Since the lower level of inclusion is 10.3 g/dl, all patient will remain above the predefined levels of the KDOQI guidelines for starting EPO treatment in CKD patients.

In order to accurately describe cardiac and renal abnormalities, amongst others, CMR and MRA, are performed, which involves IV 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 gadolineum compound gadoterate meglumine Dotarem® is used, which is more stable. No cases of NSF using Dotarem® are formally reported in The Netherlands to the adverse events database. Nonetheless, it was decided to withhold MRI 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 non-hematopoietic effects of EPO in patients with combined heart and renal failure at the level of organ function, circulating cellular and humoral mediators and markers of cardiorenal disease.

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CH.04

ANALYSIS OF MICRO-ARRAY DATA FROM SOURCES WITH SIGNIFICANT BIOLOGICAL VARIATION USING THE ILLUMINA PLATFORM

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ABSTRACT

In the last decade, microarray technology has developed from an emerging technology to a commonly used platform for the analysis of gene expression. With the introduction of bead-based technology, high quality data and reproducibility across arrays and across chips were achieved. As microarray developed and started to become more affordable, application of the technology to samples with greater biological variances has become increasingly popular. This resulted in a more challenging analysis of array data; a stricter selection of genes may lead to loss of valuable data, while a less stringent selection of genes may result in over-interpretation.

In this paper three issues are considered, focused on the Illumina platform; nevertheless, the stream of thoughts can be generalized to other platforms. To be able to analyze the data conservatively, yet to its full extent, we propose a data processing pipeline. First, we evaluated different strategies to analyze whether a gene is significantly expressed. We next evaluated how to determine whether a gene is significantly present in a group of samples when only a subset of samples reaches significant expression for a gene. Our third goal is to present a normalization procedure that is effective and reliable for Illumina, because a normalization procedure based on spotted arrays is often applied to BeadArrays and may not be optimal.

In the pipeline, determination of the significance of a gene call in a sample is different from the ranking system, used by Illumina, and is based on a statistical comparison (Student's t-test). We propose to apply a size-test to determine the minimum number of samples in a group that need to have a significant gene call in order for the gene to be called significantly expressed in that group. With the current approach we provide a less strict, yet conservative, approach to Illumina BeadArray data.

INTRODUCTION

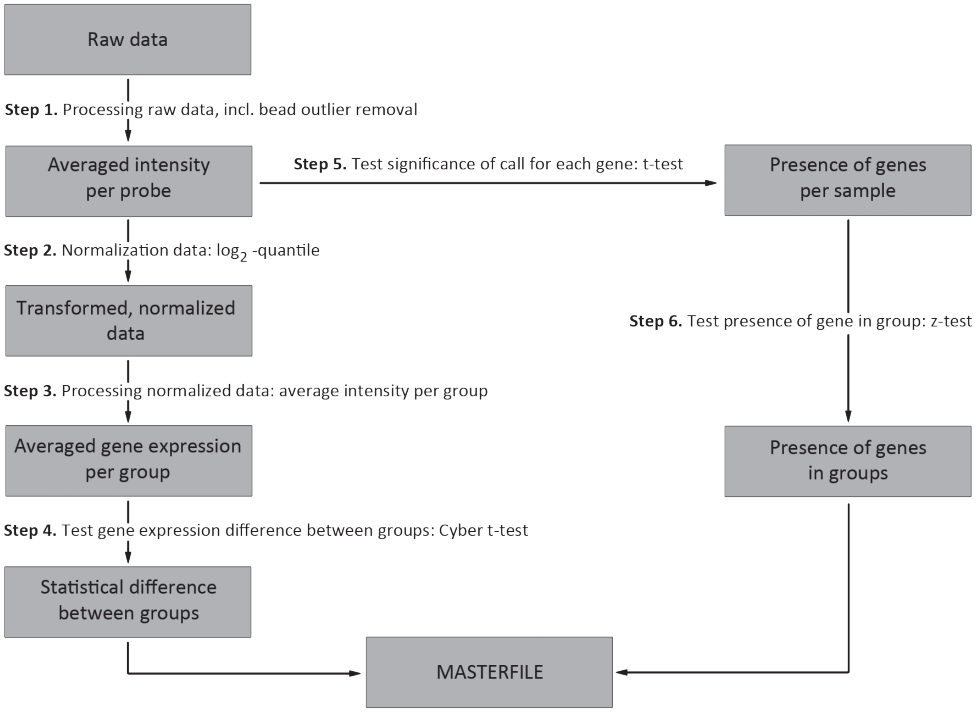
In the last decade, spotted arrays or oligonucleotide arrays (e.g. Affymetrix) on different solid surfaces became the standard in transcriptional analyses. Many methods were developed to enhance reliability and reproducibility of genomic profiles, such as dye-swap[1], spiking[2] and normalization procedures[3]. Despite these improvements, intensities measured on samples hybridized to "spotted" probes remain sensitive to technical noise, such as background signal[4, 5]. Illumina has introduced a bead-based technology with a high degree of bead redundancy, producing high quality data and ensuring reproducibility across arrays and across BeadChips[6].

Initially, microarray technology was mainly used under very well-controlled conditions, such as yeast and cell culture systems. Improvements in technology and affordability made it possible to investigate less controlled and more complex systems. However, this increases biological variation, making analysis of the array data more challenging. In particular, overly strict gene selection criteria can result in loss of data, whereas too permissive criteria come with risk of data over-interpretation. For data analyses, this relates to proper identification of genes that are expressed on the array, to determination of significant gene expression in a group of samples, and to normalization procedures. Our main goal was to critically review the current data processing procedures for the Illumina platform with respect to biological variation. Two issues were addressed in this respect. The first was the procedure to determine whether a gene is significantly present in a group of samples when only a subset of samples reaches significant expression for a gene. The second was related to the performance of normalization procedures when applied to data with relatively low signal intensity. These procedures were mainly designed for spotted arrays. Before normalization, data transformation based on Variance Stabilizing Transformation (VST) is often applied. For Illumina, this specific transformation is not necessary. Therefore, we searched for a normalization procedure that is effective and reliable for the analysis of samples with low signal intensity using the Illumina platform. Despite the fact that this paper considers these issues with a focus on the Illumina platform, the stream of thoughts can be generalized to other platforms.

OUTLIER DETECTION IN THE RAW DATA SET

The data processing involved outlier detection, determination of the significance of a call for each gene, normalization, determination of significance

FIGURE 01 OVERVIEW OF THE DATA PROCESSING PIPELINE



of gene expression in a group, and comparison of means of the groups under study. The full data-pipeline is depicted in figure 01.

For the Illumina system, each gene is probed with at least 30 beads and for the Human Ref-8 chip, each sample consists of approximately 1 million beads, interrogating about 22K genes. The measured intensities per probe should result in a normal symmetric distributed histogram. This also means that an outlier may exist within the probe set, which needs to be excluded before the average probe intensity is calculated. Generally there are two different approaches to determine an outlier: a calculation based on 1) mean and standard deviation (SD) and 2) median and median absolute deviation (MAD).

Using the median is more robust, as a mean is more sensitive to outliers. Removal of outliers causes a change in mean value, while the median is almost unaffected. As a consequence, outlier removal identified by "mean and SD" requires retesting of the data set, whereas removal of outliers identified by "median and MAD" does not. In short, MAD is a more robust estimator of scale, is a robust statistic, and is more resilient to outliers in a data set than SD. In Illumina's software (i.e. BeadStudio) each group of intensities is tested for outliers based on median and MAD. Outliers are intensities outside the area of median ± 3xMAD and thus removed. After the test, the intensities of beads per probe are averaged for further data processing. Note that the outliers in "empty" beads, the negative controls (NC), are not removed, as they are considered as background noise of the beads themselves. Outliers are frequent for each probe. In our hands close to 90% of the probes have outliers. After removal of outliers (average 3 beads) the number of beads per probe averaged 36 beads.

SIGNIFICANCE OF A CALL FOR EACH GENE IN A SAMPLE

Regarding the significance of a call, the detection is performed by discriminating the intensity from the background. In principle there are several ways to accomplish this. Ideally, any method should have 1) a high likelihood of identifying genes expressed above background, 2) a low likelihood of incorrectly identifying genes as being expressed, 3) a statistical basis. Table 01 shows several examples of gene call detection in popular platforms. In the present paper we focus on the method used by Illumina and the method used by our group.

TABLE 01 DIFFERENT CALL DETECTION METHODS FOR A GENE ON VARIOUS MICROARRAY PLATFORMS

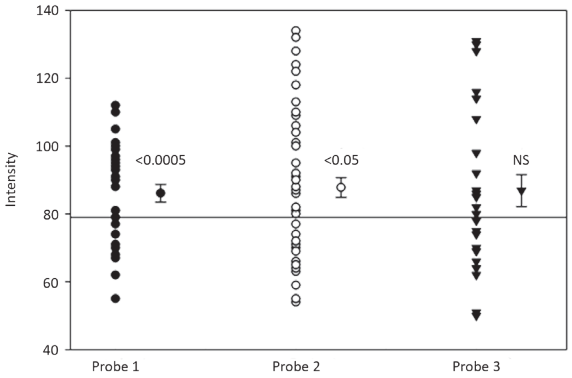
PLATFORM	METHOD	APPROACH	DETECTION CRITERIA	LIMITATION
Oligo-chip	Signal-to-noise Ratio (SNR)	Cut-off	SNR>1.5[8]	Depends on quality of hybridization; higher noise leads to more stringent selection of genes
Affymetrix	Discrimination Score (mismatch (MM) versus perfect match (PM)) and Wilcoxon score[9]	Combination between scoring and statistics	Discrimination and/ or Wilcoxon score P<0.05	Depends on probe set centrally enriched in T's and depleted in A's. Also depends on MM and PM ratio[10]
Illumina [platform's method]	P = 1-R/N[7] (explained in text)	Ranking	P<0.05 or <0.01	Depends on NC; higher number of NC leads to more stringent selection of genes
Illumina [our method]	Student's t-test	Statistic	P<0.05	Depends on variance and intensity of gene and NC; higher variances and smaller difference in intensities between a gene and NC leads to more stringent selection of genes

NC, Negative Controls

Illumina uses a nonparametric method for the computation of the detection p-value. Detection p-value is formulated as 1-R/N, where R is the rank of the gene signal relative to the signal of NC and N is number of NC[7]. Theoretically, the higher the signal level of a gene, the lower the probability that it could be related to a nonspecific source. The detection score provided by Illumina is dependent on the number of NC. In fact, according to the formula, the intensity of a gene is more readily significantly expressed above the NC signal if the number of NC is decreased. Also the approach is strict in determining significant presence of a gene. Thus, a gene is very dependent on the intensity level and the number of NC, even though the gene may have a very small variance in the intensities of the beads. A detection score that is dependent on the distribution of the intensities and the average intensity of the gene and NC (regardless of the number of NC) would be a more fitting alternative approach.

To incorporate bead intensity variation of the gene of interest into the calculation, software was developed that a) determines outliers using the same algorithm as applied by Illumina's BeadStudio, b) determines whether the distribution of the bead intensities of the gene of interest and the NC display equal variance

FIGURE 02 ILLUSTRATION OF THE DIFFERENCE IN BEAD INTENSITY VARIANCE OF THREE DIFFERENT PROBES WITH A LOW INTENSITY



NS, Not Significant

and c) performs the appropriate Student's t-test between the beads of a gene and the average intensity of all NC on the same array. In our experience, about 2/3rd of the comparisons display unequal variance, which makes such an evaluation necessary. A gene and NC may vary greatly in size and variance, thereby impacting on reliability of the t-test. Thus it is necessary to test equality between the two population variances. Figure 02 illustrates this issue using data from one of our experiments (after outlier removal per probe). In probe 1, the dataset is tight and, despite the relatively low number of beads, the average is above NC; please note that the number of probes used to calculate the NC was >700. The second probe has very substantial variation, however, the number of beads is high (n=52) and the average is also above background. In the last example, the number of beads is low (n=28), and has substantial scatter, leading to a p-value >0.05 using a t-test for samples with unequal variance.
Table 02 shows the number of genes that are significantly expressed above NC intensity using both methods and illustrates that Student's t-test was less strict and yet conservative as compared to the detection score. The software (T4Illumina) is available at www.nephrogenomics.net. Output of the software includes the number and percentage of genes expressed at a certain significance level. Indeed, the number of genes that are called significantly expressed is slightly higher when the Student t-test is applied.

TABLE 02 NUMBER OF GENES CALLED SIGNIFICANTLY PRESENT (ABOVE NC) FOR DIFFERENT P-VALUE CUT-OFFS ACCORDING TO DETECTION SCORE AND STUDENT'S T-TEST

	DETECTION SCORE (ILLUMINA)			STUDENT'S T-TEST		
SUBJECT	<0.05	<0.01	<0.001	<0.05	<0.01	<0.001
HC-1	12201	11325	10964	12396	11764	11048
HC-2	11577	10773	10465	12006	11358	10599
HC-3	11956	11174	10734	13659	13038	12328
HC-4	11229	10150	9825	13762	13118	12397
HC-5	11689	10312	10075	12670	11925	11101
HC-6	11855	10598	10207	12148	11300	10397
HC-7	11872	10663	10209	12345	11625	10815
HC-8	11599	10161	9611	12314	11538	10671
HC-9	12150	11243	10875	12961	12296	11589
HC-10	13080	11973	11543	13409	12802	12113
HC-11	12558	11475	11201	12494	11762	10927
HC-12	12404	11278	11028	11771	10997	10150
Average #	12014	10927	10561	12661	11960	11178
%	49	45	43	52	49	46

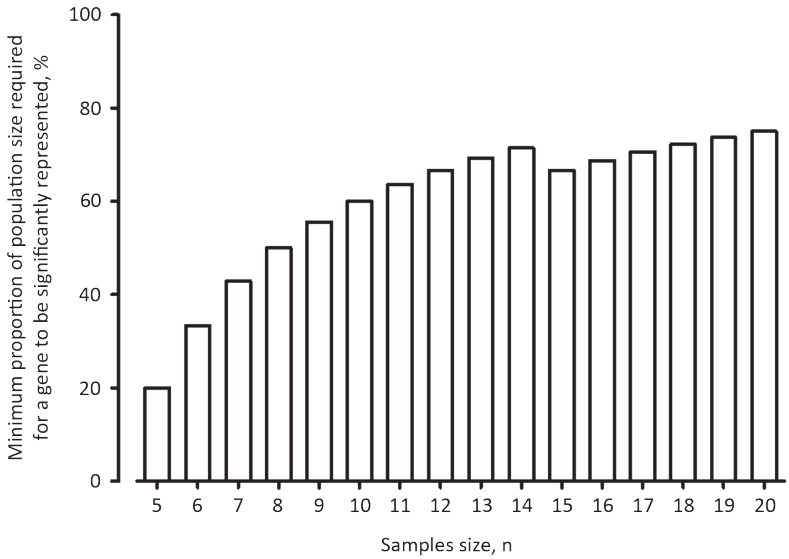
Monocytes from 12 healthy controls (HC) of the EPOCARES study population were processed on Human Ref-8 BeadChips (Illumina). Control probes are not included. Same probes are used in both methods. Data are available at NCBI Gene Expression Omnibus[11] accession number GSE17582

SIGNIFICANCE OF THE PRESENCE OF A GENE IN A GROUP OF SAMPLES WITH SIGNIFICANT BIOLOGICAL VARIATION

Particularly relevant for samples with substantial biological variation is whether a gene is considered significantly expressed in a group of samples. For example, if one third of the samples in a group show significance of a call for a gene, and in the comparator group only half of the samples shows a significant call for that same gene, it may not be justified that a test is performed to compare means. Surprisingly, this is not considered by the Illumina software. Once a decision rule is in place for this issue, three cases can occur when two different groups are compared (e.g. control *versus* treated) with regard to transcriptional activity of a gene: 1) present in both groups; 2) present in only one group and 3) not present in both groups. It is desirable to objectively determine the likelihood that the two groups of samples are representative subsets of larger populations. Two factors determine this: 1) the presence of the gene in a sample and 2) the population size. The first has been discussed above; the second factor requires a statistical approach.

One may put a strenuous restriction on the data by accepting only genes that are active in all samples of the group, but in that case valuable

FIGURE 03 REQUIRED PERCENTAGE OF SAMPLES WITH A SIGNIFICANT CALL FOR A GENE TO BE CALLED TRANSCRIPTIONALLY ACTIVE IN THE GROUP



Calculations were performed using a z-test

data may be lost. An arbitrary threshold to decide whether to continue with a comparison between groups for a specific gene is undesirable. Using a larger population size could result in a more symmetric distribution of the biological variation; however, a small size in translational studies is frequently the case. We have chosen the following approach. A z-test can be applied to determine the minimal number of samples with a significant call for the gene of interest required to be called a representative sample. This is presented in figure 03. Thus for a gene to be called present in a group with n = 6, at least two of the six samples (33%) need to be called significant, while in a larger population relatively more samples are needed to call a gene transcriptional active in the group (e.g. 13 in a group with n = 18; 72%).

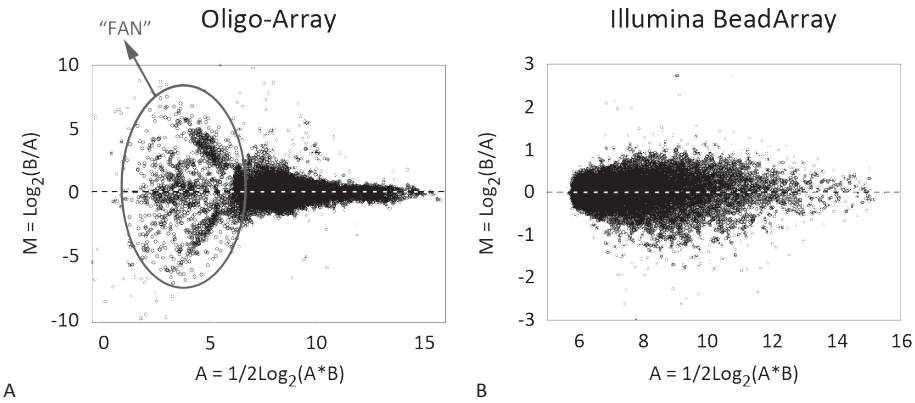
CONSEQUENCE OF TRANSFORMATIONS OF ILLUMINA DATA

When more than one sample or more than one array is used, systematic errors may be introduced. These errors are introduced by, for example, differences in quantity of the starting material, efficiency of dye incorporation and hybridization, and errors introduced by scanning. Normalization of microarray data is recommended to minimize these errors. In general, the normalization procedure is performed in two steps. First variance stabilization (transformation) is performed, followed by normalization. For this review, Quantile normalization is used; that is normalization based on the magnitude of the measures. The sample size is large in ideal circumstances, but in reality the size of a group often contains just enough samples to apply statistics. Thus the data have to be transformed into a symmetric distribution, which may also enhance interpretability. Two of the common transformations are logarithms and Variance-Stabilizing Transformation (VST).

The intensities of the probes are of several orders of magnitude, so logarithmic transform is used, however, this transformation can lead to data distortion. It has been shown that about 20-40% of the significantly differentially expressed genes are discordant, being significant only in one representation of the data and not in both, also depending on which test is used and on the threshold value for claiming significance[12]. Nevertheless, for statistics it is important to have symmetric and Gaussian-like distribution. Thus logarithmic transformed data is preferable to non-transformed data.

VST was developed especially for linearity of data. From the start of using oligonucleotides, a problem was encountered in data analysis. It seems that probes with low intensities caused a great deviation from the actual linearity of data. This is especially visible in the MA-plot[13] by two-channel microarray as shown in figure

FIGURE 04 MA-PLOT FOR DATA DERIVED FROM OLIGO-ARRAYS AND ILLUMINA PLATFORM



A B

For (A), the plot was derived from two samples of rat kidney material on 7.5k Oligo Chips manufactured in the Holstege Group of the University Medical Center Utrecht. Data have been published[16].
For (B), monocytes from two randomly chosen healthy controls of the EPOCARES study population were used. For more information about monocyte microarray data, please see table 02

04A; namely the infamous "fan". The probes in the "fan" area are unreliable. VST uses an error model that provides a bias against low intensity values, as these signals are prone to measurement noise[14]. In short, the ratio of the probes in the area of the "fan" are pulled towards the "linear" line (e.g. the "zero" line in MA-plot) and the probes with high intensities are treated with normal log_2-transformation. Thus VST can be viewed as a generalized log_2-transformation, fine-tuned for the noise characteristics of each array. The philosophy behind VST is solid. Despite discussion that VST stabilizes the variance of bead-replicates for low-ranking probes (low intensity signals) better and more efficiently for the Illumina platform[15], the VST may not be appropriate for Illumina Bead Arrays. As is shown in figure 04B, low intensity probes do not affect the linearity of the data set.

Applying VST to the whole dataset increased the average (1.6-fold) and decreased the variance of intensity of probes as compared to pure Log2 transformation. More specifically, the transformation of the average intensity of NC probes was about two-fold elevated by VST as compared to Log_2 (table 03). Further investigation revealed that changing the choice of strategy will not affect the outcome; about 97% of differentially expressed genes between two groups are found by both methods and 100% of these genes are expressed in the same direction (table 03). Regarding the MA-plot (figure 04B) and the numerical outcome, the artificial elevation of low ranking probes is not necessary.

TABLE 03 EFFECT OF LOG_2 VERSUS VST DATA TRANSFORMATION ON NEGATIVE CONTROL PROBE INTENSITIES AND DIFFERENTIAL EXPRESSION

	NEGATIVE CONTROLS (N=664)			
	Log_2- Quantile		VST- Quantile	
Group	HC	Pt	HC	Pt
Number of samples	12	18	12	18
Average intensity	6.224 ± 0.208	6.228 ± 0.211	7.346 ± 0.087	7.348 ± 0.089
	WHOLE DATASET (WITHOUT NEGATIVE CONTROLS; N=24526)			
# Differentially expressed genes	470		473	

Data from EPOCARES (see table 02) are used: monocytes of 12 HC are compared to 18 patients (Pt) with cardiorenal failure. Cyber t-test is used to determine the number of differentially expressed genes between the groups. Only genes that are transcriptionally active in at least one of the two groups are included

SUMMARY AND CONCLUSION

In the present paper we evaluated the strategy of a commonly used transcriptome analysis platform, Illlumina, with respect to the application to samples with substantial biological variation and low intensity. Three issues were addressed. The first was that determination of the significance of gene expression using the rank-based approach by Illumina is strict, so that early in the processing of data from raw to normalized one may loose genes for the remainder of the analysis. We propose to determine the significance of gene call in a sample using parametric statistics (Student's t-test) instead of ranking. Indeed we demonstrate that the Student's t-test recognizes slightly more genes as significantly expressed. Second, when working with samples with substantial biological variation, the chances that a gene is found significantly expressed to a different extend in the individual samples in one group versus the other necessitates an objective approach to determine whether the average of the groups can be compared. We propose here to use a size test. This approach reveals that with increasing group size a larger percentage of the group of samples needs to display a gene call as significantly expressed; this stabilizes at about 75% with larger group sizes. Third, VST is often applied to make microarray data more linear, which is unnecessary in Illumina as the linearity in Illumina is not affected by low-ranking probes, the statistical outcome of differentially expressed genes between two group are not affected and the low intensities of probes are greatly increased. In conclusion, we propose several adaptations in the data processing, to optimize the chances to detect genes in samples with more biological variation, while remaining conservative.

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CH.05

SHORT-TERM ERYTHROPOIETIN TREATMENT DOES NOT SUBSTANTIALLY MODULATE MONOCYTE TRANSCRIPTOMES OF PATIENTS WITH COMBINED HEART AND RENAL FAILURE

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Submitted

ABSTRACT

Objectives: We hypothesized that monocyte transcriptomes of patients with the cardiorenal syndrome (CRS) reflect the pathophysiology of the CRS and respond to short-term erythropoietin (EPO) treatment at currently recommended dose for treatment of renal anemia.

Background: CRS results in high cardiovascular morbidity and mortality. Beneficial non-hematopoietic effects of EPO treatment have been proposed. Monocytes may act as biosensors of the systemic environment.

Methods: Patients with CRS and anemia included in the EpoCaReS trial were matched to healthy controls. Patients were randomized to receive 50 IU/kg/week EPO or not. RNA from circulating $CD14^+$-monocytes was subjected to genome wide expression analysis (Illumina) at baseline and 18 days (3 EPO injections) after enrolment. Transcriptomes from patients were compared to healthy controls and effect of EPO treatment was evaluated within patients.

Results: Monocytes expressed the EPO-receptor. CRS patients showed differential expression of 471 genes, including inflammation and oxidative stress related genes. Cluster analysis did not separate patients from healthy controls. After short-term EPO treatment, every patient clustered to their own baseline transcriptome. EPO did not markedly change expression profiles on group level and individual gene responses were highly variable.

Conclusion: Monocyte transcriptomes of patients with cardiorenal failure and anemia were modestly changed compared to healthy controls and were not modulated by short-term EPO treatment at the dose recommended. Although the data indicate that EPO treatment with a standard dose has no appreciable beneficial actions on monocyte transcription profiles, it was also not associated with undesirable effects on these inflammatory cells.

INTRODUCTION

Renal dysfunction in patients with chronic heart failure (CHF) is associated with increased cardiovascular morbidity and mortality[1]. Conversely, chronic kidney disease (CKD) patients have an increased risk for myocardial infarction with higher mortality rates compared to the general population[2]. This condition in which combined cardiac and renal dysfunction aggravates failure of the individual organs has been described as the cardiorenal syndrome (CRS)[3]. In this paper, CRS is defined as the combination of CHF and CKD (CRS type 2 and 4 as described by Ronco et al.[4]). Several pathophysiological mechanisms, so-called 'cardiorenal connectors', have been recognized in combined heart and kidney failure. These include oxidative stress, inflammation, the renin-angiotensin system (RAS) and the sympathetic nervous system (SNS)[3].

Anemia may further aggravate heart and renal failure and is associated with worse outcome in CHF[5] and CKD[6]. The anemia is, amongst others, due to absolute and/or relative erythropoietin (EPO) deficiency. Erythropoiesis stimulating agents are used to treat (renal) anemia. Paradoxically, normalization of hemoglobin (Hb) in CKD patients is not associated with improved cardiovascular outcome[7, 8]. In particular, high erythropoietin (EPO) dose requirements and inability to reach target Hb even seem to be associated with adverse outcomes[9]. In addition to hematopoietic effects, EPO has non-hematopoietic anti-inflammatory and anti-oxidative effects on several organs including the kidney, brain, heart and vasculature[10, 11]. Some evidence suggests that EPO may improve cardiac and renal function in the CRS[12]. Nonetheless, as we have recently reviewed, not all reports are equivocal, and undesirable actions such as oxidative stress and hypertension have been reported[13]. The exact mechanism underlying non-hematopoietic effects of EPO is unknown.

The transcriptomes of circulating monocytes can be used as biosensor to detect changes in the systemic environment[14] and to evaluate the response to treatment[15, 16]. In addition, being inflammatory cells, monocytes are key players in the initiation and progression of atherosclerosis. Monocytes have shown to be sensitive to several of the cardiorenal connectors, like inflammatory cytokines and angiotensin II[17, 18]. We have recently described that monocytes from CKD patients display increased expression of genes coding for suppressors of cytokine signaling proteins[19, 20]. Evaluation of monocyte transcriptomes also proved valuable in the characterization of collateral artery formation in patients with

coronary artery disease[21]. As such, monocytes transcriptomes could function as a biosensor, providing an informative readout of the systemic consequences of combined heart and renal failure with anemia and the effects of short-term EPO treatment.

The hypothesis of the present study was that monocyte gene expression profiles of cardiorenal patients compared to healthy controls reflect the altered systemic environment in CRS and are responsive to short-term EPO treatment at the currently recommended dose for renal anemia. Therefore, the first aim was to explore whether CRS patients on standard treatment indeed display altered monocyte transcriptomes compared to healthy controls, with a focus on the expression of genes involved in the mediation of the cardiorenal connectors, i.e. inflammation, oxidative stress and the sympathetic nervous system. Influences on the RAS cannot be evaluated due to the administration of ACE-inhibitors, angiotensin receptor blockers and aldosterone inhibitors in our patient population. The second aim was to investigate whether short-term EPO treatment reveals non-hematopoietic effects on monocyte transcriptome level. Given the indications of recent large trials that high EPO dosages might have undesirable effects, we were not only interested in potentially beneficial effects of EPO, but also investigated whether using a sensitive readout, regular dosage of EPO might have detrimental effects on these inflammatory cells.

METHODS

STUDY DESIGN

The present study is part of the EpoCaReS trial (ClinicalTrials.gov, NCT00356733), in which CRS patients on regular treatment were randomized to receive fixed dose EPO treatment or no EPO treatment. Matched for age and gender, we selected 18 patients with mild anemia (10.3-11.9 g/dl in women and 10.3-12.6 g/dl in men), moderate renal failure (estimated creatinine clearance 20-70 ml/min calculated by Cockcroft-Gault formula) and CHF. CHF was defined as NYHA class II-IV, based on symptoms, signs and objective abnormality on echocardiography[22]. Patients with reduced ejection fraction (<50%) or left ventricular end diastolic volume index <97 ml/m^2 with evidence of diastolic LV dysfunction were included[23]. Exclusion criteria were the presence of an unstable clinical state, intolerance to EPO, kidney or heart transplantation, hyperparathyroidism, chronic inflammatory disease, malignancy, and anemia with other cause than renal failure or chronic disease. Patients were recruited from the outpatient clinics of Nephrology and Cardiology Departments at the Meander Medical Centre Amersfoort and the University Medical Center Utrecht. The institutional medical ethics committee approved the protocol and all patients gave their informed consent. All procedures were in accordance with the Helsinki Declaration.

Patients entered the study after a 4-week run-in period on standard treatment, comprising oral iron supplementation, aspirin when indicated and maximal tolerated dosages of a β-blocker, an ACE-inhibitor or an angiotensin receptor blocker. An elaborate description of the study design has been accepted for publication[24]. After enrolment, 12 out of 18 patients were randomized to receive Epoetin-β therapy (50 IU/kg/wk; Neorecormon, Roche Pharmaceuticals). Biochemical analysis and monocyte isolation for gene expression analyses were performed at baseline and after 18 days of EPO treatment (i.e. after 3 EPO injections), prior to the expected rise in Hb level. Twelve healthy age- and gender-matched persons served as baseline controls.

SAMPLE COLLECTION AND MICROARRAY PROCEDURES

Peripheral blood was collected from patients and healthy controls in EDTA containing tubes after 30 minutes in a resting position. Blood was kept on ice. Within 3 hours of blood withdrawal, $CD14^+$-monocytes were positively isolated with the use of immunomagnetic beads (Invitrogen, CA). The purity of the isolated monocyte population was on average 90% as determined by $CD14^+$-staining on flow cytometry analysis (see appendix figure 01).

mRNA was isolated from cell collections using Trizol reagent (Invitrogen/Gibco, CA) according to the manufacturer's instruction. Subsequently, mRNA was purified with NucleoSpin® RNAII (Macherey-Nagel, Düren, Germany) and samples were sent to ServiceXS (Leiden, The Netherlands) for further microarray processing. In brief, quality and integrity of RNA was checked by lab-on-chip technology (Bioanalyzer Agilent, CA). Subsequently, Illumina TotalPrep RNA Amplificationkit (Applied Biosystems/Ambion, TX) was used to create double-stranded cDNA from 500ng total RNA. After cDNA purification, in vitro transcription reaction resulted in aRNA, which was also purified. Amplified biotinylated aRNA was finally randomly hybridized to HumanRef-8 V3.0 Expression BeadChips (Illumina, CA), followed by scanning for raw gene expression intensities on Illumina's BeadArray scanner.

VALIDATION OF GENE ARRAY RESULTS BY REAL-TIME qPCR

Monocyte cDNA samples from all patients before and after 18 days EPO treatment and from healthy controls were subjected to real-time qPCR by BioMark dynamic array technology (Fluidigm, CA), performed at ServiceXS. The cDNA samples were exposed to specific target amplification, using PreAmp Master Mix and Gene Expression Assays (Taqman; Applied Biosystems, TX) for IL8, FOS, EGR1, CX3CR1, ADRB2, EPO-R and housekeeping genes GAPDH, 18S, β-Actin and RPL13a. They were subjected to a BioMark dynamic array for determination of Ct-values. Each gene was measured in triplicate for each sample. The default Taqman PCR protocol was used with an annealing temperature of 60°C and a total of 35 cycles of PCR.

STATISTICAL ANALYSES

Clinical characteristics are presented as mean±standard deviation or median (interquartile range) for respectively normally and not normally distributed data. Analysis between groups for statistically significant differences was performed by Student's t-test, Mann-Whitney U test or paired analysis when appropriate. P-values<0.05 were considered significant.

For microarray data analysis, individual bead outliers were removed from raw bead data if signal intensity was higher or lower than median intensity plus or minus 2x median absolute deviation respectively (T4Illumina, software developed by the authors). The transcription of a gene in each sample was active when the average raw intensity of the gene was significantly higher than negative controls from the same BeadArray (t-test; T4Illumina). Genes were significantly present (transcriptionally active) in a group when present in at least 8 or 13 out of 12 or 18, respectively (based on group size by Z-test, SigmaStat). After normalization procedure (Log_2-Quantile), differential expression of a gene between two groups was tested by Cyber t-test (FlexArray version 1.2). P-value<0.05 was considered significant. Genes that were significantly differentially expressed were analyzed by hierarchical clustering, with Euclidean distance as a similarity measure and clustering was based on average-linkage correlation (TIBCO Spotfire, CA). Common oxidative stress-, inflammation- and RAS-related genes were specifically addressed to search for differences between healthy controls and patients, and for the effect of short-term EPO treatment in patients.

For real-time qPCR analysis, software version 2.0.6 was used for Ct determination from the reaction chambers on the array. Linear baseline correction was applied and the Ct threshold method selected was 'Auto (Global)'. Average Ct values per gene for each sample were calculated for data that passed amplification curve quality thresholds (default value 0.65). GAPDH served as housekeeping gene, since this gene was considered most stable when comparing 18S, β-Actin, RPL13a and GAPDH in both NormFinder and GeNorm. Normalized gene expression (Ct gene of interest–Ct GAPDH; ΔCt) was related to normalized gene expression in the reference group (ΔΔCt). Fold changes were calculated by $2^{-\Delta CT}$ [19, 20]. Gene expression differences between healthy controls and patients, and effects of EPO treatment were tested by Student's t-test and paired analyses respectively. P-value<0.05 was considered significant.

ACCESSION CODES

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus[25] and are accessible through GEO Series accession number GSE17582 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17582).

RESULTS

STUDY POPULATION CHARACTERISTICS

Patients with CRS and anemia were compared to age- and gender-matched controls. Baseline characteristics are described in table 01.

Twelve out of 18 patients received EPO treatment. After 18 days, Hb was not increased significantly compared to baseline (delta Hb 0.5±1.0 g/dl in EPO-treated group vs. -0.4±1.0 g/dl in non EPO-treated group; p=0.133). The short-term EPO treatment did trigger the hematopoietic system as indicated by increased reticulocyte counts (0.05±0.02 vs. 0.07±0.02 x10e12/l; p<0.001). Whole blood mononuclear cell (MNC) counts were not different in patients compared to healthy controls (6.3(3.2) vs. 5.1(2.0) $x10^6$ MNC/ml whole blood; p=0.215) and remained unchanged after short-term EPO treatment (6.3(3.2) vs. 6.8(2.7) $x10^6$ MNC/ml whole blood; p=0.420).

EPO RECEPTOR EXPRESSION ON MONOCYTES

All monocyte samples showed a significant expression of the EPO receptor (EPO-R), reflected by Ct values of 18.0±0.5 on real-time qPCR. Slightly higher EPO-R gene expressions were found in patients compared to controls (fold change 1.2; p=0.05). EPO treatment for 18 days did not significantly alter EPO-R expressions (fold change 1.0).

TABLE 01 BASELINE CHARACTERISTICS

	HEALTHY CONTROLS (n=12)	PATIENTS (n=18)	P-VALUE
Age (years)	68±12	70±11	N.S.
Male gender (%)	8 (67%)	12 (67%)	N.S.
Body mass index (kg/m2)	23.7±1.9	26.7±4.5	0.019
Estimated creatinine clearance (ml/min)	69±19	36±11	< 0.001
Hemoglobin (g/dl)	14.0±0.8	11.8±0.9	< 0.001
Total cholesterol (mmol/l)	5.08±1.26	4.39±1.52	N.S.
HDL (mmol/l)	1.39±0.37	1.21±0.29	N.S.
LDL (mmol/l)	3.31±1.07	2.38±1.17	0.039
Triglycerides (mmol/l)	0.84±0.50	1.75±1.70	N.S.
hsCRP (mg/l)	1.0 (0.8)	4.0 (8.0)	0.007
Ejection fraction (%)	-	46±4	
Systolic blood pressure (mmHg)	129±24	129±18	N.S.
Diastolic blood pressure (mmHg)	81±7	70±8	0.001
Diabetes Mellitus (%)	-	6 (33%)	
Smoking (%)	2 (17%)	3 (17%)	N.S.
Medication			
Acetylsalicylic acid (%)	-	8 (44%)	
Statin (%)	-	10 (56%)	
Angiotensin blockade (ACEi/ARB) (%)	-	16 (89%)	
b-blockade (%)	-	13 (72%)	
Spironolactone (%)	-	4 (22%)	

Values are expressed as mean ± SD, number (percentage) or median (interquartile range).
hsCRP, high sensitive C-reactive protein; ACEi, angiotension-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; N.S., not significant

MONOCYTE GENE EXPRESSION PROFILE IN CRS PATIENTS COMPARED TO HEALTHY CONTROLS

GLOBAL GENE EXPRESSION PROFILE CHANGES, HIERARCHICAL CLUSTERING

We compared monocyte gene expression profiles of CRS patients (n=18) and healthy controls (n=12) at baseline. Out of 25,528 genes explored, 12,165 were expressed in at least one of the two groups; 471 genes were differentially expressed in CRS patients vs. healthy controls (p-value<0.05; 1.8% of total assessed genes). Fold changes were low, ranging from 0.3-1.7. Clustering of these differentially expressed genes did not separate CRS patients from healthy controls. Clustering of genes could also not be explained by gender or age of the investigated subjects.

SPECIFIC GENE EXPRESSION CHANGES

In CRS patients, 214 genes displayed increased and 257 decreased expression compared to healthy controls. Table 02 gives an overview of the 15 most induced and downregulated genes for this comparison.
A table with all genes with changed expression can be found in the appendix table 01. Patients showed lower expressions of the transcription factors EGR1 and FOS. Furthermore, patients showed decreased expression of hemoglobin-related genes HBA2 and HBB compared to healthy controls. Additional experiments with extra washing steps of isolated monocyte samples suggest that expression of these two genes can largely be attributed to reticulocyte contamination (data not shown).

We specifically addressed whether inflammation and oxidative stress in CRS patients were reflected by monocyte gene expressions. Several interesting genes involved in inflammation (i.e. IL8, IL17, IL1RAP, CX3CR1, and several TLRs) and oxidative stress (i.e. DUSP1, GPX3, DHRS9) were indeed modulated in CRS patients (table 03; see appendix table 02 for the entire panel of genes). Remarkably, some of these genes exert pro- and others anti-stimulating activities. With regard to SNS, only ADRB2 was induced in patients.

Differential expression of IL8, FOS, EGR1, CX3CR1 and ADRB2 was confirmed by qPCR (figure 01).

TABLE 02 TOP 15 OF INDUCED AND DOWNREGULATED GENES IN CRS PATIENTS VS. HEALTHY CONTROLS

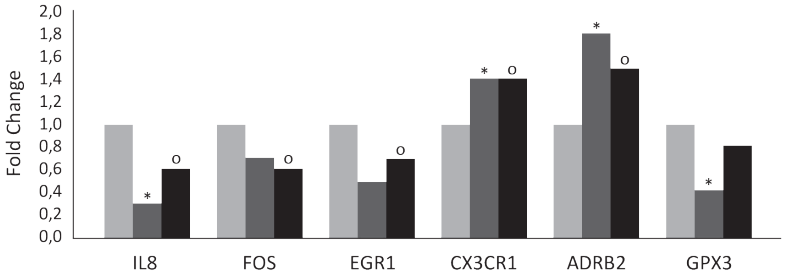
UPREGULATED (RANKED BY DESCENDING FOLD CHANGE)

ACCESSION NUMBER	SYMBOL	DESCRIPTION	FOLD CHANGE	P-VALUE
NM_018487.2	HCA112	Transmembrane protein 176A	1.695	0.037
NM_012456.1	TIMM10	Translocase of inner mitochondrial membrane 10 homolog	1.591	<0.0001
NM_017911.1	C22ORF8	Family with sequence similarity 118, member A	1.582	0.012
NM_001337.3	CX3CR1	Chemokine (C-X3-C motif) receptor 1	1.491	<0.0001
NM_006498.2	LGALS2	Lectin, galactoside-binding, soluble, 2	1.464	0.028
NM_000024.3	ADRB2	Adrenergic, b2-, receptor, surface	1.446	0.002
NM_005771.3	DHRS9	Dehydrogenase/reductase (SDR family) member 9	1.434	0.008
NM_198097.1	C7ORF28B	Chromosome 7 open reading frame 28B	1.428	0.005
NM_001343.1	DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein	1.425	<0.001
NM_016021.2	UBE2J1	Ubiquitin-conjugating enzyme E2, J1	1.424	<0.001
NM_030670.1	PTPRO	Protein tyrosine phosphatase, receptor type, variant 6	1.385	<0.0001
NM_001008566.1	TPST2	Tyrosylprotein sulfotransferase 2	1.379	0.001
NM_030671.1	PTPRO	Protein tyrosine phosphatase, receptor type, variant 5	1.372	<0.001
NR_003038.1	SNHG5	Small nucleolar RNA host gene 5	1.359	0.037
NM_080914.1	ASGR2	Asialoglycoprotein receptor 2	1.358	0.003

DOWNREGULATED (RANKED BY ASCENDING FOLD CHANGE)

ACCESSION NUMBER	SYMBOL	DESCRIPTION	FOLD CHANGE	P-VALUE
NM_000518.4	HBB	Hemoglobin, b	0.297	0.001
NM_000517.3	HBA2	Hemoglobin, a2	0.376	0.003
XM_936120.1	HLA-DQA1	PREDICTED: major histocompatibility complex, class II	0.474	0.006
NM_000584.2	IL8	Interleukin 8	0.584	<0.001
NM_005252.2	FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	0.606	0.012
NM_006732.1	FOSB	FBJ murine osteosarcoma viral oncogene homolog B	0.608	<0.0001
NM_001964.2	EGR1	Early growth response 1	0.646	0.008
NM_004417.2	DUSP1	Dual specificity phosphatase 1	0.678	0.003
NM_004666.1	VNN1	Vanin 1	0.680	0.003
NM_024933.2	FLJ12056	Ankyrin repeat domain 53	0.692	0.012
NM_005502.2	ABCA1	ATP-binding cassette, sub-family A, member 1	0.707	0.009
NM_020152.2	C21ORF7	Chromosome 21 open reading frame 7	0.710	0.009
NM_017933.3	FLJ20701	Phosphotyrosine interaction domain containing 1	0.725	<0.001
NM_002612.2	PDK4	Pyruvate dehydrogenase kinase, isozyme 4	0.736	0.017
NM_021732.1	AVPI1	Arginine vasopressin-induced 1	0.737	0.006

FIGURE 01 VALIDATION OF DIFFERENTIALLY EXPRESSED GENES ON MICROARRAY BY qPCR



**P-value<0.05 for gene expression in patients at baseline vs. healthy controls as determined with qPCR technique; °P-value<0.05 for gene expression in patients at baseline vs. healthy controls as determined with microarray technique. ADRB2, adrenergic receptor β2; CX3CR1, chemokine (C-X3-C motif) receptor 1; EGR1, early growth response 1; FOS, FBJ murine osteosarcoma viral oncogene homolog; GPX3, glutathione peroxidase 3; IL8, interleukin 8; qPCR, quantitative polymerase chain reaction*

TABLE 03 INDUCTION AND DOWNREGULATION OF OXIDATIVE STRESS AND INFLAMMATION RELATED GENES IN CRS PATIENTS VS. HEALTHY CONTROLS

INFLAMMATION RELATED GENES

TRANSCRIPT	SYMBOL	DEFINITION	FOLD CHANGE	P-VALUE
		Cytokines and cytokine receptors		
NM_000628.3	IL10RB	Interleukin 10 receptor	1.15	0.044
NM_005535.1	IL12RB1	Interleukin 12 receptor, b1	1.10	0.006
NM_001560.2	IL13RA1	Interleukin 13 receptor, a1	1.15	0.037
NM_014339.3	IL17R	Interleukin 17 receptor	1.19	0.012
NM_004633.3	IL1R2	Interleukin 1 receptor, type II	0.83	0.044
NM_002182.2	IL1RAP	Interleukin 1 receptor accessory protein	0.89	0.017
NM_173842.1	IL1RN	Interleukin 1 receptor antagonist	0.94	0.052
NM_181078.1	IL21R	Interleukin 21 receptor	1.10	0.066
NM_004843.2	IL27RA	Interleukin 27 receptor, a	1.22	0.001
NM_000584.2	IL8	Interleukin 8	0.58	0.001
NM_001557.2	IL8RB	Interleukin 8 receptor, b	0.87	0.079
		Chemokines and chemokine receptors		
NM_002982.3	CCL2	Chemokine (C-C motif) ligand 2	0.77	0.008
NM_002983.1	CCL3	Chemokine (C-C motif) ligand 3	0.86	0.027
NM_001001437.3	CCL3L3	Chemokine (C-C motif) ligand 3-like 3	0.77	0.019
NM_001337.3	CX3CR1	Chemokine (C-X3-C motif) receptor 1	1.49	<0.001
		Inflammatory response		
NM_003264.3	TLR2	Toll-like receptor 2	1.16	0.015
NM_006068.2	TLR6	Toll-like receptor 6	1.09	0.021
NM_016562.3	TLR7	Toll-like receptor 7	1.20	0.040
		Interferon transcriptional regulation		
NM_002200.3	IRF5	Interferon regulatory factor 5	1.44	0.051

OXIDATIVE STRESS RELATED GENES

Transcript	Symbol	Definition	Fold change	p-value
		Antioxidants		
NM_002084.2	GPX3	Glutathione peroxidase 3	0.88	0.017
NM_006793.2	PRDX3	Peroxiredoxin 3	0.85	0.061
NM_203472.1	SELS	Selenoprotein S	0.94	0.060
NM_005771.3	DHRS9	Dehydrogenase/reductase (SDR family) member 9	1.33	0.006
		Genes involved in ROS metabolism		
NM_024505.2	NOX5	NADPH oxidase, EF-hand calcium binding domain 5	1.05	0.052
NM_001141.1	ALOX15B	Arachidonate 15-lipoxygenase, second type	0.93	0.084
NM_004417.2	DUSP1	Dual specificity phosphatase 1	0.68	0.003

MONOCYTE GENE EXPRESSION IN CRS PATIENTS AFTER 18 DAYS OF CURRENTLY RECOMMENDED DOSE EPO TREATMENT

GLOBAL GENE EXPRESSION PROFILE CHANGES, HIERARCHICAL CLUSTERING

The effect of 18 days EPO treatment was assessed among 12 CRS patients. Out of 25,528 genes explored, 12,198 genes were expressed in the monocyte transcriptome before and/or after treatment, of which 47 genes were significantly differentially modulated by EPO (p-value<0.05; 0.2% of total assessed genes). Data are presented in the appendix table 03. Fold changes were also low in this comparison (range 0.9-3.5). Strikingly, Euclidean clustering with these genes demonstrated that every treated patient clustered to his/her own baseline gene profile (figure 02). Clustering of patients was not associated with age, gender, or the presence of diabetes. However, our intervention study was not designed to identify such associations.

SPECIFIC GENE EXPRESSION CHANGES

The 3 EPO injections only increased HBB and HBA2 expressions significantly with a fold change>1.35 when comparing group mean gene intensities. As mentioned, expression of these two genes may largely be attributed to reticulocyte contamination. Subsequently, to evaluate if short-term EPO treatment demonstrated monocytic gene modulations with regard to inflammation or oxidative stress we assessed gene expression changes in

selected gene panels. None of the genes were differentially expressed with a p-value<0.05 (data shown in the appendix table 04).

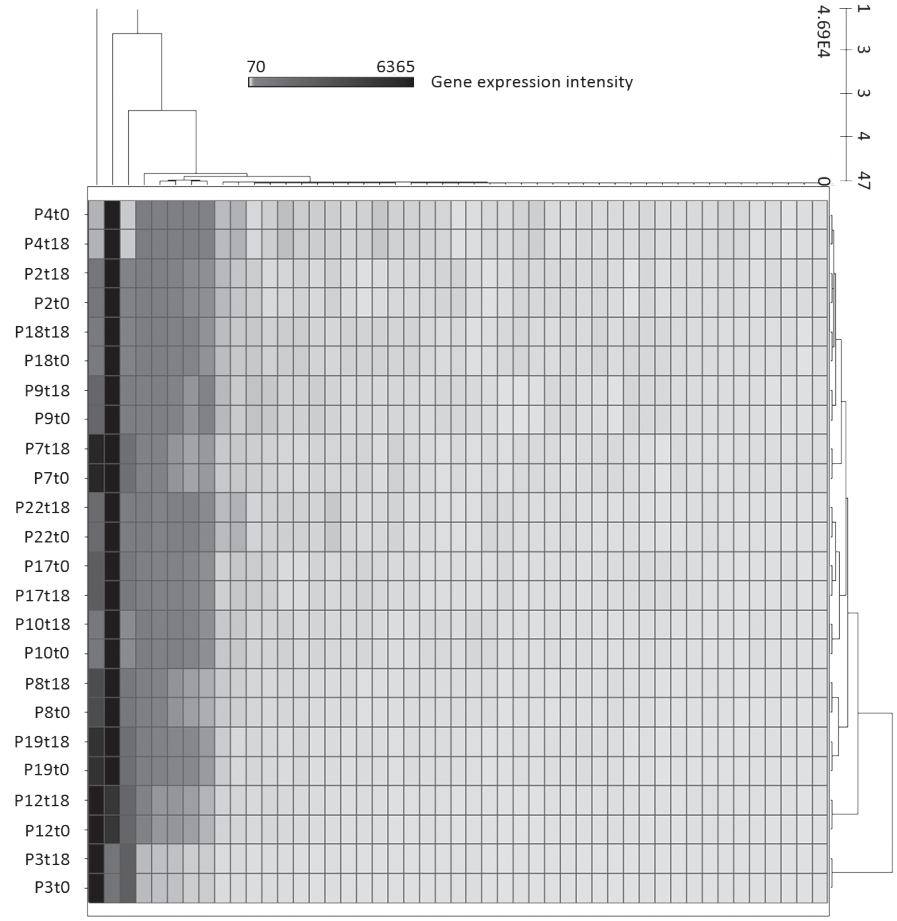
INDIVIDUAL GENE RESPONSE TO EPO

Since the expression profile of the treated group did not substantially differ from baseline, we compared gene expression modulations in the twelve individual patients. The individual gene response to EPO was remarkably variable in all patients. Only two genes (HBB, HBA2) were induced and no genes were downregulated in more than two patients after treatment.

DISCUSSION

The rationale and design of the present study is based on three of our recent studies in humans. One study showed altered leukocyte gene expression in untreated hypertensive patients, which was strongly attenuated in matched, well-treated patients[15]. In the other two papers, monocytes gene expressions in CKD[19] and ESRD[20] patients showed induction of the so-called suppressors of cytokine signaling, which modulate the Jak/Stat transcription pathway and steer the actions of IFNγ and IL6[26]. From these observations, we reasoned that monocytes, being

FIGURE 02 EUCLIDEAN CLUSTER ANALYSIS FOR PATIENTS BEFORE AND AFTER EPO TREATMENT



Patient number (P) and time point of sample collection (t0; baseline and t18; after 18 days) are listed in order of monocyte transcriptomes similarity. The closer samples are depicted to each other, the more comparable transcriptomes are.

inflammatory cells that are closely involved in atherosclerosis[27], could function as an informative readout of the systemic consequences of CRS patients with anemia and the effects of short-term EPO treatment. We have recently summarized the available support to apply transcriptomes of circulating cells to study cardiovascular risk and disease[14].

We compared monocyte transcriptomes in CRS patients to healthy controls. One of the most remarkable observations is the limited number of changes in this subpopulation of circulating cells. There are a number of potential explanations for this finding. First, the population was a carefully selected, stable group of patients with cardiorenal failure. It may be that in this specific patient group, the regular treatment, including angiotensin blockade (89%), statins (56%), acetylsalicylic acid (44%) and β-blockers (72%), has dampened or almost normalized gene expression changes, even though the inflammatory environment was not completely normalized as judged from increased hsCRP levels. Second, it may be that the monocyte has adapted to the continuous exposure of stimuli, e.g. inflammation and oxidative stress. However, if this were the case, we would expect to see more 'imprints' of such adaptations in the transcriptome (e.g. more pronounced induction of anti-oxidant genes). Third, critical changes in monocytes of cardiorenal patients may be not achieved at the level of gene transcription but at the level of protein synthesis, modification and trafficking. It is unlikely that the choice of Illumina arrays underlies the paucity of changes, since this platform provided highly reproducible results and revealed important biological processes in monocytes[28]. In our own laboratory, the same platform yielded very strong transcriptional responses of endothelial cells in culture to IFNγ and IL6 (submitted). The small changes we found are in contrast with another EpoCaRes sub-study, which evaluated the responses of plasma hepcidin to the same short-term low dose EPO stimulation (submitted). In this study we found clear responses in plasma hepcidin levels and reticulocyte numbers, clearly indicating that the dose of EPO was sufficiently strong to appreciably affect iron metabolism and erythropoiesis[29].

The pathophysiology of CRS is complex and likely involves disruption of the pro-/anti-inflammatory and pro-/anti-oxidant balance, and enhanced activity of the SNS. We investigated whether monocytes reveal imprints of such alterations. Regarding the pro-/anti-inflammatory balance, several changes in gene expression were detected in monocytes of CRS patients. This is in line with earlier studies in our group that showed modulated expression of inflammation-related genes in monocytes of CKD patients[19]. We found increased CX3CR1 expression in CRS patients. This receptor binds fractalkine (CX3CL1), a CX3C chemokine, which is expressed by activated endothelial cells and mediates adhesion and chemotaxis of CX3CR1 expressing monocytes. Experimental studies demonstrated a crucial role for CX3CR1 in the accumulation of macrophages in atherosclerotic lesions[30]. Enhanced expression of CX3CR1 in our patients may reflect activation of inflammatory processes that are critical in the initiation and progression of atherosclerosis. Transcription of some genes that stimulate inflammation (e.g. IL8, IL1RAP) was decreased in CRS patients compared to healthy controls, which may indicate a negative feedback mechanism in response to increased exposure to inflammation. All-in-all, it is quite remarkable that our analysis does not point to the cytokines IL6, IFNγ and TNFα, that have classically been associated with low grade inflammation in cardiovascular disease[31] and renal failure[32], but points to at least two other cytokines IL1 and IL8 that may be altered. Upfront, it should be emphasized that the interpretation of induction and repression of inflammatory genes is complex. For example, induced expression of a pro-inflammatory gene could represent enhanced expression of that particular pathway in the monocyte. However, increased exposure of the monocyte to this specific cytokine can also lead to a negative feedback mechanism, with decreased expression as a consequence.

Considering the pro/anti-oxidant balance in CRS patients, expression changes were modest. Downregulation of genes encoding for proteins with anti-oxidative action is suggested (e.g. GPX3, PRDX3), though others were induced (DHRS9). Decreased expression of oxidative stress responsive gene DUSP1 and markers of early activation FOS and EGR1 possibly reflects downregulation of gene expression in activated cells from our patients. This phenomenon has been reported previously in patients with coronary artery disease[33].

The only change in CRS patients related to the SNS was increased expression of ADRB2, the adrenergic receptor β2 subtype that binds epinephrine and norepinephrine[34]. This gene is particularly relevant in coupling the SNS to immune cell function[35]. It is well recognized that sympathetic hyperactivity is present in patients with heart[36] and renal failure[37]. It was expected that chronic sympathetic hyperactivity would lead to downregulation of adrenoceptor density[38], a mechanism underlying the decrease in β-adrenoceptor-mediated responsiveness characteristic[39, 40]. However, β-blockade was previously shown to increase adrenoreceptor

density in leukocytes. Patients with the highest catecholamine levels had the greatest rise in receptor density following β-blockade[38]. Albeit speculative, since 72% of our patients received β-blockade, higher ADRB2 expression levels may indicate higher baseline sympathetic activity compared to our healthy controls.

Our second aim was to evaluate short-term treatment with the currently recommended EPO dose for renal anemia in this patient population. We postulated previously that EPO may dampen activated inflammatory and oxidative stress systems in CRS patients[13]. By evaluating transcriptomes after 18 days of EPO therapy, direct effects of EPO should be discriminated from hematopoietic effects. However, EPO treatment did not substantially modulate the monocyte transcriptome in our patient group. The transcriptome after short-term EPO therapy corresponded to the baseline expression profile for every patient in our cluster analysis. This suggests that individual differences overrule the direct, short-term effects of EPO. Furthermore, we found a highly variable gene expression response to EPO in all patients, which hampers the detection of group differences. The finding that EPO response at gene expression level is so diverse may point at the variable clinical response to EPO.

Several reasons may underlie the unexpected lack in differential gene expression in response to EPO treatment. First, it was remarkable that individual responses to EPO were so variable, which aggravates the detection of global gene expression changes between groups. Despite careful selection of patients and optimally leveling out medical treatment differences, individual variations still seem to dominate the effect of EPO therapy. Microarray technology has shown to reflect the clinical response to medical therapy[15, 16]. Our study certainly does not rule out that transcriptome analysis on circulating cells could be applied to monitor early responses to EPO, however, a more sensitive cell type may be required[14]. Second, the dose and duration of EPO treatment must be considered. Various animal studies have shown protective effects of EPO in acute ischemia/reperfusion injury of the heart and kidney[10, 11]. EPO doses used in these studies are considerably higher, ranging from 3000-5000 U/kg, which is much higher than the dosages usually applied in CKD and ESRD patients. However, recent evidence from the CHOIR study shows that chronic administration of high EPO dose (mean dose 11215 IU/wk) is associated with adverse clinical events. Since we treat our patients up to 1 year, we chose a currently recommended dose for the treatment of renal anemia of 50 IU/kg/wk (i.e. approximately 3000 IU/wk), and not a short-term high dose treatment. The present data do not support a beneficial role for non-hematopoietic short-term effects of EPO. More importantly, using monocyte transcriptomes as a readout, we were unable to demonstrate any harmful effects.

In summary, we demonstrate that differences in the gene expression of monocytes, being biosensors of the pro-atherogenic environment and mediators of early atherosclerosis, are limited in our population. Nevertheless, the observed changes point at two of the systems we have proposed as important connectors in combined heart and renal failure, namely inflammation and oxidative stress. We also demonstrate that in monocytes, response in gene expression to short-term administration of the currently recommended dose of EPO is very limited, indicating that a dose that is commonly used to combat the EPO deficiency of renal anemia does not exert important non-hematopoietic effects on this pivotal cell. Fortunately, we also have been unable to identify any undesirable effects of this widely applied dose of EPO. Further studies are necessary to investigate whether other immune cells involved in the inflammatory response and in atherosclerosis may be more sensitive to this recommended dose of EPO and whether higher doses of EPO as used in cardiovascular trials do affect monocyte gene expression.

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CH.06

CIRCULATING ENDOTHELIAL PROGENITOR CELL LEVELS ARE HIGHER DURING CHILDHOOD THAN IN ADULT LIFE

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ABSTRACT

Age-related vascular dysfunction contributes to the increased cardiovascular risk in elderly. Endothelial progenitor cells (EPC), a hematopoietic stem cell (HSC) subtype, can improve vascular repair. Therefore it is hypothesized that a decrease in these circulating progenitor cells during aging plays a role in the enhanced cardiovascular risk. Until now, research has focused on EPC and HSC in the aging adult, but no studies have been conducted in children whereas animal studies specifically suggest a benefit of juvenile bone marrow. We investigated $CD34^+KDR^+$-EPC and $CD34^+$-HSC numbers by flow cytometry in healthy humans aged 1 to 81 yrs-old. An inverse relation with age was observed for EPC counts (r= -0.37, p=0.007) as well as for HSC counts (r= -0.37, p=0.008). During childhood significantly higher levels of EPC (p<0.0001) and HSC (p=0.001) were found compared to adults. These findings may have great clinical relevance since increasing circulating EPC levels is a promising therapeutic target to enhance the endogenous regenerative capacity. Better insight in the mechanisms underlying the higher EPC levels in children may provide options to increase EPC counts in adults, thereby potentiating endothelial repair mechanisms.

INTRODUCTION

Advancing age is associated with a decrease in vascular function[1, 2], which results in impaired repair of vascular lesions[3] and contributes to the increased cardiovascular risk of the elderly. Circulating bone marrow-derived endothelial progenitor cells (EPC), a subset of the hematopoietic stem cell (HSC) population, play an important role in maintenance of endothelial function and vascular repair[4, 5]. Reduced EPC levels in patients with coronary artery disease are associated with an increased risk of cardiovascular events[5, 6]. As such, EPC may constitute an endogenous vascular repair mechanism. It has been speculated that aging may lead to a decrease in circulating EPC, reducing endogenous vascular regenerative potential and facilitating the development of atherosclerosis.
Indeed, animal studies suggest that young bone marrow (BM) has atheroprotective properties that are "exhausted" with aging[7]. Chronic treatment with BM-derived progenitor cells from young mice prevented atherosclerosis progression in ApoE-/- recipients, whereas BM from old mice was far less effective. Moreover, BM transplants from young but not from aged donor mice resulted in increased neovascularization of ischemic limbs[8] and restored aging-impaired cardiac angiogenic function[9]. It is important to note that these studies used mice at a very young age (1 to 3 month-old) as BM donors. A recent study found no differences in intrinsic progenitor cell function between 4-6 month-'young' and old mice[10], which may suggest that better intrinsic EPC function is particularly present during juvenility. In humans, thus far no studies have reported on EPC levels in early youth. In healthy adults some reported lower baseline numbers of HSC and EPC with increasing age[11-14], whereas others could not find a difference in progenitor cell numbers between young and old[15-18].

METHODS

STUDY SUBJECTS

We included healthy subjects over a wide range of age. Children were recruited from the Wilhelmina Children's Hospital in Utrecht, the Netherlands. Included children were visiting the hospital for minor issues without systemic disturbances, such as hypospadias, atheroma, incontinence etc. Blood was withdrawn before surgical procedure started and/or before any medication was administered. Included adults had no history of cardiovascular disease and were not on medication. The institutional review board approved the study and all subjects or their parents gave written informed consent.

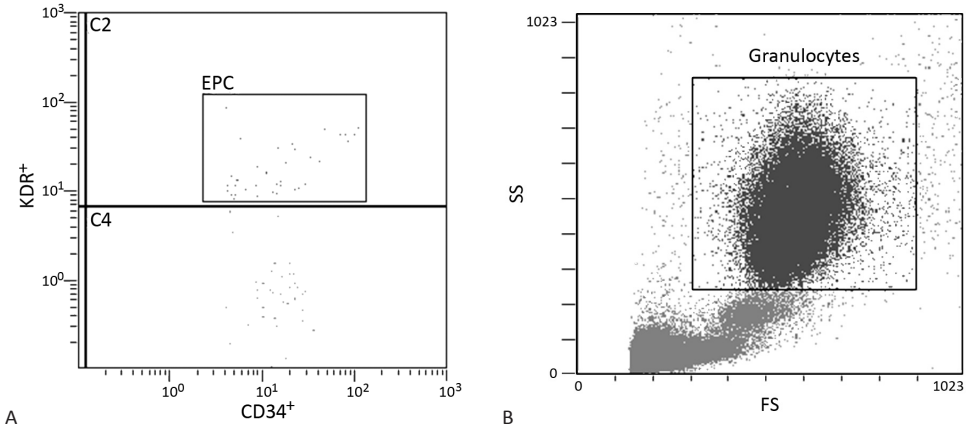
CIRCULATING PROGENITOR CELLS DETERMINED BY FLOW CYTOMETRY

Circulating EPC, defined as $CD34^+KDR^+$-cells[19], and $CD34^+$-hematopoietic stem cells were determined in peripheral blood by flow cytometry as previously reported[20]. One ml EDTA blood was collected from each subject. Samples were incubated with anti-CD34-FITC (fluorescein isothiocyanate) (BD Pharmingen, San Diego, California, USA) and anti-KDR-PE (phycoerythrin; R&D Systems, Minneapolis, Minnesota, USA) for 45 minutes. Erythrocytes were lysed in an ammonium chloride buffer. HSC and EPC were quantified in duplicate using a flow cytometer (Beckman Coulter, Fullerton, California, USA). $CD34^+$-HSCs were gated based on FITC-signal and appropriate sideward scatter in the lymphocyte range. Next, for $CD34^+KDR^+$-EPCs identification, $CD34^+$-cells were evaluated for the expression of KDR based on the presence of a concomitant PE-signal (figure 01a). Circulating EPC numbers are indicated per 10^5 granulocytes based on the foreward/sideward scatter plot (figure 01b).

STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation. Correlations were made by Spearman's rank coefficient and comparisons between groups by unpaired Student's t-test or Kruskal-Wallis one-way ANOVA when appropriate.

FIGURE 01 METHOD OF EPC DETERMINATION BY FLOW CYTOMETRY



EPC are defined as $CD34^+KDR^+$-cells (A). The number of EPC is expressed per 10^5 granulocytes, as determined on the forward (FS)/sideward (SS) scatterplot (B).

RESULTS

We consecutively included 16 healthy children and 35 healthy adults. Children had a median age of 7 years (range 1-17 yrs; 10 male/6 female) and adults had a median age of 45 years (range 22-81yrs; 16 male/19 female).

We studied levels of circulating EPC in whole blood in all subjects. We observed an inverse relation between $CD34^+KDR^+$-cell counts and age [r= -0.37, p=0.007; figure 02a]. $CD34^+$-hematopoietic stem cell levels also showed a negative association with age [r= -0.37, p=0.008; figure 02b]. Highest progenitor cell levels were found in youngest children as can be seen in figure 02. Progenitor cell levels were compared between children and adults. The number of EPC was 3 times higher in the children compared with the adult group (72±60 versus 22±22 $CD34^+KDR^+$-cells per 10^5 granulocytes, p<0.0001). The number of HSC were 2 times higher in children as compared to adults (173±133 versus 87±43 $CD34^+$-cells per 10^5 granulocytes, p=0.001).

No significant differences in EPC or HSC number were found between young (39yrs; 22-60yrs) and older adults (71yrs; 60-81yrs) (figure 03).

FIGURE 02 THE NUMBER OF CD34⁺KDR⁺-ENDOTHELIAL PROGENITOR CELLS (EPC) [A] AND CD34⁺-HEMATOPOIETIC STEM CELLS (HSC) [B] DECREASE WITH ADVANCING AGE

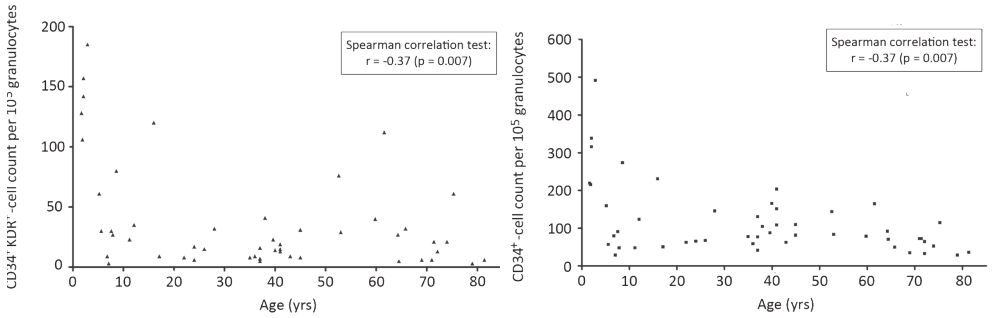
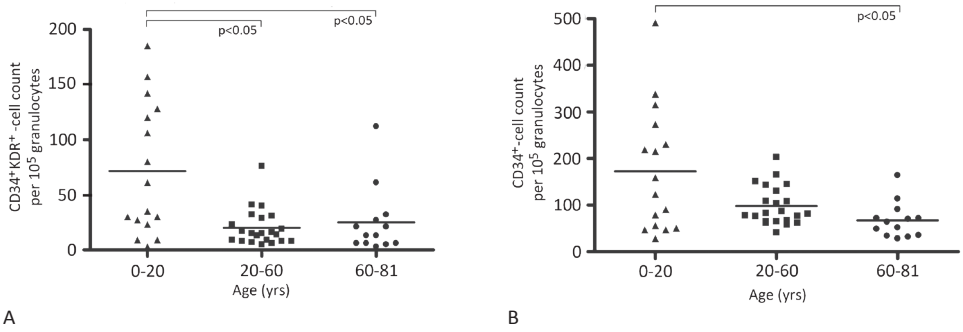


FIGURE 03 CD34⁺KDR⁺-ENDOTHELIAL PROGENITOR CELLS (EPC) [A] AND CD34⁺-HEMATOPOIETIC STEM CELLS (HSC) [B] ARE SIGNIFICANTLY HIGHER IN CHILDREN COMPARED TO ADULTS. NO DIFFERENCES WERE FOUND BETWEEN YOUNG AND OLDER ADULTS



DISCUSSION

Our data show for the first time that children have markedly more circulating EPC compared to adults, suggesting a higher vascular regenerative potential during childhood.

Highest EPC levels were demonstrated in youngest children. This is in concordance with previous animal studies, in which a difference in EPC count could only be found between the (very) young and aged subjects[7, 8]. In their interesting paper Chang et al.[10] convincingly showed that a defect in the response of aged tissue to hypoxia, resulting in impaired recruitment or mobilization of EPC, is an important cause of age-related vascular dysfunction. Based on age- and gender-mismatched BM transplantation experiments they also concluded that there are no differences in intrinsic progenitor cell function between young and old. From our data we cannot determine the mechanism underlying decreased EPC levels in adults. It may well be that decreased mobilization from the BM and recruitment as proposed by Chang et al. play a role, but diminished survival and decreased EPC availability in the BM also need to be considered. In addition, children may require enhanced recruitment to the circulation because of growth.

As improving endogenous regenerative capacity by enhancing levels of circulating EPC is increasingly recognized as potential therapeutic target in patients with cardiovascular disease our finding may have large clinical relevance. Better insight in the mechanisms underlying the higher EPC levels in children may provide options to increase EPC levels in adults, thereby potentiating endothelial repair mechanisms.

ACKNOWLEDGEMENTS

We would like to thank Mark Klein from the department of Pediatric Immunology, University Medical Center Utrecht, Wilhelmina Children's Hospital, Utrecht, Netherlands for his technical assistance.

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CH.07

CIRCULATING ENDOTHELIAL PROGENITOR CELLS ARE REDUCED IN CHILDREN WITH HEMODIALYSIS, BUT NOT PREDIALYSIS CHRONIC KIDNEY DISEASE

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ABSTRACT

In adults with chronic kidney disease (CKD) reduced levels of vasculoprotective endothelial progenitor cells (EPC) may contribute to their increased risk of cardiovascular disease. Children with CKD are also at high cardiovascular risk. However, no studies have reported on circulating EPC levels in the pediatric CKD population. We investigated $CD34^+KDR^+$-EPC numbers by flow cytometry in 15 children with predialysis CKD, 13 children on hemodialysis and 18 age-matched healthy controls. Children on hemodialysis showed 47% reduced EPC levels compared to controls, whereas no significant difference was found for predialysis CKD patients. Lower EPC levels were found in patients with higher levels of inflammatory marker hsCRP. Our data show for the first time that children on hemodialysis have reduced $CD34^+KDR^+$-EPC levels, potentially contributing to their increased cardiovascular risk. In children with predialysis CKD, a decline in renal function was not associated with reduced EPC levels, which may reflect a capacity for preservation of the endogenous repair system during relatively moderate disturbances of the systemic environment.

INTRODUCTION

Chronic kidney disease (CKD) is associated with accelerated atherosclerosis and a high risk of cardiovascular disease (CVD)[1]. Endothelial dysfunction plays a key role in the pathogenesis of CVD[2, 3]. In adult CKD patients the availability of bone marrow (BM)-derived circulating endothelial progenitor cells (EPC) -which constitute an endogenous vascular repair system-[4, 5] was reported to be reduced. This may contribute to the accelerated atherosclerosis in CKD patients[6-8]. Children with CKD also have substantially increased CVD risk. Endothelial dysfunction was shown to be present in children[9, 10]. Whether circulating EPC levels are altered in children with CKD has not been evaluated.

METHODS

STUDY SUBJECTS

We recruited children with predialysis CKD and end-stage CKD requiring hemodialysis from the outpatient clinics of Pediatric Nephrology (Wilhelmina Children's Hospital, University Medical Center Utrecht, Netherlands). Patients had no signs of active inflammation. Blood samples from hemodialysis patients were drawn immediately preceding a dialysis session. Age-matched controls were recruited among otherwise healthy children undergoing minor surgery (e.g. hypospadias, urethral valves, incontinence). Blood was withdrawn before surgical procedure started and/or before any medication was administered. The protocol was approved by the institutional medical ethics committee and all parents and patients (if >11 years of age) gave informed consent. Procedures were in accordance with the Helsinki Declaration.

CIRCULATING PROGENITOR CELLS DETERMINED BY FLOW CYTOMETRY

Circulating EPC, defined as $CD34^+KDR^+$-cells[11], and $CD34^+$-hematopoietic stem cells were determined in peripheral blood by flow cytometry as previously reported[8]. Minimal 100 µl EDTA blood was collected from each subject. Samples were incubated with anti-CD34-FITC (fluorescein isothiocyanate) (BD Pharmingen, San Diego, California, USA) and anti-KDR-PE (phycoerythrin; R&D Systems, Minneapolis, Minnesota, USA) for 45 minutes. Erythrocytes were lysed in an ammonium chloride buffer. HSC and EPC were quantified in duplicate using a flow cytometer (Beckman Coulter, Fullerton, California, USA). $CD34^+$-HSCs were gated based on FITC-signal and appropriate sideward scatter in the lymphocyte range. Next, for $CD34^+KDR^+$-EPCs identification,

$CD34^+$-cells were evaluated for the expression of KDR based on the presence of a concomitant PE-signal. Circulating EPC numbers are indicated per 10^5 granulocytes based on the foreward/sideward scatter plot.

STATISTICAL ANALYSIS

Data are presented as median (range) for age and mean±SEM for other parameters. Group differences were assessed by Mann-Whitney test and correlations were made by Spearman's rank coefficient.

RESULTS

BASELINE CHARACTERISTICS

We included 13 children with end-stage CKD on hemodialysis, 15 children with predialysis CKD and 18 age-matched healthy controls. Baseline clinical and laboratory characteristics are described in the table. Main causes for underlying CKD were obstructive uropathy and dysplastic kidneys. None of the subjects had a history of CVD. Medical therapy of CKD patients included antihypertensives (60% in predialysis patients; 23% in hemodialysis patients) and erythropoietin (20% in predialysis patients; 100% in hemodialysis patients).

PROGENITOR CELL MEASURES

White blood cell and granulocyte count in peripheral blood were not different between controls and patients on hemodialysis (7.4±1.0 vs. 7.3±0.5 $x10^6$/ml; p=0.868 and 5.0±0.8 vs. 3.6±0.3 $x10^6$/ml; p=0.167 respectively) or predialysis CKD patients (7.2±0.8 $x10^6$/ml; p=0.831 and 4.0±0.7 $x10^6$/ml; p=0.442 respectively).

$CD34^+KDR^+$-cell numbers were reduced by 47% in children on hemodialysis compared to controls (figure). No differences were found between predialysis CKD and controls. $CD34^+$-progenitor cell levels were not significantly different between controls and children on hemodialysis (118±18 vs. 75±8 cells per 10^5 granulocytes; p=0.242) or predialysis CKD (132±23 cells per 10^5 granulocytes; p=0.625). High sensitive C-reactive protein (hsCRP) was increased in hemodialysis (1.8±0.6 mg/dl; p=0.022), but not in predialysis CKD (0.6±0.2 mg/dl; p=0.459) compared to controls (0.6±0.3 mg/dl). Lower EPC levels were found in subjects with higher hsCRP (r=-0.374; p=0.019).

FIGURE CIRCULATING ENDOTHELIAL PROGENITOR CELLS IN CHILDREN WITH CKD

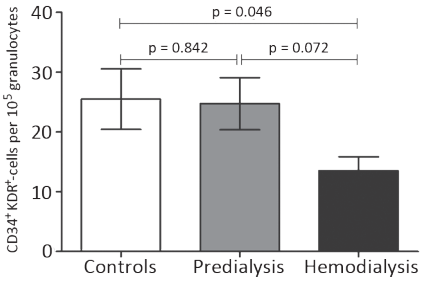

TABLE BASELINE CHARACTERISTICS

	CONTROLS	PREDIALYSIS	HEMODIALYSIS
Age, years	8 (2-17)	10 (4-18)	14 (3-18)
Male gender, n (%)	13 (72)	9 (60)	9 (69)
Serum creatinine, µmol/l	46±3	174±32*	980±60*
Creatinine clearance§, ml/min	116±6	40±4*	-
Urinary protein/creatinine ratio	0.04±0.01	0.08±0.02	-
Systolic blood pressure, mmHg	110±10	105±15	116±17
Diastolic blood pressure, mmHg	62±8	58±10	71±14
Hemoglobin, mmol/l	7.76±0.85	7.61±0.77	7.01±0.84*
Total cholesterol, mmol/l	3.67±0.59	3.88±1.29	3.48±0.92
HDL cholesterol, mmol/l	1.38±0.26	1.41±0.37	0.99±0.22*
LDL cholesterol, mmol/l	1.91±0.47	2.31±0.65	1.55±0.76
Triglycerides, mmol/l	0.85±0.50	0.95±0.40	2.07±0.97*
HbA1c, %	5.3±0.1	5.3±0.1	4.8±0.3
hsCRP, mg/l	0.6±0.3	0.6±0.2	1.8±0.6*

** P-value compared to healthy controls*
§ Creatinine clearance estimated by Schwartz formula
HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high sensitive C-reactive protein

DISCUSSION

We show for the first time that children with CKD on hemodialysis have markedly lower levels of circulating $CD34^{+}KDR^{+}$-EPC as compared to healthy age-matched controls, which is in line with previous studies in adult hemodialysis patients[8, 12] and consistent with a recent study reporting reduced EPC marker molecules in whole blood lysates of pediatric hemodialysis patients[13]. The findings are consistent with a similarly impaired vascular regenerative potential in children as in adults with end-stage CKD on hemodialysis. This may contribute to the acceleration of CVD in children on hemodialysis.

Interestingly, in children with predialysis CKD circulating EPC levels were not lower compared to healthy controls. In these children, given their marked endothelial injury[9, 10], the lack of an elevation of circulating EPC levels still may reflect an inadequate compensatory repair response. Alternatively, in this stage of CKD increased EPC mobilization and homing of EPC to the damaged vascular wall may result in lower circulating EPC numbers while endothelial repair is in progress. The discrepancy with recent studies in adults is remarkable. In adult predialysis CKD patients we[14] and others[7] recently demonstrated that EPC levels are significantly reduced as compared to healthy controls. The discrepancy in circulating EPC between children and adults with predialysis CKD may in part relate to the higher presence of comorbidity and cardiovascular risk factors -which have previously been associated with decreased circulating EPC levels[15]- in adults as compared to children. This may suggest that the predialysis uremic environment in itself does not reduce EPC levels but that the reduction in circulating EPC in adults is likely to be primarily due to concomitant presence of cardiovascular risk factors. Indeed, in adult predialysis populations EPC levels were particularly reduced in patients with a history of CVD[14] or presence of coronary artery disease[7]. Moreover, we observed a negative association between circulating EPC levels and inflammatory marker hsCRP, an independent indicator of cardiovascular risk[16]. On the other hand, the preserved EPC levels in children but not in adults with predialysis CKD could be related to a stronger vascular protective system in children, as we recently proposed based on our observations that circulating EPC levels are higher during childhood than in adult life[17]. Further insight into EPC function and mechanisms underlying reduced EPC levels in CKD children may provide valuable treatment options to reduce cardiovascular risk in this vulnerable patient population.

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CH.08

PROGENITOR CELLS AND VASCULAR FUNCTION ARE IMPAIRED IN PATIENTS WITH CHRONIC KIDNEY DISEASE

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ABSTRACT

Background: Endothelial dysfunction contributes to accelerated atherosclerosis in chronic kidney disease (CKD). Bone marrow-derived endothelial progenitor cells (EPC) constitute an endogenous vascular repair system, protecting against atherosclerosis. Smooth muscle progenitor cells (SPC) may stimulate atherosclerosis development. We hypothesized that an imbalance in EPC and SPC occurs in CKD, which may contribute to the increased cardiovascular disease (CVD) risk.

Method: EPC and SPC outgrowth from mononuclear cells (MNC), EPC migratory function and circulating $CD34^+KDR^+$-EPC were measured in 49 patients with varying degrees of CKD on regular therapy and 33 healthy volunteers. Renal function, CKD cause, CVD history and endothelial dysfunction parameters were determined as factors of influence on progenitor cells.

Results: Patients had reduced EPC outgrowth compared to controls (9(2-22) vs. 12(1-38) cells/10^3 MNC; p=0.026), independent of CKD cause and degree, whereas SPC outgrowth levels were higher in patients with more impaired kidney function (r=-0.397; p=0.008). Patients had lower $CD34^+KDR^+$-EPC compared to controls (9(0-52) vs. 19(4-110) cells/10^5 granulocytes; p=0.004). CVD history and increased endothelial dysfunction markers were related to lower EPC levels. Progenitor cell outgrowth was shifted towards SPC with progression of endothelial damage. Reduction in EPC could not be attributed to decreases in progenitor cell mobilizing factors SDF-1α and VEGF as levels increased with progressive kidney and endothelial dysfunction while EPC remained low.

Conclusion: Our data suggest that already in mild CKD, EPC mediated endogenous vascular regeneration is impaired while SPC levels increase with declining kidney function.

INTRODUCTION

Cardiovascular disease (CVD) is a major threat to patients with chronic kidney disease (CKD)[1]. Endothelial dysfunction and impaired endothelial regenerative capacity play a key role in the pathogenesis of CVD[2]. Bone marrow (BM)-derived endothelial progenitor cells (EPC) constitute an endogenous vascular repair system that protects against atherosclerosis development[3]. A decline in EPC availability or function may contribute to the pathogenesis of CVD[4]. The presence of cardiovascular risk factors or CVD has been related to reduced EPC levels and function in many, but not all studies[4-7]. Besides differentiation towards endothelial cell phenotype, BM-derived vascular progenitors may differentiate towards smooth muscle cells and myofibroblasts in the vessel wall, participating in atherosclerosis development[8, 9]. Increased levels of these smooth muscle progenitor cells (SPC) have been observed in diabetic and in CAD patients, which may contribute to vascular complications[10, 11].

In kidney disease, vascular progenitor cell availability and function may be adversely affected by accumulation of toxins, including oxidative products[12]. Several studies demonstrated reduced EPC levels in patients on dialysis[13, 14]. We reported reduced EPC but unaffected SPC outgrowth in hemodialysis patients, suggesting impaired endothelial regenerative capacity while the capacity of progenitor cells to contribute to adverse vascular remodeling is retained[15]. Interestingly, better correction of the uremic environment increases EPC levels in end-stage kidney disease[16, 17]. In kidney transplant patients a negative relation between EPC levels and graft dysfunction was observed[17]. Information on SPC and EPC in predialysis CKD is scarce.

We hypothesized that an imbalance between EPC and SPC is present in predialysis CKD. This imbalance may result in impaired regenerative and enhanced profibrotic tendency and contribute to the increased cardiovascular risk. We determined circulating $CD34^+KDR^+$-EPC and mononuclear cell (MNC) outgrowth towards EPC and SPC in patients with varying degrees and different causes of CKD. Paracrine effects of cultured EPC were tested in a scratch wound assay. We investigated whether progenitor cell number and function in CKD are related to cause and degree of kidney insufficiency and to endothelial dysfunction markers or history of CVD. Finally, we investigated whether changed levels of progenitor cell mobilizing factors underlie altered EPC levels.

SUBJECTS AND METHODS

SUBJECTS

Patients with different stages of CKD, defined as kidney damage or estimated glomerular filtration rate (eGFR) <60 mL/min/1.73m^2 for ≥3 months, and no diabetes, dialysis treatment or malignancy were consecutively recruited from the nephrology outpatient clinic, University Medical Center Utrecht (UMCU), Netherlands. Recruited patients were not allowed to have current infection. Out of 50 recruited patients, 49 were eligible for enrolment due to exclusion of 1 patient with increased inflammatory markers. Patients maintained their regular medication. Thirty-five age-matched healthy subjects were recruited (colleagues, family, spouse of patient), of whom 33 were eligible to serve as controls (2 controls excluded due to multivitamin use and increased inflammatory markers). The study protocol was approved by the local ethics committee and all subjects gave informed consent. Procedures were in accordance with the Helsinki Declaration.

Biochemical parameters were measured in fasting blood samples using standard procedures. Albuminuria (immunoturbidimetric assay) and albumin-to-creatinine ratio were assessed in morning urinary specimen. MDRD formula[18] was used to calculate eGFR.

Patients were divided into groups with an atherosclerotic or non-atherosclerotic cause of CKD as diagnosed by the patient's nephrologist. Presence of a history of CVD was defined as myocardial infarction, angina pectoris, cerebrovascular accident, transient ischemic attack, peripheral artery disease or revascularization diagnosed in medical history.

ENDOTHELIAL DYSFUNCTION

As surrogate marker of subclinical atherosclerosis, arterial stiffness was assessed by measuring augmentation index (Aix) and pulse wave velocity (PWV) using the SphygmoCor2000 system according to the manufacturer's instructions. Three good quality data runs for each measurement were averaged.

Markers reflecting endothelial activation and/or injury (E-Selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), thrombomodulin) were measured using commercially available ELISA (R&D Systems, Minneapolis, USA; Diaclone, Stamford, USA).

CIRCULATING EPC

EDTA blood was collected from fasting subjects. 100 µl blood was incubated with anti-CD34-FITC, anti-CD45-PE-Cy7 (BD Pharmingen, San Diego, USA) and anti-KDR-PE (R&D Systems) antibodies. Erythrocytes were lysed and cells were analyzed by flow cytometry (Beckman Coulter, Fullerton, USA). EPC were identified as CD34$^+$KDR$^+$-cells in the lymphocyte region of the forward/sideward scatter plot and quantified relative to 10^5 granulocytes, identified as CD45$^+$-cells with a typical granulocyte distribution. Measurements were performed in duplo and results were averaged. Isotype-stained samples served as negative controls.

OUTGROWTH OF EPC AND SPC IN CULTURE

EPC and SPC outgrowth from MNC was assessed as previously described[15]. MNC were isolated from blood samples using Ficoll density gradient separation (Histopaque 1077, Sigma, St. Louis, USA). To evaluate EPC outgrowth, 10^7 MNC/well were seeded on a human fibronectin (Sigma) coated 6-well plate in EGM-2 (Cambrex, Walkersville, USA), supplemented with accompanying aliquots, 20% fetal calf serum (Invitrogen, Carlsbad, California), 100 ng/mL recombinant VEGF-165 (R&D Systems) and antibiotics. Medium was changed after 4 days. After 7 days, cultured EPC in selected wells were placed on serum free medium (EBM-2 with hEGF, hydrocortisone, GA-1000, R^3-IGF-1, ascorbic acid, heparin and antibiotics) overnight. Conditioned medium was stored for functional experiments. Cultured EPC were detached by trypsin and cell scraping, and automatically counted using a hemocytometer.

For assessment of SPC outgrowth, 5x10^6 MNC/well were seeded on 6-well plates coated with human fibronectin and cultured in low-glucose DMEM supplemented with 20% fetal calf serum, L-glutamin (Invitrogen), 0.5 µl/mL PDGF (R&D Systems) and antibiotics. Medium was changed after 4 days. At day 8, cultured SPC were detached by trypsin and cell scraping and automatically counted using a hemocytometer.

IN VITRO SCRATCH WOUND ASSAY

The potential of EPC outgrowth to excrete paracrine factors that stimulate endothelial cell migration was assessed by in vitro scratch wound assay[19]. A mechanical scratch was created with a pipet tip in a confluent monolayer of human microvascular endothelial cells (HMECs; Centers for Disease Control and Prevention, Atlanta, USA). After washing with PBS, EPC outgrowth

conditioned medium was placed on the cells. Serum-free EPC medium served as negative control. Reference lines were made on the bottom of the wells to obtain exactly the same field during image acquisition. The scratched area was photographed using light microscope at start and after 6 hours incubation (37°C). The extent of closure after 6 hours was determined relative to the starting width of the scratch (Image-Pro plus software, Media Cybernetics 3.0). Each sample was measured in two wells and two picture fields/well were examined. Results were averaged for analysis.

VEGF/ SDF-1α PLASMA MEASUREMENTS

Plasma vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1α (SDF-1α) levels were measured by ELISA (R&D Systems). All samples were measured in duplo and averaged for analysis.

STATISTICAL ANALYSIS

Data analysis was performed using SPSS 15.0 for Windows. The Kolmogorov-Smirnov statistic test was used to explore whether data were normally distributed. Data are expressed as mean ± standard deviation for parametric data and as median (minimum-maximum) for non-parametric data. Group differences were analyzed by Student's t-test or Mann Whitney test. Multiple group comparisons were performed using ANOVA with LSD post-hoc testing for which non-parametric data were log-transformed. Fisher's exact test was

TABLE 01 PATIENT CHARACTERISTICS

	HEALTHY CONTROLS (n=33)	CKD PATIENTS (n=49)
Age, years	65(31-81)	62(30-84)
Male sex	22 (67)	24 (49)
Body mass index, kg/m²	23.8(19.6-30.7)	24.4(19.6-37.6)
eGFR, ml/min/1.73m²	80±10	37±19*
Serum creatinine, mmol/L	83(69-101)	157(76-845)*
Plasma urea, mg/dl	36.0(9.9)	61.3(58.9)*
Microalbuminuria, mg/dl	0.6 (0.2-1.9)	1.8 (0.2-63.6)*
Albumin-to-creatinine ratio (urine), mg/mmol	0.5 (0.2-3.0)	5.9 (0.2-190)*
Hemoglobin, g/dl	14.2±1.0	13.1±1.8*
Plasma total cholesterol, mg/dl	210±43	205±48
C-reactive protein, mg/L	2 (2-5)	5 (2-16)*
Systolic blood pressure (mmHg)	120 (102-140)	136 (107-197)*
Diastolic blood pressure (mmHg)	80 (60-85)	82 (68-110)*
Hypertension†	0	45 (92)
Smoker	8 (24)	8 (16)
Medication		
Erythropoietin	0	7 (14)*
Statin	0	32 (65)*
RAS blockade	0	37 (76)*
Beta blockade	0	15 (31)*
Calcium antagonist	0	8 (16)*
Diuretics	0	24 (49)*
History of CVD	0	14 (29)*
CKD cause (atherosclerotic‡/non-atherosclerotic§/unknown)	-	21 (43)/24 (49)/4 (8)

Values are n (number) (%), mean ± SD or median (minimum-maximum)
**P-value<0.05 compared to healthy controls*
†Hypertension: defined by use of antihypertensive medication, systolic or diastolic blood pressure above 140 or 90 mmHg respectively.
‡Mainly long-standing hypertension, kidney artery stenosis
§Mainly glomerulonephritis, polycystic kidney disease, nephrolithiasis, lithium-induced, membranous glomerulopathy

used to analyze whether proportions of categories varied by group. Correlations were measured by Pearson's or Spearman's correlation coefficient where appropriate. P-value<0.05 was considered statistically significant.

RESULTS

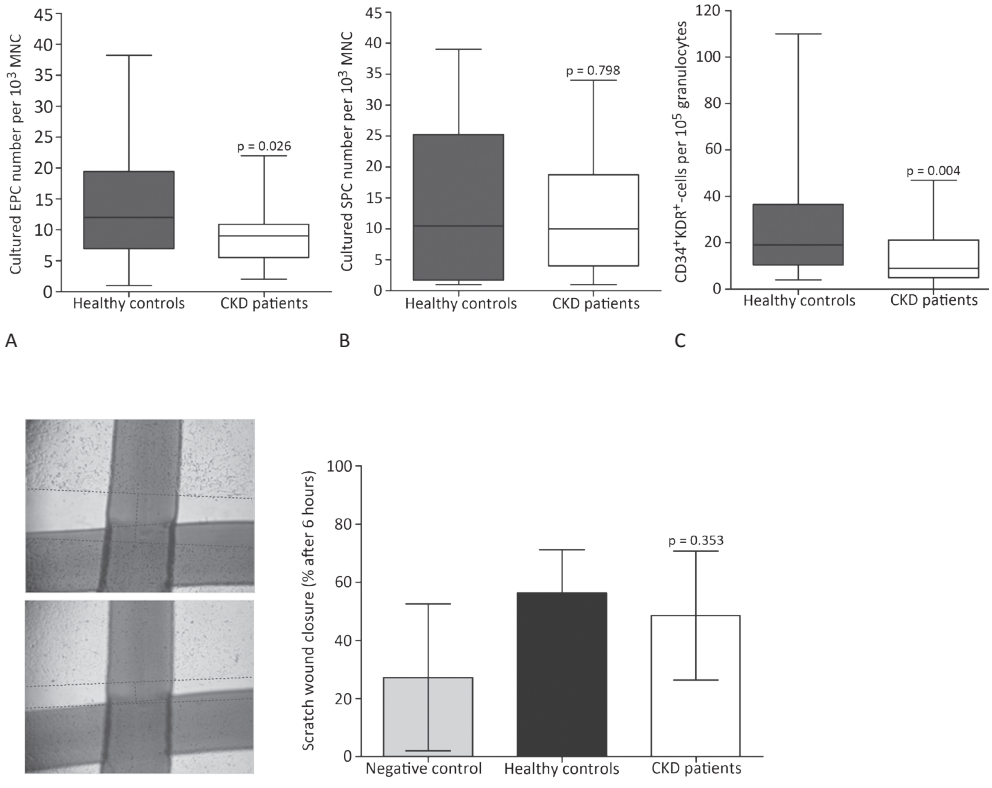
PATIENT CHARACTERISTICS

Patients with different stages of CKD were included (eGFR 60-69: n=7; eGFR 45-59: n=13; eGFR 30-44: n=12; eGFR 15-29: n=7; eGFR<15: n=10). Patient characteristics are listed in table 01.

VASCULAR PROGENITOR CELL LEVELS IN CKD

EPC outgrowth was lower in CKD versus controls (figure 01A). No difference was observed in SPC outgrowth (figure 01B). Levels of circulating $CD34^+$-hematopoietic stem cells were not significantly different between CKD and controls (56 (11-359) vs. 72 (28-162) $CD34^+$-cells/10^5 granulocytes; p=0.134). $CD34^+KDR^+$-EPC levels were lower in CKD (figure 01C). Conditioned medium from CKD patients did not induce lower migration of HMECs compared to healthy controls (figure 01D).

FIGURE 01 EPC LEVELS AND FUNCTION IN CKD PATIENTS COMPARED TO HEALTHY CONTROLS



CKD patients show decreased EPC outgrowth (A) whereas SPC outgrowth was not significantly different (B). Circulating EPC numbers are lower in CKD patients compared to healthy controls (C). Scratch width at start (D; upper picture) and closure after 6 hours incubation (D; lower picture) was measured, adjusted for image acquisition variance with help of the marker lines (cross). Migratory capacity of HMECs in conditioned EPC medium from patients was not significantly decreased compared to healthy controls (D; graph).

FACTORS THAT MAY INFLUENCE PROGENITOR CELL LEVELS IN CKD

UNDERLYING CAUSE OF CKD

EPC and SPC outgrowth numbers were not different between patients with atherosclerotic and non-atherosclerotic causes of CKD (9(4-18) vs. 8(3-22) EPC/10^3 MNC; p=0.460 and 12(2-35) vs. 10(2-34) SPC/10^3 MNC; p=0.247). Circulating EPC levels were also not different between these groups (9(0-52) vs. 9(2-47) $CD34^+KDR^+$-cells/10^5granulocytes; p=0.424).

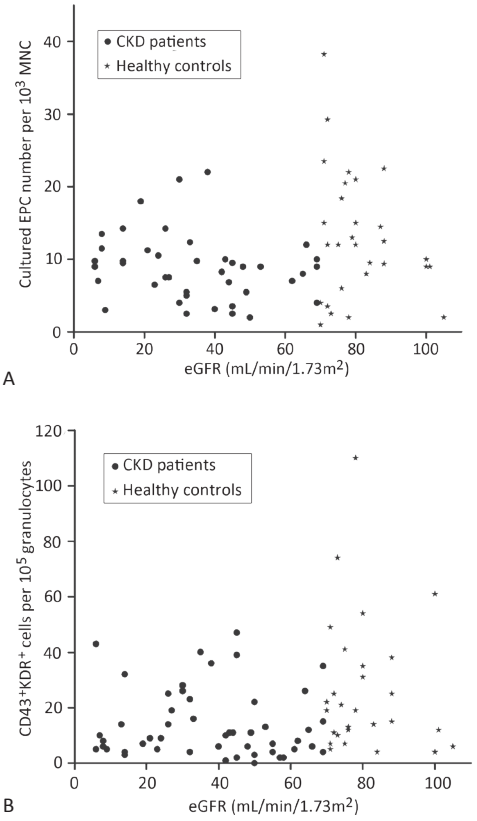
DEGREE OF KIDNEY DYSFUNCTION

Reduced EPC outgrowth was already observed in mild to moderate CKD (8(2-22) in patients with eGFR>30 mL/min/1.73m^2 vs. 12(1-38) cells/10^3 MNC in controls; p=0.021). A further decline in kidney function was not related to cultured EPC numbers (r=0.133; p=0.272; figure 02A). Among CKD patients, no correlations were found for eGFR with EPC migration capacity (r=0.161; p=0.304) or circulating $CD34^+KDR^+$-EPC levels (r=-0.138; p=0.351; figure 02B). A significant negative association was found between SPC outgrowth and eGFR (r=-0.397; p=0.008). Plasma urea, microalbuminuria and total albumin-to-creatinine ratio were also not associated with any of the progenitor cell measures (data not shown).

PRESENCE OF A HISTORY OF CARDIOVASCULAR DISEASE

CKD patients with a history of CVD showed lower $CD34^+KDR^+$-cells compared to patients without (6(0-39) vs. 12(2-52) $CD34^+KDR^+$-cells/10^5 granulocytes; p=0.053), whereas kidney function

FIGURE 02 CORRELATION OF EPC OUTGROWTH LEVELS WITH THE DEGREE OF KIDNEY DYSFUNCTION



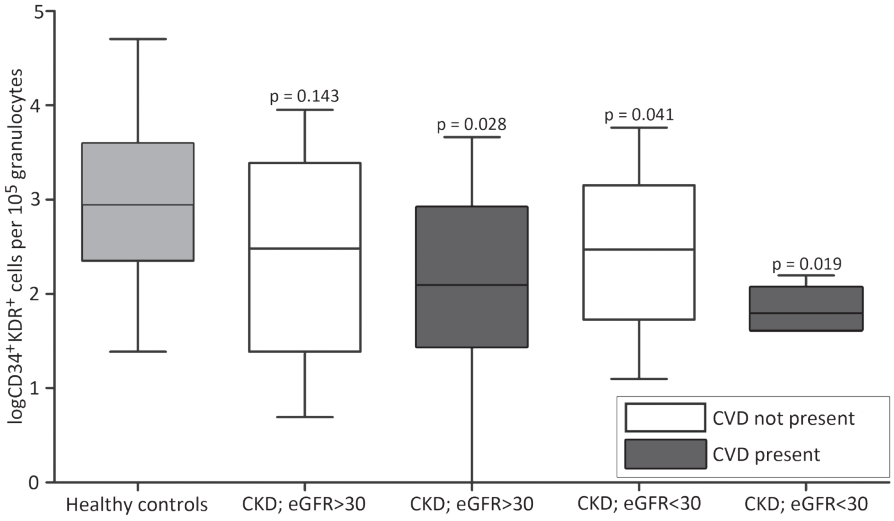
Cultured EPC levels (A) and circulating $CD34^+KDR^+$-EPC (B) are already decreased in CKD patients with mild kidney dysfunction. A further decline in eGFR was not correlated with EPC levels.

TABLE 02 ENDOTHELIAL DYSFUNCTION MARKERS ARE INCREASED IN CKD PATIENTS AND CORRELATE WITH THE DEGREE OF KIDNEY IMPAIRMENT

				eGFR (mL/min/1.73m²)	
	HEALTHY CONTROLS	CKD PATIENTS	P-VALUE†	r	P-VALUE‡
Augmentation Index	16.9±11.1	25.2±10.3	0.010*	-0.295	0.036*
PWV, m/s	7.3 (5.3-12.7)	8.7 (5.9-14.4)	0.121	-0.123	0.420
Thrombomodulin, ng/mL	0.15±0.16	0.95±0.89	<0.001*	-0.731	<0.001*
VCAM-1, ng/mL	24.4±1.0	30.1±1.7	0.007*	-0.435	<0.001*
ICAM-1, ng/mL	151±37	166±60	0.192	-0.178	0.164
E-Selectin, ng/mL	3.3±1.0	3.7±1.1	0.223	-0.201	0.114

Values are presented as mean ± SD or median (minimum-maximum); correlation values (r) are shown as Pearson's or Spearman's correlation where appropriate.
**P-value<0.05*
†P-value for univariate analysis between marker levels in CKD patients and healthy controls
‡P-value for correlation analysis between marker levels and kidney function

FIGURE 03 CIRCULATING EPC LEVELS IN CKD PATIENTS WITH A HISTORY OF CARDIOVASCULAR DISEASE



Circulating EPC levels were lower in CKD compared to healthy controls, which was more pronounced in patients with a history of CVD. Data were log-transformed and p-values are given with respect to healthy controls.

TABLE 03 VEGF AND SDF-1α PLASMA LEVELS ARE CORRELATED WITH ENDOTHELIAL DYSFUNCTION PARAMETERS

	PLASMA VEGF	PLASMA SDF-1α
Augmentation index	r =0.368, p =0.025*	r =0.247, p =0.141
PWV, m/s	r =0.300, p = 0.090	r = 0.209, p =0.243
Thrombomodulin, ng/ml	r =0.412, p =0.002*	r =0.569, p <0.001*
VCAM-1, ng/ml	r =0.353, p =0.010*	r =0.434, p =0.001*
ICAM-1, ng/ml	r =-0.082, p =0.589	r =0.184, p =0.222
E-Selectin, ng/ml	r =0.192, p =0.202	r =0.289, p =0.052

Correlation values (r) are shown as Pearson's or Spearman's correlation where appropriate.
**P-value<0.05*

was not different between the groups (36±5 vs. 38±3 mL/min/m^2; p=0.721). The difference was more pronounced in patients with eGFR <30 mL/min/1.73m^2 (figure 03).

ENDOTHELIAL DYSFUNCTION

Aix, thrombomodulin and VCAM-1 were higher in CKD compared to controls and increased with declining eGFR (table 02). A significant association was observed between cultured EPC and Aix (r=-0.37; p=0.013). The migratory capacity of HMECs was also lower in conditioned EPC medium from patients with increased VCAM-1 (r=-0.343; p=0.023) and E-Selectin levels (r=-0.262; p=0.075). Higher SPC outgrowth was associated with higher ICAM-1 levels in CKD (r=0.463; p=0.006).

MEDICATION USE

Statin and renin-angiotensin system (RAS) blocker use were associated with lower circulating EPC levels (8(0-47) vs. 15(2-52) for statins and 7(0-40) vs. 20(4-52) $CD34^+KDR^+$-cells/10^5 granulocytes for RAS-blockade; p=0.048 and p=0.009 respectively). No associations with progenitor cell levels were seen for treatment with erythropoietin, diuretics, beta-blockade or calcium receptor blockers.

VEGF AND SDF-1α IN CKD PATIENTS

VEGF and SDF-1α levels were increased in CKD compared to controls (125±29 vs. 28±24 pg/mL; p=0.003 and 3.2±0.7 vs. 2.6±0.6 ng/mL; p<0.001 respectively). VEGF and SDF-1α levels were related with degree of kidney dysfunction (r=-0.48; p<0.001 and r=-0.72; p<0.001 respectively) and presence of endothelial dysfunction (table 03). EPC outgrowth and migratory capacity were most reduced in subjects with highest VEGF levels (r=-0.288, p=0.028 and r=-0.418, p=0.007 respectively).

DISCUSSION

Our study shows that predialysis CKD patients on regular medical therapy have lower levels of circulating EPC and reduced EPC outgrowth compared to healthy controls. This reduction in EPC did not depend on cause or degree of kidney dysfunction. SPC outgrowth gradually increased with declining kidney function in CKD. These data suggest that in a uremic environment EPC mediated endogenous vascular regeneration may be impaired, whereas SPC mediated development of atherosclerosis may be enhanced. Reduction in EPC levels could not be attributed to reduced VEGF or SDF-1α levels.

Few studies reported on EPC and SPC levels in CKD. Previous studies demonstrated reduced EPC levels and function as well as an imbalance between EPC and SPC in hemodialysis patients[13-15]. These data suggested an adverse effect of uremia on vascular progenitor cells. However, dialysis sessions, in itself related to reduced EPC levels, and the overall worse condition and (cardiovascular) comorbidity of dialysis patients may have influenced these results. Several studies suggested that reduction of uremia by kidney transplantation improved EPC numbers and function[17, 20]. Furthermore, EPC levels were related to graft function by some[17, 20], but not others[21]. The effects of immunosuppressive therapy and former exposure to long-term dialysis treatment complicate interpretation of these findings. Surdacki et al[22] studied a very specific population of patients with stable angina and severe angiographic CAD with strict criteria on medication use and comorbidity. They found lower $CD34^+KDR^+$-EPC counts in patients with impaired kidney function within their selected population. In this study, patients in lower eGFR groups also had more severe CAD which may have influenced the results. No EPC or SPC outgrowth numbers were reported in this study. Krenning et al[23] reported lower numbers of $CD34^+$-hematopoietic stem cells in CKD patients more comparable to our population, but did not assess $CD34^+KDR^+$-EPC levels, the type of EPC that was predictive for cardiovascular events in CAD patients[4]. They observed decreased endothelial outgrowth of MNC obtained from CKD patients on PCLdiUPy biomaterial and suggested a limitation for use of EPC derived from CKD patients in regenerative medicine. Although we did not observe significantly lower $CD34^+$-cells, we found reduced numbers of circulating $CD34^+KDR^+$-EPC. This reduction was even present in mild CKD. Furthermore, our data indicate that already in mild kidney dysfunction a ~25% decrease in EPC outgrowth on fibronectin-coated plates occurs. These findings are important as low $CD34^+KDR^+$-EPC numbers and reduced EPC outgrowth using comparable culture methods were previously associated with increased cardiovascular risks[4]. Our observation that a small decline in eGFR compared to our controls was associated with low EPC levels may indicate that minor loss of kidney function relates to reduced EPC levels. Exact indication of the tipping point at which progenitor cell changes occur would require larger sample size. Of note, we may have underestimated eGFR in our controls as the MDRD-formula is less accurate in healthy populations. We observed preserved SPC levels together with diminished EPC in CKD. Together, our findings indicate that uremia not only has a negative influence on progenitor cells in end-stage kidney disease[15], but also in earlier stages of kidney dysfunction. This may contribute to the accelerated atherosclerosis in CKD patients. Additionally, these alterations in vascular progenitor cell levels may advance progression of CKD as it has been reported that EPC contribute to glomerular endothelial repair[24] whereas SPC may enhance glomerulosclerosis[25].

We did not find a correlation between EPC levels and the degree of kidney dysfunction within our CKD group. We investigated progenitor cells in patients on regular medication, which provides valuable information, since CKD patients are at increased risk for CVD despite current medical treatment regimens. However, medication use could have influenced the relation between EPC levels and the degree of kidney dysfunction. Erythropoietin is a well-known EPC mobilizing agent[13]. Excluding erythropoietin-treated patients from our analysis did not influence our results, however, we cannot exclude that selection bias of other medication could have influenced our results. Indeed, we demonstrated lower $CD34^+KDR^+$-EPC levels in patients on statin or RAS blockade, which have previously been reported to increase EPC numbers[26, 27]. Possibly, EPC levels were even lower in absence of medical treatment. In addition, we cannot exclude that our patients with severe CKD were in a relatively good condition.

We did not find a relation between the underlying cause of CKD and levels of progenitor cells. Previous studies in dialysis patients[14, 15] also did not detect differences in EPC level when comparing patients with diabetic and non-diabetic causes. Since atherosclerosis may underlie, but also may result from CKD, absolute separation of atherosclerotic and non-atherosclerotic CKD remains difficult. Of note, most of the non-atherosclerotic CKD patients suffered from cystic kidney disease, recurrent nephrolithiasis or lithium-induced CKD. These data suggest that the effect of other factors than CKD degree or cause are dominant in the determination of EPC recruitment, mobilization and function.

Several studies have shown that the presence of cardiovascular risk factors or CVD is an important determinant of reduced EPC levels[4, 6]. However, others did not observe such inverse relations or even reported a positive relation between EPC number and vascular risk factors[5, 7], which could reflect a protective compensatory response to the vascular risk burden. We found that CKD patients with a history of CVD had reduced $CD34^{+}KDR^{+}$-EPC numbers as compared to patients without such history. This is in line with previously reported associations between EPC levels and history of CVD in patients on peritoneal dialysis[21]. Furthermore, EPC outgrowth and function were negatively associated with endothelial dysfunction parameters in our subjects and increased levels of outgrowth SPC were found in patients with higher endothelial dysfunction markers. The combination of endothelial dysfunction with lack of a compensatory response but even reduced EPC levels, reflecting impaired endothelial repair, and enhanced numbers of SPC may accelerate atherosclerosis in CKD. Our population consisted of patients under current treatment regimen with minimal exclusion criteria, thus representing the CKD population, but heterogeneous in its composition, comorbidity, medication and other influencing factors. An important limitation of our study is that influences of reduced eGFR, (cardiovascular) comorbidity and medication on EPC and SPC cannot be fully separated from each other. However, cardiovascular risk factors can be cause and result of renal insufficiency, which complicates such discerning analyses. In addition, cardiovascular risk indicators may manifest differently in CKD patients and may not correlate with CVD events as in subjects without CKD[28].

The cross-sectional nature of our study does not allow definitive conclusions on the mechanism underlying diminished EPC levels in CKD. We investigated whether a defect of EPC mobilizing factors in response to endothelial injury could explain the reduced EPC levels in CKD. We found that with progression of CKD and endothelial dysfunction, important stimuli SDF-1α and VEGF gradually increased while EPC levels remained low. Moreover, EPC outgrowth was most reduced in subjects with highest VEGF levels. Low circulating EPC pools could result from increased homing of EPC to injured tissue mediated by SDF-1α and VEGF[7]. SDF-1α and VEGF may be accumulated due to reduced renal clearance, which may result in continuous stimuli and eventually resistance of EPC. Alternatively, there could be a common underlying mechanism for endothelial dysfunction and impaired EPC mobilization. Impaired nitric oxide (NO) availability[29] in CKD may underlie endothelial dysfunction and impaired EPC mobilization despite upregulation of SDF-1α and VEGF, as both processes are NO dependent[30]. Increased SDF-1α levels together with endothelial NO-synthase deficiency can also result in enhanced SPC levels[31], thereby contributing to neointimal lesion formation.

We used eGFR calculated by the MDRD equation to correlate the degree of uremia to EPC levels. The eGFR represents the collection of a whole variety of accumulated uremic toxins. Whereas a decrease in eGFR is associated with an increased risk for cardiovascular events[32], it is not known which uremic toxin importantly influences EPC availability and function. Plasma urea concentration, microalbuminuria and total albumin-to-creatinine ratio as other markers for kidney function were also not associated with EPC levels in our study. More insight in toxic substances influencing EPC in CKD may provide a more specific uremic marker set to correlate with EPC levels to monitor and predict CVD risk.

In conclusion, CKD patients on regular medication have lower circulating EPC levels and reduced EPC outgrowth already in mild CKD, whereas outgrowth towards SPC is increased with decline in kidney function. Moreover, lower EPC numbers were found in patients with a history of CVD and endothelial dysfunction. EPC reduction could not be attributed to impaired SDF-1α and VEGF levels.

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CH.09

LONG-TERM ERYTHROPOIETIN TREATMENT PROTECTS AGAINST FURTHER REDUCTION OF CIRCULATING EPC LEVELS IN CARDIORENAL SYNDROME PATIENTS

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ABSTRACT

Aims: Patients with the cardiorenal syndrome (CRS) have high cardiovascular morbidity and mortality. Endothelial progenitor cells (EPC) constitute an endogenous vascular repair system, protecting against the development of atherosclerosis. Erythropoietin (EPO) treatment may have beneficial effects by mobilizing EPC from the bone marrow. We hypothesized that CRS patients have decreased EPC levels that can be augmented by EPO therapy.

Methods and Results: This study is part of the EPOCARES trial (clinicaltrials.gov) which investigates EPO effects (50IU/kg/wk) in patients with CRS and anemia. Circulating $CD34^+KDR^+$-EPC, cultured EPC outgrowth and function were measured at baseline, after 18 days and after 52 weeks. Patients showed lower $CD34^+KDR^+$-cell numbers compared to healthy controls (6(12) vs. 19(19) cells/10^5 granulocytes; p=0.010), despite increased levels of stromal cell-derived factor-1α (3.1(0.8) vs. 2.6(0.3) ng/mL; p=0.001). EPC outgrowth and function were not different between patients and controls. EPC levels did not change after 18 days with or without EPO treatment. However, $CD34^+KDR^+$-cells significantly declined after 52 weeks in the non-treated group (p=0.028), while levels were maintained in EPO-treated patients (p=0.248).

Conclusion: CRS patients showed reduced $CD34^+KDR^+$-EPC levels compared to healthy controls, consistent with a reduced vascular regenerative potential and despite upregulated SDF-1α levels. Levels remained unchanged in EPO-treated and non-treated patients after 18 days. However, $CD34^+KDR^+$-EPC levels showed a further reduction over a 52-weeks period in non-treated patients, whereas long-term EPO treatment prevented this decline. Long-term EPO therapy may therefore protect against aggravation of atherosclerosis in the cardiorenal patient.

INTRODUCTION

Patients with the cardiorenal syndrome (CRS), characterized by presence of both chronic heart failure (CHF) and chronic kidney disease (CKD) and often accompanied by anemia[1], have a remarkably high cardiovascular morbidity and mortality[2]. Atherosclerosis is the main cause of cardiovascular disease (CVD). Endothelial dysfunction and impaired endothelial regenerative capacity play a key role in the pathogenesis of atherosclerosis. Bone marrow (BM)-derived circulating endothelial progenitor cells (EPC) constitute an endogenous vascular repair system that may protect against atherosclerosis development. EPC can be mobilized from the BM to sites of endothelial damage upon release of growth factors and cytokines and may incorporate into the endothelial layer or excrete paracrine factors that stimulate proliferation of resident endothelial cells[3]. Reduced EPC availability or function may contribute to the pathogenesis of CVD.

Low EPC levels predicted the risk of cardiovascular events in patients with coronary artery disease[4]. Furthermore, disease conditions with high cardiovascular risk, including end-stage renal disease, have been associated with reduced EPC levels and function[5, 6]. Others did not observe such inverse relations or even reported a positive relation between EPC number and vascular risk factors[7, 8]. In CHF, a negative correlation between cultured EPC and functional New York Heart Association (NYHA) class has been reported[9]. However, others found higher levels of EPC in mild CHF, together with increased levels of EPC mobilizing factors, whereas EPC levels in severe disease were decreased, despite similar increases in SDF-1α and VEGF[10, 11]. The latter observation suggests a protective compensatory response to the vascular risk burden in mild CHF, but exhaustion or suppression of BM progenitor cells in advanced CHF. Increased inflammation in severe CHF, known to exert a suppressive effect on hematopoiesis[12], may contribute to the impaired BM EPC response at this advanced stage[11]. We hypothesized that in CRS, the combined presence of even mild stages of CHF, CKD and anemia is associated with impaired levels of circulating EPC, due to accumulation of uremic toxins, decreased nitric oxide availability and increased inflammation[13]. We compared levels of circulating $CD34^+KDR^+$-EPC, $CD34^+$-hematopoietic stem cells (HSC) and cultured EPC outgrowth number and function in CRS patients and age- and gender-matched healthy controls. VEGF and SDF-1α were measured as EPC mobilizing factors.

Restoring reduced levels of circulating EPC

may enhance vascular protection and reduce the progression of CVD. We hypothesized that EPO treatment can improve EPC levels in CRS patients, which may be related to a reduction in cardiovascular risk. Beneficial effects of EPO on the cardiovascular system have been suggested from small clinical studies in patients with CKD[14] or CHF[15], although this could not be confirmed in larger randomized trials[16, 17]. Short-term, high-dose EPO therapy mobilized EPC from the BM to the peripheral blood in animal studies[18-20]. In patients with myocardial infarction, circulating $CD34^+$-HSC increased after a single high dose EPO injection[21]. Regular dose EPO treatment increased EPC levels in advanced CKD[22] and promoted proliferative and adhesive capacities of EPC in CHF patients on the longer term[23]. No randomized controlled studies are available on EPC and regular EPO dose effects in patients with CRS. We evaluated whether short- (18 days) and long-term (52wk) EPO therapy improved EPC number and function in CRS patients.

METHODS

STUDY SUBJECTS

We studied a consecutively included subgroup of CRS patients from the EPOCARES trial (ClinicalTrials.gov, NCT00356733), and age- and gender-matched healthy controls. Patients were recruited from the outpatient clinics of Nephrology and Cardiology Departments at the University Medical Centre Utrecht and the Meander Medical Centre Amersfoort. The protocol was approved by the institutional medical ethics committee and all patients gave their informed consent. All procedures were in accordance with the Helsinki Declaration.

Patients with mild anemia (6.4-7.4 mmol/L in women, 6.4-7.8 mmol/L in men), moderate CKD and CHF were included. CKD was defined as estimated creatinine clearance 20-70 ml/min (Cockcroft-Gault formula). CHF was defined as functional NYHA class II-IV, based on symptoms, signs and objective abnormality on echocardiography[24, 25]. Patients with reduced ejection fraction (<50%) or left ventricular end-diastolic volume index <97 mL/m^2 with evidence of diastolic left ventricular dysfunction entered the study[26]. Exclusion criteria were presence of an unstable clinical state, known intolerance to EPO, kidney or heart transplantation, hyperparathyroidism, chronic inflammatory disease, malignancy, and anemia with other known cause than renal failure or chronic disease.

Patients were subjected to a run-in period on standard treatment comprising of oral iron supplementation, aspirin when indicated, and maximal tolerated dosages of a β-blocker, ACE-inhibitor or angiotensin receptor blocker. Patients were included if they had been clinically stable on this treatment for at least four weeks.

STUDY DESIGN

EPC levels and function were compared between 45 CRS patients at baseline and 20 healthy controls. The effects of EPO treatment on EPC levels and function were evaluated in an open-label, randomized design. Patients were randomly assigned to receive EPO treatment (50IU/kg/wk; Neorecormon, Roche Pharmaceuticals, Woerden, The Netherlands) for one year or standard treatment without EPO. Short-term effects of treatment with EPO (n=30) vs. no EPO (n=15) were evaluated after 18 days, when EPO treatment was not yet expected to result in a hematopoietic response. Blood was collected 3 days after the 3rd EPO injection. Long-term effects of EPO treatment were assessed after 52 weeks in a subgroup of the EPO-treated patients, who were allowed to increase in hemoglobin (Hb) levels up to a target level of 8.5 mmol/L for men and 8.3 mmol/L for women (n=10), and a non-EPO treated group (n=9).

PLASMA MEASUREMENTS

Biochemical parameters (creatinine, urea, Hb, lipids) were measured using standard laboratory procedures. Serum EPO levels at baseline were measured by sandwich chemiluminescent immunoassay (IMMULITE 2000 platform, Siemens Healthcare Diagnostics, Breda, Netherlands). Markers reflecting endothelial dysfunction or inflammation were measured using enzyme immunoassay kits (ELISA) (serum levels of E-Selectin and plasma levels of VCAM-1, interleukin (IL)-6 (R&D Systems, MN, USA) and thrombomodulin (Diaclone, CT, USA)) or using multiplex immunoassay[27] (ICAM-1 and monocyte chemotactic protein-1 (MCP-1) (Bio-Rad Laboratories, CA, USA)). High sensitivity C-reactive protein (hsCRP) was determined by particle-enhanced immunonephelometry (standard Cardio-Phase hsCRP for BNII, Dade Behring Holding GmbH, Liederbach, Germany). Plasma levels of progenitor cell mobilizing factors VEGF and SDF-1α were measured by ELISA (R&D Systems). NT-proBNP was measured using electrochemiluminescense immunoassay (Cobas CA6000, Roche, Mannheim, Germany). Samples were measured in duplo and averaged for analysis.

CIRCULATING EPC LEVELS

EDTA blood was collected in the morning from fasting subjects. To determine circulating EPC levels, 100 µl blood was incubated with anti-CD34-FITC (BD Pharmingen, CA, USA), anti-KDR-PE (R&D Systems) and anti-CD45-PE-Cy7 (BD Pharmingen) antibodies for 45 minutes. Erythrocytes were lysed in an ammonium chloride buffer and remaining cells were washed and analyzed by flow cytometry (Beckman Coulter, CA, USA). Circulating HSC and EPC were identified as $CD34^+$ and $CD34^+KDR^+$-cells in the lymphocyte region of the forward/sideward scatter plot. Cell numbers were quantified relative to 10^5 granulocytes, identified as $CD45^+$-cells with a typical granulocyte distribution. Measurements were performed in duplo and results were averaged. Isotype-stained samples served as negative controls.

OUTGROWTH OF EPC IN CULTURE

EPC outgrowth from mononuclear cells (MNC) was assessed as described before[6]. In brief, MNC were isolated from EDTA blood samples using Ficoll density gradient separation (Histopaque 1077, Sigma, MO, USA). To evaluate EPC outgrowth in culture, 10^7 MNC/well were seeded on a human fibronectin (Sigma) coated 6-well plate in EGM-2 (Cambrex, MD, USA), supplemented with accompanying aliquots, 20% fetal calf serum (Invitrogen, CA, California), 100 ng/ml recombinant VEGF-165 (R&D Systems) and antibiotics. Medium was changed after 4 days to wash non-adherent cells away. After 7 days, cultured EPC in selected wells were placed on serum free medium (EBM-2 with hEGF, hydrocortisone, GA-1000, R^3-IGF-1, ascorbic acid, heparin and antibiotics) overnight. Conditioned medium was stored for functional experiments and cultured EPC were detached by trypsin and cell scraping, and automatically counted using a hemocytometer.

IN VITRO SCRATCH WOUND ASSAY

The potential of EPC outgrowth to excrete paracrine factors that stimulate endothelial cell migration was assessed by in vitro scratch wound assay[28]. Human microvascular endothelial cells (HMECs; Centers for Disease Control and Prevention, GA, USA) were grown to confluence on fibronectin coated 48-well plates in MCDB-131 supplemented with 10% fetal calf serum, 5% L-glutamine, 0.1% hEGF, 0.1% hydrocortisone and antibiotics. A mechanical scratch was created in a straight line with a p200 pipet tip. The cell monolayer was carefully washed with phosphate buffered saline. EPC outgrowth conditioned medium was placed on the cells. Serum free EPC medium served as negative control. Reference lines were made with a fine tip marker on the bottom of the wells to obtain exactly the same field during the image acquisition. The scratched area was photographed using a light microscope at start and after 6 hours of incubation at 37°C. The extent of closure after 6 hours was determined relative to the starting width of the scratch using Image-Pro plus software (Media Cybernetics, version 3.0). Each sample was measured in two separate wells and two picture fields per well were examined. Results were averaged for data analysis.

STATISTICAL ANALYSIS

Data analysis was performed using SPSS version 15.0 for Windows. The Kolmogorov-Smirnov statistic test was used to explore whether data were normally distributed. Data are expressed as mean±standard deviation for parametric data and as median (interquartile range) for non-parametric data. Differences between groups were analyzed using Student's t-test or Mann Whitney test. Fisher's exact test was used to analyze whether proportions of categories varied by group. Multiple group comparisons were performed using ANOVA with LSD post-hoc testing for which non-parametric data were log-transformed. At baseline, correlations were studied between progenitor cell levels and kidney function (creatinine clearance, serum urea), cardiac function (NT-proBNP, ejection fraction), Hb level, serum EPO levels, cardiovascular risk parameters (age, blood pressure, cholesterol, body mass index, diabetes mellitus, inflammation markers) and endothelial dysfunction markers (vascular cell adhesion molecule-1(VCAM-1), intercellular adhesion molecule-1 (ICAM-1), thrombomodulin and eSelectin). Correlations were measured by Pearson's or Spearman's correlation coefficient where appropriate. Multivariate determinants of log-transformed progenitor cell counts were identified by stepwise multiple linear regression analysis. P-value <0.05 was considered statistically significant.

RESULTS

BASELINE CHARACTERISTICS

Baseline characteristics of CRS patients and controls are summarized in table 01. Patients were mildly anemic, had reduced creatinine clearance, lower diastolic blood pressure, and reduced ejection fraction. CRS patients demonstrated increased hsCRP levels and higher levels of markers for endothelial dysfunction VCAM-1, ICAM-1, thrombomodulin and eSelectin compared to healthy controls.

TABLE 01 BASELINE CHARACTERISTICS STUDY POPULATION

	HEALTHY CONTROLS	CRS PATIENTS	P-VALUE
Age, years	72.0(5.9)	74.6(11.9)	0.132
Male sex, n (%)	15(75)	31(69)	0.425
Body mass index, kg/m²	23.3±2.3	26.2±4.4	<0.001
Systolic blood pressure, mmHg	127±20	125±19	0.605
Diastolic blood pressure, mmHg	79±9	66±9	<0.001
Plasma total cholesterol, mmol/L	4.8±0.9	4.1±1.1	0.028
HDL cholesterol, mmol/L	1.4±0.4	1.2±0.3	0.013
LDL cholesterol, mmol/L	3.1±0.7	2.3±0.9	<0.001
Haemoglobin, mmol/L	8.6(0.7)	7.3(0.9)	<0.001
Serum EPO level, IU/L	NA	11.8±6.2	-
Estimated creatinine clearance, ml/min	66(22)	33(22)	<0.001
Serum urea, mmol/L	5.1(2.3)	14.0(10.2)	<0.001
NT-proBNP, pg/ml	NA	1794(2916)	-
Ejection fraction, %	NA	43.9±2.4	-
hsCRP, mg/L	1.04(2.53)	4.90(8.93)	0.003
Diabetes mellitus, n (%)	0	14(31)	-
Smoker, n (%)	6(30)	5(11)	0.068
Medication			
Statin, n (%)	0	31(69)	-
Angiotensin blocker (ACEi/ARB), n (%)	0	41(91)	-
Beta blocker, n (%)	0	29(64)	-
Aldactone, n(%)	0	12(27)	-
Endothelial dysfunction parameters			
ICAM-1, ng/ml	213(56)	297(188)	0.001
VCAM-1, ng/ml	23.3(4.5)	101.2(65.5)	<0.001
Thrombomodulin, ng/ml	<0.01(0.15)	0.96(1.34)	<0.001
eSelectin, ng/ml	3.32±0.81	4.16±1.43	0.051

Values are presented as n (number) (%), mean ± SD or median (interquartile range).
** P-value<0.05 compared to healthy controls; NA, not available.*
CRS, cardiorenal syndrome; HDL/LDL, high/low-density lipoprotein; hsCRP, high sensitive C-reactive protein; NT-proBNP, N-Terminal Pro B-Type Natriuretic Peptide; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin-receptor blocker; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1

HEMOGLOBIN LEVELS DURING THE STUDY

Short-term EPO therapy did not significantly change Hb levels (7.5(1.2) vs. 7.3(1.0) mmol/L; p=0.061). However, there was a significant increase in reticulocyte count (0.066±0.004 vs. 0.045±0.003 $x10^{12}$/L; p<0.001) compared to baseline. EPO treatment resulted in significantly increased Hb levels after 52 weeks compared to baseline (8.4(0.8) vs. 6.9(0.8) mmol/L; p=0.012). Patients not treated with EPO remained anemic at all time-points (7.4(0.4) at baseline, 7.2(0.8) at 18 days, 7.4(0.9) mmol/L at 52 wk; p-value not significant between time-points).

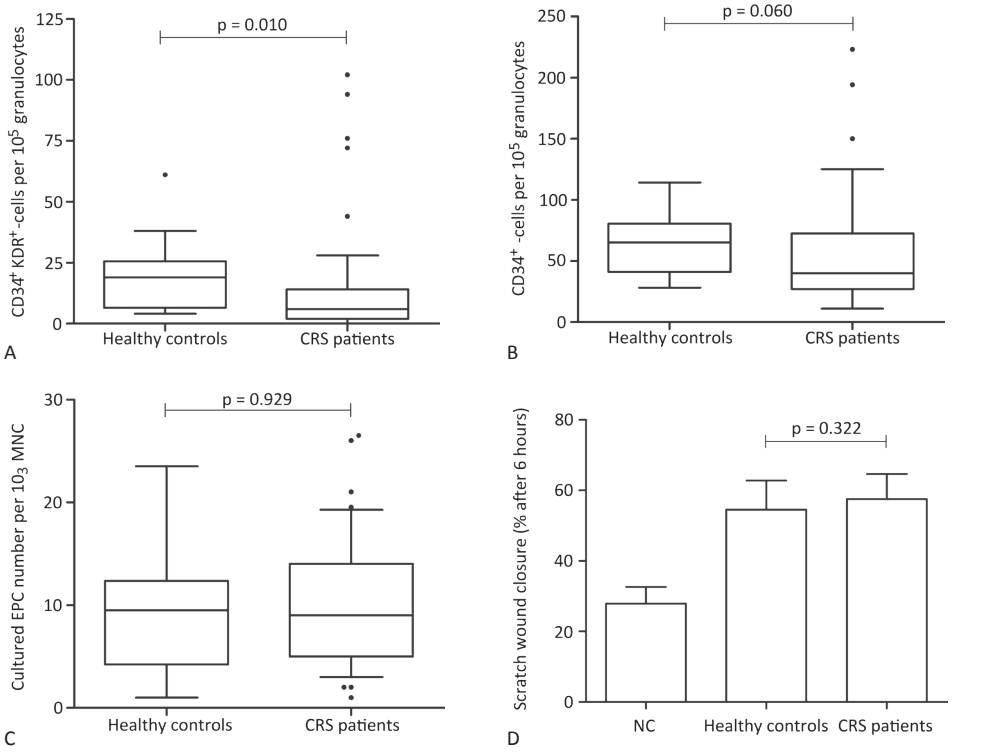
CIRCULATING EPC LEVELS ARE REDUCED IN CRS PATIENTS COMPARED TO HEALTHY CONTROLS

$CD34^+KDR^+$-EPC were 68% lower in CRS patients at baseline compared to healthy controls (figure 01A). CRS patients tended to have lower $CD34^+$-HSC (38% reduction; figure 01B). No differences were found in EPC outgrowth (figure 01C). In a scratch wound assay, we observed no significant difference between conditioned medium from EPC outgrowth cultures from CRS patients and controls for their capacity to stimulate endothelial cell migration (figure 01D).

Univariate analysis revealed lower $CD34^+KDR^+$-EPC levels in subjects with advanced age, lower Hb and estimated creatinine clearance and higher IL-6 levels (table 02).

Circulating EPC levels tended to reduce with declining plasma LDL. $CD34^+$-HSC were also negatively correlated with age and IL-6 levels and tended to decrease in patients with lower estimated creatinine clearance and higher serum urea. $CD34^+$-HSC were increased in patients with

FIGURE 01 PROGENITOR CELL LEVELS AND FUNCTION IN CRS PATIENTS COMPARED TO HEALTHY CONTROLS



Comparison of circulating CD34⁺KDR⁺-cell levels (A), CD34⁺-cell levels (B), cultured endothelial progenitor cell (EPC) number (C) and function as determined by scratch wound analysis (D) between CRS patients (n=45) and healthy controls (n=20). NC, negative control

higher plasma LDL. After multivariate regression analysis of log-transformed CD34⁺KDR⁺-EPC levels for age, Hb, estimated creatinine clearance and IL-6, only Hb remained an independent predictor of circulating EPC levels (β=0.323; 95% CI 0.021-0.538; p=0.034). Only IL-6 (β=-0.443; 95% CI -0.135-0.029; p=0.003) was an independent predictor of log-transformed CD34⁺-HSC in multivariate regression analysis for age, IL-6 and plasma LDL levels.

No correlations were found for CD34⁺- and CD34⁺KDR⁺-cells with serum EPO levels. Presence of diabetes, statin use or β-blockade treatment were not associated with circulating progenitor cell counts. CD34⁺-cell levels tended to correlate negatively with all levels of measured endothelial dysfunction parameters at baseline. Reduced CD34⁺KDR⁺-cell levels were also associated with increased VCAM-1 levels, but not with other endothelial dysfunction measures (table 02). EPC outgrowth level and function were not associated with any of the markers.

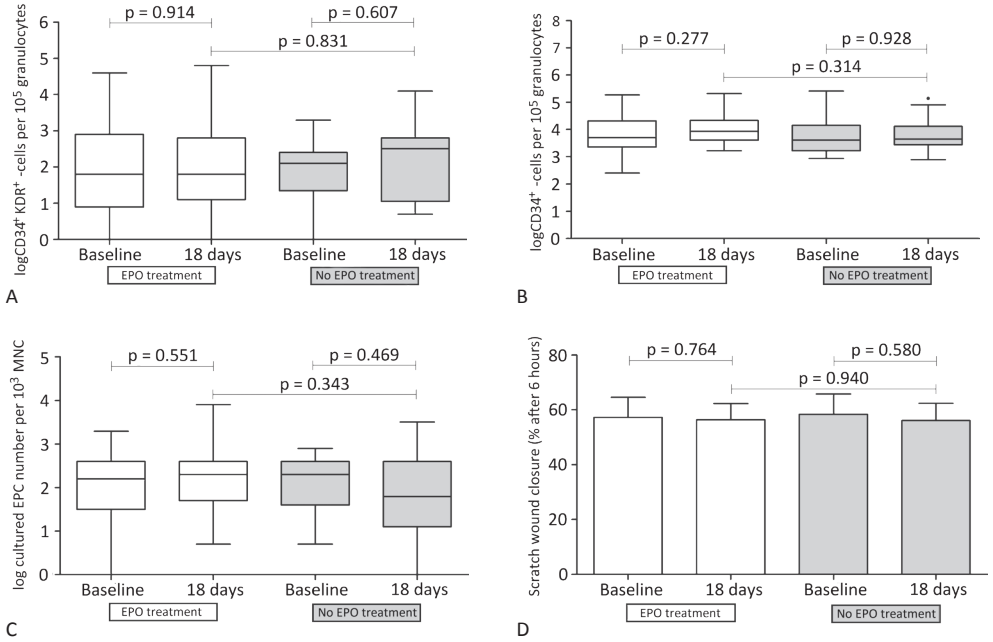
SDF-1α levels were significantly enhanced in CRS patients as compared to controls (3.1(0.8) vs. 2.6(0.3) pg/mL; p=0.001). SDF-1α levels correlated with estimated creatinine clearance (r= -0.361; p=0.004), serum urea (r= 0.527; p<0.001), Hb (r= -0.276; p=0.03) and NT-proBNP (r= 0.503; p=0.001), but not with ejection fraction (r= -0.190; p=0.307). SDF-1α levels increased with deterioration of endothelial dysfunction measured by ICAM-1 (r=0.437; p=0.002), VCAM-1 (r=0.553; p<0.001) and thrombomodulin (r=0.494; p<0.001), but not with eSelectin (r=0.203; p=0.162). ICAM-1 (β=0.313; 95% CI 0.000-0.002; p=0.041) and VCAM-1 (β=0.441; 95% CI 1.888-9.813; p=0.005) remained independent predictors of SDF-1α in multivariate regression analysis for significant univariate determinants.

TABLE 02 UNIVARIATE CORRELATIONS OF CIRCULATING HSC AND EPC WITH CLINICAL PARAMETERS

	$CD34^+$	$CD34^+KDR^+$
Clinical parameters		
Age	r=-0.273; p=0.038*	r=-0.352; p=0.007*
Body mass index	r=-0.067; p=0.618	r=-0.013; p=0.925
Systolic blood pressure	r=-0.026; p=0.847	r=-0.068; p=0.613
Diastolic blood pressure	r=0.078; p=0.559	r=0.194; p=0.145
Total plasma cholesterol	r=0.241; p=0.069	r=0.239; p=0.071
HDL cholesterol	r=0.197; p=0.138	r=-0.058; p=0.651
LDL cholesterol	r=0.285; p=0.035*	r=0.264; p=0.051
Hemoglobin	r=0.211; p=0.112	r=0.362; p=0.005*
Serum EPO level	r=0.207; p=0.200	r=-0.017; p=0.919
Estimated creatinine clearance	r=0.241; p=0.068	r=0.328; p=0.012
Serum urea	r=-0.258; p=0.053	r=-0.175; p=0.192
NT-proBNP	r=-0.085; p=0.602	r=-0.154; p=0.343
Ejection Fraction	r=0.058; p=0.766	r=0.035; p=0.856
hsCRP	r=-0.185; p=0.229	r=-0.155; p=0.316
IL-6	r=-0.454; p=0.002*	r=-0.302; p=0.044*
MCP-1	r=-0.212; p=0.162	r=-0.107; p=0.486
Endothelial dysfunction parameters		
ICAM-1	r=-0.368 p=0.013*	r=-0.251 p=0.156
VCAM-1	r=-0.271 p=0.072	r=-0.377 p=0.011*
Thrombomodulin	r=-0.247 p=0.087	r=-0.021 p=0.887
e-Selectin	r=-0.354 p=0.017*	r=-0.210 p=0.167

*Correlation values are shown as Spearman's correlation. * P-value<0.05*
HDL/LDL, high/low-density lipoprotein; hsCRP, high sensitivity C-reactive protein; IL-6, interleukin 6; MCP-1, monocyte chemotactic protein-1; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1

FIGURE 02 SHORT-TERM EFFECTS OF EPO TREATMENT ON CIRCULATING EPC IN CRS PATIENTS



Comparison of circulating $CD34^+KDR^+$-cell levels (A), $CD34^+$-cell levels (B), cultured endothelial progenitor cell (EPC) number (C) and function as determined by scratch wound analysis (D) between CRS patients treated with EPO for 18 days (n=30, open bars) and non-EPO treated patients (n=15, filled bars).

We observed no relation between levels of circulating $CD34^+KDR^+$-cells and SDF-1α levels in the total patient population (r=0.127; p=0.352). However, SDF-1α was correlated with $CD34^+KDR^+$-cells in patients with milder CHF indicated by low levels (below median) of NT-proBNP (r=0.469; p=0.05) or high (above median) ejection fraction (r=0.651; p=0.022). Correlations were absent or even reversed in more severe CHF (r=0.104; p=0.661 in patients with NT-proBNP levels above median and r=-0.461; p=0.083 in patients with ejection fraction below median). SDF-1α and $CD34^+KDR^+$-cell correlations were absent in patients above as well as below median estimated creatinine clearance. Levels of VEGF were not different between CRS patients and controls (24.8(16.4) vs. 22.5(7.1) pg/mL; p=0.212).

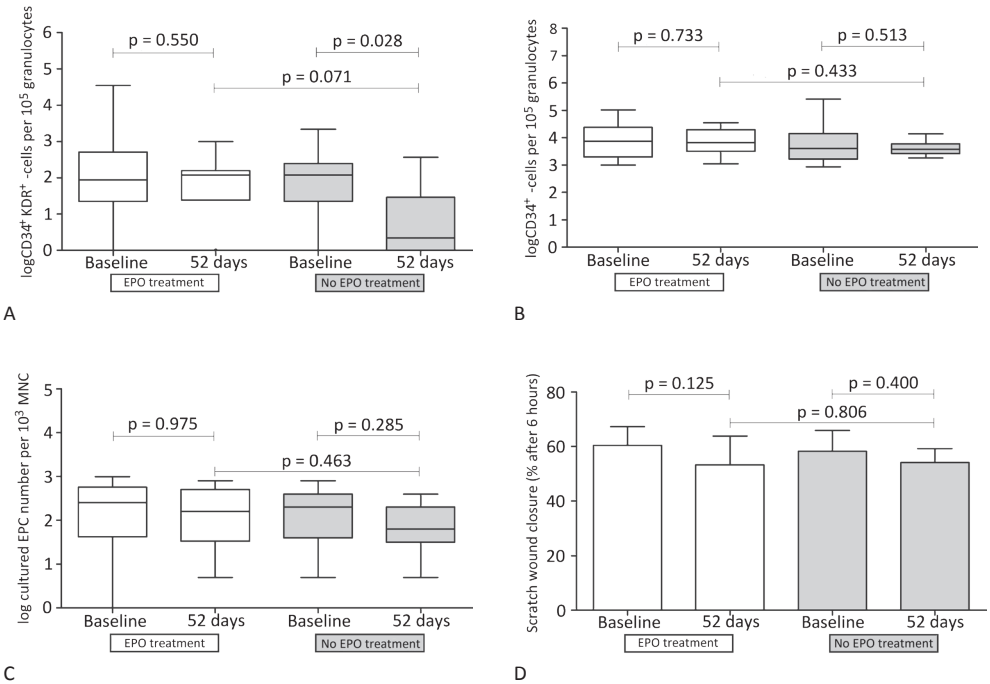
SHORT-TERM EPO TREATMENT HAS NO EFFECT ON EPC IN CRS PATIENTS

Short-term (18 days) EPO treatment had no effect on levels of $CD34^+$- or $CD34^+KDR^+$-cells, EPC outgrowth and migratory function as compared to baseline (figure 02). Progenitor cell levels and function also did not significantly change in the non EPO-treated group as compared to baseline. No significant differences in $CD34^+$-, $CD34^+KDR^+$-cells and EPC outgrowth and function were observed between the EPO-treated and non EPO-treated group.

LONG-TERM EPO TREATMENT PREVENTS A DECLINE IN EPC IN CRS PATIENTS

Long-term (52 weeks) EPO treatment did not alter $CD34^+KDR^+$-EPC, $CD34^+$-HSC levels, EPC outgrowth or EPC function as compared to baseline. However, in patients not treated with EPO a significant decline in $CD34^+KDR^+$-EPC was found after 52 weeks (figure 03A). As a result, $CD34^+KDR^+$-EPC tended to be lower in patients without EPO therapy after 52 weeks compared to those who received EPO therapy, whereas levels were not different between the groups at baseline (p=0.638). No differences in $CD34^+$-HSC, EPC outgrowth and function were observed in the group without EPO treatment after 52 weeks as compared to baseline (figure 03B-D).

FIGURE 03 LONG-TERM EFFECTS OF EPO TREATMENT ON CIRCULATING EPC IN CRS PATIENTS



Comparison of circulating $CD34^+KDR^+$-cell levels (A), $CD34^+$-cell levels (B), cultured endothelial progenitor cell (EPC) number (C) and function as determined by scratch wound analysis (D) between CRS patients treated with EPO for 52 weeks (n=10, open bars) and non-EPO treated patients (n=9, filled bars).

DISCUSSION

The present data show that patients with CRS and anemia who receive standard medication have reduced levels of circulating $CD34^+KDR^+$-EPC as compared to healthy controls. Levels were reduced despite increased levels of progenitor cell mobilizing factor SDF-1α. We observed no effect of short-term, 18 days EPO therapy on EPC levels. However, long-term, 52 weeks EPO therapy stabilized $CD34^+KDR^+$-EPC levels, whereas EPC levels decreased over this time period in patients without EPO.

Our study is the first to report reduced circulating progenitor cells in patients with combined CHF, CKD and anemia. Studies on circulating EPC in patients with CHF report variable results[10, 11, 29, 30]. We observed that in the presence of CHF, CKD and anemia $CD34^+KDR^+$-cell levels were decreased independent of cardiac disease severity, as assessed by ejection fraction or NT-proBNP levels. Plasma levels of NT-proBNP may be influenced by renal dysfunction, but remain a powerful reflection of cardiac status[31]. At the tissue level EPC recruitment depends on ischemia- or endothelial injury-induced upregulation of SDF-1α[32]. In our study, levels of SDF-1α correlated with renal function, heart function and markers of endothelial dysfunction, indicating that signals for progenitor cell mobilization are intact. However, this did not result in an efficient increase in circulating EPC levels when CRS patients were compared to controls. This may be explained by impaired EPC production due to uraemia and other cardiovascular risk factors and/or increased homing to injured endothelium. In agreement with previous results[11], in CRS patients with mild CHF, increased SDF-1α levels were associated with higher $CD34^+KDR^+$-EPC levels, suggesting that in milder CHF stages SDF-1α can lead to EPC mobilisation. In contrast, in severe CHF the association between SDF-1α and EPC levels disappeared or even reversed, suggesting an impaired EPC mobilizing response to SDF-1α in this stage. This is consistent with the biphasic response reported in severe CHF[10, 11] and may be related to exhaustion of hematopoietic progenitor cells at the BM level or inflammatory factors exerting BM suppressive effects in advanced CHF stages[11, 33]. Indeed, our data show a significant inverse relation between levels of IL-6 and circulating $CD34^+$-HSC numbers. Inflammatory cytokines are known to exert a suppressive effect on erythropoiesis. Consistently, in our patients with mild anemia we observed lower Hb levels when hsCRP levels were higher. We observed no correlations between serum EPO levels and either Hb levels or endothelial progenitor cell counts. Interestingly, $CD34^+KDR^+$-EPC levels correlated positively with Hb levels, suggesting a common BM suppressive effect on both EPC production or mobilization and erythropoiesis.

In our study short-term EPO administration (3 doses in 18 days) did not influence hematopoietic stem cell or EPC levels. This contrasts with previous clinical reports in CKD or post myocardial infarction patients[21, 22, 34]. In part this discrepancy may be explained by differences in EPO dosage, as EPC response to EPO has been shown to be dose-dependent[18]. Since the potential clinical problems caused by long-term EPO therapy are presumably dose-related[35], we treated our patients with a relatively low EPO dosage as currently recommended (50 IU/kg/wk). This dose was sufficient to enhance reticulocyte levels. It could be that our patients with combined CKD and CHF were not able to increase EPC levels in response to EPO due to exhaustion or suppression of these progenitors at the BM level[11, 33]. In line with this hyporesponsiveness of EPC on EPO, we observed no relations between serum EPO levels and EPC counts, whereas endogenous EPO levels were previously reported to be positively associated with EPC levels in patients with CHF[36] or CKD[37].

We evaluated long-term effects of EPO on EPC in a randomized controlled interventional trial. To our knowledge, no previous studies have reported on one-year follow-up of EPC levels in a high CVD risk population. In patients not treated with EPO, we observed a 68% reduction in circulating $CD34^+KDR^+$-EPC levels after one year follow-up compared to baseline. Interestingly, we found that long-term EPO treatment prevented further reduction in EPC levels over one year. One previous study reported no difference in EPC levels between CHF patients who had been treated with EPO for 2.5 years and a non EPO-treated control group. However, this observation may be confounded by the cross-sectional study design and retrospective matching of the control group[23]. Our longitudinal, randomized study suggests a protective effect of EPO on EPC levels, consistent with maintained vascular protection. Our current data do not allow definitive mechanistic conclusions but may suggest a role for nitric oxide (NO). CRS is associated with impaired NO availability[38], whereas NO and the NO producing enzyme eNOS are essential for EPC mobilisation[39]. EPO has been reported to increase eNOS activity[40] and in animal studies EPO-induced mobilization of EPC was shown to depend upon eNOS activation[20]. Our finding that EPC reduced over time whereas HSC were not affected seems consistent with the observation that NO supported mobilization of EPC but not of hematopoietic stem cells in a mouse model[41]. Alternatively, an effect on apoptosis of progenitor cells may be involved. Increased

apoptosis of progenitor cells in CHF[42] and CKD[43] has been suggested while EPO treatment has been shown to reduce apoptotic cell death via STAT5, PI3/Akt and NFκB, thereby inducing anti-apoptotic factors[44].

In contrast to circulating $CD34^+KDR^+$-EPC, EPC outgrowth and function were not different between CRS patients and healthy controls and were not changed by EPO treatment. It could be that CRS affects mainly the 'true' progenitor cells in the hematopoietic/endothelial lineage as it affects progenitors in the erythropoietic lineage in the BM. $CD34^+KDR^+$-EPC represent a defined subset of true BM-derived progenitor cells with the ability for clonal expansion into endothelial cell colonies from single cells[45]. Cultured EPC are mostly derived from CD34-negative cells, particularly $CD14^+$-monocytes[46] and can adopt an endothelial phenotype after culture, but are not able to clonally expand. Earlier studies reported decreased levels of EPC outgrowth and colony forming units in CHF[9, 11, 33] or CKD[47] and increased number and function upon EPO treatment[23]. Besides study population differences, differences in culture method may contribute to the observed discordances[9, 11, 23, 47]. However, our methodology is the same as in previous studies in which we found both reduced $CD34^+KDR^+$-EPC outgrowth in patients with CKD[48] and ESRD[6], and comparable to methods used in several articles reporting reduced cultured EPC in CHF[33] and CKD[49-51].

In conclusion, CRS patients showed reduced $CD34^+KDR^+$-EPC levels compared to healthy controls, consistent with a reduced vascular regenerative potential and despite increased SDF-1α levels. A further reduction over a 52-week period was prevented by long-term EPO treatment. Long-term EPO therapy may therefore protect against aggravation of atherosclerosis in the cardiorenal patient.

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CHAPTER 10 Summary and perspectives

CH.10

SUMMARY & PERSPECTIVES

The presence of combined chronic heart failure (CHF) and chronic kidney disease (CKD), i.e. the cardiorenal syndrome (CRS), is a rising problem with high cardiovascular morbidity and mortality[1-6]. In CRS, disease progression in both organs is amplified[7-10]. In this thesis, we aimed to gain insight into pathophysiologic mechanisms of CRS by analyzing monocyte transcriptomes as biosensors of the altered systemic environment and by characterizing vascular progenitor cell levels and function, as key players of endothelial repair in CKD and in CRS patients. Beneficial effects of erythropoietin (EPO) treatment on the cardiovascular system have previously been shown in some, but not all studies[11-13]. Mechanisms underlying EPO effects are not fully understood. Effects of short-term EPO treatment on monocyte transcriptomes and effects of short- and long-term EPO treatment on vascular progenitor cell levels and function were evaluated in CRS patients to identify underlying mechanisms of (non-) hematopoietic EPO actions.

THE CARDIORENAL SYNDROME

The pathophysiology of combined heart and renal failure is complex and has been proposed to be driven by the cardiorenal connectors, i.e. disruption of the pro-/anti-inflammatory balance, disruption of the pro-/anti-oxidant balance, enhanced activity of the renin angiotensin system and of the sympathetic nervous system[14]. The presence of anemia, among others due to an absolute or relative EPO deficiency, has been associated with deterioration of organ failure and increased mortality rates[15-18]. EPO therapy to resolve anemia has shown beneficial effects on the cardiovascular system in small studies[12, 19-23], but this could not be confirmed in larger randomized trials[11, 13, 24]. A detailed analysis of the literature on potential mechanisms underlying effects of EPO was presented in ***chapter 2***. Increased nitric oxide[25] and decreased reactive oxygen species production[26] were shown upon EPO-treatment, indicating anti-oxidative capacity. Anti-inflammatory actions of EPO have also been observed in experimental models[27, 28]. However, other studies showed no or adverse effects of EPO treatment on the cardiorenal connectors[29], which is in line with controversial results from clinical studies. The review in this chapter underlined that EPO elicits hematopoietic as well as non-hematopoietic effects that may influence the cardiorenal connectors and concluded that, to elucidate which cellular pathways are induced by EPO in patients, and to distinguish hematopoietic from non-hematopoietic effects of EPO, a clinical study was urgently needed.

We set up an open-label, randomized clinical study 'Erythropoietin in the Cardiorenal System' (EPOCARES) in which we aimed to identify pathophysiologic mechanisms of CRS and to distinguish hematopoietic from non-hematopoietic effects of EPO treatment ***(chapter 3)***. After a 4-week run-in period on standard treatment, patients with CRS and anemia were assigned to receive a fixed dose (50 IU/kg/week) of 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 level to a maximum of 13.7 g/dL in men and 13.4 g/dL in women; or to receive standard care without EPO (control group). Follow-up was 12 months. The unique aspect of this study is that it attempts to separate chronic from acute and hematopoietic from non-hematopoietic actions of EPO. The latter could not be distinguished in former studies investigating effects of EPO treatment, because no patient subgroups were included that were maintained at stable Hb levels. We investigated whether monocytes, as biosensors of the internal environment on the one hand and as mediator cells in atherosclerotic and inflammatory processes on the other hand, revealed imprint of the altered systemic environment in CRS compared to healthy controls. Monocyte transcriptome analyses could thereby provide insight into pathophysiologic mechanisms of CRS and identify new intervention strategies in this difficult-to-treat patient population.

THE CARDIORENAL SYNDROME: MONOCYTE TRANSCRIPTOMES AND EFFECTS OF EPO TREATMENT

In ***chapter 4***, we proposed a pipeline to analyze microarray data with substantial biological variation and low signal intensity. In this pipeline, statistical Student's t-test, to determine the significance of a gene call in a sample, resulted in slightly more genes as significantly expressed compared to the ranking system used by Illumina. To take biological variation into consideration, we proposed to use a size-test to determine the minimal number of samples in a group that needs to have a significant gene call in order for the gene to be called significantly expressed in that group. This data processing pipeline provides a less strict, yet conservative, approach to Illumina BeadArray data. With use of this pipeline, we report in ***chapter 5*** that CRS patients in the EPOCARES study showed modest differences in monocyte gene expression compared to healthy controls. Several reasons, including inter-individual variance, effects of standard medical treatment, adaptation of monocytes to its environment or the choice of cell type may underlie the limited gene expression differences. Even so, the observed gene expression changes pointed at

modulated inflammation and oxidative stress, two of the systems we have proposed as important connectors in combined heart and renal failure. Observed gene expression differences were small, but even modest alterations in gene expression may be clinically relevant as has previously been demonstrated[30].

We investigated the transcriptomic response of monocytes to short-term EPO therapy. In ***chapter 5***, we demonstrated that the response to administration of three EPO injections was very limited, indicating that a dose that is commonly used to combat renal anemia did not exert notable short-term effects on the transcriptome in this cell type. Importantly, we also did not demonstrate potentially harmful effects on gene expression level. Transcriptomes of each subject clustered to their own baseline transcriptome, indicating that biological and technical variance was small and that individual differences dominate direct EPO effects. In addition, inter-individual transcriptome response to EPO was variable, hampering detection of EPO effects at the group level. The combination of a regular EPO dose to treat renal anemia and short exposure time may underlie the lack of short-term monocyte transcriptome changes. Alternatively, monocytes may not be a representative cell type to reflect systemic alterations and EPO effects in this complex patient population. It has been suggested that non-hematopoietic effects of EPO are exerted via a heterodimeric EPO receptor complex, comprising one EPO-receptor unit and a pair of β-common receptor subunits (also known as CD131), in contrast to hematopoietic effects of EPO which are effectuated through activation of homodimeric EPO-receptors. Activation of the EPO-receptor heterocomplex requires higher concentrations of EPO compared to the homodimeric complex, because its binding affinity is much lower than the blood circulating concentration of EPO. Moreover, the limited reaction of monocytes we found possibly reflects absence of intracellular signaling after ligand–receptor interaction, low EPO-receptor density, or non-functional EPO-receptor at the cell surface in monocytes[31]. Gene expression profiling of circulating cells remains promising provided that a representative cell type for the specific disease is chosen. Further research on other circulating cells may provide a more sensitive cell type to gain insights into the underlying mechanisms of EPO. Several leukocyte subpopulations (e.g. lymphocytes and granulocytes) involved in atherosclerotic processes may be of interest, but profiling of circulating vascular progenitor cells also may give insight in pathophysiologic processes[32].

VASCULAR PROGENITOR CELLS IN HEART AND KIDNEY FAILURE

Atherosclerosis is the main cause underlying cardiovascular morbidity and mortality. Endothelial dysfunction is an initial step in the development of atherosclerosis. Maintenance of endothelial integrity is therefore essential in the protection against cardiovascular disease. Circulating BM-derived endothelial progenitor cells (EPC) form an endogenous endothelial repair system by homing to and incorporating into the injured endothelial layer or by paracrine stimulation of resident endothelial cells[33, 34]. Reduced EPC availability or function may contribute to the pathogenesis of cardiovascular disease and has been associated with higher risk of cardiovascular events[35-37].

The physiologic process of aging is associated with endothelial dysfunction, which results in impaired repair of vascular lesions and contributes to the increased cardiovascular risk of the elderly[38-40]. In ***chapter 6*** we investigated circulating EPC levels in advancing age and demonstrated that in a healthy population, children have markedly more circulating EPC compared to adults, suggesting a higher vascular regenerative potential during childhood which is in line with the increased cardiovascular risk during aging. Future research is needed to gain better insight in the mechanisms underlying the higher EPC levels in children, which may provide options to increase EPC levels in adults, thereby potentiating endothelial repair mechanisms.

In ***chapter 7***, we evaluated EPC levels in children with CKD who are at increased risk of CVD. In line with previous studies in adults[41], children on hemodialysis showed reduced levels of $CD34^{+}KDR^{+}$-EPC compared to healthy children, which may contribute to accelerated atherosclerosis development. Predialysis CKD children did not demonstrate altered EPC levels which contrasts with our (chapter 8) and others' observation[42] in predialysis CKD adults. The lower presence of comorbidity and cardiovascular risk factors in children may underlie preserved EPC levels, which is supported by our finding that EPC were particularly reduced in CKD adults with a history of CVD. In addition, children may have a stronger vascular protective system which is in concordance with chapter 6 in which we described higher EPC levels during childhood compared to adult life.

Besides differentiation towards an endothelial cell phenotype, BM-derived vascular progenitor cells may differentiate towards smooth muscle like cells in the vessel wall[43-45]. These smooth muscle

progenitor cells (SPC) may play a detrimental role in initiating and/or contributing to atherosclerosis development[46, 47]. Previous studies in patients with end-stage renal disease showed an imbalance between EPC and SPC levels[41]. In ***chapter 8***, we investigated whether an imbalance of EPC and SPC in various degrees of CKD is present, which may contribute to their increased cardiovascular risk. We found that already in mild CKD, patients on regular medication showed lower circulating EPC levels and reduced EPC outgrowth, whereas outgrowth towards SPC was increased with decline in kidney function. These data suggest that in a uremic environment EPC mediated endogenous vascular regeneration may be impaired, whereas SPC mediated development of atherosclerosis may be enhanced. Previous studies showed reduced EPC levels in the presence of cardiovascular risk factors or CVD[35-37, 48-50], whereas others could not confirm this inverse relation or even reported a positive association between EPC levels and vascular risk factors[50-52]. We found lower EPC levels in CKD patients with a history of CVD and increased endothelial dysfunction markers. The combination of endothelial dysfunction with lack of a compensatory response, but even reduced EPC levels, reflecting impaired endothelial repair and enhanced numbers of SPC may accelerate atherosclerosis in CKD.

In patients with combined cardiac and renal failure, EPC levels were also reduced compared to healthy controls ***(chapter 9)***, which is in concordance with the high cardiovascular morbidity and mortality in CRS patient, despite current medical treatment regimens. Previous research demonstrated increased EPC levels in mild CHF and decreased levels in severe CHF. This suggested a protective compensatory response to the vascular risk burden in mild CHF, but exhaustion or suppression of BM progenitor cells in advanced CHF[53, 54]. In agreement with these studies, our CRS patients with mild CHF showed higher $CD34^+KDR^+$-EPC levels with increased SDF-1α levels, suggesting that in milder CHF stages SDF-1α can lead to EPC mobilisation. In contrast, in CRS patients with severe CHF the association between SDF-1α and EPC levels disappeared, suggesting an impaired EPC mobilizing response to SDF-1α in this stage.

The mechanism underlying decreased EPC levels in kidney and heart failure cannot be deducted from our data. $CD34^+KDR^+$- EPC levels were reduced despite augmented levels of progenitor cell mobilizing factor SDF-1α, which increased with increasing markers of endothelial injury in our CKD and CRS populations. A combination of factors, such as inflammation and uremic toxins, may have lead to disturbed EPC levels by reducing BM responsiveness, EPC mobilization, differentiation or survival.

VASCULAR PROGENITOR CELLS IN HEART AND KIDNEY FAILURE: EFFECTS OF EPO TREATMENT

In previous animal and human studies, EPO has shown the ability to mobilize EPC, to improve EPC function and to exert anti-apoptotic effects[55-62]. We investigated effects of short- and long-term EPO treatment, at a regular dose to treat renal anemia, on EPC level and function in CRS patients ***(chapter 9)***. In line with limited short-term EPO effects on monocyte transcriptomes, no changes in EPC levels or function were observed after 3 EPO injections. After 52 weeks however, EPO stabilized $CD34^+KDR^+$-EPC levels, while levels decreased in patients without EPO therapy. These data suggest that reduction of circulating EPC levels in CRS patients, which may contribute to the increased CVD risk, can be decelerated by long-term EPO treatment.

The effect of EPO therapy on EPC levels in our CRS population was modest compared to previously described effects in literature[55, 58-60, 63]. One of the reasons underlying this limited change in EPC levels might be the relatively low EPO dose we used. In addition, it might be that CRS patients are not able to mobilize EPC despite presence of exogenous EPC-recruiting EPO due to BM exhaustion or suppression in response to continuous BM stimulation, or due to lack of NO-availability which is required for EPC mobilization.

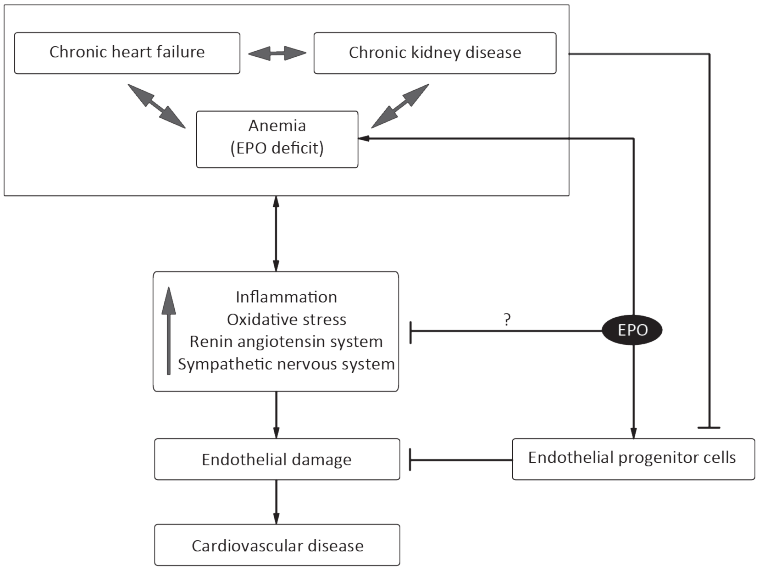
OVERALL CONSIDERATIONS

We found modest effects of short-term EPO treatment on monocyte transcriptomes and progenitor cell levels in our CRS patient population. This contrasts with previous research that showed increased progenitor cell mobilization in patients with myocardial infarction[63] and in several animal studies after brief EPO therapy[55-57, 64]. Decreased inflammatory (monocyte) activity, e.g. diminished IL-6 and TNF-α production and less macrophage influx, was also demonstrated after short-term EPO treatment in experimental studies[27, 65-68]. Most of these studies were however performed in acute disease models[27, 56, 57, 64, 65, 68] and all used substantially higher EPO dose (range 300-5000 IU/kg/wk) compared to the dose we used (50 IU/kg/wk). Supported by previously reported EPO dose-dependent EPC mobilization[55], it might be that a higher EPO dose is required to increase EPC levels and to substantially modulate monocyte transcriptomes during this short exposure time. Possibly, EPO dose we applied was insufficient to achieve such effects. The regular EPO dose to treat

renal anemia we used in our study was chosen for several reasons.

We aimed to discern hematopoietic from non-hematopoietic EPO effects in a mechanistic feasibility study. Of note, our study was not aiming at Hb target effects. A fixed EPO dose was required in both EPO-treated groups to compare Hb-dependent with Hb-independent effects. Therefore, a regular EPO dose was chosen that would be sufficient to increase Hb levels, but that did not require too many phlebotomies to maintain baseline Hb levels in a subgroup of patients. Moreover, we treated our patients for up to one year. Chronic EPO treatment regimen has been in the heat of debate because 2 large randomized trials investigating anemia correction by EPO treatment to achieve high compared to low Hb targets in CKD and end-stage renal disease patients showed detrimental effects[13, 24] and one other randomized trial (CREATE) showed no beneficial effects of higher Hb targets in CKD patients[11]. These findings are strengthened by the recently published largest randomized, double-blind, placebo-controlled study (TREAT) thus far, in which EPO treatment had no effect on death or cardiovascular risk and was associated with an increased risk of stroke[69]. Guidelines have been modified to restrict EPO treatment to a target Hb of 11-12 g/dL, with a maximum of 13.0 g/dL. Remarkably, in the two studies that showed harmful EPO effects, EPO dose was much higher (>23000 IU/week in Besarab's study[24] and 11215 IU/week in the CHOIR study[13]) compared to the CREATE study (5000 IU/week) and to our study (3000 IU/week). Secondary analysis of the CHOIR study revealed that high EPO dose requirement with inability to achieve Hb targets was associated with increased risk of adverse events[70]. In addition, use of high EPO dose in hemodialysis patients has been associated with higher death risk in a large observational study[71]. Our study targets up to 13.5 g/dL, but differs from these large-scale studies in that EPO is administered in a fixed, regular dose to treat renal anemia, avoiding dose augmentations if targets are not achieved. It remains to be investigated whether longer exposure to safe, low dose EPO-treatment has an effect on the cardiorenal connectors (figure).

FIGURE POSSIBLE INTERVENTION TARGETS OF EPO IN THE CARDIORENAL SYNDROME



Increased presence of a.o. inflammation, oxidative stress and decreased levels of EPC may contribute to the increased cardiovascular risk in CRS patients. EPO may intervene by reducing anemia, or by non-hematopoietic effects such as inhibition of inflammation and stimulation of EPC mobilization.

Moreover, in this thesis we found decreased levels of circulating EPC during aging, in children with end-stage CKD, in adults with predialysis CKD and a history of CVD and in combined cardiac and renal failure. Our data suggest that renal dysfunction in particular in presence of other cardiovascular risk factors (e.g. inflammation) is associated with decreased EPC levels and may contribute to accelerated atherosclerosis. Interestingly, 52 weeks of EPO treatment had beneficial effects on EPC levels in CRS patients of our EPOCARES study, indicating that a low, safe dose may have an effect after longer exposure (figure). Whether stabilization of EPC levels by this EPO dose results in improved clinical outcomes compared to non EPO-treated patients with reduced EPC levels remains to be investigated.

In conclusion, beneficial but also potentially harmful effects of EPO treatment have been reported in literature. This thesis gave more insight into the pathophysiology of CRS and effects of EPO therapy by characterizing monocyte transcriptomes in a clinical setting. Limited effects of low EPO dose to treat renal anemia were observed. Of note, at this regular EPO dose no harmful effects could be detected. In addition, this thesis gave insight into vascular progenitor cell availability in high cardiovascular risk conditions including aging, CKD and the CRS. Our findings open a new field of possible interventions to reduce cardiovascular risk in these populations. The mechanisms underlying reduced EPC availability in these conditions remain to be clarified in order to design effective treatment strategies. Regular dose EPO treatment demonstrated promising favorable long-term results on EPC levels in CRS patients.

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APPENDICES CH.02

ERYTHROPOIETIN AND THE CARDIORENAL SYNDROME: CELLULAR MECHANISMS ON THE CARDIORENAL CONNECTORS

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APPENDIX TABLE 02A EFFECTS OF EPO SHIFTING NO/ROS BALANCE TOWARDS NO

TISSUE/OBJECT	EPO STIMULATION: DOSE + DURATION	EFFECT OF EPO	REFERENCE
Rat neuronal cell	10^{-14}- 10^{-10} M; 4 hrs	NO ↑	Koshimura[1]
HUVEC HUVEC, HPAEC, HDEC	4U/ml; 24 hrs 4U/ml/day; 6 days	NOS ↑ NOS ↑	Banerjee[2]
HUVEC, THBMEC in hypoxia	5U/ml; 48 hrs 5U/ml; 1 hr	eNOS ↑ NO ↑ (also in normoxia)	Beleslin-Cokic[3]
Cardiomyocyte in anoxia/ reoxygenation	5U/ml; 4 hrs	eNOS ↑, NO ↑	Rui[4]
Healthy rat	2U/ml/day s.c. 14 days	Aorta: eNOS ↑, NO ↑ NO-dependent relaxation aorta	Kanagy[5]
Mouse Parkinson model	16U/4µl bilateral injection above SN	Nitrate levels in SN and striatum	Genc[6]
Human erythrocyte + phenylhydrazine	0,2U/ml	OH* ↓	Choudhury[7]
Goat erythrocyte + copper (II) ascorbate	1U/2ml; 1,5 hr 0.2U/2ml; 1,5 hr 1, 2U/2ml; 1,5 hr	MDA ↓, Catalase ↑, GPx ↑ OH* ↓	Chattopadhyay[8]
Mouse Parkinson model	16U/4µl bilateral injection above SN	GPx activity in SN ↑	Genc[9]
Astroglial cell	0.1, 1, 5U/ml; 48 hrs	GPx activity ↑	
Ischemic brain injured rat	1000U/kg i.p.	GPx activity ↑+ TBARS ↓	Kumral[10]
Traumatic brain injured rat	5000U/kg i.p.	MDA ↓	Ozturk[11]
Traumatic brain injured rat	1000U/kg i.p.	TBARS ↓ in heart tissue	Emir[12]
Hemodialysis pt	50U/kg 3x/wk; 8 months	Monocyte heme-oxygenase-1 ↑, plasma anti-oxidant power ↑	Calo[13]
Hemodialysis pt	109+/-56U/kg/wk i.v.; 4 months	Catalase ↑, SOD ↑	Delmas-Beauvieux[14]
Hemodialysis pt	30U/kg 3x/wk s.c. 6 months	In plasma + red blood cell: GPx ↑, SOD ↑, thiol groups ↑	Mimic-Oka[15]
Hemodialysis pt	100U/kg 3x/wk; 3 months	MDA ↓, Catalase ↑, SOD ↑	Inal[16]
Hemodialysis pt	Epo dose adjusted to Ht; 6 months	MDA ↓ at complete correction of anemia	Ludat[17]
CRF pt	50U/kg 1x/wk s.c.; 4 months	Prevention of ↓ PAF-AH and GPx	Papavasiliou[18]

NO *Nitric oxide*
ROS *Reactive oxygen species*
eNOS *Endothelial nitric oxide synthase*
HUVEC *Human umbilical vein endothelial cell*
HPAEC *Human pulmonary artery endothelial cell*
HDEC *Human dermis endothelial cell*
THBMEC *Transformed human bone marrow endothelial cell*
SN *Substantia nigra*
*OH** *Hydroxyl radical*
MDA *Malondialdehyde; end-product of lipid peroxidation*
GPx *Glutathione peroxidase; an anti-oxidant defence enzyme*
TBARS *Thiobarbituric acid-reactive substance; index of ROS and lipid peroxidation*
SOD *Superoxide dismutase, an anti-oxidant defence enzyme*
PAF-AH *Platelet-activated factor acetylhydrolase; degrades PAF and oxidized phospholipids*

APPENDIX TABLE 02B EFFECTS OF EPO SHIFTING NO/ROS BALANCE TOWARDS ROS

TISSUE/OBJECT	EPO STIMULATION: DOSE + DURATION	EFFECT OF EPO	REFERENCE
HCAEC	5, 20U/ml; 24 hrs	eNOS ↓, NO ↓	Wang[19]
Human healthy cutaneous vessel	25, 50, 100U/min	Vasoconstriction, probably due to NO ↓	Buemi[20]
HUVEC	0.1-200U/ml; 24 hrs	ADMA ↑, associated with NO↓, ROS ↑	Scalera[21]
IL-1b treated rat VSMC	250U/ml; 24 hrs	cGMP ↓, nitrite ↓, iNOS ↓	Kusano[22]
CRF rats	150U/kg 2x/wk; 6 weeks	Vasodilatation on NO ↓	Vaziri[23]
Acetylcholine-treated rabbits	400U/kg/2 days s.c.; 1 week	Vasodilatation ↓, probably due to NOS ↓	Noguchi[24]
Hemodialysis children	50-100U/kg 3x/wk in wk 1-12; followed by dose adjustment according to Ht; 1 yr	Oxidative stress erythrocyte ↑	Nemeth[25]
Hemodialysis pt	30U/kg 3x/wk s.c. 3 months	In red blood cells: MDA ↑	Mimic-Oka[15]
Hemodialysis pt	88+/-24U/kg/wk s.c.; 6 months; followed by 6 months Vitamin E supplementation	Vitamin E ↓, MDA↑ With Vitamin E supplementation: MDA ↓	Cristol[26]

NO *Nitric oxide*
ROS *Reactive oxygen species*
iNOS *Intrinsic nitric oxide synthase*
eNOS *Endothelial nitric oxide synthase*
HCAEC *Human coronary artery endothelial cell*
HUVEC *Human umbilical vein endothelial cell*
ADMA *Asymmetric dimethylarginine; intrinsic NOS inhibitor*
VSMC *Vascular smooth muscle cell*
cGMP *Cyclic guanosine 3',5'-cyclic monophosphate; induced by NO*
MDA *Malondialdehyde; end-product of lipid peroxidation*

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APPENDICES CH.05

SHORT-TERM ERYTHROPOIETIN TREATMENT DOES NOT SUBSTANTIALLY MODULATE MONOCYTE TRANSCRIPTOMES OF PATIENTS WITH COMBINED HEART AND RENAL FAILURE

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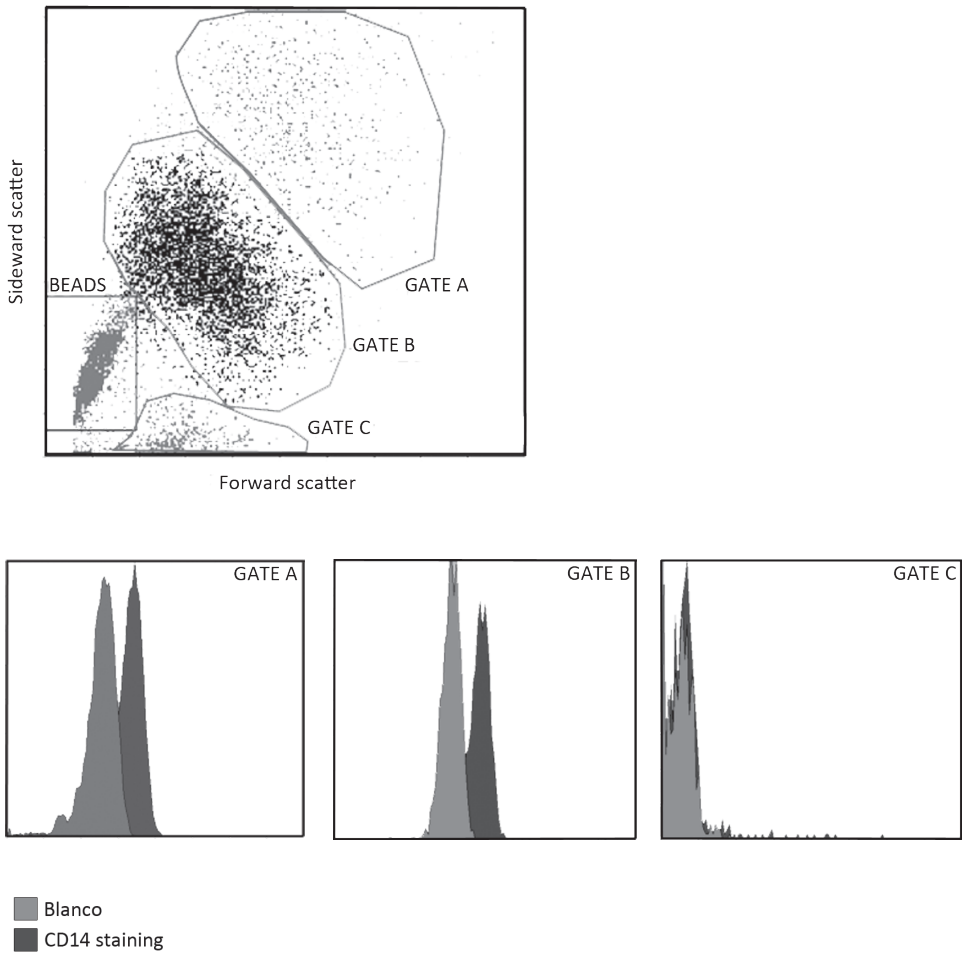
Submitted

APPENDIX FIGURE 01 MONOCYTE SAMPLE PURITY WAS DETERMINED BY CD14^{+} STAINING USING FLOW CYTOMETRY



Monocytes isolated with CD14^{+}-dynabeads are plotted in the forward/sideward scatter. Separate beads and three cell populations can be discerned. PECy5 conjugated CD14-staining of the isolated cell population revealed CD14^{+}-cells in gate A and B with multiple or single beads attached to the cells respectively. Gate C is CD14^{-} and accounts for <10% contamination of the isolated cell population.

APPENDIX TABLE 01 MONOCYTE GENE EXPRESSION DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

TRANSCRIPT	SYMBOL	DEFINITION	CONTROLS	PATIENTS	FOLD CHANGE	P-VALUE
UPREGULATED			log intensity	log intensity		
NM_018487.2	HCA112	TRANSMEMBRANE PROTEIN 176A	8.33	9.09	1.70	0.0373
NM_012456.1	TIMM10	TRANSLOCASE OF INNER MITOCHONDRIAL MEMBRANE 10 HOMOLOG	7.70	8.37	1.59	0.0000
NM_017911.1	C22ORF8	FAMILY WITH SEQUENCE SIMILARITY 118, MEMBER A	6.89	7.55	1.58	0.0118
NM_001337.3	CX3CR1	CHEMOKINE (C-X3-C MOTIF) RECEPTOR 1	10.86	11.43	1.49	0.0002
NM_006498.2	LGALS2	LECTIN, GALACTOSIDE-BINDING, SOLUBLE, 2	8.20	8.75	1.46	0.0284
NM_000024.3	ADRB2	ADRENERGIC, BETA-2-, RECEPTOR, SURFACE	8.47	9.00	1.45	0.0015
NM_005771.3	DHRS9	DEHYDROGENASE/REDUCTASE (SDR FAMILY) MEMBER 9	8.38	8.90	1.43	0.0080
NM_198097.1	C7ORF28B	CHROMOSOME 7 OPEN READING FRAME 28B (C7ORF28B),	8.40	8.92	1.43	0.0053
NM_001343.1	DAB2	DISABLED HOMOLOG 2, MITOGEN-RESPONSIVE PHOSPHOPROTEIN	6.92	7.44	1.43	0.0003
NM_016021.2	UBE2J1	UBIQUITIN-CONJUGATING ENZYME E2, J1	8.42	8.93	1.42	0.0006
NM_030670.1	PTPRO	PROTEIN TYROSINE PHOSPHATASE, RECEPTOR TYPE, O	6.91	7.38	1.39	0.0000
NM_001008566.1	TPST2	TYROSYLPROTEIN SULFOTRANSFERASE 2	9.73	10.20	1.38	0.0014
NM_030671.1	PTPRO	PROTEIN TYROSINE PHOSPHATASE, RECEPTOR TYPE, O	7.37	7.82	1.37	0.0006
NR_003038.1	SNHG5	SMALL NUCLEOLAR RNA HOST GENE (NON-PROTEIN CODING) 5	10.13	10.58	1.36	0.0365
NM_080914.1	ASGR2	ASIALOGLYCOPROTEIN RECEPTOR 2	9.44	9.88	1.36	0.0029
NM_016613.4	C4ORF18	CHROMOSOME 4 OPEN READING FRAME 18	8.93	9.36	1.35	0.0158
NM_016613.4	C4ORF18	CHROMOSOME 4 OPEN READING FRAME 18	9.57	10.00	1.35	0.0033
NM_152594.1	SPRED1	SPROUTY-RELATED, EVH1 DOMAIN CONTAINING 1	8.11	8.54	1.34	0.0040
NM_005771.3	DHRS9	DEHYDROGENASE/REDUCTASE (SDR FAMILY) MEMBER 9	7.13	7.54	1.33	0.0057
NM_144580.1	C1ORF85	CHROMOSOME 1 OPEN READING FRAME 85	9.54	9.94	1.32	0.0099
NM_021244.2	RRAGD	RAS-RELATED GTP BINDING D	7.85	8.24	1.31	0.0137
NM_004383.1	CSK	C-SRC TYROSINE KINASE	10.59	10.98	1.31	0.0001
NM_004949.2	DSC2	DESMOCOLLIN 2	7.42	7.81	1.31	0.0207
NM_031890.2	CECR6	CAT EYE SYNDROME CHROMOSOME REGION, CANDIDATE 6	7.75	8.14	1.30	0.0003
NM_080914.1	ASGR2	ASIALOGLYCOPROTEIN RECEPTOR 2	9.32	9.70	1.30	0.0167
NM_000129.2	F13A1	COAGULATION FACTOR XIII, A1 POLYPEPTIDE	8.85	9.21	1.29	0.0127
NM_003494.2	DYSF	DYSFERLIN, LIMB GIRDLE MUSCULAR DYSTROPHY 2B (AUTOSOMAL RECESSIVE)	8.67	9.03	1.28	0.0064
NM_013385.2	PSCD4	PLECKSTRIN HOMOLOGY, SEC7 AND COILED-COIL DOMAINS 4	11.30	11.65	1.27	0.0015
NM_002935.2	RNASE3	RIBONUCLEASE, RNASE A FAMILY, 3 (EOSINOPHIL CATIONIC PROTEIN)	6.72	7.07	1.27	0.0093
NM_004462.3	FDFT1	FARNESYL-DIPHOSPHATE FARNESYLTRANSFERASE 1	9.74	10.08	1.27	0.0014
NM_198595.1	AFAP	ACTIN FILAMENT ASSOCIATED PROTEIN	6.46	6.79	1.26	0.0034
NM_020223.1	FAM20C	FAMILY WITH SEQUENCE SIMILARITY 20, MEMBER C	7.73	8.07	1.26	0.0017
NM_018476.3	BEX1	BRAIN EXPRESSED, X-LINKED 1	6.48	6.81	1.26	0.0089
NM_182616.1	C15ORF38	CHROMOSOME 15 OPEN READING FRAME 38	7.45	7.78	1.26	0.0211
NM_013451.2	FER1L3	FER-1-LIKE 3, MYOFERLIN	8.84	9.17	1.26	0.0030

APPENDIX TABLE 01 MONOCYTE GENE EXPRESSION DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

TRANSCRIPT	SYMBOL	DEFINITION	CONTROLS	PATIENTS	FOLD CHANGE	P-VALUE
UPREGULATED			log intensity	log intensity		
NM_005902.3	SMAD3	SMAD FAMILY MEMBER 3	7.78	8.11	1.25	0.0005
NM_000820.1	GAS6	GROWTH ARREST-SPECIFIC 6	6.86	7.19	1.25	0.0013
NM_003516.2	HIST2H2AA3	HISTONE CLUSTER 2, H2AA3	9.07	9.40	1.25	0.0156
XM_942084.1	GAS6	PREDICTED: GROWTH ARREST-SPECIFIC 6	7.17	7.49	1.25	0.0025
NM_002934.2	RNASE2	RIBONUCLEASE, RNASE A FAMILY, 2 (LIVER, EOSINOPHIL-DERIVED NEUROTOXIN)	10.51	10.83	1.24	0.0461
NM_000491.2	C1QB	COMPLEMENT COMPONENT 1, Q SUBCOMPONENT, BETA POLYPEPTIDE	6.87	7.19	1.24	0.0344
NM_080759.3	DACH1	DACHSHUND HOMOLOG 1	6.83	7.13	1.24	0.0010
NM_006270.2	RRAS	RELATED RAS VIRAL (R-RAS) ONCOGENE HOMOLOG	9.30	9.61	1.23	0.0064
NM_004364.2	CEBPA	CCAAT/ENHANCER BINDING PROTEIN (C/EBP), ALPHA	9.56	9.86	1.23	0.0066
NM_005817.3	M6PRBP1	MANNOSE-6-PHOSPHATE RECEPTOR BINDING PROTEIN 1	10.66	10.95	1.23	0.0030
NM_173078.2	SLITRK4	SLIT AND NTRK-LIKE FAMILY, MEMBER 4	7.87	8.17	1.23	0.0065
NM_021965.3	PGM5	PHOSPHOGLUCOMUTASE 5	6.79	7.08	1.23	0.0430
NM_000355.2	TCN2	TRANSCOBALAMIN II; MACROCYTIC ANEMIA	7.30	7.59	1.23	0.0129
NM_006745.3	SC4MOL	STEROL-C4-METHYL OXIDASE-LIKE	7.13	7.42	1.22	0.0024
NM_001004340.1	FCGR1B	FC FRAGMENT OF IGG, HIGH AFFINITY IB, RECEPTOR (CD64)	8.33	8.62	1.22	0.0488
NM_001006932.1	RPS6KA2	RIBOSOMAL PROTEIN S6 KINASE, 90KDA, POLYPEPTIDE 2	6.45	6.74	1.22	0.0346
NM_080593.1	HIST1H2BK	HISTONE CLUSTER 1, H2BK	9.78	10.07	1.22	0.0153
NM_001008485.1	SLC41A3	SOLUTE CARRIER FAMILY 41, MEMBER 3	8.03	8.32	1.22	0.0200
NM_004843.2	IL27RA	INTERLEUKIN 27 RECEPTOR, ALPHA	8.79	9.08	1.22	0.0012
NM_173511.2	ALS2CR13	AMYOTROPHIC LATERAL SCLEROSIS 2 (JUVENILE) CHROMOSOME REGION, CANDIDATE 13	9.75	10.03	1.21	0.0382
NM_005885.2	MARCH6	MEMBRANE-ASSOCIATED RING FINGER (C3HC4)	7.45	7.73	1.21	0.0244
NM_001425.1	EMP3	EPITHELIAL MEMBRANE PROTEIN 3	12.05	12.32	1.21	0.0051
NM_014584.1	ERO1L	ERO1-LIKE	7.65	7.92	1.21	0.0022
NM_000270.1	NP	NUCLEOSIDE PHOSPHORYLASE	9.27	9.55	1.21	0.0254
NM_004672.3	MAP3K6	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 6	8.30	8.57	1.21	0.0180
NM_002209.1	ITGAL	INTEGRIN, ALPHA L (ANTIGEN CD11A (P180)	10.19	10.46	1.21	0.0047
NM_080792.1	PTPNS1	PROTEIN TYROSINE PHOSPHATASE, NON-RECEPTOR TYPE SUBSTRATE 1	11.70	11.97	1.21	0.0144
NM_013337.2	TIMM22	TRANSLOCASE OF INNER MITOCHONDRIAL MEMBRANE 22 HOMOLOG	8.39	8.66	1.21	0.0284
NM_152773.2	MGC33212	HYPOTHETICAL PROTEIN	7.04	7.31	1.20	0.0106
NM_153280.1	UBE1	UBIQUITIN-ACTIVATING ENZYME E1	9.08	9.35	1.20	0.0083
NM_004099.4	STOM	STOMATIN	8.74	9.01	1.20	0.0292
NM_005828.2	WDR68	WD REPEAT DOMAIN 68	9.11	9.37	1.20	0.0142
NM_004090.2	DUSP3	DUAL SPECIFICITY PHOSPHATASE 3	9.69	9.96	1.20	0.0498
NM_000110.2	DPYD	DIHYDROPYRIMIDINE DEHYDROGENASE	8.66	8.93	1.20	0.0257
NM_000067.1	CA2	CARBONIC ANHYDRASE II	7.09	7.35	1.20	0.0058

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NM_016562.3	TLR7	TOLL-LIKE RECEPTOR 7	9.03	9.30	1.20	0.0403
NM_006942.1	SOX15	SRY (SEX DETERMINING REGION Y)-BOX 15	6.72	6.97	1.20	0.0120
NM_032350.3	MGC11257	HYPOTHETICAL PROTEIN	9.47	9.73	1.20	0.0283
NM_001386.4	DPYSL2	DIHYDROPYRIMIDINASE-LIKE 2	11.47	11.73	1.20	0.0320
NM_014339.3	IL17R	INTERLEUKIN 17 RECEPTOR	8.26	8.52	1.19	0.0117
NM_021708.1	LAIR1	LEUKOCYTE-ASSOCIATED IG-LIKE RECEPTOR 1	7.25	7.50	1.19	0.0370
NM_182827.1	FKBP9L	FK506 BINDING PROTEIN 9-LIKE	6.74	6.99	1.19	0.0017
NM_001014763.1	ETFB	ELECTRON-TRANSFER-FLAVOPROTEIN, BETA POLYPEPTIDE	9.73	9.98	1.19	0.0259
NM_015179.2	KIAA0690	RIBOSOMAL RNA PROCESSING 12 HOMOLOG	8.13	8.38	1.19	0.0418
NM_005319.3	HIST1H1C	HISTONE CLUSTER 1, H1C	6.80	7.05	1.19	0.0027
NM_003129.3	SQLE	SQUALENE EPOXIDASE	6.93	7.17	1.18	0.0134
NM_138452.1	DHRS1	DEHYDROGENASE/REDUCTASE (SDR FAMILY) MEMBER 1	8.82	9.06	1.18	0.0087
NM_001002235.1	SERPINA1	SERPIN PEPTIDASE INHIBITOR, CLADE A, MEMBER 1	12.02	12.26	1.18	0.0426
NM_018440.3	PAG1	PHOSPHOPROTEIN ASSOCIATED WITH GLYCOSPHINGOLIPID MICRODOMAINS 1	8.16	8.40	1.18	0.0299
NM_133171.2	ELMO2	ENGULFMENT AND CELL MOTILITY 2	8.64	8.88	1.18	0.0366
NM_000391.2	TPP1	TRIPEPTIDYL PEPTIDASE I	10.76	10.99	1.18	0.0169
NM_001009566.1	CLSTN1	CALSYNTENIN 1	8.61	8.85	1.18	0.0094
NM_006367.2	CAP1	CAP, ADENYLATE CYCLASE-ASSOCIATED PROTEIN 1	12.37	12.60	1.18	0.0318
NM_014449.1	GPR162	G PROTEIN-COUPLED RECEPTOR 162	10.37	10.60	1.17	0.0318
NM_013410.2	AK3L1	ADENYLATE KINASE 3-LIKE 1	6.95	7.18	1.17	0.0026
NM_025201.3	PLEKHQ1	PLECKSTRIN HOMOLOGY DOMAIN CONTAINING, FAMILY Q	8.55	8.78	1.17	0.0473
NM_004740.3	TIAF1	TGFB1-INDUCED ANTI-APOPTOTIC FACTOR 1	9.41	9.64	1.17	0.0237
NM_014877.2	HELZ	HELICASE WITH ZINC FINGER	8.34	8.57	1.17	0.0125
NM_002468.2	MYD88	MYELOID DIFFERENTIATION PRIMARY RESPONSE GENE (88)	10.10	10.33	1.17	0.0449
NM_018653.3	GPRC5C	G PROTEIN-COUPLED RECEPTOR, FAMILY C, GROUP 5, MEMBER C	6.49	6.71	1.17	0.0073
NM_198565.1	LRRC33	LEUCINE RICH REPEAT CONTAINING 33	8.80	9.02	1.17	0.0268
NM_002562.4	P2RX7	PURINERGIC RECEPTOR P2X, LIGAND-GATED ION CHANNEL, 7	9.07	9.29	1.17	0.0255
NM_170600.1	SH2D3C	SH2 DOMAIN CONTAINING 3C	8.95	9.17	1.16	0.0254
NM_001008213.1	OPTN	OPTINEURIN	6.61	6.82	1.16	0.0046
NM_080549.2	PTPN6	PROTEIN TYROSINE PHOSPHATASE, NON-RECEPTOR TYPE 6	10.65	10.87	1.16	0.0238
NM_138570.1	MGC15523	HYPOTHETICAL PROTEIN	7.19	7.41	1.16	0.0404
NM_024321.3	MGC10433	HYPOTHETICAL PROTEIN	8.09	8.31	1.16	0.0213
NM_020148.1	SPIRE1	SPIRE HOMOLOG 1	7.14	7.36	1.16	0.0416
NM_003264.3	TLR2	TOLL-LIKE RECEPTOR 2	7.44	7.66	1.16	0.0149
NM_018982.3	YIPF1	YIP1 DOMAIN FAMILY, MEMBER 1	8.89	9.10	1.16	0.0256
NM_015150.1	RAFTLIN	RAFTLIN, LIPID RAFT LINKER 1	9.33	9.54	1.16	0.0458
NM_003896.2	ST3GAL5	ST3 BETA-GALACTOSIDE ALPHA-2,3-SIALYLTRANSFERASE 5	8.64	8.85	1.16	0.0470

APPENDIX TABLE 01 MONOCYTE GENE EXPRESSION DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

TRANSCRIPT	SYMBOL	DEFINITION	CONTROLS	PATIENTS	FOLD CHANGE	P-VALUE
UPREGULATED			log intensity	log intensity		
NM_025144.2	ALPK1	ALPHA-KINASE 1	7.73	7.94	1.16	0.0457
NM_022833.2	C9ORF88	CHROMOSOME 9 OPEN READING FRAME 88	10.72	10.93	1.16	0.0454
NM_014063.5	DBNL	DREBRIN-LIKE	11.68	11.89	1.16	0.0375
NM_007200.3	AKAP13	A KINASE (PRKA) ANCHOR PROTEIN 13	10.54	10.74	1.16	0.0422
NM_015219.2	EXOC7	EXOCYST COMPLEX COMPONENT 7	8.83	9.04	1.16	0.0102
NM_018326.2	GIMAP4	GTPASE, IMAP FAMILY MEMBER 4	12.37	12.58	1.16	0.0388
NM_032804.5	C10ORF22	CHROMOSOME 10 OPEN READING FRAME 22	7.59	7.80	1.15	0.0183
NM_001259.5	CDK6	CYCLIN-DEPENDENT KINASE 6	6.90	7.10	1.15	0.0104
NM_001014431.1	AKT1	V-AKT MURINE THYMOMA VIRAL ONCOGENE HOMOLOG 1	9.71	9.91	1.15	0.0370
NM_007061.3	CDC42EP1	CDC42 EFFECTOR PROTEIN (RHO GTPASE BINDING) 1	6.88	7.08	1.15	0.0447
NM_016063.1	HDDC2	HD DOMAIN CONTAINING 2	9.06	9.26	1.15	0.0372
NM_176787.3	PIGN	PHOSPHATIDYLINOSITOL GLYCAN ANCHOR BIOSYNTHESIS, CLASS N	7.39	7.59	1.15	0.0280
NM_007165.4	SF3A2	SPLICING FACTOR 3A, SUBUNIT 2, 66KDA	9.10	9.30	1.15	0.0476
NM_003481.1	USP5	UBIQUITIN SPECIFIC PEPTIDASE 5 (ISOPEPTIDASE T)	8.46	8.67	1.15	0.0317
NM_180981.1	MRPL52	MITOCHONDRIAL RIBOSOMAL PROTEIN L52	7.71	7.91	1.15	0.0206
NM_002068.1	GNA15	GUANINE NUCLEOTIDE BINDING PROTEIN	9.38	9.59	1.15	0.0373
NM_005481.2	THRAP5	MEDIATOR COMPLEX SUBUNIT 16	8.80	9.00	1.15	0.0334
NM_080686.1	BAT2	HLA-B ASSOCIATED TRANSCRIPT 2	8.10	8.30	1.15	0.0235
NM_001560.2	IL13RA1	INTERLEUKIN 13 RECEPTOR, ALPHA 1	10.73	10.93	1.15	0.0369
NM_000628.3	IL10RB	INTERLEUKIN 10 RECEPTOR, BETA	11.01	11.20	1.15	0.0442
NM_024841.2	FLJ14213	HYPOTHETICAL PROTEIN FLJ14213	6.95	7.15	1.15	0.0120
NM_006761.3	YWHAE	TYROSINE 3-MONOOXYGENASE/TRYPTOPHAN 5-MONOOXYGENASE ACTIVATION PROTEIN	7.22	7.42	1.14	0.0416
NM_003809.2	TNFSF12	TUMOR NECROSIS FACTOR (LIGAND) SUPERFAMILY, MEMBER 12	9.15	9.34	1.14	0.0493
NM_138499.2	PWWP2	PWWP DOMAIN CONTAINING 2	7.65	7.84	1.14	0.0187
NM_025078.3	PQLC1	PQ LOOP REPEAT CONTAINING 1	10.49	10.69	1.14	0.0400
NM_018103.3	LRRC8D	LEUCINE RICH REPEAT CONTAINING 8 FAMILY, MEMBER D	7.59	7.79	1.14	0.0451
NM_014505.4	KCNMB4	POTASSIUM LARGE CONDUCTANCE CALCIUM-ACTIVATED CHANNEL, SUBFAMILY M, BETA MEMBER 4	6.38	6.57	1.14	0.0047
NM_021019.3	MYL6	MYOSIN, LIGHT CHAIN 6, ALKALI, SMOOTH MUSCLE AND NON-MUSCLE	13.26	13.45	1.14	0.0294
NM_024422.2	DSC2	DESMOCOLLIN 2	6.56	6.75	1.14	0.0165
NM_030914.1	C9ORF74	UBIQUITIN RELATED MODIFIER 1 HOMOLOG	9.46	9.65	1.14	0.0353
NM_004924.3	ACTN4	ACTININ, ALPHA 4	8.80	8.99	1.14	0.0360
NM_138578.1	BCL2L1	BCL2-LIKE 1	8.03	8.21	1.14	0.0401
NM_001101.2	ACTB	ACTIN, BETA	14.26	14.45	1.14	0.0374
NM_052875.2	VPS26B	VACUOLAR PROTEIN SORTING 26 HOMOLOG B	7.75	7.94	1.14	0.0292
NM_198540.2	B3GALT7	UDP-GLCNAC:BETAGAL BETA-1,3-N-ACETYLGLUCOSAMINYLTRANSFERASE 8	6.95	7.13	1.14	0.0163

APPENDIX TABLE 01 MONOCYTE GENE EXPRESSION DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

NM_180991.4	SLCO4C1	SOLUTE CARRIER ORGANIC ANION TRANSPORTER FAMILY, MEMBER 4C1	6.35	6.53	1.13	0.0005
NM_183041.1	DTNBP1	DYSTROBREVIN BINDING PROTEIN 1	7.30	7.48	1.13	0.0310
NM_003127.1	SPTAN1	SPECTRIN, ALPHA, NON-ERYTHROCYTIC 1	8.14	8.32	1.13	0.0440
NM_018174.4	BPY2IP1	MICROTUBULE-ASSOCIATED PROTEIN 1S	7.45	7.64	1.13	0.0084
NM_014712.1	SETD1A	SET DOMAIN CONTAINING 1A	7.86	8.04	1.13	0.0144
NM_012095.4	AP3M1	ADAPTOR-RELATED PROTEIN COMPLEX 3, MU 1 SUBUNIT	8.19	8.37	1.13	0.0257
NM_015459.3	DKFZP564J0863	DKFZP564J0863 PROTEIN	7.96	8.14	1.13	0.0267
NM_022048.2	CSNK1G1	CASEIN KINASE 1, GAMMA 1	7.75	7.93	1.13	0.0330
NM_020451.2	SEPN1	SELENOPROTEIN N, 1	6.91	7.09	1.13	0.0152
NM_014800.8	ELMO1	ENGULFMENT AND CELL MOTILITY 1	8.18	8.36	1.13	0.0243
NM_000675.3	ADORA2A	ADENOSINE A2A RECEPTOR	6.77	6.94	1.13	0.0249
NM_018054.4	ARHGAP17	RHO GTPASE ACTIVATING PROTEIN 17	7.46	7.63	1.13	0.0333
NM_015609.1	C1ORF144	CHROMOSOME 1 OPEN READING FRAME 144	8.55	8.72	1.12	0.0443
NM_006116.2	MAP3K7IP1	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 7 INTERACTING PROTEIN 1	7.60	7.77	1.12	0.0282
NM_024293.2	C2ORF17	CHROMOSOME 2 OPEN READING FRAME 17	7.85	8.02	1.12	0.0456
NM_001037633.1	SIL1	SIL1 HOMOLOG, ENDOPLASMIC RETICULUM CHAPERONE	7.55	7.72	1.12	0.0488
NM_007259.2	VPS45A	VACUOLAR PROTEIN SORTING 45A	8.58	8.75	1.12	0.0482
NM_012320.3	LYPLA3	LYSOPHOSPHOLIPASE 3 (LYSOSOMAL PHOSPHOLIPASE A2)	7.05	7.22	1.12	0.0218
NM_033446.1	C9ORF28	FAMILY WITH SEQUENCE SIMILARITY 125, MEMBER B	7.13	7.30	1.12	0.0344
NM_032730.3	RTN4IP1	RETICULON 4 INTERACTING PROTEIN 1	6.85	7.01	1.12	0.0124
XM_946079.1	C7ORF20	PREDICTED: CHROMOSOME 7 OPEN READING FRAME 20	7.25	7.41	1.12	0.0490
NM_024517.1	PHF2	PHD FINGER PROTEIN 2	8.08	8.24	1.12	0.0418
NM_030805.1	LMAN2L	LECTIN, MANNOSE-BINDING 2-LIKE	7.34	7.50	1.12	0.0473
NM_024025.1	DUSP26	DUAL SPECIFICITY PHOSPHATASE 26 (PUTATIVE)	7.25	7.41	1.12	0.0406
NM_183425.1	RBM38	RNA BINDING MOTIF PROTEIN 38	6.83	6.99	1.12	0.0324
NM_145800.2	Sep-06	SEPTIN 6	6.94	7.10	1.11	0.0360
NM_005477.1	HCN4	HYPERPOLARIZATION ACTIVATED CYCLIC NUCLEOTIDE-GATED POTASSIUM CHANNEL 4	6.44	6.59	1.11	0.0353
NM_139021.1	MAPK15	MITOGEN-ACTIVATED PROTEIN KINASE 15	6.52	6.67	1.11	0.0318
NM_202468.1	GIPC1	GIPC PDZ DOMAIN CONTAINING FAMILY, MEMBER 1	6.68	6.83	1.11	0.0167
NM_004960.2	FUS	FUSION (INVOLVED IN T(12;16) IN MALIGNANT LIPOSARCOMA)	6.79	6.94	1.11	0.0119
NM_020314.3	MGC16824	ESOPHAGEAL CANCER ASSOCIATED PROTEIN	6.71	6.86	1.11	0.0248
NM_004798.2	KIF3B	KINESIN FAMILY MEMBER 3B	7.74	7.89	1.11	0.0458
NM_052848.1	MGC20255	COILED-COIL DOMAIN CONTAINING 97	7.51	7.66	1.11	0.0421
NM_018243.2	Sep-11	SEPTIN 11	7.02	7.16	1.11	0.0307
NM_001001939.1	PCBD1	G-PYRUVOYL-TETRAHYDROPTERIN SYNTHASE/DIMERIZATION COFACTOR OF HEPATOCYTE NUCLEAR FACTOR 1α	7.40	7.54	1.11	0.0474
NM_003816.2	ADAM9	ADAM METALLOPEPTIDASE DOMAIN 9 (MELTRIN GAMMA)	6.89	7.04	1.10	0.0312
NM_005535.1	IL12RB1	INTERLEUKIN 12 RECEPTOR, BETA 1	6.57	6.71	1.10	0.0057

APPENDIX TABLE 01 MONOCYTE GENE EXPRESSION DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

TRANSCRIPT	SYMBOL	DEFINITION	CONTROLS	PATIENTS	FOLD CHANGE	P-VALUE
UPREGULATED			log intensity	log intensity		
NM_019843.2	EIF4ENIF1	EUKARYOTIC TRANSLATION INITIATION FACTOR 4E NUCLEAR IMPORT FACTOR 1	7.29	7.43	1.10	0.0377
NM_004957.4	FPGS	FOLYLPOLYGLUTAMATE SYNTHASE	6.45	6.59	1.10	0.0056
NM_013347.1	RPA4	REPLICATION PROTEIN A4, 34KDA	6.73	6.87	1.10	0.0394
NM_014508.2	APOBEC3C	APOLIPOPROTEIN B MRNA EDITING ENZYME, CATALYTIC POLYPEPTIDE-LIKE 3C	6.99	7.13	1.10	0.0333
NM_031466.3	NIBP	NIK AND IKK{BETA} BINDING PROTEIN	7.10	7.24	1.10	0.0318
NM_213568.1	SLC39A3	SOLUTE CARRIER FAMILY 39 (ZINC TRANSPORTER), MEMBER 3	6.43	6.56	1.10	0.0054
NM_001001795.1	MGC70857	SIMILAR TO RIKEN CDNA C030006K11 GENE	7.10	7.24	1.10	0.0382
NM_018645.3	HES6	HAIRY AND ENHANCER OF SPLIT 6	6.77	6.90	1.10	0.0325
NM_147202.1	C9ORF25	CHROMOSOME 9 OPEN READING FRAME 25	6.79	6.92	1.10	0.0166
NM_152267.2	RNF185	RING FINGER PROTEIN 185	7.07	7.20	1.10	0.0429
NM_000297.2	PKD2	POLYCYSTIC KIDNEY DISEASE 2 (AUTOSOMAL DOMINANT)	6.94	7.07	1.10	0.0425
NM_032932.3	RAB11FIP4	RAB11 FAMILY INTERACTING PROTEIN 4 (CLASS II)	6.54	6.67	1.09	0.0439
NM_006068.2	TLR6	TOLL-LIKE RECEPTOR 6	6.69	6.82	1.09	0.0214
NM_005311.3	GRB10	GROWTH FACTOR RECEPTOR-BOUND PROTEIN 10	6.76	6.89	1.09	0.0328
NM_000755.2	CRAT	CARNITINE ACETYLTRANSFERASE	6.46	6.59	1.09	0.0126
NM_032222.1	FLJ22374	HYPOTHETICAL PROTEIN	6.44	6.56	1.09	0.0115
NM_033211.2	LOC90355	CHROMOSOME 5 OPEN READING FRAME 30	6.47	6.60	1.09	0.0160
NM_015441.1	OLFML2B	OLFACTOMEDIN-LIKE 2B	6.59	6.71	1.09	0.0394
NM_001007232.1	INCA	INHIBITORY CASPASE RECRUITMENT DOMAIN (CARD) PROTEIN	6.55	6.67	1.09	0.0214
NM_022086.6	ELMO2	ENGULFMENT AND CELL MOTILITY 2 (CED-12 HOMOLOG)	6.47	6.59	1.09	0.0298
NM_014925.2	R3HDM2	R3H DOMAIN CONTAINING 2	6.85	6.97	1.08	0.0433
NM_138422.1	LOC113179	TRNA-SPECIFIC ADENOSINE DEAMINASE 3	6.54	6.65	1.08	0.0121
NM_001042535.1	CENTG3	CENTAURIN, GAMMA 3	6.48	6.60	1.08	0.0202
NM_001012288.1	CRLF2	CYTOKINE RECEPTOR-LIKE FACTOR 2	6.48	6.60	1.08	0.0353
NM_001031854.1	LOC390110	HYPOTHETICAL PROTEIN	6.43	6.54	1.08	0.0184
NM_001149.2	ANK3	ANKYRIN 3, NODE OF RANVIER (ANKYRIN G)	6.39	6.51	1.08	0.0096
NM_152455.2	ZNF690	ZINC FINGER PROTEIN 690	6.38	6.49	1.08	0.0257
NM_004565.1	PEX14	PEROXISOMAL BIOGENESIS FACTOR 14	6.46	6.56	1.07	0.0400
NM_022896.1	LPIN3	LIPIN 3	6.45	6.56	1.07	0.0415
NM_145344.1	APOL1	APOLIPOPROTEIN L, 1	6.50	6.60	1.07	0.0378
NM_001040694.1	INCENP	INNER CENTROMERE PROTEIN ANTIGENS 135/155KDA	6.52	6.61	1.07	0.0460
NM_020341.2	PAK7	P21(CDKN1A)-ACTIVATED KINASE 7	6.44	6.53	1.06	0.0423
NM_020536.2	CSRP2BP	CSRP2 BINDING PROTEIN	6.43	6.52	1.06	0.0391
NM_017793.1	RPP25	RIBONUCLEASE P 25KDA SUBUNIT	6.50	6.59	1.06	0.0461
NM_176795.2	HRAS	V-HA-RAS HARVEY RAT SARCOMA VIRAL ONCOGENE HOMOLOG	6.40	6.49	1.06	0.0465

APPENDIX TABLE 01 MONOCYTE GENE EXPRESSION DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

DOWNREGULATED						
NM_000518.4	HBB	HEMOGLOBIN, BETA	11.48	9.73	0.30	0.0010
NM_000517.3	HBA2	HEMOGLOBIN, ALPHA 2	9.85	8.43	0.38	0.0026
XM_936120.1	HLA-DQA1	PREDICTED: MAJOR HISTOCOMPATIBILITY COMPLEX, CLASS II, DQ ALPHA 1	11.71	10.64	0.47	0.0056
NM_000584.2	IL8	INTERLEUKIN 8	7.79	7.01	0.58	0.0007
NM_005252.2	FOS	V-FOS FBJ MURINE OSTEOSARCOMA VIRAL ONCOGENE HOMOLOG	9.72	9.00	0.61	0.0116
NM_006732.1	FOSB	FBJ MURINE OSTEOSARCOMA VIRAL ONCOGENE HOMOLOG B	8.41	7.69	0.61	0.0000
NM_001964.2	EGR1	EARLY GROWTH RESPONSE 1	8.12	7.49	0.65	0.0076
NM_004417.2	DUSP1	DUAL SPECIFICITY PHOSPHATASE 1	11.75	11.19	0.68	0.0030
NM_004666.1	VNN1	VANIN 1	8.75	8.19	0.68	0.0028
NM_005502.2	ABCA1	ATP-BINDING CASSETTE, SUB-FAMILY A (ABC1), MEMBER 1	8.76	8.26	0.71	0.0085
NM_020152.2	C21ORF7	CHROMOSOME 21 OPEN READING FRAME 7	8.44	7.94	0.71	0.0092
NM_017933.3	FLJ20701	PHOSPHOTYROSINE INTERACTION DOMAIN CONTAINING 1	8.91	8.45	0.72	0.0008
NM_002612.2	PDK4	PYRUVATE DEHYDROGENASE KINASE, ISOZYME 4	9.31	8.87	0.74	0.0166
NM_021732.1	AVPI1	ARGININE VASOPRESSIN-INDUCED 1	8.11	7.67	0.74	0.0055
NM_014887.1	PFAAP5	PHOSPHONOFORMATE IMMUNO-ASSOCIATED PROTEIN 5	8.70	8.27	0.74	0.0004
NM_002201.4	ISG20	INTERFERON STIMULATED EXONUCLEASE GENE 20KDA	9.39	8.97	0.75	0.0057
NM_015263.1	DMXL2	DMX-LIKE 2	9.79	9.38	0.76	0.0134
NM_003937.2	KYNU	KYNURENINASE (L-KYNURENINE HYDROLASE)	9.76	9.35	0.76	0.0028
NM_138373.3	MYADM	MYELOID-ASSOCIATED DIFFERENTIATION MARKER	8.76	8.36	0.76	0.0025
NM_006343.2	MERTK	C-MER PROTO-ONCOGENE TYROSINE KINASE	8.55	8.15	0.76	0.0230
NM_001001437.3	CCL3L3	CHEMOKINE (C-C MOTIF) LIGAND 3-LIKE 3	7.05	6.67	0.77	0.0193
NM_014468.2	VENTX	VENT HOMEOBOX HOMOLOG (XENOPUS LAEVIS)	9.38	9.00	0.77	0.0052
NM_002982.3	CCL2	CHEMOKINE (C-C MOTIF) LIGAND 2	7.26	6.88	0.77	0.0081
NM_001004305.1	LOC284757	HYPOTHETICAL PROTEIN	6.91	6.54	0.77	0.0038
NM_017652.1	ZNF586	ZINC FINGER PROTEIN 586	8.81	8.46	0.78	0.0043
NM_003663.3	CGGBP1	CGG TRIPLET REPEAT BINDING PROTEIN 1	9.60	9.25	0.78	0.0052
NM_003937.2	KYNU	KYNURENINASE (L-KYNURENINE HYDROLASE)	10.22	9.87	0.79	0.0015
NM_054114.3	TAGAP	T-CELL ACTIVATION GTPASE ACTIVATING PROTEIN	8.37	8.03	0.79	0.0175
NM_001641.2	APEX1	APEX NUCLEASE (MULTIFUNCTIONAL DNA REPAIR ENZYME) 1	9.25	8.91	0.79	0.0016
NM_001020820.1	MYADM	MYELOID-ASSOCIATED DIFFERENTIATION MARKER	12.04	11.71	0.79	0.0195
NM_014585.3	SLC40A1	SOLUTE CARRIER FAMILY 40 (IRON-REGULATED TRANSPORTER), MEMBER 1	9.65	9.32	0.79	0.0203
NM_021960.3	MCL1	MYELOID CELL LEUKEMIA SEQUENCE 1 (BCL2-RELATED) VARIANT 1	10.25	9.91	0.79	0.0259
NM_002120.2	HLA-DOB	MAJOR HISTOCOMPATIBILITY COMPLEX, CLASS II, DO BETA	7.17	6.84	0.79	0.0173
NM_022495.3	C14ORF135	CHROMOSOME 14 OPEN READING FRAME 135	8.71	8.38	0.80	0.0037
NM_020070.2	IGLL1	IMMUNOGLOBULIN LAMBDA-LIKE POLYPEPTIDE 1	7.15	6.82	0.80	0.0126
NM_002600.3	PDE4B	PHOSPHODIESTERASE 4B, CAMP-SPECIFIC	7.98	7.65	0.80	0.0083

APPENDIX TABLE 01 MONOCYTE GENE EXPRESSION DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

TRANSCRIPT	SYMBOL	DEFINITION	CONTROLS	PATIENTS	FOLD CHANGE	P-VALUE
DOWNREGULATED			log intensity	log intensity		
NM_001023582.1	RPGR	RETINITIS PIGMENTOSA GTPASE REGULATOR	7.37	7.04	0.80	0.0015
NM_001530.2	HIF1A	HYPOXIA-INDUCIBLE FACTOR 1, ALPHA SUBUNIT	7.86	7.53	0.80	0.0102
NM_001080498.1	EMR4	EGF-LIKE MODULE CONTAINING, MUCIN-LIKE, HORMONE RECEPTOR-LIKE 4	6.78	6.46	0.80	0.0227
NM_032472.3	PPIL3	PEPTIDYLPROLYL ISOMERASE (CYCLOPHILIN)-LIKE 3	10.19	9.87	0.80	0.0438
NM_018398.2	CACNA2D3	CALCIUM CHANNEL, VOLTAGE-DEPENDENT, ALPHA 2/DELTA 3 SUBUNIT	8.44	8.12	0.80	0.0140
NM_153322.1	PMP22	PERIPHERAL MYELIN PROTEIN 22	7.00	6.69	0.80	0.0086
NM_152716.1	FLJ36874	FLJ36874 PROTEIN	10.49	10.18	0.81	0.0100
NM_001001974.1	PLEKHA1	PLECKSTRIN HOMOLOGY DOMAIN CONTAINING, FAMILY A, MEMBER 1	7.92	7.60	0.81	0.0034
NM_001080392.1	KIAA1147	KIAA1147	9.03	8.73	0.81	0.0429
NM_003913.3	PRPF4B	PRP4 PRE-MRNA PROCESSING FACTOR 4 HOMOLOG B	8.67	8.36	0.81	0.0137
NM_014059.1	RGC32	RESPONSE GENE TO COMPLEMENT 32	7.71	7.41	0.81	0.0036
NM_032308.1	RPAIN	RPA INTERACTING PROTEIN	10.37	10.07	0.81	0.0175
NM_014886.2	TINP1	TGF BETA-INDUCIBLE NUCLEAR PROTEIN 1	11.65	11.35	0.81	0.0306
NM_005087.2	FXR1	FRAGILE X MENTAL RETARDATION, AUTOSOMAL HOMOLOG 1	8.97	8.67	0.81	0.0034
NM_002157.1	HSPE1	HEAT SHOCK 10KDA PROTEIN 1	8.36	8.07	0.82	0.0042
NM_005463.2	HNRPDL	HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN D-LIKE	9.72	9.43	0.82	0.0128
NM_004986.2	KTN1	KINECTIN 1 (KINESIN RECEPTOR)	8.83	8.55	0.82	0.0043
NM_001079539.1	XBP1	X-BOX BINDING PROTEIN 1	10.64	10.36	0.82	0.0369
NM_018664.1	SNFT	JUN DIMERIZATION PROTEIN P21SNFT	8.15	7.86	0.82	0.0215
NM_002101.3	GYPC	GLYCOPHORIN C	8.45	8.16	0.82	0.0063
NR_002166.1	SEDLP	SPONDYLOEPIPHYSEAL DYSPLASIA, LATE, PSEUDOGENE	6.98	6.70	0.82	0.0003
NM_018643.2	TREM1	TRIGGERING RECEPTOR EXPRESSED ON MYELOID CELLS 1	7.76	7.48	0.82	0.0206
NM_175738.3	RAB37	RAB37, MEMBER RAS ONCOGENE FAMILY	8.89	8.62	0.82	0.0178
NM_016316.1	REV1L	REV1-LIKE	8.66	8.38	0.82	0.0022
NM_004506.2	HSF2	HEAT SHOCK TRANSCRIPTION FACTOR 2	7.09	6.82	0.83	0.0008
NM_173343.1	IL1R2	INTERLEUKIN 1 RECEPTOR, TYPE II	7.61	7.33	0.83	0.0442
NM_001012734.1	AGPAT4	1-ACYLGLYCEROL-3-PHOSPHATE O-ACYLTRANSFERASE 4	8.22	7.94	0.83	0.0189
NM_023039.2	ANKRA2	ANKYRIN REPEAT, FAMILY A	8.82	8.55	0.83	0.0186
NM_207304.1	MBNL2	MUSCLEBLIND-LIKE 2	8.99	8.72	0.83	0.0168
NM_015308.1	FNBP4	FORMIN BINDING PROTEIN 4	11.03	10.77	0.83	0.0377
NM_001024070.1	GCH1	GTP CYCLOHYDROLASE 1	7.78	7.52	0.83	0.0436
NM_139075.1	TPCN2	TWO PORE SEGMENT CHANNEL 2	8.83	8.56	0.83	0.0176
NR_002944.2	HNRPA1L-2	HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN A1 PSEUDOGENE	8.68	8.42	0.83	0.0254
NM_001010.2	RPS6	RIBOSOMAL PROTEIN S6	12.41	12.15	0.83	0.0478
NM_080649.1	APEX1	APEX NUCLEASE	10.71	10.45	0.84	0.0428

APPENDIX TABLE 01 MONOCYTE GENE EXPRESSION DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

NM_203433.1	DSCR2	Down syndrome critical region gene 2	7.75	7.49	0.84	0.0099	
NM_001621.3	AHR	aryl hydrocarbon receptor	7.53	7.28	0.84	0.0350	
NM_144778.2	MBNL2	muscleblind-like 2	8.09	7.84	0.84	0.0079	
NM_005792.1	MPHOSPH6	M-phase phosphoprotein 6	6.75	6.50	0.84	0.0000	
NM_001280.1	CIRBP	cold inducible RNA binding protein	9.76	9.50	0.84	0.0241	
NM_138810.2	TAGAP	T-cell activation GTPase activating protein	8.10	7.85	0.84	0.0421	
NM_032592.1	PHACS	1-aminocyclopropane-1-carboxylate synthase	7.53	7.28	0.84	0.0368	
NM_005385.3	NKTR	natural killer-tumor recognition sequence	9.96	9.71	0.84	0.0206	
NM_001078645.1	CDC16	cell division cycle 16 homolog	10.58	10.33	0.84	0.0406	
NM_139343.1	BIN1	bridging integrator 1	7.14	6.90	0.84	0.0179	
NM_016818.2	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	6.71	6.46	0.84	0.0107	
NM_016006.3	ABHD5	abhydrolase domain containing 5	8.15	7.90	0.84	0.0202	
NM_016474.3	C3ORF19	chromosome 3 open reading frame 19	9.77	9.52	0.84	0.0459	
NM_015225.1	KIAA0367	KIAA0367	6.83	6.58	0.84	0.0273	
NM_016594.1	FKBP11	FK506 binding protein 11, 19 kDa	8.27	8.03	0.84	0.0107	
NM_021078.1	GCN5L2	GCN5 general control of amino-acid synthesis 5-like 2	8.96	8.71	0.84	0.0161	
NM_018032.3	LUC7L	LUC7-like (S. cerevisiae)	8.25	8.01	0.84	0.0115	
NM_003972.2	BTAF1	BTAF1 RNA polymerase II, B-TFIID transcription factor-associated, 170kDa	10.08	9.84	0.84	0.0253	
NM_004848.1	C1ORF38	chromosome 1 open reading frame 38	8.05	7.81	0.85	0.0070	
NM_006321.1	ARIH2	ariadne homolog 2	9.54	9.30	0.85	0.0313	
NM_015130.1	TBC1D9	TBC1 domain family, member 9	9.44	9.20	0.85	0.0263	
NM_012433.2	SF3B1	splicing factor 3b, subunit 1, 155kDa	10.70	10.46	0.85	0.0200	
NM_007079.2	PTP4A3	protein tyrosine phosphatase type IVA, member 3	6.93	6.69	0.85	0.0080	
NM_006779.2	CDC42EP2	CDC42 effector protein (Rho GTPase binding) 2	8.22	7.99	0.85	0.0235	
NM_001006623.1	WDR33	WD repeat domain 33	7.86	7.62	0.85	0.0288	
NM_001033853.1	RPL3	ribosomal protein L3	12.07	11.83	0.85	0.0411	
NM_004137.2	KCNMB1	potassium large conductance calcium-activated channel, subfamily M, beta member 1	9.67	9.44	0.85	0.0359	
NM_006469.4	IVNS1ABP	influenza virus NS1A binding protein	8.72	8.48	0.85	0.0315	
NM_024558.1	C14ORF138	chromosome 14 open reading frame 138	7.64	7.40	0.85	0.0368	
NM_153682.1	DSCR5	Down syndrome critical region gene 5	7.53	7.30	0.85	0.0267	
NM_004641.2	MLLT10	myeloid/lymphoid or mixed-lineage leukemia	8.82	8.59	0.85	0.0117	
NM_024692.3	RSNL2	CAP-GLY domain containing linker protein family, member 4	8.32	8.09	0.85	0.0209	
NM_016463.5	CXXC5	CXXC finger 5	8.48	8.25	0.86	0.0340	
NM_181873.1	MTMR11	myotubularin related protein 11	10.88	10.65	0.86	0.0399	
NM_172364.3	CACNA2D4	calcium channel, voltage-dependent, alpha 2/delta subunit 4	8.02	7.80	0.86	0.0318	
NM_000256.2	MYBPC3	myosin binding protein C	8.23	8.01	0.86	0.0347	
NM_002285.2	AFF3	AF4/FMR2 family, member 3	6.88	6.66	0.86	0.0035	

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TRANSCRIPT	SYMBOL	DEFINITION	CONTROLS	PATIENTS	FOLD CHANGE	P-VALUE
DOWNREGULATED			log intensity	log intensity		
NM_001441.1	FAAH	FATTY ACID AMIDE HYDROLASE	7.26	7.04	0.86	0.0234
NM_006874.2	ELF2	E74-LIKE FACTOR 2 (ETS DOMAIN TRANSCRIPTION FACTOR)	9.10	8.88	0.86	0.0427
NM_133173.2	APBB3	AMYLOID BETA (A4) PRECURSOR PROTEIN-BINDING, FAMILY B, MEMBER 3	8.66	8.44	0.86	0.0091
NM_005646.2	TARBP1	TAR (HIV-1) RNA BINDING PROTEIN 1	7.20	6.98	0.86	0.0122
NM_020472.1	PIGA	PHOSPHATIDYLINOSITOL GLYCAN, CLASS A	8.55	8.33	0.86	0.0446
NM_017426.2	NUP54	NUCLEOPORIN 54KDA	8.60	8.38	0.86	0.0416
NM_139353.1	TAF1C	TATA BOX BINDING PROTEIN (TBP)-ASSOCIATED FACTOR, RNA POLYMERASE I, C	10.08	9.87	0.86	0.0359
NM_001770.3	CD19	CD19 ANTIGEN	6.83	6.61	0.86	0.0477
NM_006469.4	IVNS1ABP	INFLUENZA VIRUS NS1A BINDING PROTEIN	8.95	8.74	0.86	0.0432
NM_015508.2	TIPARP	TCDD-INDUCIBLE POLY(ADP-RIBOSE) POLYMERASE	9.21	9.00	0.86	0.0478
NM_015151.2	DIP2A	DIP2 DISCO-INTERACTING PROTEIN 2 HOMOLOG A	7.53	7.32	0.86	0.0129
NM_016640.3	MRPS30	MITOCHONDRIAL RIBOSOMAL PROTEIN S30	8.94	8.73	0.86	0.0486
NM_001004307.1	MGC33556	HYPOTHETICAL LOC339541	7.91	7.70	0.86	0.0326
NM_016424.3	CROP	CISPLATIN RESISTANCE-ASSOCIATED OVEREXPRESSED PROTEIN	8.28	8.07	0.86	0.0333
NM_002161.2	IARS	ISOLEUCINE-TRNA SYNTHETASE	7.40	7.19	0.86	0.0263
NM_002896.1	RBM4	RNA BINDING MOTIF PROTEIN 4	9.38	9.17	0.86	0.0401
NM_002598.2	PDCD2	PROGRAMMED CELL DEATH 2	9.26	9.05	0.86	0.0395
NM_001017.2	RPS13	RIBOSOMAL PROTEIN S13	13.05	12.84	0.86	0.0439
NM_024610.3	HSPBAP1	HSPB (HEAT SHOCK 27KDA) ASSOCIATED PROTEIN 1	9.36	9.15	0.86	0.0169
NM_002983.1	CCL3	CHEMOKINE (C-C MOTIF) LIGAND 3	6.84	6.63	0.86	0.0270
NM_020666.2	CLK4	CDC-LIKE KINASE 4	8.19	7.98	0.87	0.0217
NM_024820.2	DENND1A	DENN/MADD DOMAIN CONTAINING 1A	8.35	8.14	0.87	0.0473
NM_004520.1	KIF2	KINESIN HEAVY CHAIN MEMBER 2A	7.62	7.41	0.87	0.0430
NM_001675.2	ATF4	ACTIVATING TRANSCRIPTION FACTOR 4	6.71	6.51	0.87	0.0018
NM_021178.2	CCNB1IP1	CYCLIN B1 INTERACTING PROTEIN 1	7.26	7.05	0.87	0.0200
NM_001023571.1	IQCB1	IQ MOTIF CONTAINING B1	8.18	7.98	0.87	0.0467
NM_003119.2	SPG7	SPASTIC PARAPLEGIA 7	10.46	10.26	0.87	0.0290
NM_006122.2	MAN2A2	MANNOSIDASE, ALPHA, CLASS 2A, MEMBER 2	7.77	7.57	0.87	0.0417
NM_020338.2	RAI17	ZINC FINGER, MIZ-TYPE CONTAINING 1	10.95	10.75	0.87	0.0496
NM_022828.2	YTHDC2	YTH DOMAIN CONTAINING 2	7.22	7.02	0.87	0.0049
NM_014412.2	CACYBP	CALCYCLIN BINDING PROTEIN	7.36	7.16	0.87	0.0166
NM_006051.2	APBB3	AMYLOID BETA (A4) PRECURSOR PROTEIN-BINDING, FAMILY B, MEMBER 3	9.53	9.33	0.87	0.0354
NM_139022.2	TSPAN32	TETRASPANIN 32	8.45	8.25	0.87	0.0446
NM_031284.3	ADPGK	ADP-DEPENDENT GLUCOKINASE	9.81	9.61	0.87	0.0262
NM_006447.2	USP16	UBIQUITIN SPECIFIC PEPTIDASE 16	8.98	8.78	0.87	0.0404

APPENDIX TABLE 01 MONOCYTE GENE EXPRESSION DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

NM_052869.1	TTYH2	TWEETY HOMOLOG 2	8.35	8.15	0.87	0.0372	
NM_019600.1	KIAA1370	KIAA1370	7.61	7.41	0.87	0.0152	
NM_198055.1	ZNF42	MYELOID ZINC FINGER 1	9.46	9.26	0.87	0.0297	
NM_003756.1	EIF3S3	EUKARYOTIC TRANSLATION INITIATION FACTOR 3, SUBUNIT 3 GAMMA, 40KDA	10.81	10.61	0.87	0.0480	
NM_024561.3	NARG1L	NMDA RECEPTOR REGULATED 1-LIKE	7.34	7.15	0.87	0.0099	
NM_001010909.1	C6ORF205	CHROMOSOME 6 OPEN READING FRAME 205	6.89	6.69	0.87	0.0031	
NM_001080498.1	EMR4	EGF-LIKE MODULE CONTAINING, MUCIN-LIKE, HORMONE RECEPTOR-LIKE 4	6.48	6.29	0.87	0.0172	
NM_002015.2	FOXO1A	FORKHEAD BOX O1A	8.43	8.23	0.87	0.0410	
NM_021209.3	CARD12	NLR FAMILY, CARD DOMAIN CONTAINING 4	8.89	8.69	0.87	0.0283	
NM_003633.1	ENC1	ECTODERMAL-NEURAL CORTEX	8.51	8.32	0.87	0.0357	
NR_002200.1	FTHL2	FERRITIN, HEAVY POLYPEPTIDE-LIKE 2	7.57	7.37	0.87	0.0463	
NM_001032287.1	NR2C1	NUCLEAR RECEPTOR SUBFAMILY 2, GROUP C, MEMBER 1	7.40	7.21	0.87	0.0186	
NM_006190.3	ORC2L	ORIGIN RECOGNITION COMPLEX, SUBUNIT 2-LIKE	7.66	7.47	0.88	0.0433	
NM_054016.1	FUSIP1	FUS INTERACTING PROTEIN (SERINE/ARGININE-RICH) 1	7.31	7.11	0.88	0.0127	
NM_002892.2	ARID4A	AT RICH INTERACTIVE DOMAIN 4A	8.02	7.83	0.88	0.0158	
NM_030948.1	PHACTR1	PHOSPHATASE AND ACTIN REGULATOR 1	6.72	6.53	0.88	0.0014	
NM_014773.2	KIAA0141	KIAA0141	8.90	8.72	0.88	0.0416	
NM_133436.1	ASNS	ASPARAGINE SYNTHETASE	7.19	7.00	0.88	0.0182	
NM_182627.1	WDR53	WD REPEAT DOMAIN 53	6.97	6.78	0.88	0.0121	
NM_013283.3	MAT2B	METHIONINE ADENOSYLTRANSFERASE II, BETA	6.81	6.63	0.88	0.0291	
NM_001277.2	CHKA	CHOLINE KINASE ALPHA	7.88	7.70	0.88	0.0415	
NM_001675.2	ATF4	ACTIVATING TRANSCRIPTION FACTOR 4	12.07	11.89	0.88	0.0404	
NM_080687.1	UPF3A	UPF3 REGULATOR OF NONSENSE TRANSCRIPTS HOMOLOG A	7.75	7.57	0.88	0.0423	
NM_032529.1	KIAA1875	KIAA1875	7.14	6.96	0.88	0.0279	
NM_020650.2	RCN3	RETICULOCALBIN 3, EF-HAND CALCIUM BINDING DOMAIN	7.55	7.37	0.88	0.0159	
NM_080632.1	UPF3B	UPF3 REGULATOR OF NONSENSE TRANSCRIPTS HOMOLOG B	7.86	7.68	0.88	0.0262	
NM_001031712.1	C6ORF75	CHROMOSOME 6 OPEN READING FRAME 75	7.07	6.89	0.88	0.0335	
NM_012411.2	PTPN22	PROTEIN TYROSINE PHOSPHATASE, NON-RECEPTOR TYPE 22	7.32	7.15	0.88	0.0369	
NM_002084.2	GPX3	GLUTATHIONE PEROXIDASE 3 (PLASMA)	6.79	6.61	0.88	0.0170	
NM_004075.2	CRY1	CRYPTOCHROME 1 (PHOTOLYASE-LIKE)	7.42	7.25	0.89	0.0156	
NM_002600.2	PDE4B	PHOSPHODIESTERASE 4B, CAMP-SPECIFIC	6.77	6.59	0.89	0.0128	
NM_012393.1	PFAS	PHOSPHORIBOSYLFORMYLGLYCINAMIDINE SYNTHASE	7.17	6.99	0.89	0.0415	
NM_005690.2	DNM1L	DYNAMIN 1-LIKE	7.33	7.16	0.89	0.0199	
NM_015654.3	NAT9	N-ACETYLTRANSFERASE 9	7.99	7.81	0.89	0.0489	
NM_017736.3	THUMPD1	THUMP DOMAIN CONTAINING 1	8.63	8.46	0.89	0.0314	
NM_001033577.1	ZNHIT3	ZINC FINGER, HIT TYPE 3	8.36	8.19	0.89	0.0412	
NM_002512.2	NME2	NON-METASTATIC CELLS 2	7.13	6.97	0.89	0.0330	

APPENDIX TABLE 01 MONOCYTE GENE EXPRESSION DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

TRANSCRIPT	SYMBOL	DEFINITION	CONTROLS	PATIENTS	FOLD CHANGE	P-VALUE
DOWNREGULATED			log intensity	log intensity		
NM_003353.2	UCN	UROCORTIN	6.57	6.40	0.89	0.0042
NM_001039649.1	ZMYM5	ZINC FINGER, MYM-TYPE 5	7.02	6.85	0.89	0.0119
NM_002182.2	IL1RAP	INTERLEUKIN 1 RECEPTOR ACCESSORY PROTEIN	6.94	6.77	0.89	0.0168
NM_213603.2	LOC285989	ZINC FINGER PROTEIN 789	7.33	7.17	0.89	0.0410
NM_024120.3	C20ORF7	CHROMOSOME 20 OPEN READING FRAME 7	6.86	6.70	0.89	0.0152
NM_182646.1	CPEB2	CYTOPLASMIC POLYADENYLATION ELEMENT BINDING PROTEIN 2	7.81	7.65	0.89	0.0342
NM_052926.1	PNMA5	PARANEOPLASTIC ANTIGEN LIKE 5	6.64	6.47	0.89	0.0077
NM_182686.1	KIAA0319L	KIAA0319-LIKE	7.02	6.86	0.90	0.0334
NM_020311.1	CMKOR1	CHEMOKINE ORPHAN RECEPTOR 1	6.61	6.46	0.90	0.0052
NM_018082.3	POLR3B	POLYMERASE (RNA) III (DNA DIRECTED) POLYPEPTIDE B	7.24	7.08	0.90	0.0336
NM_025249.1	KIAA1683	KIAA1683	6.68	6.52	0.90	0.0254
NM_006372.3	SYNCRIP	SYNAPTOTAGMIN BINDING, CYTOPLASMIC RNA INTERACTING PROTEIN	8.79	8.64	0.90	0.0448
NM_018590.3	GALNACT-2	CHONDROITIN SULFATE GALNACT-2	6.93	6.77	0.90	0.0395
NM_016389.2	IVNS1ABP	INFLUENZA VIRUS NS1A BINDING PROTEIN	6.71	6.55	0.90	0.0263
NM_025000.1	FLJ13096	HYPOTHETICAL PROTEIN	6.48	6.32	0.90	0.0017
NM_006575.3	MAP4K5	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE KINASE 5	6.48	6.33	0.90	0.0134
NM_001008401.1	FLJ16231	FLJ16231 PROTEIN	6.55	6.40	0.90	0.0199
NM_015326.2	SRGAP2	SLIT-ROBO RHO GTPASE ACTIVATING PROTEIN 2	6.70	6.55	0.90	0.0249
NM_013364.2	PNMA3	PARANEOPLASTIC ANTIGEN MA3	6.48	6.33	0.90	0.0098
NM_181701.2	QSCN6L1	QUIESCIN Q6-LIKE 1	7.30	7.15	0.90	0.0423
NM_001292.1	CLK3	CDC-LIKE KINASE 3	6.69	6.54	0.90	0.0128
NM_007362.2	NCBP2	NUCLEAR CAP BINDING PROTEIN SUBUNIT 2, 20KDA	7.55	7.40	0.90	0.0485
NM_015387.2	PREI3	PREIMPLANTATION PROTEIN 3	6.54	6.39	0.90	0.0023
NM_004733.2	SLC33A1	SOLUTE CARRIER FAMILY 33 (ACETYL-COA TRANSPORTER), MEMBER 1	6.77	6.62	0.90	0.0354
NM_001229.2	CASP9	CASPASE 9, APOPTOSIS-RELATED CYSTEINE PEPTIDASE	6.53	6.38	0.91	0.0027
NM_001029840.1	C3ORF23	CHROMOSOME 3 OPEN READING FRAME 23	6.60	6.46	0.91	0.0051
NM_001907.1	CTRL	CHYMOTRYPSIN-LIKE	7.13	6.98	0.91	0.0295
NM_002114.1	HIVEP1	HUMAN IMMUNODEFICIENCY VIRUS TYPE I ENHANCER BINDING PROTEIN 1	7.07	6.93	0.91	0.0377
NM_016437.1	TUBG2	TUBULIN, GAMMA 2	7.20	7.06	0.91	0.0276
NM_014970.2	KIFAP3	KINESIN-ASSOCIATED PROTEIN 3	6.98	6.84	0.91	0.0289
NM_000056.2	BCKDHB	BRANCHED CHAIN KETO ACID DEHYDROGENASE E1	6.53	6.40	0.91	0.0024
NM_018991.2	DKFZP434A0131	DKFZP434A0131 PROTEIN	6.45	6.31	0.91	0.0101
NM_030971.3	SFXN3	SIDEROFLEXIN 3	6.50	6.37	0.91	0.0027
NM_182926.1	KTN1	KINECTIN 1 (KINESIN RECEPTOR)	6.81	6.67	0.91	0.0353
NM_007045.2	FGFR1OP	FGFR1 ONCOGENE PARTNER	6.84	6.70	0.91	0.0421

APPENDIX TABLE 01 MONOCYTE GENE EXPRESSION DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

NR_002448.1	SNORD36A	SMALL NUCLEOLAR RNA, C/D BOX 36A	6.68	6.55	0.91	0.0055
NM_172107.1	KCNQ2	POTASSIUM VOLTAGE-GATED CHANNEL, KQT-LIKE SUBFAMILY, MEMBER 2	6.62	6.49	0.91	0.0230
NM_015419.1	MXRA5	MATRIX-REMODELLING ASSOCIATED 5	6.64	6.50	0.91	0.0135
NM_198460.1	GBP6	GUANYLATE BINDING PROTEIN FAMILY, MEMBER 6	6.47	6.34	0.91	0.0121
NM_001032282.1	KLF10	KRUPPEL-LIKE FACTOR 10	6.73	6.60	0.91	0.0340
NM_033402.2	LRRCC1	LEUCINE RICH REPEAT AND COILED-COIL DOMAIN CONTAINING 1	6.53	6.40	0.91	0.0043
NM_005698.2	SCAMP3	SECRETORY CARRIER MEMBRANE PROTEIN 3	6.64	6.51	0.91	0.0182
NM_023000.2	ARID4A	AT RICH INTERACTIVE DOMAIN 4A (RBP1-LIKE)	6.73	6.60	0.91	0.0327
NM_002814.2	PSMD10	PROTEASOME (PROSOME, MACROPAIN) 26S SUBUNIT, NON-ATPASE, 10	6.89	6.77	0.92	0.0482
NM_001951.2	E2F5	E2F TRANSCRIPTION FACTOR 5, P130-BINDING	6.58	6.45	0.92	0.0086
NM_152221.2	CSNK1E	CASEIN KINASE 1, EPSILON	6.59	6.46	0.92	0.0167
NM_007214.3	SEC63	SEC63 HOMOLOG	6.64	6.52	0.92	0.0128
NM_003035.2	STIL	SCL/TAL1 INTERRUPTING LOCUS	6.63	6.51	0.92	0.0252
NM_006996.1	SLC19A2	SOLUTE CARRIER FAMILY 19 (THIAMINE TRANSPORTER), MEMBER 2	6.50	6.37	0.92	0.0045
NM_016488.5	PPHLN1	PERIPHILIN 1	6.78	6.66	0.92	0.0297
NM_052963.1	TOP1MT	TOPOISOMERASE (DNA) I, MITOCHONDRIAL	6.89	6.76	0.92	0.0492
NM_030965.1	ST6GALNAC5	ST6-N-ACETYLGALACTOSAMINIDE ALPHA-2,6-SIALYLTRANSFERASE 5	6.56	6.44	0.92	0.0242
NM_014245.2	RNF7	RING FINGER PROTEIN 7	6.55	6.42	0.92	0.0209
NM_006080.1	SEMA3A	SEMA DOMAIN, IMMUNOGLOBULIN DOMAIN (IG), SHORT BASIC DOMAIN, 3A	6.66	6.54	0.92	0.0138
NM_017984.2	ZCWPW1	ZINC FINGER, CW TYPE WITH PWWP DOMAIN 1	6.65	6.53	0.92	0.0206
NM_006813.1	PNRC1	PROLINE-RICH NUCLEAR RECEPTOR COACTIVATOR 1	6.63	6.52	0.92	0.0229
NM_015316.1	PPP1R13B	PROTEIN PHOSPHATASE 1, REGULATORY (INHIBITOR) SUBUNIT 13B	6.65	6.53	0.92	0.0331
NM_006205.1	PDE6H	PHOSPHODIESTERASE 6H, CGMP-SPECIFIC, CONE, GAMMA	6.51	6.39	0.92	0.0199
NM_032207.1	FLJ21742	HYPOTHETICAL PROTEIN	6.71	6.60	0.92	0.0389
NM_005371.3	METTL1	METHYLTRANSFERASE LIKE 1	6.62	6.51	0.93	0.0457
NM_021777.2	ADAM28	ADAM METALLOPEPTIDASE DOMAIN 28	6.51	6.40	0.93	0.0370
NM_198149.1	TMEM58	TRANSMEMBRANE PROTEIN 58	6.49	6.38	0.93	0.0095
NM_182966.1	NEDD9	NEURAL PRECURSOR CELL EXPRESSED, DEVELOPMENTALLY DOWN-REGULATED 9	6.51	6.40	0.93	0.0495
NM_001042610.1	DBNDD1	DYSBINDIN (DYSTROBREVIN BINDING PROTEIN 1) DOMAIN CONTAINING 1	6.60	6.49	0.93	0.0358
NM_080680.1	COL11A2	COLLAGEN, TYPE XI, ALPHA 2	6.58	6.47	0.93	0.0306
NM_005995.2	TBX10	T-BOX 10	6.55	6.44	0.93	0.0438
NM_000864.3	HTR1D	5-HYDROXYTRYPTAMINE (SEROTONIN) RECEPTOR 1D	6.53	6.42	0.93	0.0213
NM_203411.1	TMEM88	TRANSMEMBRANE PROTEIN 88	6.61	6.51	0.93	0.0347
NM_173803.2	FLJ39599	MPV17 MITOCHONDRIAL MEMBRANE PROTEIN-LIKE	6.59	6.49	0.93	0.0464
NM_001010870.1	TDRD6	TUDOR DOMAIN CONTAINING 6	6.63	6.53	0.93	0.0392
NM_021777.2	ADAM28	ADAM METALLOPEPTIDASE DOMAIN 28	6.45	6.35	0.94	0.0362
NM_001018116.1	LOC347273	SIMILAR TO RIKEN CDNA 2310039E09	6.53	6.44	0.94	0.0159

APPENDIX TABLE 01 MONOCYTE GENE EXPRESSION DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

TRANSCRIPT	SYMBOL	DEFINITION	CONTROLS	PATIENTS	FOLD CHANGE	P-VALUE
DOWNREGULATED			log intensity	log intensity		
NM_153605.2	DKFZP667G2110	HYPOTHETICAL PROTEIN	6.46	6.37	0.94	0.0461
NM_001009957.1	ZNF655	ZINC FINGER PROTEIN 655	6.51	6.42	0.94	0.0493
NM_001080829.1	LOC390688	CDC37-LIKE	6.46	6.37	0.94	0.0454
NM_033118.2	MYLK2	MYOSIN LIGHT CHAIN KINASE 2, SKELETAL MUSCLE	6.44	6.36	0.94	0.0428
XM_926644.1	LOC643298	PREDICTED: SIMILAR TO THYROID HORMONE RECEPTOR-ASSOCIATED PROTEIN COMPLEX	6.49	6.41	0.94	0.0485

APPENDIX TABLE 02 CARDIORENAL CONNECTOR MONOCYTE GENE EXPRESSION PANEL: DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

INFLAMMATION RELATED GENES

SYMBOL	FOLD CHANGE	P-VALUE
IL10	0.91	0.369
IL10RA	0.91	0.222
IL10RB	1.15	0.044
IL12A	not expressed	not expressed
IL12RB1	1.10	0.006
IL13RA1	1.15	0.037
IL17C	1.03	0.471
IL17D	1.04	0.404
IL17E	not expressed	not expressed
IL17R	1.19	0.012
IL1B	0.82	0.080
IL1F7	0.96	0.664
IL1F8	not expressed	not expressed
IL1R2	0.83	0.044
IL1RAP	0.89	0.017
IL1RL1	0.97	0.382
IL1RN	0.94	0.052
IL21	not expressed	not expressed
IL21R	1.10	0.066
IL27	1.03	0.472
IL27RA	1.22	0.001
IL2RG	not expressed	not expressed
IL4I1	not expressed	not expressed
IL4R	1.12	0.151
IL5RA	1.03	0.587
IL6	not expressed	not expressed
IL6R	0.92	0.317
IL8	0.58	0.001
IL8RB	0.87	0.079
IFNA10	1.06	0.577
IFNA14	not expressed	not expressed
IFNA21	1.01	0.824
IFNA8	1.00	0.945
IFNAR1	1.06	0.530
IFNAR2	1.07	0.410
IFNGR1	1.10	0.123
IFNGR2	1.08	0.298
TNF	1.06	0.360
CCL19	0.97	0.419
CCL2	0.77	0.008
CCL21	not expressed	not expressed
CCL23	1.02	0.618
CCL25	1.03	0.368
CCL26	0.99	0.813
CCL27	not expressed	not expressed
CCL28	not expressed	not expressed
CCL3	0.86	0.027
CCL3L1	not expressed	not expressed
CCL3L3	0.77	0.019
CCL4L1	not expressed	not expressed
CCL5	0.98	0.870
CCL8	0.89	0.097
CXCL10	0.94	0.506
CXCL12	not expressed	not expressed
CXCL14	0.98	0.680
CXCL16	0.90	0.218
CXCL9	not expressed	not expressed
CXCR4	0.90	0.221
CCR1	1.09	0.206
CCR10	not expressed	not expressed
CCR2	1.11	0.274
CCR6	1.02	0.791
CCR7	not expressed	not expressed
CX3CR1	1.49	0.0002
CCXCR1	not expressed	not expressed
TLR1	1.05	0.615
TLR10	not expressed	not expressed
TLR2	1.16	0.015
TLR4	0.99	0.864
TLR5	0.97	0.741
TLR6	1.09	0.021
TLR7	1.20	0.040
TLR8	1.03	0.407
TLR9	not expressed	not expressed
IRF1	1.00	0.988
IRF3	1.04	0.536
IRF4	1.02	0.798
IRF5	1.44	0.051
IRF7	1.09	0.359
IRF8	1.11	0.136

APPENDIX TABLE 02 CARDIORENAL CONNECTOR MONOCYTE GENE EXPRESSION PANEL: DIFFERENCES IN CRS PATIENTS AT BASELINE (N=18) COMPARED TO HEALTHY CONTROLS (N=12)

OXIDATIVE STRESS RELATED GENES

SYMBOL	FOLD CHANGE	P-VALUE
GPX1	0.93	0.119
GPX1	1.12	0.175
GPX2	1.02	0.470
GPX3	0.88	0.017
GPX4	0.98	0.747
GPX4	1.01	0.940
GPX6	1.01	0.741
GPX7	0.95	0.380
PRDX1	0.98	0.756
PRDX1	0.98	0.862
PRDX2	0.89	0.167
PRDX3	0.85	0.061
PRDX4	1.04	0.548
PRDX5	0.91	0.277
PRDX5	0.98	0.776
PRDX5	1.02	0.779
PRDX6	0.99	0.916
TXNRD1	1.05	0.390
TXNRD1	0.99	0.901
TXNRD2	1.04	0.618
SRXN1	1.01	0.897
SELS	0.94	0.060
DHRS9	1.33	0.006
GSR	0.97	0.356
SOD1	1.02	0.800
SOD2	0.93	0.213
SOD2	0.95	0.582
SOD2	0.99	0.901
CCS	0.92	0.182
CCS	1.06	0.356
CYBA	1.08	0.167
DUOX1	1.01	0.752
NCF1	0.93	0.376
NCF2	1.00	0.964
NOS3	0.98	0.372
NOX4	0.99	0.719
NOX5	1.05	0.052
PREX1	1.09	0.212
ALOX15	0.98	0.458
ALOX15B	0.93	0.084
ALOX15B	0.97	0.401
ALOX15B	0.99	0.832
ALOX5	1.03	0.322
ALOX5	1.04	0.634
ALOX5AP	1.11	0.283
ALOXE3	1.01	0.741
DUSP1	0.68	0.003
MPO	0.92	0.175

RENIN ANGIOTENSIN SYSTEM RELATED GENES

SYMBOL	FOLD CHANGE	P-VALUE
AGTR1	not expressed	not expressed
AGT	not expressed	not expressed
REN	0.99	0.809
ACE	not expressed	not expressed
RENBP	1.18	0.052

APPENDIX TABLE 03 MONOCYTE GENE EXPRESSION CHANGES IN CRS PATIENTS (N=12) AFTER 2 WEEKS OF EPO TREATMENT

TRANSCRIPT	SYMBOL	DEFINITION	PATIENTS AT BASELINE	PATIENTS AFTER 2WK EPO	FOLD CHANGE	P-VALUE
UPREGULATED			log mean intensity	log mean intensity		
NM_000518.4	HBB	HEMOGLOBIN, BETA	9.78	11.59	3.50	0.0043
NM_000517.3	HBA2	HEMOGLOBIN, ALPHA 2	8.50	9.97	2.76	0.0097
NM_015208.2	ANKRD12	ANKYRIN REPEAT DOMAIN 12	8.31	8.56	1.19	0.0313
NM_002295.4	RPSA	RIBOSOMAL PROTEIN SA	12.78	13.02	1.18	0.0270
NM_016075.2	VPS36	VACUOLAR PROTEIN SORTING 36 HOMOLOG	8.86	9.08	1.16	0.0444
NM_001014451.1	DDX19B	DEAD (ASP-GLU-ALA-AS) BOX POLYPEPTIDE 19B	9.25	9.46	1.16	0.0438
NM_014138.3	TMEM29	TRANSMEMBRANE PROTEIN 29	8.66	8.87	1.16	0.0473
NM_015336.1	ZDHHC17	ZINC FINGER, DHHC-TYPE CONTAINING 17	8.23	8.44	1.15	0.0499
NM_018494.3	LRDD	LEUCINE-RICH REPEATS AND DEATH DOMAIN CONTAINING	6.76	6.95	1.14	0.0113
NM_020449.1	THOC2	THO COMPLEX 2	7.14	7.32	1.14	0.0472
NM_005065.3	SEL1L	SEL-1 SUPPRESSOR OF LIN-12-LIKE	6.53	6.71	1.13	0.0044
NM_012460.2	TIMM9	TRANSLOCASE OF INNER MITOCHONDRIAL MEMBRANE 9 HOMOLOG	6.85	7.03	1.13	0.0335
NM_004075.2	CRY1	CRYPTOCHROME 1	7.22	7.39	1.12	0.0417
NM_018074.3	FLJ10374	HYPOTHETICAL PROTEIN	6.37	6.53	1.12	0.0099
NM_012329.2	MMD	MONOCYTE TO MACROPHAGE DIFFERENTIATION-ASSOCIATED	6.82	6.98	1.12	0.0359
NM_024906.1	SCD5	STEAROYL-COA DESATURASE 5	6.69	6.85	1.12	0.0179
NM_017709.2	FAM46C	FAMILY WITH SEQUENCE SIMILARITY 46, MEMBER C	6.42	6.56	1.10	0.0441
NM_017866.4	TMEM70	TRANSMEMBRANE PROTEIN 70	6.48	6.62	1.10	0.0339
NM_152221.2	CSNK1E	CASEIN KINASE 1, EPSILON	6.43	6.56	1.10	0.0192
NM_000645.2	AGL	AMYLO-1, 6-GLUCOSIDASE, 4-ALPHA-GLUCANOTRANSFERASE	6.76	6.89	1.09	0.0389
NM_014915.1	ANKRD26	ANKYRIN REPEAT DOMAIN 26	6.41	6.54	1.09	0.0442
NM_054111.2	IHPK3	INOSITOL HEXAPHOSPHATE KINASE 3	6.43	6.55	1.09	0.0358
NM_138334.1	JOSD2	JOSEPHIN DOMAIN CONTAINING 2	6.73	6.85	1.09	0.0478
NM_006444.1	SMC2L1	SMC2 STRUCTURAL MAINTENANCE OF CHROMOSOMES 2-LIKE 1	6.43	6.55	1.09	0.0139
NM_024818.2	UBE1DC1	UBIQUITIN-ACTIVATING ENZYME E1-DOMAIN CONTAINING 1	6.45	6.57	1.09	0.0467
NM_130443.1	DPP3	DIPEPTIDYLPEPTIDASE 3	6.37	6.49	1.09	0.0430
NM_198148.1	CPXM2	CARBOXYPEPTIDASE X (M14 FAMILY), MEMBER 2	6.47	6.58	1.08	0.0443
NM_001519.2	BRF1	BRF1 HOMOLOG, SUBUNIT OF RNA POLYMERASE III TRANSCRIPTION INITIATION FACTOR IIIB	6.47	6.59	1.08	0.0389
NM_005670.3	EPM2A	EPILEPSY, PROGRESSIVE MYOCLONUS TYPE 2A, LAFORA DISEASE (LAFORIN)	6.43	6.54	1.08	0.0216
NM_012263.4	TTLL1	TUBULIN TYROSINE LIGASE-LIKE FAMILY, MEMBER 1	6.41	6.50	1.07	0.0466
NM_152602.1	ZNF433	ZINC FINGER PROTEIN 433	6.39	6.47	1.06	0.0493

APPENDIX TABLE 03 MONOCYTE GENE EXPRESSION CHANGES IN CRS PATIENTS (N=12) AFTER 2 WEEKS OF EPO TREATMENT

TRANSCRIPT	SYMBOL	DEFINITION	PATIENTS AT BASELINE	PATIENTS AFTER 2WK EPO	FOLD CHANGE	P-VALUE
DOWNREGULATED			log mean intensity	log mean intensity		
NM_001042771.1	LCK	LYMPHOCYTE-SPECIFIC PROTEIN TYROSINE KINASE	6.93	6.76	0.89	0.0243
NM_130459.1	TOR2A	TORSIN FAMILY 2, MEMBER A	6.87	6.72	0.90	0.0340
NM_007028.3	TRIM31	TRIPARTITE MOTIF-CONTAINING 31	6.73	6.58	0.90	0.0134
NM_198843.1	SFTPB	SURFACTANT, PULMONARY-ASSOCIATED PROTEIN B	6.59	6.44	0.91	0.0118
NM_139248.2	LIPH	LIPASE, MEMBER H	6.55	6.41	0.91	0.0319
NM_001042478.1	AJAP1	ADHERENS JUNCTION ASSOCIATED PROTEIN 1	6.79	6.65	0.91	0.0317
NM_006829.2	C10ORF116	CHROMOSOME 10 OPEN READING FRAME 116	6.81	6.69	0.92	0.0476
NM_018706.4	DHTKD1	DEHYDROGENASE E1 AND TRANSKETOLASE DOMAIN CONTAINING 1	6.59	6.47	0.92	0.0412
NM_173174.1	PTK2B	PTK2B PROTEIN TYROSINE KINASE 2 BETA	6.57	6.46	0.92	0.0189
XM_942851.1	CHIC1	PREDICTED: CYSTEINE-RICH HYDROPHOBIC DOMAIN 1, TRANSCRIPT VARIANT 6	6.57	6.45	0.92	0.0283
NM_001001342.1	BLOC1S2	BIOGENESIS OF LYSOSOME-RELATED ORGANELLES COMPLEX-1, SUBUNIT 26.64		6.53	0.93	0.0347
NM_194463.1	RNF128	RING FINGER PROTEIN 128	6.58	6.47	0.93	0.0350
NM_176795.2	HRAS	V-HA-RAS HARVEY RAT SARCOMA VIRAL ONCOGENE HOMOLOG	6.49	6.39	0.93	0.0240
NM_001080451.1	SERPINA11	SERPIN PEPTIDASE INHIBITOR, CLADE A	6.49	6.39	0.93	0.0335

APPENDIX TABLE 04 CARDIORENAL CONNECTOR MONOCYTE GENE EXPRESSION PANEL: CHANGES IN CRS PATIENTS (N=12) AFTER 2 WEEKS OF EPO TREATMENT

INFLAMMATION RELATED GENES								
SYMBOL	**FOLD CHANGE**	**P-VALUE**	**SYMBOL**	**FOLD CHANGE**	**P-VALUE**	**SYMBOL**	**FOLD CHANGE**	**P-VALUE**
IL10	1.06	0.580	CCL19	1.01	0.741	IRF4	0.99	0.900
IL10RA	0.96	0.654	CCL2	1.07	0.332	IRF5	0.96	0.790
IL10RB	1.02	0.750	CCL21	not expressed	not expressed	IRF7	1.03	0.390
IL12RB1	0.95	0.180	CCL23	0.96	0.463	IRF8	0.97	0.627
IL13RA1	0.98	0.804	CCL25	0.98	0.496	CD40	0.99	0.788
IL17C	0.98	0.741	CCL26	1.01	0.879	CD40LG	not expressed	not expressed
IL17D	0.96	0.491	CCL27	not expressed	not expressed			
IL17E	not expressed	not expressed	CCL3	1.02	0.740			
IL17R	1.01	0.915	CCL3L1	not expressed	not expressed			
IL1F7	0.99	0.959	CCL3L3	1.05	0.554			
IL1R2	0.95	0.588	CCL5	1.26	0.236			
IL1RAP	0.99	0.794	CCL8	1.07	0.330			
IL1RL1	not expressed	not expressed	CXCL10	1.10	0.318			
IL1RN	0.98	0.862	CXCL12	not expressed	not expressed			
IL21	not expressed	not expressed	CXCL14	0.97	0.594			
IL21R	0.97	0.314	CXCL16	0.94	0.431			
IL27	0.92	0.110	CXCL9	not expressed	not expressed			
IL27RA	0.94	0.353	CXCR4	0.93	0.368			
IL2RG	not expressed	not expressed	CCR1	0.97	0.691			
IL4I1	not expressed	not expressed	CCR10	not expressed	not expressed			
IL4R	0.98	0.761	CCR2	1.02	0.840			
IL5RA	1.04	0.231	CCR6	1.01	0.908			
IL6	not expressed	not expressed	CX3CR1	1.09	0.338			
IL6R	0.94	0.521	CCXCR1	not expressed	not expressed			
IL8	0.97	0.850	TLR1	0.98	0.879			
IL8RB	0.98	0.858	TLR10	0.97	0.300			
IFNA10	1.02	0.872	TLR2	0.99	0.928			
IFNA14	not expressed	not expressed	TLR4	1.08	0.292			
IFNA21	0.98	0.537	TLR5	1.04	0.729			
IFNA8	0.98	0.762	TLR6	0.95	0.269			
IFNAR1	0.95	0.574	TLR7	1.06	0.479			
IFNAR2	0.95	0.793	TLR8	1.09	0.382			
IFNGR1	1.03	0.727	TLR9	not expressed	not expressed			
IFNGR2	1.04	0.597	IRF1	0.96	0.640			
TNF	0.98	0.788	IRF3	0.97	0.653			

APPENDIX TABLE 04 CARDIORENAL CONNECTOR MONOCYTE GENE EXPRESSION PANEL: CHANGES IN CRS PATIENTS (N=12) AFTER 2 WEEKS OF EPO TREATMENT

OXIDATIVE STRESS RELATED GENES

SYMBOL	FOLD CHANGE	P-VALUE
GPX1	0.94	0.495
GPX1	1.03	0.517
GPX2	1.01	0.735
GPX3	0.99	0.872
GPX4	0.95	0.604
GPX4	0.99	0.909
GPX6	0.96	0.264
GPX7	1.01	0.883
PRDX1	0.96	0.633
PRDX1	1.01	0.939
PRDX2	1.01	0.873
PRDX3	1.10	0.340
PRDX4	0.99	0.887
PRDX5	1.10	0.278
PRDX5	1.03	0.687
PRDX5	1.02	0.785
PRDX6	0.99	0.893
TXNRD1	0.94	0.441
TXNRD1	0.98	0.712
TXNRD2	0.97	0.696
SRXN1	0.99	0.928
SELS	1.07	0.370
DHRS9	1.04	0.224
GSR	1.02	0.734
SOD1	0.93	0.295
SOD2	1.03	0.587
SOD2	1.03	0.783
SOD2	0.99	0.919
CCS	1.03	0.717
CCS	0.98	0.759
CYBA	0.99	0.900
DUOX1	0.98	0.462
NCF1	1.01	0.909
NCF2	0.98	0.883
NOX4	not expressed	not expressed
NOX5	not expressed	not expressed
PREX1	0.92	0.310
ALOX15	not expressed	not expressed
ALOX15B	0.96	0.146
ALOX15B	1.02	0.692
ALOX5	0.96	0.640
ALOX5	0.99	0.843
ALOX5AP	0.95	0.629
ALOXE3	0.94	0.095
DUSP1	1.00	0.988
MPO	1.04	0.501

RENIN ANGIOTENSIN SYSTEM RELATED GENES

SYMBOL	FOLD CHANGE	P-VALUE
AGTR1	not expressed	not expressed
AGT	not expressed	not expressed
REN	not expressed	not expressed
ACE	not expressed	not expressed
RENBP	1.02	0.807

NEDERLANDSE SAMENVATTING

ACHTERGROND

CHRONISCH NIERFALEN VERHOOGT HET RISICO OP HART- EN VAATZIEKTEN

Chronisch nierfalen is wereldwijd een veel en steeds vaker voorkomend probleem. Wanneer de functie van de nieren erg is afgenomen kunnen patiënten afhankelijk worden van nierfunctievervangende therapie, zoals hemodialyse. Patiënten met chronisch nierfalen hebben daarnaast een opvallend hoog risico op hart- en vaatziekten, wat hun belangrijkste doodsoorzaak vormt. Deze hart- en vaatziekten omvatten o.a. hartinfarct, herseninfarct en hartfalen en worden voornamelijk veroorzaakt door slagaderverkalking, ook wel atherosclerose genoemd.

Een van de eerste stappen in de ontwikkeling van atherosclerose is dat er schade optreedt van de binnenste cellaag van het bloedvat, het endotheel. Deze schade kan veroorzaakt worden door bijv. roken of een hoge bloeddruk. Er vindt een ontstekingsproces plaats: componenten uit het bloed, zoals cholesterol en ontstekingscellen (o.a. **monocyten**), infiltreren in de vaatwand en als reactie hierop vindt er een ophoping plaats van gladde spiercellen.

> *<u>Monocyten:</u> witte bloedcellen die een belangrijk deel uitmaken van het immuunsysteem in het menselijk lichaam.*
> *Monocyten zijn tevens betrokken bij de ontwikkeling van atherosclerose.*

Dit proces leidt uiteindelijk tot een verdikking van de wand, de atherosclerotische plaque, die het bloedvat geleidelijk kan vernauwen of zelfs helemaal afsluiten.

De plaque kan ook scheuren waardoor de inhoud ervan vrijkomt en elders in het vat een verstopping kan geven. Bij nierpatiënten treedt atherosclerose frequent op door verhoogde aanwezigheid van risicofactoren zoals hoge bloeddruk, suikerziekte en hogere leeftijd. Verder leidt de verminderde nierfunctie zelf tot meer ontsteking (inflammatie) en vrije radicalen (oxidatieve stress), die endotheelschade kunnen veroorzaken. Het behoud en herstel van de integriteit van het endotheel is essentieel in het voorkomen van het ontstaan en/of verergeren van atherosclerose.

GECOMBINEERD CHRONISCH HART- EN NIERFALEN

Patiënten met chronisch nierfalen ontwikkelen dus vaker chronisch hartfalen. Vice versa hebben patiënten met chronisch hartfalen vaak een verminderde nierfunctie. Deze frequent voorkomende combinatie van hart- en nierfalen wordt ook wel het *cardiorenaal syndroom (CRS)* genoemd. Het falen van zowel hart als nieren verergert door het gecombineerd voorkomen van de twee. CRS patiënten hebben dan ook een hoog risico op sterfte.

Het is niet volledig duidelijk hoe het komt dat hart- en nierfalen vaak samen voorkomen en elkaar verergeren. Veranderingen in o.a. de vaatweerstand en bloeddruk spelen wellicht een rol. Daarnaast zijn er 4 factoren geïdentificeerd die verstoord zijn in zowel hart- als nierfalen. Dit zijn verhoogde aanwezigheid van inflammatie (1) en oxidatieve stress (2) en een versterkte activatie van het bloeddrukregulerende renine angiotensine systeem (3) en sympathisch zenuwstelsel (4). Een verstoring van één factor kan resulteren in verstoring van de andere factoren: een vicieuze cirkel ontstaat wat kan bijdragen aan de verergering van CRS.

Vaak gaat CRS gepaard met bloedarmoede, omdat het lichaam ongevoelig is geworden voor het hormoon **erythropoietine** (EPO), of omdat EPO onvoldoende wordt gemaakt door de zieke nieren.

> *<u>Erythropoietine:</u> hormoon dat voornamelijk door de nier wordt geproduceerd en de aanmaak van rode bloedcellen in het beenmerg stimuleert (een hematopoietisch effect).*
> *De stof kan synthetisch worden geproduceerd en wordt als medicijn gebruikt voor bijv. nierpatiënten die bloedarmoede hebben doordat de nieren te weinig erythropoietine produceren.*

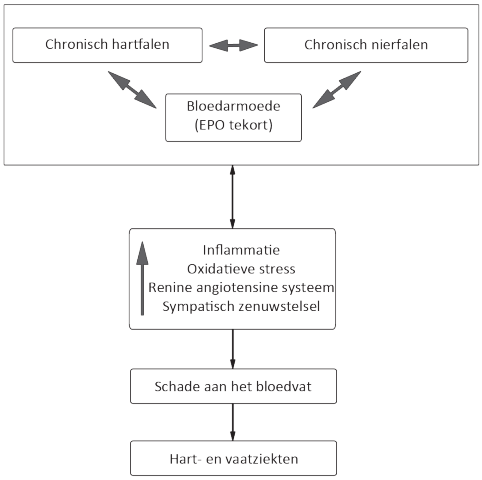
De bloedarmoede is geassocieerd met een verergering van hart- en nierfalen en met een slechtere prognose voor overleving.

In figuur 01 wordt het CRS en de gevolgen hiervan schematisch weergegeven.

EFFECTEN VAN EPO BEHANDELING BIJ PATIËNTEN MET CRS

Aangezien bloedarmoede met hogere sterfte is geassocieerd in chronisch hart- en nierfalen, heeft men onderzoek gedaan naar het effect van EPO therapie. Verschillende kleine studies lieten een positief effect zien op hart- en nierfunctie, maar grotere studies lieten geen of zelfs een nadelig effect zien na correctie van de bloedarmoede met EPO. Het is onduidelijk wat de werking van EPO precies is en hoe deze uiteenlopende studieresultaten te verklaren zijn. Er zijn

FIGUUR 01 HET CARDIORENAAL SYNDROOM RESULTEERT IN EEN HOOG RISICO OP OVERLIJDEN AAN HART- EN VAATZIEKTEN



aanwijzingen dat EPO niet alleen de aanmaak van rode bloedcellen stimuleert (hematopoietische effecten), maar ook andere effecten kan hebben gericht tegen bijv. inflammatie en oxidatieve stress (niet-hematopoietische effecten).

CIRCULERENDE CELLEN IN HART- EN NIERFALEN

In dit proefschrift ligt de focus op 2 typen cellen die in het bloed circuleren en een belangrijke rol spelen in hart- en nierfalen: monocyten en uit het beenmerg afkomstige voorlopercellen.

Monocyten zijn nauw betrokken in inflammatoire processen en dragen zo bij aan de progressie van falen van hart en/of nier en aan de ontwikkeling en verergering van atherosclerose. Deze cellen kunnen dus worden gezien als belangrijke spelers in weefsel- en vaatschade. Omdat monocyten door de bloedbaan circuleren worden zij voortdurend blootgesteld aan hun omgeving, het intern systemisch milieu. Veranderingen in dit intern milieu, bijv. een verhoging van inflammatoire factoren, kunnen leiden tot aanpassingen of reacties in monocyten.

Om het gedrag van monocyten te bestuderen kan gebruik worden gemaakt van **genexpressie analyse**. Eerder onderzoek heeft laten zien dat je met behulp van genexpressie analyse ziektegerelateerde veranderingen kan detecteren. Bovendien kan de methode gebruikt worden om effecten van behandeling te bestuderen.

> *Genexpressie analyse:* Een gen is een stukje van het DNA dat codeert voor een eiwit met een bepaalde functie. Hoe meer van het gen wordt afgeschreven ('tot expressie komt'), hoe meer nieuw eiwit kan worden gevormd.
> Er zijn meerdere methoden om genexpressie te meten. De microarray techniek is een globale methode die genexpressie van wel 25.000 genen tegelijk kan gemeten, terwijl de RT-PCR techniek de expressie per gen bepaalt.

Een voorbeeld hiervan is dat in patiënten met een hoge bloeddruk bepaalde genen verhoogd tot expressie komen, wat na behandeling met medicatie weer normaliseert. In dit proefschrift wordt de *microarray techniek* (zie kader) gebruikt om meer inzicht te krijgen in CRS gerelateerde genexpressie veranderingen in monocyten en om het effect van EPO behandeling in deze CRS patiënten te bestuderen.

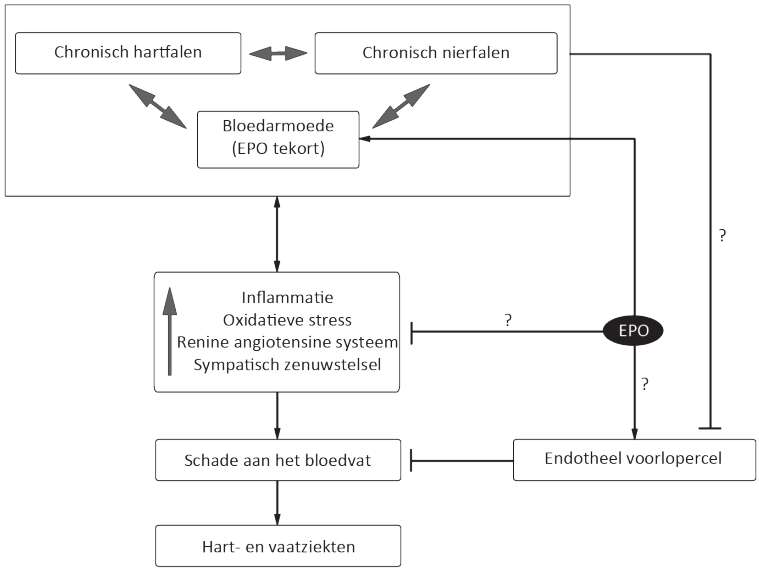
Uit het beenmerg afkomstige endotheel voorloper cellen (EPC) spelen een belangrijke rol in het herstel van endotheelschade. Door signalen afkomstig van beschadigde vaten of weefsel worden EPC uit het beenmerg gemobiliseerd om vervolgens op de plaats van schade uit te rijpen tot endotheelcel. Daarnaast kunnen EPC via uitscheiding van factoren endotheelcellen ter plekke van de schade stimuleren om het vat te herstellen. Eerder onderzoek heeft aangetoond dat lagere EPC aantallen gerelateerd zijn met een verminderde endotheelfunctie, het eerste stadium van atherosclerose. Lage EPC aantallen bleken dan ook een voorspellende waarde te hebben voor het optreden van hart- en vaatziekten en sterfte. Het is bekend dat volwassenen met eindstadium chronisch nierfalen verlaagde EPC aantallen hebben, die ook minder goed functioneren, wat kan bijdragen aan hun verhoogde risico op hart- en vaatziekten. In dit proefschrift bestuderen wij EPC in andere hoog risico groepen, zoals de ouder wordende mens, kinderen met chronisch nierfalen, volwassen patiënten met chronisch nierfalen in de predialyse fase en CRS patiënten.

Naast voorlopercellen voor endotheel circuleren er ook beenmergafkomstige voorlopercellen die kunnen uitgroeien tot gladde spiercellen in de vaatwand. Deze

gladde spier voorlopercellen (SPC) zijn mogelijk juist ongunstig omdat zij het ontwikkelen van atherosclerose kunnen bevorderen. In hemodialyse patiënten is de balans tussen de beschermende EPC en de tot atherosclerose bijdragende SPC verschoven richting SPC. Het is nog niet duidelijk hoe deze voorlopercellen zich in predialyse patiënten presenteren.

De ontdekking van EPC en hun capaciteit om vaatschade te herstellen biedt nieuwe therapeutische mogelijkheden. Een mogelijke factor die het aantal EPCs en hun functie zou kunnen verbeteren is EPO. Verschillende experimentele modellen hebben laten zien dat EPO mobilisatie van EPC van het beenmerg naar de bloedbaan kan stimuleren. In figuur 02 wordt een overzicht gegeven van mogelijke effecten van EPO bij CRS patiënten.

FIGUUR 02 MOGELIJKE AANGRIJPINGSPUNTEN VAN EPO IN HET CARDIORENAAL SYNDROOM



CRS patiënten hebben een hoog risico op sterfte t.g.v. hart- en vaatziekten door factoren als verhoogde inflammatie en oxidatieve stress, maar mogelijk ook door reductie van vaatbeschermende endotheel voorlopercellen. EPO behandeling kan gunstige uitwerking hebben in de CRS patiënt door opheffen van de bloedarmoede, door niet-hematopoietische effecten zoals remming van inflammatie of door stimulatie van endotheel voorlopercellen.

DOEL VAN DIT PROEFSCHRIFT

In dit proefschrift hebben wij genexpressie profielen van monocyten (als biosensor van het interne milieu) en aantallen en functie van EPC (als centrale speler in herstel van endotheelschade) vergeleken tussen CRS patiënten en gezonde controles om zo meer inzicht te krijgen in het mechanisme onderliggend aan het cardiorenaal syndroom. Aangezien EPO wisselende uitwerkingen heeft laten zien op het hart- en vaatstelsel hebben wij effecten van EPO therapie in CRS patiënten op deze monocyten en EPC onderzocht.

Verder wordt in dit proefschrift onderzocht of EPC aantal en functie veranderd zijn in fysiologische processen zoals ouder worden, maar ook in patiënten met mild en ernstig nierfalen, wat mogelijk bijdraagt aan versnelde ontwikkeling van atherosclerose en het verhoogde risico op hart- en vaatziekten.

RESULTATEN BESCHREVEN IN DIT PROEFSCHRIFT

In **hoofdstuk 2** wordt een overzicht gegeven van de huidige literatuur over effecten van EPO op de factoren inflammatie, oxidatieve stress, renine angiotensine systeem en sympathisch zenuwstelsel, die mogelijk bijdragen aan verergering van hart- en nierfalen (zie figuur 01). Verschillende experimentele studies hebben remmende effecten van EPO op inflammatie en oxidatieve stress gevonden, maar anderen vonden dit niet of zelfs tegenovergestelde effecten. Dit komt overeen met de uiteenlopende resultaten uit patiënten studies. Het is duidelijk dat EPO hematopoietische en niet-hematopoietische effecten kan hebben, maar om achter het werkingsmechanisme van EPO te komen is een nieuwe patiëntenstudie nodig.

Wij hebben de studie 'ErythroPOietine in het CardioREnaal Syndroom' (EPOCARES) opgezet om het mechanisme onderliggend aan het CRS op te helderen en om hematopoietische van niet-hematopoietische effecten van EPO te onderscheiden. De opzet van deze studie is beschreven in **hoofdstuk 3.** Patiënten met gecombineerd hartfalen, nierfalen en bloedarmoede zijn geïncludeerd in de studie. Door middel van loting worden de patiënten ingedeeld in drie behandelgroepen: een groep ontvangt een vaste EPO dosis per week maar wordt de eerste zes maanden op hetzelfde bloedgehalte gehouden met behulp van aderlating, een groep ontvangt een vaste dosis EPO en mag stijgen in bloedgehalte tot een bepaald maximum, en een groep ontvangt standaard zorg zonder EPO behandeling. De patiënten worden een jaar lang gevolgd. In deze studie kunnen acute van chronische effecten en hematopoietische van niet-hematopoietische effecten worden gescheiden.

Met microarray techniek hebben wij de genexpressie in monocyten van CRS patiënten uit de EPOCARES studie in kaart gebracht. Om deze uitgebreide dataset optimaal te bestuderen in een groep waarin veel variatie tussen patiënten zit hebben wij een methode voor data analyse voorgesteld in **hoofdstuk 4**. Met behulp van deze analyse methode laten wij in **hoofdstuk 5** zien dat de CRS patiënten bescheiden veranderingen hebben in monocyte genexpressies vergeleken met gezonde controles. Mogelijk is de monocyte niet de meest geschikte cel om veranderingen van het intern milieu in de patiënt te bestuderen of heeft medicamenteuze behandeling effect gehad op genexpressie. Het zou ook kunnen dat de verschillen tussen individuen zo groot zijn dat het lastig wordt om veranderingen op groepsniveau op te pikken. Evengoed wijzen de verschillen die we zien naar twee van de connectoren tussen hart en nier, namelijk inflammatie en oxidatieve stress. Ondanks de kleine expressieverschillen kan dit wel klinisch relevant zijn.

Vervolgens hebben we het korte termijn effect van EPO therapie -dat wil zeggen na 2 weken behandeling met EPO- op monocyt genexpressie bestudeerd in CRS patiënten. We zagen slechts weinig effect van EPO, wat aanduidt dat kortdurende toediening van deze dosis EPO, die vaak gebruikt wordt om bloedarmoede bij nierpatiënten te bestrijden, geen substantieel (maar ook geen nadelig) effect heeft op de monocyt. Het opvallende was dat het genexpressie profiel na EPO behandeling in elke patiënt erg overeenkwam met het genexpressie profiel van vóór de behandeling, wat betekent dat de technische en biologische variatie klein is. De combinatie van dosis en duur van EPO behandeling of de keuze van het onderzochte celtype kan de oorzaak zijn van het gebrek aan verschil in genexpressie. Onderzoek heeft aangetoond dat niet-hematopoietische effecten van EPO via een ander soort receptor, een heterocomplex, lopen dan hematopoietische effecten. Voordat EPO een effect kan uitoefenen moet deze eerst binden aan de EPO receptor. Deze binding gaat minder gemakkelijk aan het heterocomplex, dus mogelijk zijn er hogere concentraties van EPO nodig om een effect te zien. De laatste tijd zijn er echter studies gepubliceerd die lieten zien dat hoge dosis EPO, in combinatie met slechte gevoeligheid van de patiënt voor EPO, geassocieerd is met nadelige effecten. Dit wordt in de kliniek dus liever vermeden. Microarray techniek blijft een interessante methode om effecten van therapie te bestuderen, maar mogelijk zijn andere celtypen gevoeliger om deze effecten te vinden.

In hoofdstuk 6 t/m 9 hebben we endotheel voorlopercellen beschreven in verschillende populaties met verhoogd risico op hart- en vaatziekten. Als mensen ouder worden, wordt de functie van het endotheel vaak minder waardoor sneller hart- en vaatziekten optreden. In **hoofdstuk 6** hebben wij circulerende EPC aantallen onderzocht in gezonde mensen van jong tot oud. Wij toonden aan dat kinderen aanzienlijk meer EPC hebben vergeleken met volwassenen, wat suggereert dat kinderen beter in staat zijn om schade aan vaten te herstellen. Dit komt overeen met het verhoogde risico op hart- en vaatziekten bij toename van leeftijd. Er is meer onderzoek nodig naar het mechanisme onderliggend aan de hogere EPC aantallen in kinderen, want dan kunnen gerichte therapieën worden ontworpen om EPC in volwassenen te verbeteren.

Vervolgens hebben wij in **hoofdstuk 7** EPC

aantallen bestudeerd in kinderen met chronisch nierfalen, die al vroeg endotheelschade en een hoog risico op hart- en vaatziekten hebben. Net als eerder in volwassenen al is aangetoond zagen wij dat kinderen die hemodialyseren ten gevolge van eindstadium nierfalen lagere aantallen EPC hebben dan gezonde leeftijdsgenoten, wat kan bijdragen aan hun versnelde ontwikkeling van atherosclerose. In kinderen met nierfalen in de predialyse fase hebben wij echter geen veranderde EPC aantallen gevonden, wat tegenstrijdig is met resultaten van studies in volwassenen met predialyse nierfalen. Het zou kunnen dat EPC nog op peil zijn in kinderen met predialyse nierfalen omdat zij minder vaak andere ziekten en risicofactoren hebben vergeleken met volwassen. Deze gedachte wordt ook gesteund door onze in hoofdstuk 8 beschreven bevinding dat EPC aantallen voornamelijk verlaagd zijn in predialyse volwassenen die in het verleden al een uiting van hart- en vaatziekten hebben gehad. Daarnaast kan het ook zo zijn dat kinderen beschikken over een sterker herstelsysteem voor vaatschade, zoals in hoofdstuk 6 ook wel gesuggereerd werd n.a.v. verhoogde EPC aantallen in gezonde kinderen vergeleken met volwassenen.

Eerder onderzoek heeft aangetoond dat de balans tussen EPC en SPC in volwassenen met eindstadium nierfalen verschoven is richting SPC, die kan bijdragen aan de ontwikkeling van atherosclerose. In **hoofdstuk 8** hebben we onderzocht of dit ook het geval is in volwassenen met eerdere stadia van CKD. Wij vonden dat patiënten met mild nierfalen al lagere EPC aantallen hadden, terwijl de uitgroei van voorlopercellen richting SPC toenam met afname van nierfunctie. EPC aantallen waren vooral afgenomen in patiënten die al een keer een uiting van hart- en vaatziekten, bv. een hartinfarct, hadden gehad en in patiënten die in het bloed meer aanwijzingen voor endotheelschade hadden. De combinatie van endotheelschade en het gebrek aan compensatoire mobilisatie van EPC (en zelfs lagere EPC aantallen) wijst op een tekortschietend herstelsysteem voor de vaten. Samen met de stijging van SPC aantallen bij verergering van nierfalen kan dit bijdragen aan de toegenomen atherosclerose in predialyse nierpatiënten.

Ook in CRS patiënten uit onze EPOCARES studie vonden wij afgenomen EPC aantallen vergeleken met gezonden, ondanks dat patiënten zo optimaal mogelijk behandeld werden met medicatie (**hoofdstuk 9**). Eerder onderzoek toonde aan dat patiënten met een milde vorm van hartfalen verhoogde EPC aantallen hadden, terwijl deze verlaagd waren bij ernstiger hartfalen. Dit zou kunnen duiden op een compensatoire mobilisatie van EPC in reactie op vaatschade in mild hartfalen, maar uitputting en/of onderdrukking van beenmerg voorlopercellen in ernstiger hartfalen. Bevindingen in ons onderzoek ondersteunen deze gedachte. Wij toonden aan dat SDF-1α, een stof die EPC mobilisatie stimuleert, geassocieerd was met hogere EPC aantallen in de circulatie van patiënten met mild hartfalen. Dit suggereert dat EPC in mild hartfalen nog kunnen mobiliseren als hiervoor signalen aanwezig zijn. In ernstiger hartfalen daarentegen was er geen relatie tussen SDF-1α en EPC aantallen, wat kan betekenen dat mobilisatie van EPC van het beenmerg naar de circulatie in dit stadium van hartfalen gestoord is. Aangezien EPO in andere studies heeft laten zien dat het EPC aantallen en functie kan verbeteren, hebben wij vervolgens in hoofdstuk 9 beschreven wat het effect is van korte en lange termijn EPO therapie op EPC aantallen in de circulatie van CRS patiënten. Op korte termijn zagen wij geen effect van EPO op EPC aantallen. Na een jaar zagen wij echter dat EPC aantallen waren afgenomen in patiënten die niet met EPO waren behandeld, terwijl deze stabiel waren gebleven in met EPO behandelde patiënten. Dit suggereert dat de EPC afname, die kan bijdragen aan het verhoogde risico op hart- en vaatziekten, geremd kan worden met lange termijn EPO behandeling.

Wij kunnen op dit moment geen uitspraak doen over het mechanisme dat ten grondslag ligt aan de verlaagde EPC aantallen in hart- en nierfalen. Wel hebben wij gezien dat EPC aantallen verlaagd zijn ondanks stijging van SDF-1α in de circulatie, die zoals verwacht toenam met verergering van endotheelschade in onze patiënten met nierfalen of het cardiorenaal syndroom. Een combinatie van factoren, zoals inflammatie en ophoping van toxische stoffen door nierfalen, zou EPC kunnen beïnvloeden door bv. afname van beenmerg gevoeligheid, uitputting van het beenmerg, verslechterde EPC mobilisatie naar de circulatie of verlaagde overleving of uitgroei van voorlopercellen tot EPC.

De verlaagde EPC aantallen die wij vonden in populaties met een hoog risico op hart- en vaatziekten bieden een handreiking voor nieuwe behandelstrategieën. Daarvoor is er nog onderzoek nodig naar het mechanisme dat ten grondslag ligt aan de verlaagde EPC aantallen. Een klinisch toepasbare dosis EPO behandeling op lange termijn liet gunstige effecten zien op EPC aantallen in CRS patiënten. Om EPO therapie effectief in te kunnen zetten is meer onderzoek nodig naar effecten van EPO op het ziektebeloop, naar andere doseringen van EPO, en naar karakterisatie van patiënten die wel en niet goed op EPO reageren.

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Kim Ellis Jie is geboren op 13 mei 1983 in Leiden en groeide samen met haar ouders Kenneth en Agnes, broer Michael en zusje Janice op in Gouda. Tijdens haar basis- en middelbare school heeft zij met veel plezier geturnd op nationaal niveau. Na het behalen van haar VWO diploma in 2000 startte zij haar studie geneeskunde aan de Universiteit Utrecht, waarvoor zij o.a. co-schappen heeft gelopen in Kuala Lumpur (Maleisië) en Paramaribo (Suriname).

Haar wetenschappelijke stage op de afdeling Nefrologie en Hypertensie in het UMC Utrecht wekte haar interesse voor het onderzoek naar hart- en nierfalen. In 2006 ontving zij het Alexander Suerman stipendium van de Raad van Bestuur van het UMC Utrecht, waardoor zij direct na haar studie kon beginnen aan promotie-onderzoek o.l.v. prof. dr. Pieter Doevendans, prof. dr. Marianne Verhaar, dr. Branko Braam en dr. Carlo Gaillard, wat resulteerde in dit proefschrift.

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