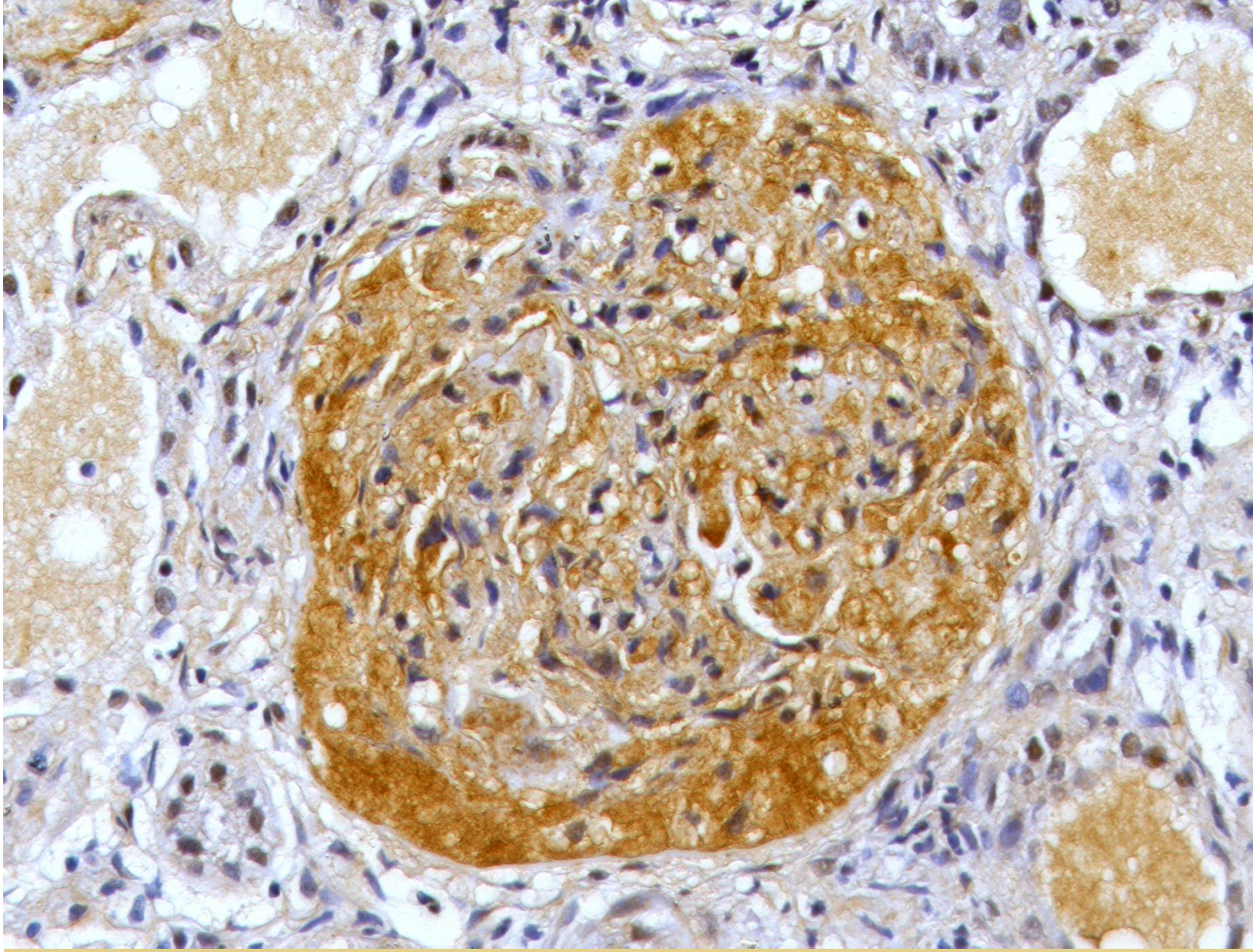

Ana Patrícia Amieiro Garrido

RESISTANCE TO RECOMBINANT HUMAN ERYTHROPOIETIN THERAPY - STUDIES IN EXPERIMENTAL MODELS OF ANEMIA AND CHRONIC KIDNEY DISEASE

Doctoral thesis in Health Sciences (Biomedical Sciences), supervised by Doctor Flávio Reis and Professor Alice Santos Silva presented to the Faculty of Medicine of the University of Coimbra

July 2014

· U C ·

UNIVERSIDADE DE COIMBRA

***Cover illustration*:** Microscopy image (from the author) of connective tissue growth factor immunostaining in the glomerulus of a chronic renal failure rat kidney.

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RESISTÊNCIA À TERAPÊUTICA COM ERITROPOIETINA RECOMBINANTE HUMANA – ESTUDOS EM MODELOS EXPERIMENTAIS DE ANEMIA E DOENÇA RENAL CRÓNICA

Julho/2014

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UNIVERSIDADE DE COIMBRA

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Abbreviations

18S	18S ribosomal subunit
Abs	Antibodies
ACR	Albumin to creatinine ratio
Actb	Beta actin
ADMA	Asymmetrical dimethylarginine
AER	Albumin excretion rate
ALT	Alanine transaminase
ANF	Atrial natriuretic factor
ARNT	Aryl hydrocarbon receptor nuclear translocator
AST	Aspartate transaminase
ATG	Antithymocyte globulin
BFU-E	Burst forming unit-erythroid
BHK	Baby hamster kidney
BMP6	Bone morphogenetic protein 6
BP	Blood pressure
BUN	Blood urea nitrogen
BW	Body weight
CBP	CREB-binding protein
CERA	Continuous erythropoietin receptor activator
CFU-E	Colony forming unit-erythroid
CHMP	Committee for Medicinal Products for Human Use
CHO	Chinese hamster ovary
CHOIR	Correction of Hemoglobin Outcomes in Renal Insufficiency
CK	Creatine kinase
CKD	Chronic kidney disease mineral bone disorder
CKD-MBD	Chronic kidney disease
CREATE	Cardiovascular Risk Reduction by Early Anemia Treatment with Epoetin Beta
CRF	Chronic renal failure
CTGF	Connective tissue growth factor

CytC	Cytocrome C
DAB	Diaminobenzidine
DBP	Diastolic blood pressure
DMT1	Divalent metal transporter 1
eGFR	Estimated glomerular filtration rate
EGLN	Egl nine homolog
EH	Erythroid hypoplasia
EMA	European Medicines Agency
EPARs	European Public Assessment Reports
EPAS1	Endotelial PAS domain protein 1
EPO	Erythropoietin
EPOR	Erythropoietin receptor
ERI	Erythropoietin resistance index
ESAs	Erythropoiesis stimulant agents
ESRD	End stage renal disease
ET-1	Endotelin-1
EU	European Union
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FIH	Factor inhibiting HIF
G-CSF	Granulocyte colony-stimulating factor
GDF15	Growth differentiation factor 15
GFR	Glomerular filtration rate
GM-CSF	Granulocyte macrophage colony-stimulating factor
H&E	Hematoxylin and eosin
Hamp	Hepcidin antimicrobial peptide
Hb	Hemoglobin
Hct	Hematocrit
HD	Hemodialysis
HFE	Hemochromatosis

HIF-1α	Hypoxia inducible factor 1 alpha
HIF-1β	Hypoxia inducible factor 1 beta
HIF-2α	Hypoxia inducible factor 2 alpha
HIF-2β	Hypoxia inducible factor 2 beta
HJV	Hemojuvelin
HR	Heart rate
HREs	Hypoxia response elements
HRP	Horseradish peroxidase
HRQOL	Health related quality of life
hsCRP	High sensitive C reactive protein
HW	Heart weight
IFN-γ	Interferon-γ
IFTA	Interstitial fibrosis and tubular atrophy
IGF-1	Insulin like growth factor 1
IL-1	Interleukin-1
IL-1β	Interleukin-1 beta
IL-3	Interleukin-3
IL-5	Interleukin-5
IL-6	Interleukin-6
INN	International Non-Proprietary Name
JAK-2	Janus kinase 2
KDIGO	Kidney Disease: Improving Global Outcomes
KW	Kidney weight
LVH	Left ventricular hypertrophy
LW	Liver weight
MAPK	Mitogen activated protein kinase
MBP	Mean blood pressure
MCH	Mean cell hemoglobin
MCHC	Mean cell hemoglobin concentration
MCV	Mean cell volume
MEC	Minimum effective dose

NF-KB	Nuclear transcription factor kappa B
NKF KDOQI	National Kidney Foundation Kidney Disease Outcomes Quality Initiative
NO	Oxide nitric
ODD	O_2 dependent degradation domain
PAS	Periodic acid of Shiff
PBS	Phosphate buffered saline
PD	Peritoneal dialysis
PDW	Platelet distribution width
PGF2α	Prostaglandin 2α
PGI2	Prostacyclin 2
PHD	Prolyl-4-hydroxylase domain
PHZ	Phenylhydrazine
PI-3K	Phosphatidyl-inositol 3-kinase
PLT	Platelets
pmp	*Per* million population
PRCA	Pure red cell aplasia
RBC	Red blood cells
RDW	RBC distribution width
REPC	Renal EPO-producing cells
RET	Reticulocytes
rHuEPO	Recombinant human erythropoietin
RRT	Renal replacement therapy
SBP	Systolic blood pressure
SCF	Stem cell factor
SEM	Standard error of means
SLC40A1	Solute carrier family 40, member 1 (ferroportin)
SPSS	Statistical Package For Social Sciences
sTfR	Soluble transferrin receptor
sTfR2	Soluble transferrin receptor 2
TBM	Tubular basement membrane
TF	Transferrin

TfR1	Transferrin receptor 1
TGF-β1	Transforming growth factor beta 1
TGs	Triglycerides
TMB	Tetramethylbenzidine
TMPRSS6	Matriptase-2
TNF-α	Tumor necrosis factor alfa
Total-c	Total cholesterol
TREAT	Trial to Reduce Cardiovascular Events with Aranesp Therapy
TSAT	Transferrin saturation
TSP-1	Thrombospondin-1
TWSG1	Twisted gastrulation protein 1
TXB2	Thromboxane B2
US	United States
VEGF	Vascular endothelial growth factor
VHL	Von Hippel–Lindau tumor suppressor
WBC	White blood cells
WHO	World Health Organization

Resumo

A doença renal crónica (DRC) é uma doença que afeta cerca de 7% das pessoas com mais de 30 anos, traduzindo-se em mais de 70 milhões de pessoas em países desenvolvidos por todo o mundo. A anemia é uma complicação da DRC que se desenvolve no início da doença, aumentando a sua frequência e gravidade com o declínio da função renal. Esta patologia tem sido principalmente associada à diminuição da produção de eritropoietina, através da falência renal e de outros fatores, tais como a inflamação crónica, a perda de sangue e deficiências em vitaminas. A terapêutica com eritropoietina recombinante humana (rHuEPO) para tratar a anemia associada à DRC reduziu os requisitos de transfusões e melhorou a qualidade de vida dos doentes. No entanto, existe uma grande variabilidade na resposta ao tratamento, e cerca de 10% dos doentes desenvolvem resistência à terapêutica com EPO, agravando o seu prognóstico. Apesar da investigação dos últimos anos, os mecanismos subjacentes ao desenvolvimento de resistência à rHuEPO permanecem ainda por elucidar. Modelos animais poderão ser ferramentas úteis para clarificar as características celulares e moleculares desta condição.

Os nossos objetivos foram, usando um modelo animal de DRC induzida por nefrectomia (5/6), esclarecer os mecanismos subjacentes ao desenvolvimento de anemia e à evolução de lesão renal; estudar o efeito da rHuEPO *per se*; compreender a variabilidade na resposta/resistência ao tratamento com rHuEPO, focando a eritropoiese, o metabolismo de ferro, assim como as lesões renais, a hipóxia, a inflamação e a fibrose, no sangue, nos tecidos e a nível celular; finalmente, desenvolver modelos animais experimentais que possam ser úteis noutros estudos de anemia associada à DRC.

Tal como esperado, o nosso modelo desenvolveu uma anemia precoce logo após a nefrectomia que persistiu durante o estudo. A anemia persistente não foi devida a uma deficiência de EPO pois o rim remanescente dos ratos foi ainda capaz de produzir EPO (níveis aumentados em soro), e o fígado pareceu aumentar a expressão génica de *EPO*, sugerindo que o rim remanescente, ou mesmo os tecidos extra-renais, foram capazes de compensar a produção de EPO. Além disso, a redução na expressão da hepcidina, provavelmente induzida por hipoxia, deve favorecer a absorção de ferro. Os nossos resultados sugerem que a persistência da anemia pode resultar de distúrbios no

metabolismo do ferro e, eventualmente, de uma alterada actividade/função da EPO que pode resultar do dano nas células do rim, e da inflamação local desenvolvida.

A fim de elucidar os mecanismos subjacentes à persistência da anemia, foi utilizado o modelo animal previamente caracterizado, para avaliar o impacto de duas doses de rHuEPO (50 e 200 UI) nos mecanismos mencionados, nomeadamente na (dis)funcionalidade da EPO, no (dis)metabolismo e disponibilidade do ferro, na hipóxia renal, na inflamação e na fibrose do rim remanescente.

Neste modelo de DRC, a anemia desenvolvida a longo prazo foi devida a uma atividade eritropoiética reduzida, uma vez que ratos com DRC foram capazes de corrigir a anemia quando tratados com rHuEPO; a inflamação local renal e o tipo (ligeiro ou avançado) de lesões renais são cruciais para o resultado desta anemia. O tratamento de ratos com DRC com rHuEPO leva ao desenvolvimento de anticorpos anti-rHuEPO e, por conseguinte, à anemia, após 9 semanas de tratamento. Além disso, o impacto deste tratamento depende da dose de rHuEPO usada, que deve ser suficiente para corrigir a anemia, prevenindo o rim e a hipertrofia cardíaca, mas não demasiado elevada pois conduzirá a um agravamento de lesões renais glomerulares e tubulointersticiais.

Adicionalmente, a possibilidade do desenvolvimento de resistência à EPO exógena (tratamento com rHuEPO) foi também considerada e analisada pelo desenvolvimento de um modelo de rato de hipoplasia eritróide mediada por anticorpos induzida por rHuEPO, através de um tratamento a longo prazo com uma alta dose de rHuEPO (200 UI/Kg de peso corporal/semana) durante 9 semanas em ratos Wistar. Os resultados obtidos sugeriram que, neste modelo, os anticorpos anti-rHuEPO inibiram tanto a rHuEPO como EPO endógena levando à anemia; adicionalmente, o ferro estava aumentado, induzindo a expressão da hepcidina hepática, apesar da não evidência de inflamação, sendo ele o modulador chave de síntese hepcidina.

Os resultados desta tese dão-nos mais conhecimento sobre os mecanismos subjacentes a diferentes respostas do tratamento com rHuEPO observado nos doentes com DRC, assim como sobre o desenvolvimento de resistência à rHuEPO; além disso, pode abrir novas janelas para identificar alvos terapêuticos putativos para esta condição, bem como para a resistência à rHuEPO, que ocorre em cerca de 10% dos doentes com DRC. Conseguimos também desenvolver dois modelos experimentais, de DRC e de PRCA, que são ferramentas úteis para futuros estudos, incluindo farmacológicos, nesta área.

Abstract

Chronic kidney disease (CKD) is a debilitating disease affecting about 7% of people with more than 30 years and older, which translates to more than 70 million people in developed countries worldwide. Anemia is a common complication of CKD that develops early in the course of the disease, increasing its frequency and severity with the decline of renal function. It has been associated, mainly, with a reduced production of erythropoietin by failing kidneys and with other factors, including chronic inflammation, blood loss and vitamin deficiencies. Since the introduction of recombinant human erythropoietin (rHuEPO), erythropoiesis-stimulating agents (ESAs) have been used to treat CKD associated-anemia, reducing the requirements for transfusion and improving the quality of life in these patients. Nevertheless, there is a marked variability in the response to the treatment and about 10% of patients develop resistance to rHuEPO therapy, aggravating their prognosis. Despite the intense research during the last years, the mechanisms underlying the development of resistance to rHuEPO therapy remain unsolved. Animal models could be useful tools to clarify the cellular and molecular features of this condition.

Our aims were, by using the remnant kidney rat model of CKD induced by (5/6) nephrectomy, to clarify the mechanisms underlying the development of anemia and the evolution of renal damage; to study the effect of rHuEPO *per se*; to understand the variability in the response/resistance to rHuEPO therapy, focusing on erythropoiesis, iron metabolism as well as on kidney lesion, hypoxia, inflammation and fibrosis, at blood, tissue and cellular levels; finally, to develop experimental animal models that can be useful in further studies on CKD associated-anemia.

As expected, our model developed anemia early after nephrectomy, which persisted throughout the study. The persistent anemia was not due to EPO deficiency because the remnant kidney of rats was still able to produce EPO (increased serum EPO levels), and the liver seems to increase *EPO* gene expression, suggesting that the remnant kidney or even extra-renal tissues were able to compensate EPO production. In addition, the reduced expression of hepcidin, probably induced by hypoxia, should favour iron absorption. Our data suggest that the persistence of anemia may result from disturbances

in iron metabolism and, eventually, by an altered activity/function of EPO that might result from kidney cell damage and from the developed local inflammatory milieu.

In order to contribute to elucidate the mechanisms underlying the persistence of anemia, we used the animal model of CKD induced by (5/6) nephrectomy, previously characterized, to evaluate the impact of two doses of rHuEPO (50 and 200IU) on the physiological/pathophysiological procedures above mentioned, namely EPO (dys)functionality, iron (dys)metabolism and availability, kidney hypoxia, inflammation and fibrosis of the remnant kidney.

In this rat model of CKD, the anemia developed with long-term CRF is due to the synthesis of EPO with a reduced erythropoietic activity, as the CRF rats were able to overcome the anemia when treated with rHuEPO; the local kidney inflammation and the type (mild or advanced) of kidney lesions are crucial to the outcome of the anemia. A long-term treatment of CRF rats with rHuEPO leads to the development of rHuEPO antibodies and, therefore, to anemia, after about 9 weeks of treatment. In addition, the impact of this treatment depends on the rHuEPO dose, which should be enough to correct anemia, preventing kidney and heart hypertrophy, but not too high that will lead to worsening of advanced glomerular and tubulointerstitial kidney lesions.

In addition, the possibility of development of resistance to exogenous EPO (rHuEPO therapy) was also considered and analyzed by the development of a rat model of antibody-mediated EH induced by rHuEPO, by long-term treatment with a high dose of rHuEPO (200 IU/Kg bw/week) during 9 weeks in Wistar rats. The results obtained suggested that in this model the formation of anti-rHuEPO antibodies inhibited both rHuEPO and endogenous EPO leading to anemia; in addition, iron was increased, triggering hepatic hepcidin expression, despite no evidence of inflammation, and is the key modulator of hepcidin synthesis.

The findings of this thesis give further insights into the mechanisms behind the different responses to rHuEPO treatment observed in chronic kidney disease patients, as well as to the development of resistance to rHuEPO; moreover, it might open new windows to identify putative therapeutic targets for this condition, as well as for rHuEPO resistance, which occurs in about 10% of CKD patients. We managed also to develop two experimental models, of CKD and of PRCA, that will provide useful tools for further studies in this research area and, eventually, in pharmacological studies.

Chapter I

Introduction

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1. The Kidney

1.1. Basic kidney anatomy and physiology

Each kidney contains about 1 million functional units, the nephrons, consisting of numerous specialized cell types originating from distinct embryological lineages. Each nephron contains a filtrating body, the glomerulus, and a long tubule made of a dozen differentiated segments. The final parts of these tubules are interconnected to form the collecting ducts, which open into the renal pelvis. The basic principles of kidney physiology dictate the central role played by this organ in excretory, metabolic, and endocrine functions. Although the kidneys excrete only about 1.5 L of urine daily, they are perfused with 20% of the cardiac output and form about 180 L of ultrafiltrate every day, which is profoundly modified during tubular passage. This function exposes the kidney to the interior milieu unlike any other organ and at the same time makes the body composition very sensitive to changes in kidney function. The glomerular filtration barrier consists of the fenestrated endothelial cells, an elaborate basement membrane, and an intricate layer formed by the foot processes of highly differentiated epithelial cells called podocytes (Figure I-1).[1]

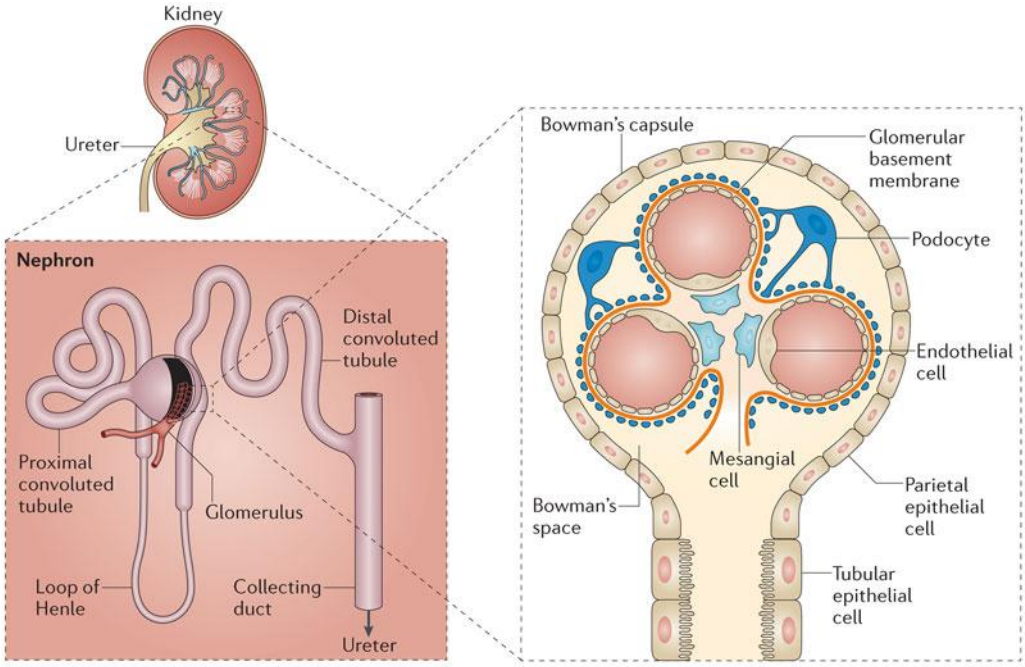

Figure I-1. Basic kidney anatomy and physiology. (*Taken from* Kurts et al., 2013[2]).

The filtration barrier is permeable to water, small solutes, and low-molecular-weight proteins up to the mass of albumin, but largely precludes the filtration of plasma proteins with a mass of more than 60–70 kDa, especially if they are negatively charged. Thus, the glomerular filtration rate, the product of the filtration area, the hydraulic permeability, and the net ultrafiltration pressure, yields a large ultrafiltrate containing plasma solutes and several grams of low-molecular-weight proteins. Any disturbance in glomerular hemodynamics or structure can result in reduced glomerular filtration rate or increased leakage of proteins into the urine—two classic signs of renal disease. Along the tubular segments of the nephron, the ultrafiltrate undergoes a series of modifications with massive reabsorption of low-molecular-weight proteins, solutes, and water, secretion of a gel-like protein called uromodulin (Tamm-Horsfall protein) and elimination of excess potassium, acids, and bases (Figure I-1).[3]

The processes affecting urine composition are mediated by polarised transport systems operating in the epithelial cells lining the tubules. The proximal tubule reabsorbs the bulk (about two-thirds) of filtered solutes and water. In addition, the cells lining these tubules possess multiligand receptors involved in the endocytic uptake of filtered proteins that include hormones, carrier proteins (eg, for vitamins), and enzymes. Once reabsorbed, these proteins are metabolised by proximal tubular cells, as the human urine is virtually devoid of plasma proteins under physiological conditions. This massive uptake of proteins plays an important role in metabolic clearance, hormone homoeostasis, and conservation of essential vitamins (vitamin D, vitamin A, and vitamin B12), and provides a protein-free milieu for the cells lining distal nephron segments.[4]

Organic molecules and drug metabolites are secreted into the urine via specific transporters located in the last part of the proximal tubule. The tubule segments forming the loop of Henle generate the hypertonic environment in the medulla, necessary for concentrating the urine, and mediate the paracellular reabsorption of Ca^{2+} and Mg^{2+} under the control of the Ca^{2+}/Mg^{2+}-sensing receptor.[5] Specialised epithelial cells, located at the junction between the thick ascending limb of the loop of Henle and the distal nephron (macula densa) sense the tubular NaCl concentration and interact with renin-containing granular cells in the afferent arterioles to regulate the glomerular blood flow through a mechanism called tubuloglomerular feedback.[6] The distal nephron, which includes the distal convoluted tubule, the connecting tubule, and the collecting

ducts, is responsive to aldosterone and vasopressin and regulates the final urine composition and concentration.[7] Excretory, metabolic, and endocrine functions of the kidney mediate essential interactions with several organs, sustaining an array of vital functions (Figure I-2), including regulation of body water and thirst, blood pressure, ventilation, drug metabolisation, potassium balance, erythropoiesis, calcium and phosphate metabolism, and acid-base homoeostasis.

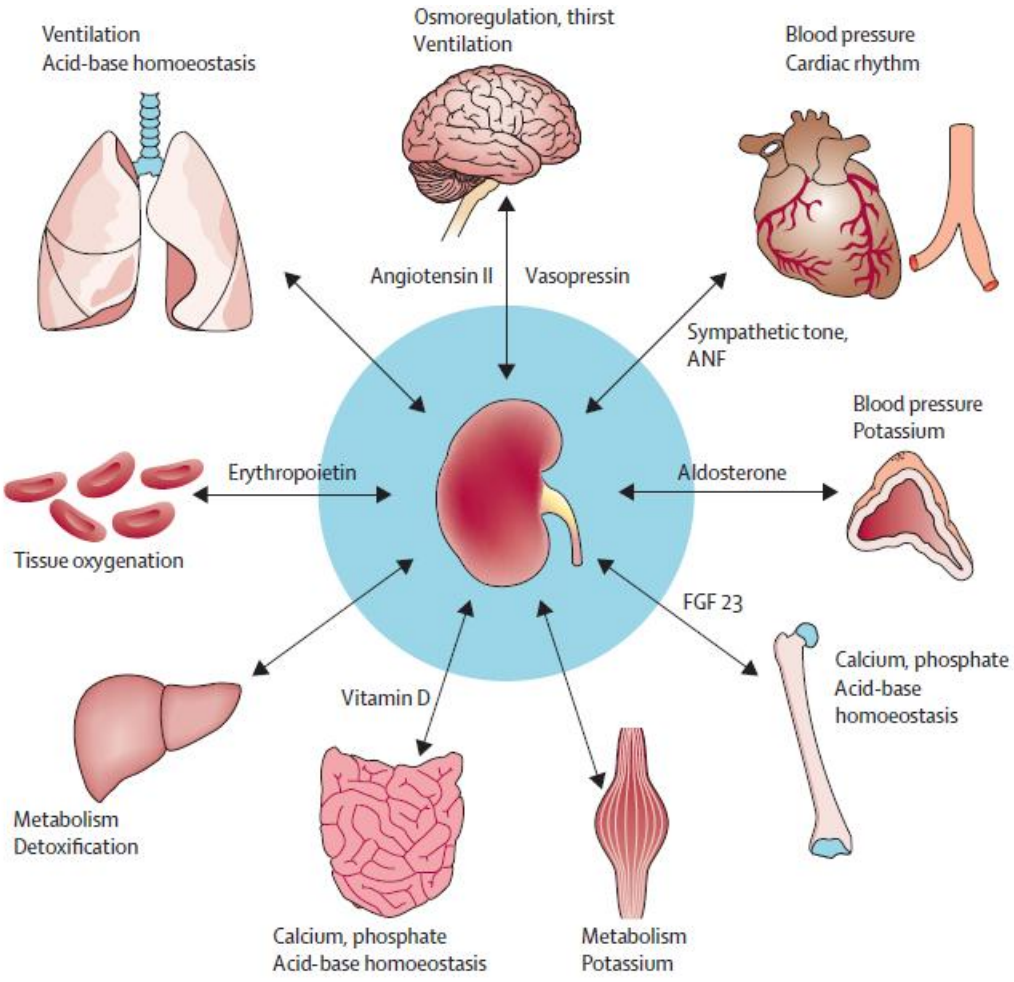

Figure I-2. Effect of kidney function on essential homoeostatic processes. FGF, fibroblast growth factor; ANF, atrial natriuretic factor. (*Taken from* Eckardt et al., 2013 [8]).

The regulation of NaCl excretion, which is crucial for extracellular fluid volume and blood pressure control, is influenced by the renin-angiotensin-aldosterone system, the atrial natriuretic peptide, the sympathetic nervous system and, to a lesser extent, the antidiuretic hormone arginine vasopressin.[9] Renal excretion of potassium is mainly dependent on the distal tubular flow rate and the release of aldosterone and its action

on the principal cells of the distal nephron. The regulation of body water content (osmoregulation) involves the action of vasopressin on aquaporin mediated transport of water.[10,11] The kidney is also a main site of systemic oxygen sensing, regulating the oxygen supply in red blood cells and, hence, tissue oxygen supply, through hypoxia-inducible erythropoietin production in peritubular fibroblasts of the renal cortex.[12] Apart from control of body homoeostasis, the kidney is also involved in immune function. Dendritic cells and macrophages, which form a network in the renal interstitium, contribute to innate and adaptive immunity and are increasingly recognised for their sentinel role against kidney injury and infection and their potential contribution to progression of kidney disease.[13]

2. Chronic kidney disease

2.1. Overview and definition, classification and staging of chronic kidney disease

Chronic kidney disease (CKD) is a general term for heterogeneous disorders affecting the structure and function of the kidney. The variation in disease expression is related partly to the cause and pathology, severity, and rate of progression. Since the introduction of the conceptual model, definition, and staging of CKD 12 years ago,[14-16] the guidelines have recommended a shift from the concept of kidney disease as a life-threatening disorder affecting few people who need care by nephrologists, to a common disorder of varying severity that not only merits attention by general internists, but also needs a concerted public health approach for prevention, early detection, and management.[17] Although guidelines have had an important effect on clinical practice, research, and public health, they have also generated controversy.[8,18-20]

Figure I-3 shows a conceptual model for the development, progression, and complications of CKD. The model includes antecedents associated with increased risk, disease stages, and complications, including death. Risks can be categorised either as susceptibility to kidney disease because of sociodemographic and genetic factors, or as exposure to factors that can lead to disease. Early stages of disease are often asymptomatic, are detected during the assessment of comorbid disorders, and can be reversible. Rapidly progressive diseases can lead to kidney failure within months;

however, most diseases evolve over decades and some patients do not progress during many years of follow-up. [8,14,20]

In 2002, the clinical practice guidelines of US National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) defined CKD as kidney damage or glomerular filtration rate lower than 60 mL/min per 1.73 m^2 for 3 months or longer, and proposed a classification scheme based on estimated glomerular filtration rate (eGFR).[14] Later analyses have shown that albuminuria also has an important effect on outcomes, [21] which prompted the Kidney Disease: Improving Global Outcomes (KDIGO) Work Group on Evaluation and Management of Chronic Kidney Disease to include albuminuria in the revised 2012 classification.[22] The causes of CKD are also included in the new scheme because they can affect outcomes and the choice of treatments. Early identification of CKD is needed to prevent disease progression and reduce the risk of cardiovascular morbidity and mortality. Public health approaches to enabling early identification are, therefore, receiving increasing attention.

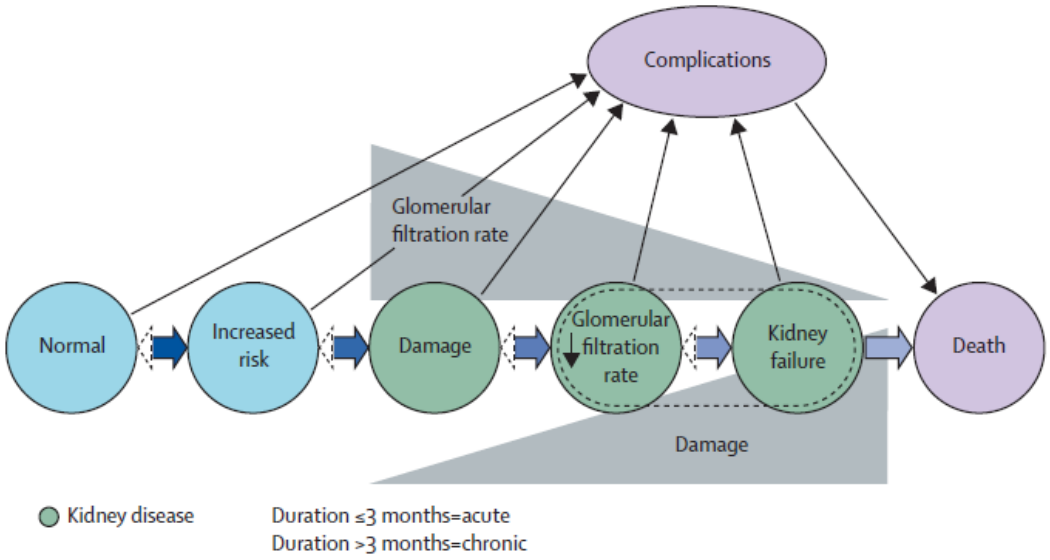

Figure I-3. Conceptual model for the development, progression and complications of CKD. Factors associated with increased risk of kidney disease (blue), stages of disease (green), and complications (including death; purple). Horizontal arrows show transitions between stages (kidney outcomes). Solid arrows pointing from left to right show progression of kidney disease. Grey triangles show continuous nature of changes in glomerular filtration rate and kidney damage. (*Adapted from* Levey et al., 2012 and Eckardt et al., 2013 [8,20]).

The diagnosis, treatment, and management of CKD depend on classification and staging of the disease as set forth by international, country-specific, and other clinical guidelines.[14,23-26] As evidenced by a variety of definitions and staging systems in the scientific literature, identification of optimal patient care strategies and interpretation of data are complicated. To date, the most frequently cited and used CKD staging system is that developed by the National Kidney Foundation, Dialysis Outcomes Quality Initiative.[14,17,27]

Although measurement of eGFR is considered the gold standard for diagnosing and evaluating progression of CKD, there is a movement among clinicians and researchers to improve clinical guidelines specifically related to diagnosis, classification, and staging. The following frequently reported criticisms of current guidelines have prompted the discussion for modification of current clinical guidelines.[16]

• A diagnosis based on current eGFR estimation formulas is imprecise.

• There is an absence of risk-stratification across patients; those at high risk for disease progression are not identified, and stage 3 is too broad (eg, stage 3 should be stratified into 3a (eGFR 45–59 mL/min/1.73 m^2) and 3b (eGFR 30–44 mL/min/1.73 m^2)).

• Patient variability (age, sex, race, ethnicity) is not considered in current methods of evaluation (eg, eGFR and proteinuria estimation, at risk for progression, prognosis).

In an effort to collaborate and provide a foundational set of international CKD guidelines that address these criticisms, KDIGO formed a dedicated workgroup. Publication of the KDIGO "Clinical practice guideline on CKD classification and management" was anticipated in 2012. The proposed guideline structure and associated discussion points were as follows: [22]

• *Guideline 1:* Definition and stages of CKD. Stages modified and enriched to include different degrees of proteinuria; splitting stage 3 into 3a and 3b; define differences between kidney damage and disease (Table I-1).

• *Guideline 2:* Identification and evaluation of CKD. eGFR, high-risk population evaluation, appropriateness and frequency of testing.

• *Guideline 3:* Estimation of glomerular filtration rate (GFR). New equations that address "within-individual" biological variability, age, and ethnicity.

Table I-1. Classification of chronic kidney disease based on GFR.

Stage	Description	By severity	
		eGFR (mL/min/1.73 m^2)	Related terms
G1	Kidney damage with normal or ↑ eGFR	≥ 90	Albuminuria, proteinuria, hematuria
G2	Kidney damage with mild ↓e GFR *	60–89	Albuminuria, proteinuria, hematuria
G3a	Mild to moderate ↓eGFR	45–59	Chronic renal insufficiency, early renal insufficiency
G3b	Moderate to severe ↓eGFR	30-44	
G4	Severe ↓ eGFR	15–29	Chronic renal insufficiency, late renal insufficiency, pre-ESRD
G5	Kidney failure	< 15 (or dialysis)	Renal failure, uremia, ESRD

ESRD, end-stage renal disease; eGFR, estimated glomerular filtration rate.*Relative to young adult level. In the absence of evidence of kidney damage, neither GFR category G1 nor G2 fulfill the criteria for CKD. (*Adapted from* KDIGO, 2012 [22]).

• *Guideline 4:* Estimation of proteinuria. Regional variability in methods; change lexicon from microalbuminuria to albuminuria mild, moderate, or severe (Table I-2).

• *Guideline 5:* Definition of progression of CKD. Definition of change in eGFR, acute versus chronic change, duration and stability of change, prognostic indicators, frequency of testing.

Table I-2. Classification of chronic kidney disease based on albuminuria.

Category	AER	ACR (approximate equivalent)		Related terms
	(mg/24 hours)	(mg/mmol)	(mg/g)	
A1	<30	<3	<30	Normal to mild increase
A2	30-300	3-30	30-300	Moderate increase *
A3	>300	>30	>300	Severe increase

AER, albumin excretion rate; ACR, albumin-to-creatinine ratio.*Relative to young adult level. (*Adapted from* KDIGO, 2012[22]).

Because of the complex nature of CKD, emphasis on well-defined disease classification and staging is paramount for optimal patient care. The evidence presented here elucidates several clinical unmet needs: for example, well-defined guidelines for use by primary care physicians, internists, and nephrologists; early identification,

prevention, and management of patients at risk for CKD (pre-CKD); “on-time” referral to a nephrologist when patients with early CKD are progressing to intermediate or advanced stages of CKD; optimal management for patients with confirmed CKD and those at risk for accelerated progression (eg, complex cases with multiple comorbidities); and educational programs and tools developed for primary care physicians, internists and nephrologists, to address a host of topics (eg, clinical guidelines, population-specific treatment, and management).

Findings from experimental and clinical studies have suggested an important role for proteinuria in the pathogenesis of disease progression.[28] Epidemiological studies have shown graded relations between increased albuminuria and mortality, and kidney outcomes in diverse study populations, in addition to, and independent of, low GFR and risk factors for cardiovascular disease.[21,29,30] In view of these findings, an international conference recommended modification of disease classification to indicate prognosis by the addition of stages based on albuminuria, and an update of the 2002 guidelines (Figure I-4).[20]

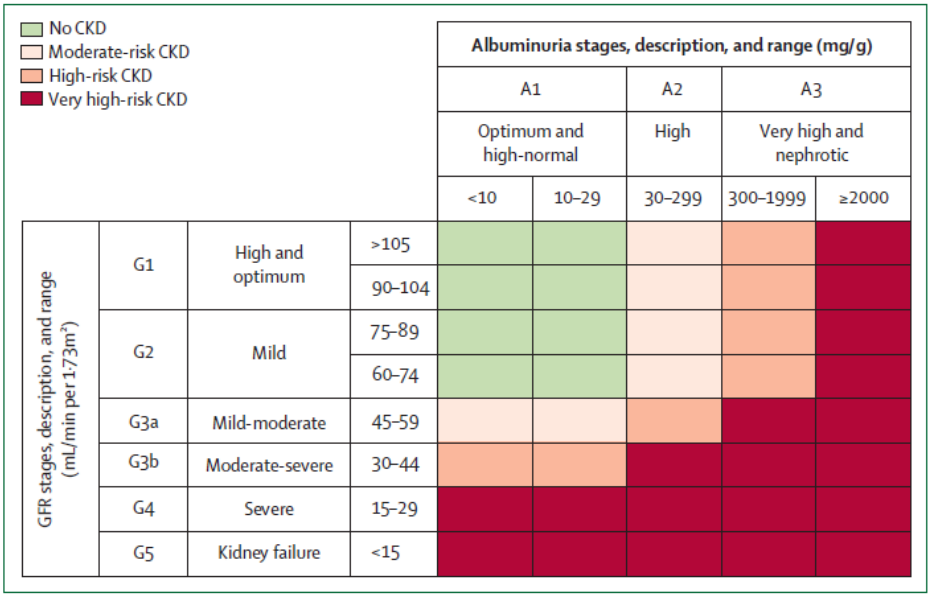

Figure I-4. Prognosis of chronic kidney disease by GFR and albuminuria. Colours show how adjusted relative risk is ranked for five outcomes from a meta-analysis of general population cohorts: all-cause mortality, cardiovascular mortality, kidney failure treated by dialysis and transplantation, acute kidney injury, and progression of kidney disease. For categories with GFR >15 mL/min per 1.73 m^2 and albuminuria <2000 mg/g, ranks were averaged across outcomes. Mean rank numbers: 1–8=green, 9–14=pink, 15–21=orange, and 22–28=red. (*Taken from* Levey et al.,2012[20]).

Kidney failure is traditionally regarded as the most serious outcome of CKD and symptoms are usually caused by complications of reduced kidney function. When symptoms are severe they can be treated only by dialysis and transplantation; kidney failure treated this way is known as end-stage renal disease (ESRD). Other outcomes include complications of reduced GFR, such as increased risk of cardiovascular disease, acute kidney injury, infection, cognitive impairment, and impaired physical function. Complications can occur at any stage, which often lead to death with no progression to kidney failure, and can arise from adverse effects of interventions to prevent or treat the disease.[20]

2.2. Epidemiology of CKD

According to the 2010 Global Burden of Disease study, CKD was ranked 27th in the list of causes of total number of global deaths in 1990 (age-standardised annual death rate of 15.7 per 100 000), but rose to 18th in 2010 (annual death rate 16.3 per 100 000).[31] An analysis of data on cause of death in the USA and Australia by Rao et al. (2012) showed that a substantial proportion of individuals who had died from diabetes had renal failure, but the cause of death was coded as diabetes without complication. Reported mortality from diabetes-related renal disease was estimated to be four to nine times less than the actual rate.[32]

The incidence and prevalence of end-stage kidney disease differ substantially across countries and regions (Figure I-5). More than 80% of all patients receiving treatment for ESRD are estimated to be in affluent countries with large elderly populations and universal access to health care.[33] The lowest bars reported from poor countries are largely due to patients not being accepted into renal replacement therapy (RRT) programmes, although where economies are growing, the numbers of patients being accepted for RRT are rising strikingly.[34]

Projected worldwide population changes suggest that the potential number of cases of ESRD will increase disproportionately in developing countries, such as China and India, where the numbers of elderly people are expanding. This effect will be enhanced further if the trends of increasing hypertension and diabetes prevalence persist,

competing causes of death—such as stroke and cardiovascular diseases—are reduced, and access to treatment improves. In contrast to clinically apparent advanced-stage CKD, a precise calculation of the burden of less symptomatic or asymptomatic early-stage chronic kidney disease, which accounts for 80–90% of all cases, is difficult.[22] The estimates are usually based on a single-time measurement rather than on sustained demonstration of abnormality.

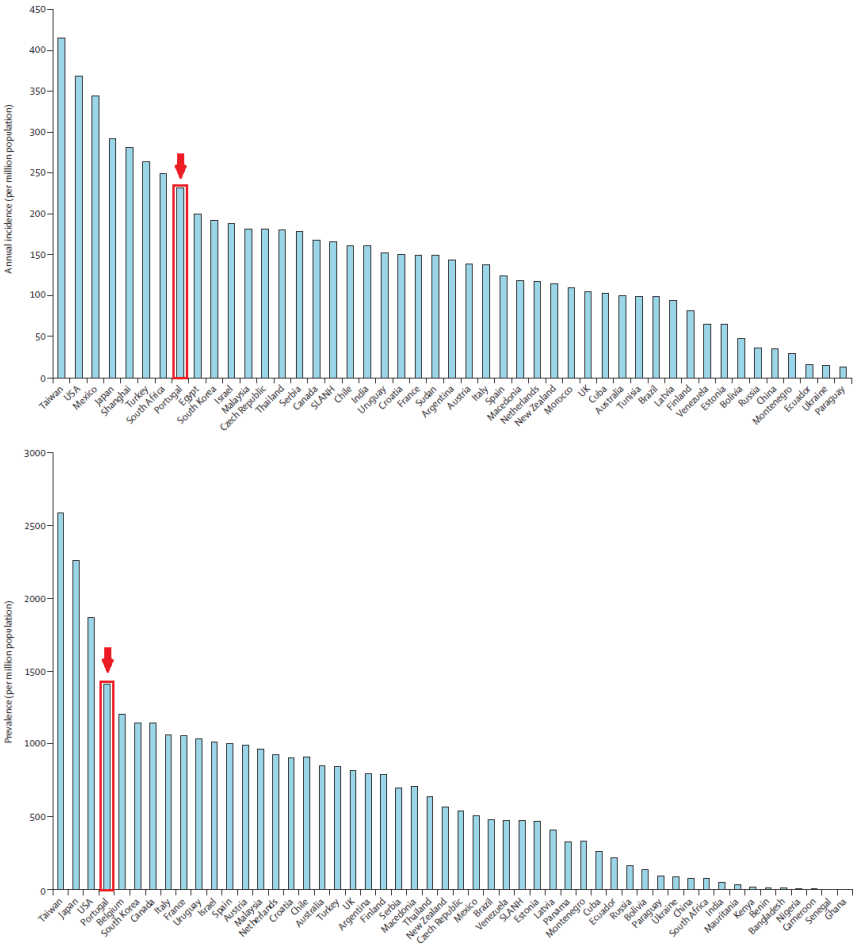

Figure I-5. Annual incidence (A) and prevalence (B) rates of end-stage kidney disease in different countries. (*Adapted from* Jha et al., 2013 [35]).

CKD is a debilitating disease affecting approximately 7% of all people aged 30 years and older, which translates to more than 70 million people in developed countries worldwide.[36] This number is likely to be much higher given the unknown prevalence in underdeveloped countries. The increased prevalence of diabetes, hypertension, and obesity and an aging population will perpetuate the rise of CKD.[37,38] Patients have been,

and continue to be, diagnosed with CKD later in the disease cycle, and, therefore, they have to be prepared for life on dialysis or to undergo kidney transplant. However, with better screening, early management, and innovative pharmacologic therapies, the disease progression may be delayed and patients with CKD may enjoy healthier and more productive lives.[39] Data from Portuguese Society of Nephrology[40] shows the incidence in hemodialysis (HD) and peritoneal dialysis (PD) in last year 7 years, with an increase in last year (Figure I-6).

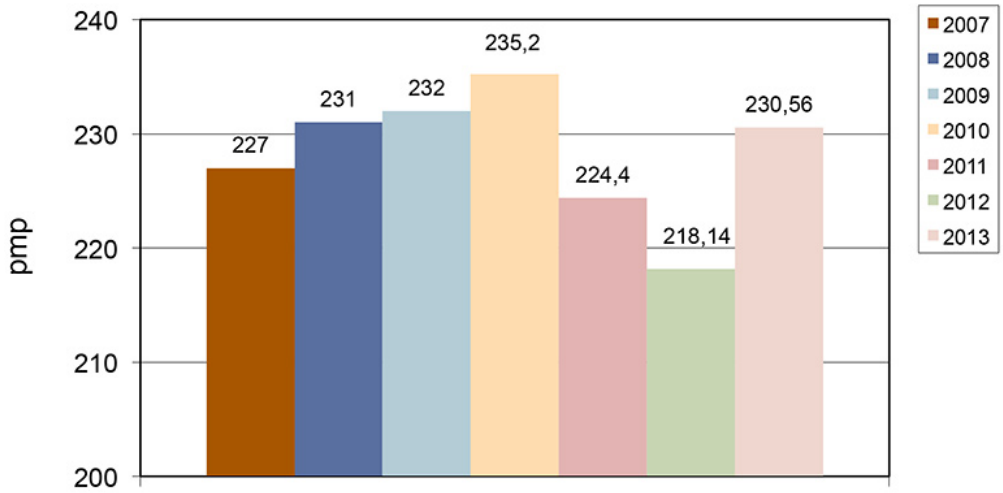

Figure I-6. Global data of incidence of HD and DP in Portugal during 2007-2013. pmp, *per* million population (*Taken from* Portuguese Society of Nephrology[40]).

Prevalence estimates for several modifiable risk factors affecting initiation and/or progression of disease have also increased. National Health and Nutrition Examination Survey 2001–2008 data report that diabetes, hypertension, cardiovascular disease, and congestive heart failure are more prevalent in patients with eGFRs, of 60 mL/min/1.73 m^2; additionally, the prevalence of hypertension is twofold and the prevalence of cardiovascular disease is fivefold greater compared with those with eGFR 60 mL/min/1.73 m^2. As the prevalence of diabetes, hypertension, and other risk factors rise, so does the severity of CKD. For example, the frequency of diabetes increased more than five times by CKD stage and eGFR category. Diabetes occurred in an estimated 7% of patients in stage 1/2, 18% of patients in early stage 3 (3a), 27% of patients in late stage 3 (3b), and 40% of patients in stages 4 and 5. Given the increase in CKD prevalence

and associated risk factors, more global epidemiological research is needed to better characterize the international burden of CKD. With improved patient-level definitions of CKD, large-scale epidemiological studies may present more adequately representative populations with CKD across countries.[39]

2.3. Major pathophysiological contributors/complications associated to CKD

Several factors contribute to the high risk of death in CKD (Figure I-7). Common risk factors for mortality in CKD patients include diabetes, hypertension, overweight, atherosclerosis, lipid disorders, smoking, and possibly salt and phosphate intake.[41] Risk factors such as coronary artery disease and heart failure, left ventricular hypertrophy, electrolyte shifts, and vascular calcification might be important contributors to sudden death in CKD patients.[42,43]

Cardiovascular disease is the single most common cause of death in patients with CKD and patients with late stage CKD (3b, 4 and 5) are at very high risk of cardiovascular events (eg, a 40-year-old dialysis patient has the same cardiovascular risk as an 80-year-old patient without CKD). There is both an increased burden of traditional risk factors but also a constellation of novel risk factors. It appears that as CKD progresses, the associated CVD changes from typical atherosclerotic disease to a form of arterial stiffness is accompanied by structural heart disease so that dialysis patients die of similar causes as patients with advanced heart failure.[44] Atherosclerosis is primarily a disease of the intima; although not a specific consequence of CKD, it is strongly aggravated by CKD.[45]

Dialysis techniques cannot replace all the different physiological functions of the kidney. Dialysis-related factors, such as the use of central venous catheters for hemodialysis, increase the risk of death from infection and cardiovascular causes.[46]

Transplantation related factors, such as the use of immunosuppressive drugs, increase the risk of infection and cancer and impair the cardiovascular risk profile.[47] These risk factors contribute to processes precipitating death. CKD results in accelerated ageing, particularly of the cardiovascular system.[48]

Uraemic toxins, including trimethylamine-N-oxide, have been linked to cardiovascular risk in the general population,[49,50] whereas phosphate accumulation is thought to be a key driver of CKD mineral bone disorder.[51]

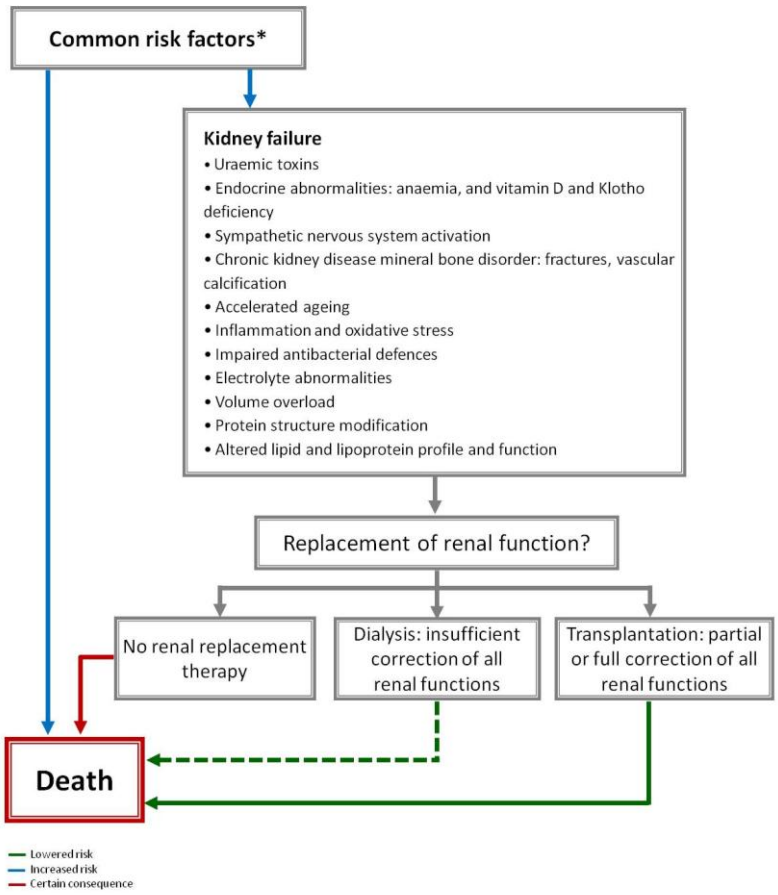

Figure I-7. Pathophysiological links between potential contributors to mortality in CKD. Coloured lines represent a direct effect on mortality. Line thickness shows the magnitude of the effect. Dotted lines show a lesser effect than solid lines. *Diabetes, hypertension, overweight, atherosclerosis, lipid disorders, smoking, and possibly salt and phosphate intake. (*Adapted from* Ortiz et al., 2014[41]).

CKD induces a complex disturbance in mineral bone metabolism which is incompletely understood. As GFR falls, the kidney's ability to excrete the phosphate absorbed in the diet is exceeded and hence serum phosphate starts to rise. This, in conjunction with reducing renal 1α-hydroxylase activity (which converts 25-hydroxy-vitamin D to the highly active 1,25-dihydroxy-vitamin D), leads to secondary hyperparathyroidism. Homeostatic PTH secretion is stimulated by high phosphate

concentrations, low concentrations of calcium and 1,25-dihydroxy-vitamin D, all attempting to normalize plasma calcium and phosphate. Although these biochemical changes may be evident in the later phases of stage 3 CKD, symptomatic bone disease (eg, with pain or pathological fractures) does not develop until patients have been on dialysis for many years. However, in children it has a significant effect on bone growth, mineralization and maturation at earlier stages of CKD.

Currently, hyperphosphatemia is treated by dietary restriction and (when this is insufficient or unsuccessful) whith dietary phosphate binders which bind phosphate in the intestine and prevent its absorption. Some of the biochemical disturbances caused by CKD mineral bone disorder (CKD-MBD) are associated with mortality in dialysis and CKD patients. It is thought that this is mediated by vascular calcification which leads to arterial stiffening and structural heart disease. There is observational evidence that treatment of hyperphosphatemia with calcium-containing phosphate binders may in fact worsen vascular calcification (and hence increase the risk of cardiovascular disease) although trials are again lacking demonstrating a benefit of calcium-free phosphate binders over calcium.

As the kidneys are central to acid-base balance, a metabolic acidosis develops which can interact with other complications (eg, worsen anemia, mineral bone disease and hyperkalemia). It is treated with oral sodium bicarbonate taken four times a day.[52] The sodium load of this treatment may worsen salt and water retention and hence hypertension; refractory acidosis is, therefore, one indication for starting dialysis. There are several other complications of CKD, such as hyperprolactinemia, low testosterone levels, sexual dysfunction, reduced fertility, erectile dysfunction, dysmenorrhea, neuropathy.[44]

Sympathetic nerve activity, which contributes to hypertension and cardiovascular events, is markedly increased in CKD, due to activation of central sympathetic tone by diseased kidneys.[53,54] Nocturnal hypoxemia due to sleep apnoea triggers sympathetic overactivity and is an independent predictor of cardiovascular death in patients on dialysis. Increased concentrations of asymmetric dimethyl arginine, an inhibitor of nitric oxide synthase, are associated with increased all-cause death. Both asymmetric dimethylarginine and sympathetic activity are part of the same pathophysiological pathway, which leads to an increased risk of death in CKD patients.[55]

The immune system is also altered in CKD. Innate immune activation leads to systemic inflammation whereas immune suppression predisposes to infection and cancer. Inflammation is associated with protein energy wasting and increased mortality. Left ventricular overload and excess sodium might be proinflammatory.[41]

Table I-3. Common chronic kidney disease (CKD) risk categories and risk factors.

Risk category	Risk factors
Susceptibility	Older age, reduction in kidney mass, low birth weight, family history of CKD, US racial or minority status
Initiation	**Diabetes, hypertension,** autoimmune disease, systemic infections; urinary tract infections, stones, or obstruction; drug toxicity
Progression	**Higher proteinuria, hypertension, poor glycemic control in diabetes, smoking, obesity, dyslipidemia, cardiovascular disease, high dietary-protein intake, decreased nephron number**
End-stage decline	**Lower dialysis dose, temporary vascular access for hemodialysis, anemia,** lower serum albumin, **late referral to nephrologist,** mineral and bone disorders, metabolic acidosis

Note: Modifiable risk factors are presented in bold. (*Taken from* Braun et al., 2012[39]).

The intertwined nature of risk factors and comorbid illnesses complicates the characterization of CKD. These terms are often used interchangeably due to the continuous and progressive nature of this disease. Some patients may have risk factors for developing CKD, while others may have risk factors contributing to the progression of CKD. Some comorbid illnesses are risk factors for both the initiation and the progression of disease. However, some risk factors are simply nonmodifiable patient characteristics. Table I-3 presents common risk categories and associated factors. As noted, some conditions fit across categories and the presence of multiple risk factors and/or comorbid illnesses leads to progression of CKD and increased mortality. Importantly, several of the debilitating risk factors and comorbidities are modifiable, and disease progression may be delayed with active patient–clinician collaboration and appropriate treatment.[39] More attention to and early active management of modifiable risk factors and comorbidities are necessary to thwart rapid disease progression.

3. Anemia secondary to stage 5 of CKD

3.1. Erythropoiesis and cytokine regulation

In mammals, definitive erythropoiesis occurs first in the fetal liver with progenitor cells from the yolk sac.[56] Within the fetal liver and the adult bone marrow, hematopoietic cells are formed continuously from a small population of pluripotent stem cells that generate progenitors committed to one or a few hematopoietic lineages (Figure I-8). In the erythroid lineage, the earliest committed progenitors identified ex vivo are the slowly proliferating burst forming unit-erythroid (BFU-E). Early BFU-E cells divide and further differentiate through the mature BFU-E stage into rapidly dividing colony-forming unit-erythroid (CFU-E).[57] CFU-E progenitors divide 3 to 5 times giving rise to pro-erythroblast, the first erythrocyte precursor that will differentiate in different erythroblasts, undergoing many substantial changes, including a decrease in cell size, chromatin condensation, hemoglobinization, and enucleation, giving rise to the reticulocyte that will mature into the erythrocyte.

A primary function of RBCs is to transport O_2 from the lungs to O_2-dependent tissues. Changes in O_2 levels may lead both acute and long-term physiologic adaptations that might include an increase in respiratory and heart rate, vasoconstriction, and changes in blood volume. Erythropoiesis is a longer-term adaptation to boost O_2-carrying capacity by increasing the concentration of RBCs, and thus, hemoglobin (Hb) concentration. RBCs are the most abundant (~99%) circulating cells in the bloodstream, representing 40% to 45% of total blood volume. In a healthy human with approximately 5L blood, this represents approximately $2.5x10^{13}$ cells, a quantity substantial enough to provide the large O_2 transport capacity needed to support aerobic respiration. In humans, the RBC lifespan is ~100 to 120 days, with a daily loss of ~0.8% to 1.0% of circulating RBCs. To match this loss, the body assumes a normal, prodigious production capacity of ~$2.5x10^{11}$ cells/day. RBC production results from a tightly controlled proliferation and differentiation pathway (Figure I-8). Early hematopoietic progenitors differentiate into burst-forming unit–erythroid cells, in which EPORs appear for the first time; however, EPO is not required at this stage.[58]

Burst-forming unit–erythroid cells differentiate into colony-forming unit–erythroid cells, which are dependent on EPO for survival, and there is a corresponding

rise in expression of EPORs.[59,60] Continued stimulation with EPO triggers differentiation into erythroblasts, which enucleate to form reticulocytes and after a few days show loss of reticulin, resulting in RBCs. Reticulocytes and RBCs stop expressing EPOR and cease being responsive to EPO.[60]

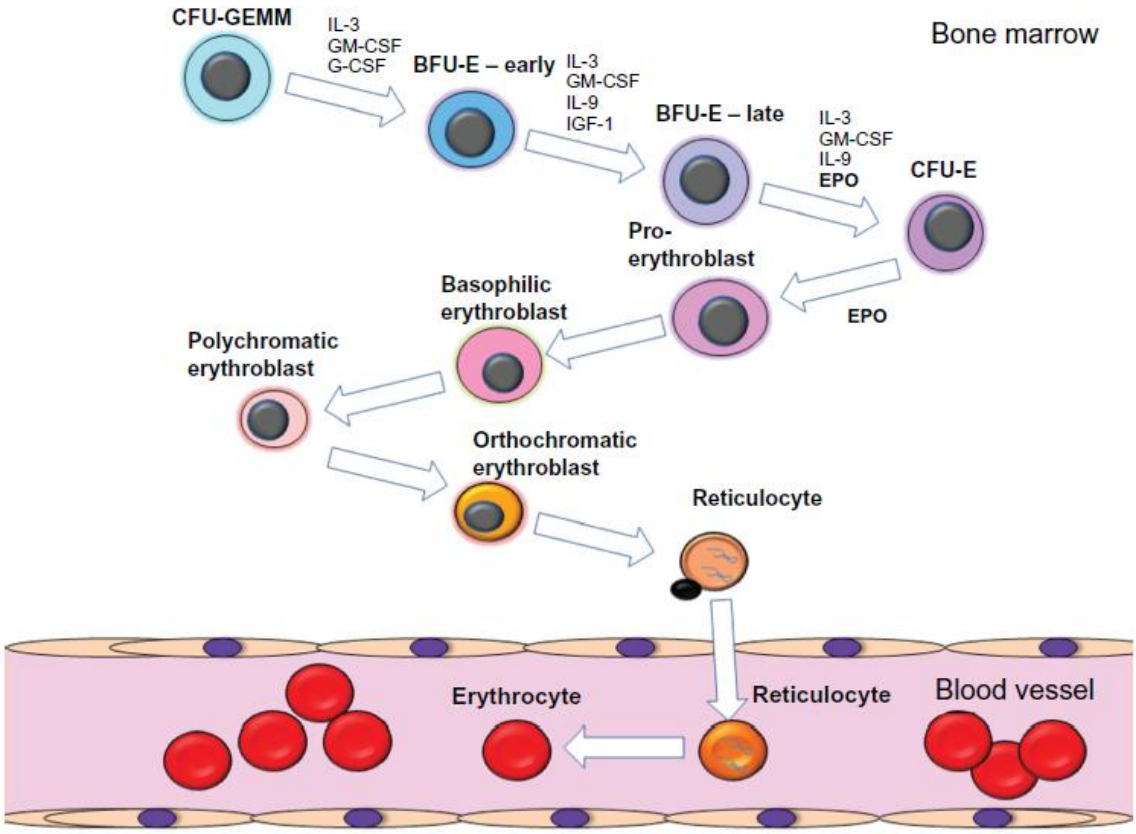

Figure I-8. Schematic diagram of the process of erythropoiesis. The various stages of erythroid differentiation are shown including the key cytokines that are involved in the proliferation, survival and differentiation of the erythroid progenitors. (*Taken from* Sinclair 2013 [61]).

Erythropoietin is essential for the survival, proliferation, and differentiation of erythrocyte progenitors in bone marrow. Erythrocyte production is continuously adjusted to regulate the loss of senescent red blood cells and to guarantee optimal tissue oxygenation. Erythropoiesis is regulated by several cytokines, such as granulocyte colony-stimulating factor (G-CSF), interleukin 6 (IL-6), stem cell factor (SCF), IL-1, IL-3, IL-4, IL-9, IL-11, granulocyte macrophage colony-stimulating factor (GM-CSF), insulin- like growth factor (IGF-1), and, of course, EPO.[62]

The normally low concentration of EPO enables only a small percentage of progenitors to survive and proliferate, whereas the remaining progenitors undergo apoptosis. In contrast, when the concentration of EPO rises in blood (either endogenously or exogenously), many more burst-forming unit erythroid (BFU-E) and CFU-E escape from apoptosis and proliferate to result in the growth and maturation of proerythroblasts.[63]

EPO acts synergistically with SCF, GM-CSF, IL-3, IL- 4, IL-9, and IGF-1 to induce red cell precursors to proliferate and mature from the stage of BFU-E and CFU-E cells to the normoblast stage.[58,64] Thus, EPO is the critical growth factor that acts on bone marrow erythroid progenitor cells to prevent them from undergoing apoptosis.[62]

A reduction in the number of red blood cells does not directly stimulate EPO synthesis and release. Instead, EPO production is controlled by the systemic availability of oxygen in a tightly regulated feedback loop. The oxygen-dependent control of EPO formation requires sensing mechanisms that perceive changes in the oxygen supply and translate them into alterations of EPO gene activity in the liver and kidneys. These mechanisms are the key element in the feedback control of erythropoiesis and are sensitive to conditions that affect arterial pO_2 and tissue and venous pO_2. EPO levels remain constant under physiological conditions, whereas in anemias and reduced renal oxygen supply EPO secretion increases with a resultant enhancement of erythrocyte production.[65] In contrast, decreased EPO concentrations are typical of conditions with an increased oxygen supply or a reduced oxygen demand.[66]

Erythropoiesis primarily occurs in the kidney, but other organs (liver, brain) also produce EPO. Interstitial fibroblasts produce EPO in the kidney, while hepatocytes produce EPO in the liver. Initially, EPO is synthesized as a 193-amino-acid precursor. A 27-amino-acid signal peptide and C-terminal arginine are removed, and carbohydrate is added to three N-linked glycosylation sites and one O-linked glycosylation site. The secreted protein contains 165 amino acids and is heavily glycosylated, with ~40% of its mass composed of carbohydrate. The structure of EPO is a compact globular bundle that contains four α helices.

Generally, serum EPO concentrations of 10 to 25 mU/mL maintain Hb levels within the normal range of 12 to 17 g/dL. The terminal half-life (t ½) of EPO is ~5 hours, which requires an average EPO production rate of ~2 U/kg/day. The EPO production rate

per cell appears constant, with fluctuations in EPO synthesis resulting from changes in the number of cells producing the molecule. In cases of severe anemia, circulating EPO levels can increase up to 1000-fold because of a logarithmic increase in the number of cells producing EPO. Other factors affecting EPO levels and, therefore, erythropoiesis, include iron availability, nutritional status, disease or comorbidities, environmental conditions, and genetic factors (congenital polycythemias).[57]

A direct correlation exists between RBC production and serum EPO concentrations. However, the rate of erythropoiesis change (~4-fold) is small compared to the larger change in EPO concentrations (~1000-fold).[67-69] Thus, the magnitude of the increase in RBC concentration is primarily controlled by the length of time EPO concentrations are maintained, and not by the EPO concentration level *per se* (Figure I-9).

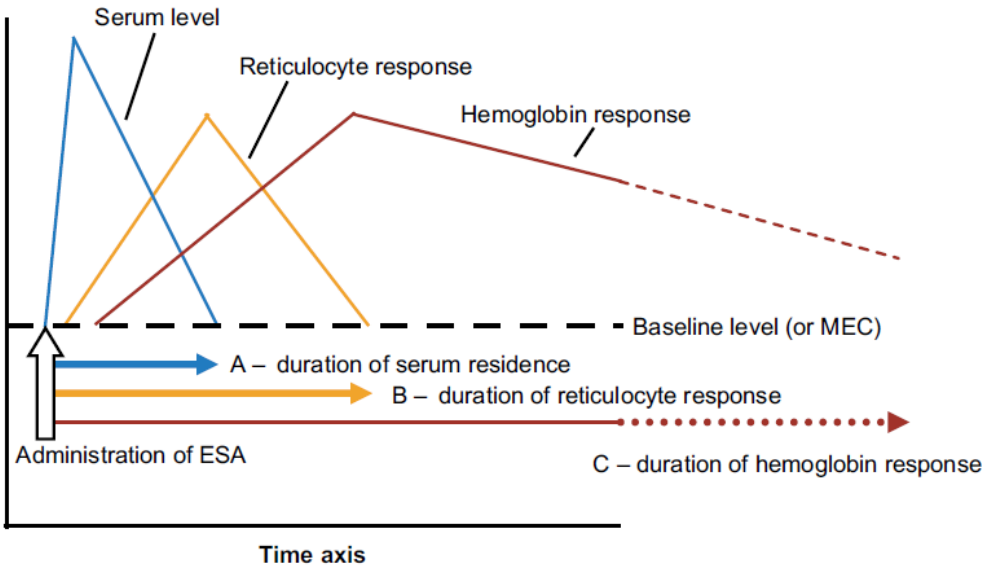

Figure I-9. Disproportion between half-life of recombinant human erythropoietin and lifespan of red blood cells. MEC, minimum effective dose (*Taken from* Elliott et el., 2008[57]).

Increased EPO synthesis has a prolonged effect due to the disproportionate relationship between EPO t ½ and RBC lifespan. Thirty minutes of hypoxia can result in production of EPO (t ½~5 hours).[70] In turn, EPO stimulates formation of enucleated reticulocytes (t ½ 51–5 days), which rapidly mature into RBCs that have a long lifespan (100-120 days). Thus, a short duration of EPO exposure results in a prolonged increase in RBC concentration.[57]

3.1.1. Erythropoietin receptor signaling

EPO promotes the viability, proliferation and differentiation of mammalian erythroid progenitor cells *via* signals transduced by the specific cell surface EPO receptor (EPOR). The EPOR is a 508 amino acid protein and is a member of the cytokine receptor superfamily. Bone marrow erythroid progenitors are the major EPO target cells. During erythroid differentiation, cells become progressively more sensitive to EPO due to the appearance of EPORs, with late progenitors CFU-E and proerythroblasts having the highest EPOR expression (~1000 receptors/cell).[71,72] Interestingly, normal levels of progenitors are still observed, indicating that EPOR signaling is dispensable at the earlier stages of erythropoiesis, including BFU-E formation.[58] Upon EPO binding, preformed receptor dimers undergo a conformational change which activates three major signal transduction pathways (JAK-STAT, PI3K-Akt, Ras-MAPK) (Figure I-10).[73]

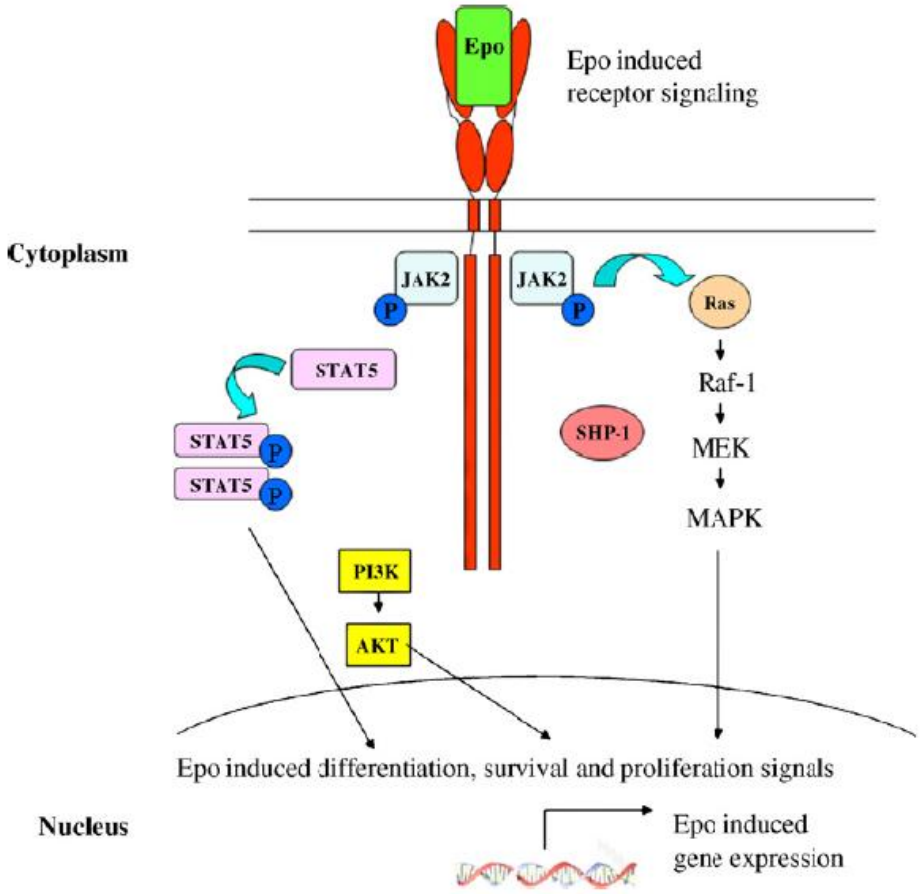

Figure I-10. EPO signaling pathways. The three main EPO signaling pathways are highlighted. (*Adapted from* Hodges et al., 2007 [74]).

As shown in Figure I-10, EPO binding causes the intracellular activation of EPOR-associated Janus kinase 2 (JAK-2). In turn, JAK-2, EPOR and other signaling proteins are tyrosine-phosphorylated.[75] Further, JAK-2 is an essential chaperone for transferring EPOR to the cell surface [76]. The phosphorylated EPOR provides docking sites for proteins containing SRC homology 2 (SH2) domains. As referred, EPOR signaling involves signal transducers and activators of transcription, such as STAT-5, phosphatidyl-inositol 3-kinase (PI-3K)/AKT (protein kinase B), and SHC/mitogen-activated protein kinase (MAPK). The action of EPO is terminated when the EPOR is dephosphorylated by the tyrosine phosphatase SHP-1 (Src homology phosphatase-1) and the EPO/EPOR-complex is internalized. In vitro studies suggest that about 60% of EPO is resecreted, and 40% undergoes proteasomal degradation after internalization.[77] EPOR mediated EPO uptake by its target cells is considered a major mechanism of the degradation of circulating EPO.[78]

3.1.2. Oxygen sensing pathway

EPO production is primarily stimulated by hypoxia, which, depending on severity, increases serum EPO levels up to several hundred-fold.[79] Hipoxia inducible factor (HIF) is a heterodimeric basic helix-loop-helix (bHLH) transcription factor that belongs to the PAS (PER/aryl hydrocarbon receptor nuclear translocator (ARNT)/single minded (SIM)) family of transcription factors. It consists of an O2-sensitive α-subunit and a constitutively expressed β-subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT).[80-82] Three HIF α-subunits are known, HIF-1α, HIF-2α and HIF-3α. HIF-1 was first isolated from human Hep3B hepatoma cells sing DNA sequences that were derived from the 3'-hypoxia enhancer of the EPO gene.[83,84] Together with HIF-2α (also known as endothelial PAS domain protein 1 (EPAS1) or HIF like factor, (HLF)), HIF-1α facilitates O2 delivery and cellular adaptation to hypoxia by stimulating a wide spectrum of biological processes that include angiogenesis, anaerobic glucose metabolism, mitochondrial biogenesis and others.[85] HIF-regulated genes are induced following the binding of HIF heterodimers to specific DNA consensus sequences and recruitment of transcriptional

co-factors. HIF-specific DNA elements are found in the regulatory regions of many O2-sensitive genes and are referred to as hypoxia-response elements (HREs) (Figure I-11).

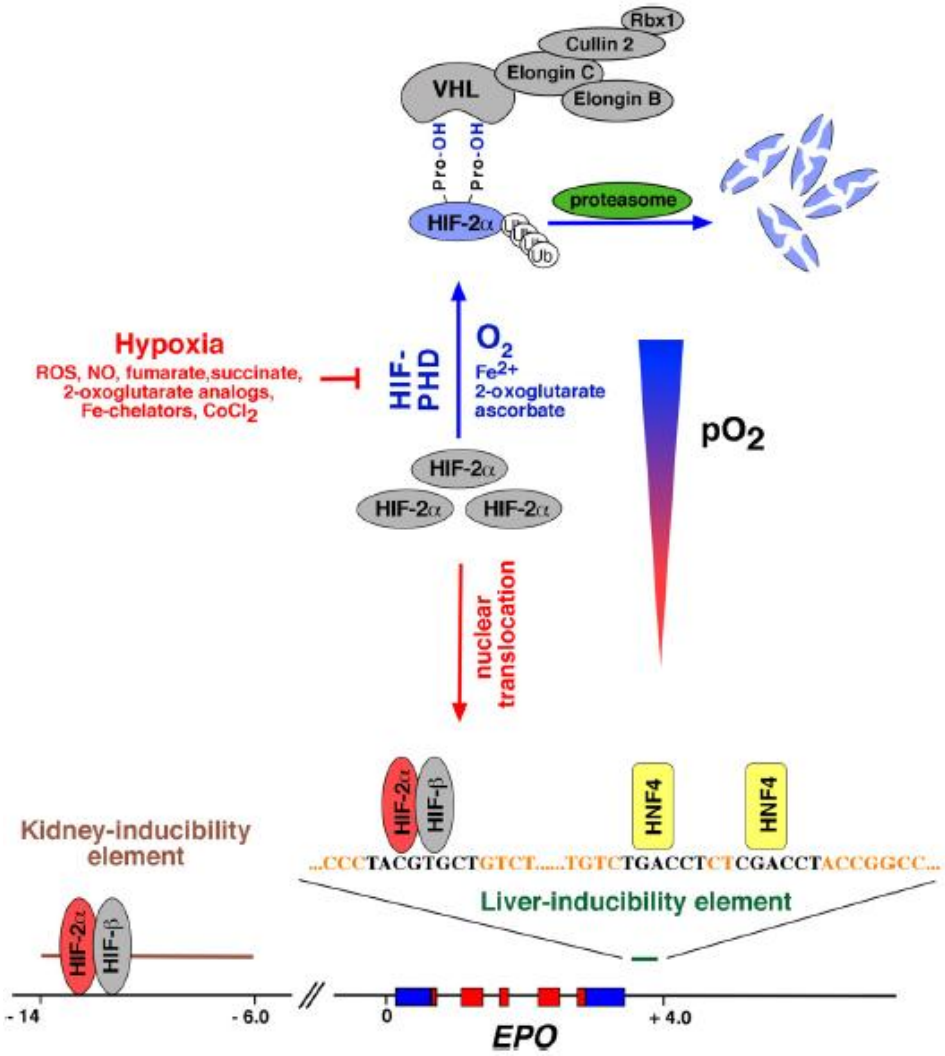

Figure I-11. EPO is HIF-2-regulated. Shown is an overview of EPO gene regulation by HIF-2.The VHL-E3-ubiquitin ligase complex targets hydroxylated HIF-2α for proteasomal degradation. Hydroxylated HIF-α binds to the β-domain of VHL. The C-terminal α-domain links VHL to the E3-ligase via elongin C. HIF-2α hydroxylation is carried out by O2- and iron-dependent HIF prolyl-4-hydroxylases (HIF-PHD). In the absence of molecular O_2, HIF-2α, which is constitutively synthesized, is no longer degraded and translocates to the nucleus where it forms a heterodimer with HIF-β. HIF-2α/β heterodimers bind to the HIF consensus binding site 5'-RCGTG-3' and increase EPO transcription in the presence of transcriptional coactivators, such as CREB-binding protein (CBP) and p300. Hypoxic induction of EPO in the liver is mediated by the liver-inducibility element located in the 3'-end of the EPO gene. The hypoxic induction of EPO in REPC requires the kidney inducibility element. Nitric oxide, reactive O_2 species, succinate and fumarate, cobalt chloride and iron chelators such as desferrioxamine inhibit HIF-PHDs, which results in increased EPO transcription. CoCl2, cobalt chloride; Fe^{2+}, ferrous iron; HNF4, hepatocyte nuclear factor 4; NO, nitric oxide; ROS, reactive oxygen species; ub, ubiquitin (*Taken from* Haase, 2013[86]).

While hypoxic suppression of certain genes has been found to be associated with HIF-1 and/or HIF-2 activation, it is unlikely that HIF acts as a direct transcriptional repressor.[87] Under normoxia, all three HIF α-subunits are targeted for rapid proteasomal degradation by the von Hippel–Lindau tumor suppressor (VHL), which acts as the substrate recognition component of an E3 ubiquitin ligase.[88,89] Whereas HIF-1 and HIF-2 heterodimers function as transcriptional activators, splice variants of HIF-3α have been shown to be inhibitory.[90,91] Although HIF-1 and HIF-2 share many transcriptional targets, certain genes and processes do not appear to be co-regulated. For example, anaerobic glycolysis appears to be predominantly controlled by HIF-1, whereas EPO synthesis and iron metabolism have emerged as HIF-2-regulated processes.[92-95]

Under normal O_2 conditions HIF-α-subunits are rapidly degraded following ubiquitylation by the VHL-E3 ubiquitin ligase complex, precluding the formation of transcriptionally active heterodimers. VHL-mediated poly-ubiquitylation requires hydroxylation of specific proline residues (Pro402 and Pro564 in human HIF-1α; Pro405 and Pro531 in human HIF-2α), which are localized within its O_2-dependent degradation domain (ODD).[96-99] Hydroxylation of HIF-α is carried out by three major 2-oxoglutarate (2OG)-dependent oxygenases (prolyl-4-hydroxylase domain (PHD) proteins), PHD1, PHD2 and PHD3, also known as egl nine homolog (EGLN) 2, EGLN1, and EGLN3, respectively. These enzymes belong to a larger family of proteins, in humans there are over 60 members, which couple the oxidative decarboxylation of 2OG to various chemical processes, which aside from O_2-sensing, include collagen synthesis and fatty acid metabolism. In mammals, these reactions produce succinate and CO_2 and appear to be limited to hydroxylation and demethylation initiated by hydroxylation.[100] HIF 2OG oxygenases function as O_2 sensors as they require molecular O_2 for catalysis. Under hypoxia, hydroxylation is inhibited and HIF signaling is activated.[101]

To add complexity to the regulation of this pathway, HIF increases transcription of PHD2 and PHD3. Furthermore, protein turnover of PHD1 and PHD3 is hypoxically regulated by Siah proteins, which themselves are hypoxia-inducible. All three PHDs are expressed in the kidney where they control HIF activity.[102,103]

The transcriptional activity of HIF is modulated by a second hypoxic switch, which operates within the carboxy-terminal transactivation domain of HIF-α. The factor inhibiting HIF (FIH) is a 2OG oxygenase that catalyzes the hydroxylation of an asparagine

residue within the C-terminal transactivation domain of HIF-α, thereby inhibiting the binding of co-activators CREB-binding protein (CBP) and p300 to the HIF transcriptional complex. Conversely, FIH inactivation facilitates CBP/p300 recruitment and results in increased HIF target gene expression under hypoxia. In the kidney, FIH has been detected in renal EPO-producing cells (REPC), podocytes and in the distal tubule.[104,105] While the role of PHDs and FIH in the regulation of HIF activity is well established, alternative hydroxylation targets have been identified and are likely to have impact on hypoxia and EPO responses in the kidney.[106,107] Furthermore, it is likely that renal EPO synthesis is modulated by epigenetic changes that are carried out by non-HIF 2OG oxygenases. Although nothing is known about their role in renal physiology, 2OG oxygenases, catalyze the demethylation of methylated histones, and are likely to provide additional functional links between alterations in renal pO_2 levels and gene expression. [101,108] Although in vitro approaches identified HIF-1 as the transcription factor responsible for the hypoxic induction of EPO,[101] HIF-2 has now emerged as the main regulator of EPO production in vivo. [86]

3.2. Stage 5 of anemia of CKD

3.2.1. Diagnosis and definition

The definition of anemia in CKD patients has been changing across guidelines over the last few years. In 2004 the Revised European Best Practice Guidelines on Anemia indicated as a definition for anemia in CKD the following: “In patients living below 1500 m, Hb values were considered below normal if they were <11.5 g/dL in women and <13.5 g/ dL in men (<12 g/dL in those aged >70 years)”.[109] This definition had the advantage of differentiating the definition of anemia between older or younger males but did not differentiate between post-menopausal and younger women. It had perhaps the disadvantage of missing anemia diagnosis in male patients >70 years, given a relatively low threshold in this category.

In 2006 the KDOQI guidelines on anemia suggested that the diagnosis of anemia should be made, and further evaluation should be undertaken, when Hb concentrations were <13.5 g/dL in adult males and <12.0 g/dL in adult females.[110] This definition was

obtained from the mean Hb of the lowest fifth percentile of the sex-specific general adult population and assumes a lack of adjustment downward for age in males and an adjustment upward for iron deficiency in females.[110] This definition is simple and easy to remember, increasing the likelihood that physicians may apply it in everyday clinical practice.

The new KDIGO suggestion[22] is based on the World Health Organization (WHO) definition of anemia.[111] It is true that this definition has been applied across populations, but it also true that it has been derived from very few data using older methodologies by a WHO expert committee.[112] Its primary aim was to screen for malnutrition and it is therefore perhaps inappropriately applied to the general population of developed countries, and especially so for a population of patients affected by a chronic disease. Given that the majority of the European population is of Caucasian ethnicity, it should also be taken into account that Caucasian men have an Hb set point that is 1–2 g/dL higher than African Americans.[113] Similar differences have been described between Caucasian, African American and Asian women. According to the Scripp-Kaiser database, the lower limit of normality of Hb values should be of 13.7 g/dL for Caucasian men aged between 20 and 60 years and 13.2 g/dL for older men. For women of all ages the set point should be 12.2 g/dL.[114]

3.2.2. Etiology

Although anemia in patients with CKD may develop in response to a wide variety of causes, erythropoietin deficiency is the primary cause of anemia associated with CKD. Erythropoietin is the hormone responsible for maintaining the proliferation and differentiation of erythroid progenitor cells in the bone marrow. Loss of peritubular cells leads to an inappropriately low level of circulating erythropoietin. Other factors in the genesis of renal anemia include functional or absolute iron deficiency, blood loss (either occult or overt), the presence of uremic inhibitors (for example, parathyroid hormone, inflammatory cytokines), reduced half-life of circulating blood cells, and deficiencies of folate or Vitamin B12.[115]

3.2.2.1. Insufficient erythropoietin production

A reduced production of erythropoietin (EPO) is the main cause of the development of renal anemia. The main stimulus to increase synthesis of EPO is tissue hypoxia, which normally leads to an exponential increase in serum EPO levels.[116,117] This feedback is affected in patients with pathological conditions involving the kidneys, as the developing anemia is not adequately compensated by a sufficient increase in the EPO production.[118-120]

Normal serum EPO concentrations in humans vary between 10 to 30 mU/mL (2 to 7 pmol/L), as determined by radioimmunoassay. EPO concentrations are increased under a variety of conditions, largely reflecting alterations of oxygen delivery to tissues. The expected compensatory response to anemia is an increased rate of erythropoiesis, with an inverse relationship between the concentration of the hormone and the Hb concentration.[121] Although the anemia of chronic renal failure is a complex disorder in which many factors may play a role, the main defect is absolute or relative EPO deficiency. In most patients with substantially impaired renal function, EPO production is impaired at any given hematocrit concentration.[122] Why EPO production remains inappropriately low in patients with CKD remains unclear, this could be because the EPO-producing cells are lost, because they experience higher levels of oxygen for a given hematocrit level, or because the relationship between local oxygenation and EPO production is altered.[123] Several lines of evidence suggest that diseased kidneys are hypoxic and that this chronic decrease in oxygen availability, in fact, may contribute to disease progression.[124] A defect in EPO synthesis, rather than a lack of the hypoxic stimulus, may lead to the failure of EPO production.

3.2.2.2. Disturbances in iron metabolism

Iron deficiency, or its limited availability for erythropoiesis, is an important pathogenetic mechanism for the development of renal anemia. Hepcidin is currently believed to play a crucial role in the regulation of iron metabolism. This hormone was discovered by Krause et al. (2000)[125] and independently by Park et al. (2001).[126] Bioactive hepcidin is composed of 25 amino acids and is synthesized mainly in hepatocytes (Figure I-12). The regulation of iron absorption and its distribution into the

tissues is mediated by regulation of the expression of its receptor, ferroportin, which is known to be the sole cellular iron exporter.

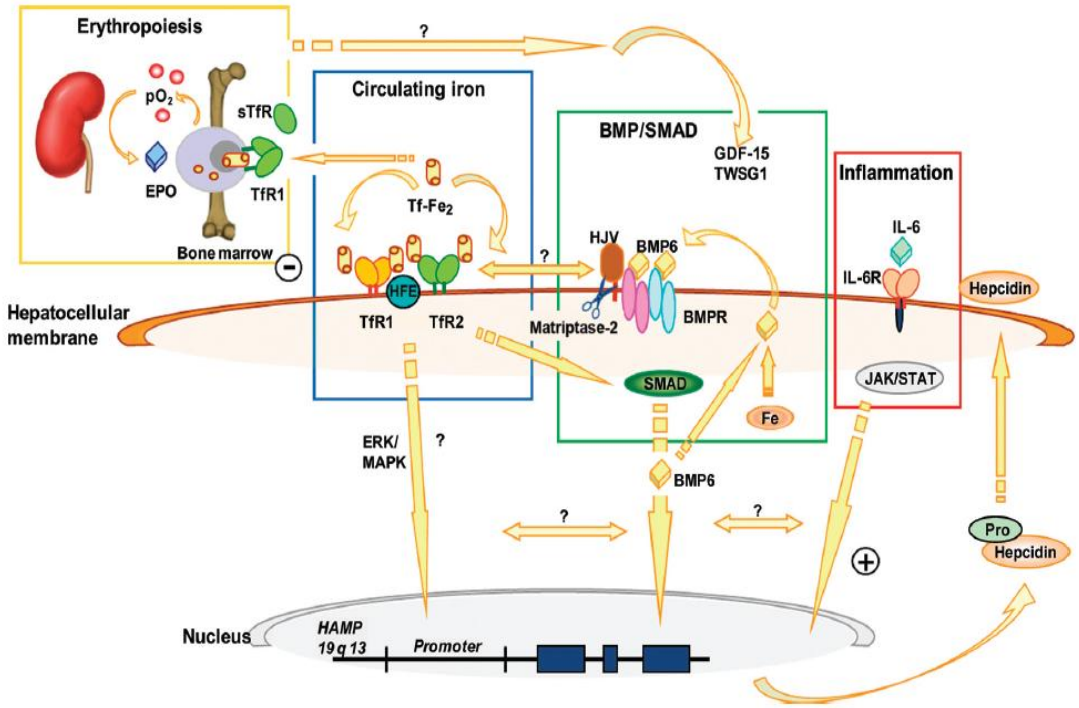

Figure I-12. Molecular and functional pathways of hepatocyte hepcidin synthesis. Three molecular pathways can be distinguished: the HFE/TfR2, BMP/SMAD, and JAK/STAT. This proposed model depicts 2 iron signals to hepcidin, 1 mediated by intracellular iron stores (Fe) and the other by circulating iron (Tf-Fe2). Hepatocellular iron stores increase the expression of BMP-6, which serves as an autocrine factor by interacting with surface BMP receptors. HJV is a BMP coreceptor that augments BMP binding. The consequent activation of intracellular SMAD proteins transduces a signal to increase hepcidin transcription. HJV is subject to cleavage by furin, which is regulated by iron and hypoxia, to form a soluble component (sHJV) [127]. sHJV can subsequently act as a decoy coreceptor and antagonist of BMP-6 induced hepcidin synthesis. Under low iron conditions membrane bound HJV is also cleaved by matriptase-2 (scissors) again weakening the BMP-6 signal. Extracellular Tf-Fe2 mediates a second iron signal. In this scheme, Tf-Fe2 displaces HFE from TfR1. HFE is then liberated to interact with TfR2. The HFE–TfR2 complex activates hepcidin transcription via BMP/SMAD signaling. Several studies have provided indirect evidence for the involvement of the hepatic ERK/MAPK signaling pathway in hepcidin regulation by iron through TfR2 and/or HFE.[128] Hypoxia influences liver-specific stabilization of HIF-1, which induces matriptase-2 and the subsequent cleavage of HJV [129]. The latter pathway may be synergistic to the increased release of sHJV upon its cleavage by furin under hypoxic conditions. Erythropoiesis is controlled in part by EPO production in the kidney and communicates with the hepatocyte by the proteins GDF15 and TWSG1, which inhibit the BMP/SMAD signaling to hepcidin. Inflammatory stimuli, such as IL-6, induce hepcidin synthesis through the janus kinase/signal transducer and activator of transcription-3 (JAK/STAT) pathway. These pathways have recently been reviewed.[128] pO2, partial oxygen pressure; sTfR: soluble TfR; BMPR, BMP receptor; IL-6R: IL-6 receptor. (*Taken from* Kroot et al., 2011[130]).

Hepcidin synthesis is induced by excess iron levels or inflammation, while it is inhibited by increased erythropoiesis or hypoxia. Increased hepcidin levels are known to block iron absorption from food in the intestines and its release from iron stores. The activation of erythropoiesis logically leads to the suppression of hepcidin production and subsequent mobilization of iron from its stores. Synthesis of hepcidin is stimulated by proinflammatory cytokines, in particular IL-6.[131] However, based on current knowledge, the evaluation of hepcidin for the diagnosis of iron deficiency in dialyzed patients is not used in routine clinical practice.[132]

Daily iron loss is small in normal individuals. Iron is lost by the desquamation of skin epithelial cells, intestinal tract mucosa, growth of hair and nails, and through the bile, sweat, and urine. The total iron loss in adult males as measured by radioisotope techniques is 0.5 to 1.5 mg daily. The expected daily iron loss in women is higher than in men.[133] Under normal circumstances, physiological iron loss is fully compensated by iron reabsorption from food. In dialyzed patients, however, physiological iron loss is increased by a number of other circumstances that could easily induce iron deficiency. The main factor that leads to iron deficiency in dialyzed patients is excessive blood loss during dialysis, especially the significant amount of residual blood that remains inside the dialyzer, dialysis sets, and needles after each hemodialysis. It is generally accepted that the average annual loss of iron in dialyzed patients is 1.5-2.0 g.[133,134]

The normal daily intake of iron is 10 to 15 mg and the maximum absorption is about 20%. Therefore, the amount of iron that the body of dialyzed patients can absorb from regular food is not able to compensate for these substantial losses. Thus, dialyzed patients, who receive no iron supplementation therapy, can very easily develop a negative iron balance and depletion of tissue iron stores.[135,136] The body of a healthy adult normally contains 4-5 g of iron. Iron deficiency is defined as a condition where the amount of iron in the body decreases to a level that is characterized by a depletion of iron stores in bone marrow, liver and spleen and it, usually, develops when occurs an imbalance between the iron supply to the tissues and the real needs.[133]

Under normal circumstances, storage iron accounts for approximately 30% of the body's total iron, and it is stored in the macrophages of the reticuloendothelial system, the bone marrow, liver, and spleen, and in the liver parenchymal cells. Iron is stored as ferritin or as hemosiderin. Ferritin is composed of an inorganic core called holoferritin,

which is surrounded by a protein envelope. Hemosiderin granules are large aggregates of ferritin molecules with higher iron content. Hemosiderin further contains fats and, unlike ferritin, it is soluble in water. Under normal circumstances, iron is primarily stored as ferritin, and the ferritin/hemosiderin ratio is 60:40.[120]

To evaluate the functional iron status, we can use a variety of other laboratory parameters, such as iron plasma concentrations (pFe), transferrin, and transferrin saturation (TSAT), ferritin and soluble transferrin receptors. Low pFe levels may have different causes and in differential diagnosis is very important to differentiate low pFe levels due to an actual iron deficiency from reactive hyposideremia due to a disturbed iron absorption or iron mobilization, as is the case in infectious processes, tissue damage, and malignant tumors.[137] Organic and ionized forms of iron are highly toxic and rather insoluble and, for this reason, the transport of iron in the body is bound to a protein component, transferrin. Transferrin is a glycoprotein with a molecular weight of 79550 Da. The main site of transferrin production is the liver. Transferrin has two binding sites for iron, which allow the existence of apoferric, or diferric transferrin. In addition to iron bound in the oxidized form, transferrin can, also, bind other metal ions, of which binding to aluminum may have adverse effects on the erythropoiesis in dialyzed patients. In patients with sideropenic anemia, the ability of the serum to bind iron is increased, and iron deficiency leads to an icrease in the synthesis of transferrin. Transferrin is known to control the internal transfer of iron from the site of absorption to the places of iron consumption or storage, and back, when utilizing the iron released from macrophages or from the disintegrating red blood cells.[138] Circulating iron bound to transferrin in the plasma is transported into cells via receptors expressed on the cell membranes, triggering receptor-mediated endocytosis. In the acidic environment of the cell endossoma, iron is released from transferrin and in the mitochondria, after reduction by mitochondrial ferrochelatase, is incorporated into the heme. The final phase is exocytosis of apotransferrin back into the blood plasma. Iron deficiency results in a compensatory increase in the number of transferrin receptors in the erythroid cells. The serum concentration of these transferrin receptors is also increased and its evaluation is already performed in hematologic studies, as its value is independent of inflammation, unlike transferrin and ferritin.[139] Since pFe and transferrin are subject of numerous reactive changes under various pathological conditions, the relationship

between these two values, known as TSAT, is considered to be a more accurate indicator of the metabolic status of iron. Under physiological conditions, the binding capacity of transferrin is 20-45% saturated with iron. Decreased transferrin saturation is one of the indicators of iron deficiency. Iron is most efficiently uptaken by erythroblasts at 30-60% transferrin saturation. If the saturation exceeds 60%, iron is stored in the mononuclear phagocyte system cells.[135]

3.2.2.3. Other factors contributing to the development of anemia

Several other factors have been identified that may further exacerbate anemia in patients with impaired renal function, such as inflammatory processes or tumors, vitamin B12 and folic acid deficiencies, drugs or uremic inhibitors of erythropoiesis.

Suppression of erythropoiesis occurs during severe infectious processes, inflammatory diseases, and malignancies. Inflammation is often accompanied by malnutrition and both have a significant role in the development of anemia, usually known as the MIA syndrome (malnutrition, inflammation and anemia). The main causes are: increased production of cytokines, such as, IL-1, IL-6, TNF-alpha and interferon-gamma that block the release of iron from iron stores (through an increase in hepcidin synthesis triggered by IL-6), reduce the production of endogenous EPO, and inhibit erythropoiesis. Inflammation increases iron uptake within cells of the reticuloendothelial system. This leads to limitation of iron availability for erythroid progenitor cells and subsequently to decreased erythropoiesis. A key role in this chronic inflammation-related anemia is played by hepcidin, whose synthesis and serum levels increase up to 100-fold during inflammation. In the laboratory, this condition is confirmed by increased serum ferritin concentrations, and reduced pFe, transferrin levels and TSAT.[140,141]

In patients with renal disease, reduced intravascular survival of red blood cells is caused by chronic hemolysis, which is the result of accumulation of hypothetical uremic toxins in the blood. The most important uremic toxins in the pathophysiology of renal anemia are polyamines, such as spermine, spermidine, putrescine and cadaverine. Polyamines are organic cations involved in cell growth and maturation that reduce the proliferative activity of erythroid cells in the bone marrow by a direct toxic effect that is independent on their interaction with EPO.[142] Renal anemia may be exacerbated by a

lack of folic acid or vitamin B12. Deficiency of vitamin B12 or folic acid is uncommon, occurring in less than 10% of dialyzed patients; in these cases, the anemia has a macrocytic pattern. Folic acid deficiency may occur particularly in dialyzed patients, because folic acid is dialyzable; serum folate levels are also decreased after dialysis. The loss of folic acid during the dialysis outweighs the loss of folic acid in the urine of normal people, which is about 10 μg/24h. The minimum recommended daily dose of folic acid in dialyzed patients is 50 μg.[143,144]

A decrease in hemoglobin levels may occur in aluminum intoxication. The anemia induced by the accumulation of aluminum has a microcytic and hypochromic pattern. Aluminum competes with iron for the binding on the transferrin molecule, increasing its binding to free receptors, in particular in patients with iron deficiency. In addition, aluminum has been demonstrated to suppress the cytosolic uroporphyrinogen decarboxylase and mitochondrial ferrochelatase, disturbing heme synthesis.[145]

Hypoproliferative anemia may also be exacerbated by fibrous osteodystrophy, which is usually caused by severe secondary hyperparathyroidism.[146] Excessive parathormone levels are known to reduce EPO production, increase hemolysis, and suppress progenitor of red blood cell line in the bone marrow.[147]

Anemia may be further aggravated by drugs, particularly by ACE inhibitors. ACE inhibitors suppress the synthesis of erythropoietin in a dose-dependent manner via enhanced oxygenation in the peritubular region due to decrease in vascular resistance in efferent arterioles, decreased serum concentration of IGF-1 and increased apoptosis of progenitor erythroid cells $CD34^+$.[148,149]

Drugs are an important contributor to anemia in recipients of renal allografts. After kidney transplantation anemia is a highly prevalent disorder affecting approximately 40% of patients and has been associated with a decline of graft function. Immunossuppressors play an important additional role. Azathioprine, mycophenolate mofetil and mycophenolate sodium have a direct antiproliferative effect on bone marrow cells. Sirolimus and everolimus (mTOR inhibitors) can lead to microcytic anemia. Cyclosporine A and mTOR inhibitors are known to induce hemolytic uremic syndrome and hemolytic anemia in transplant patiens. Another immunosuppressive agent, antithymocyte globulin (ATG) can decrease hemoglobin concentration via toxic effect on bone marrow.[150]

3.3. Treatment of anemia in CKD - The KDOQI and KDIGO guidelines

Renal anemia is treated by erythropoiesis-estimulanting agents (ESAs), to stimulate red blood cell production, and with iron supplementation, as recommended.[109] Iron is a crucial element of treatment, whether to address absolute iron deficiency (a lack of stored iron characterised by low TSAT <20% and serum ferritin levels below <100 ng/ml) or functional iron deficiency (inadequate release of iron from iron stores, characterised by low TSAT <20% and serum ferritin levels above ≥100 ng/ml).[151] Previous treatment guidelines issued by the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) recommended that iron supplementation therapy should be sufficient to maintain iron stores, prevent iron-deficient erythropoiesis and achieve and maintain target haemoglobin levels. Whilst delivery of iron by the intravenous (i.v.) route was regarded as optimal for patients on hemodialysis, for those not requiring hemodialysis both oral and i.v. administration were deemed appropriate.[110] Oral iron is self-administered at home, whereas i.v. iron must be administered by a healthcare professional. The regimens for administration of i.v. iron highlight how treatment of renal anemia in people not requiring hemodialysis can impact on their life. Limitations on the maximum dose of iron administered by i.v. injection can mean that administration is divided over several appointments over a period of one to five weeks. Depending on the preparation, appointments may reach four hours in duration, partly due to restrictions on the rate of injection and, in some cases, requirement for a test dose or postdose observation period. These test doses and observation periods stem from the possibility for anaphylactic reactions with i.v. iron preparations; these are of particular concern in high molecular weight iron dextran formulations, although warnings are found in the prescribing information of many formulations.[152] Put into the context of a patient's daily routine, the implication of these regimens is that, as well as the time spent being treated, a patient must spend time travelling to and from the clinic or hospital at which treatment is administered. This must be repeated for as many appointments as is necessary for the patient to receive the appropriate total dose of iron to replenish their iron stores; and then again, in the future, when iron levels require further repletion. However, it is important to balance these implications with the potential benefits of i.v. iron. For example, a recent meta-

analysis highlighted that up to 60% of people with CKD not undergoing hemodialysis can reach their haemoglobin target and maintain it by i.v. iron repletion.[153] This is supported by the recommendations in the KDIGO Clinical Practice Guidelines on Anemia in CKD[22] that recommend initial IV iron for individuals requiring an increase in haemoglobin concentration.

KDIGO (Kidney Disease Improving Global Outcomes) working group published in August 2012 new recommendations for treating anemia in patients with CKD. The new recommendations have created many changes affecting clinical practice, in comparison to the previous ones. Newly recommended were higher serum levels of ferritin (500 μg/L) and for transferin saturation (30%). ESAs should be administered only following suitable supplementation of iron levels. Another important change is the lower recommended Hb concentration for which an intervention using ESAs is indicated. ESAs should be administered when Hb levels are in the range 9-10 g/dL. In patients with symptoms of the anemic syndrome, ESAs can be applied with Hb $\geq$ 10 g/dL. It is generally recommended that during maintenance ESAs therapy, the Hb level should not exceed 12 g/dL in adult patients.[22]

The conclusions and subsequent analyses of CHOIR,[154] CREATE[155] and TREAT[156] trials were key considerations in the development of the KDIGO Guidelines.[22] Published in August 2012, with the aim of assisting clinical decision making, the guidelines evaluate the strength of current evidence regarding the diagnosis and evaluation of anemia in CKD as well as its treatment by red cell transfusion, iron supplementation and ESAs.

4. Recombinant human erythropoietin

The human EPO gene was cloned in 1985,[157,158] allowing for clinical development of recombinant human erythropoietin (rHuEPO), a biotechnological advance that revolutionized anemia treatment. Endogenous EPO and rHuEPO share the same amino acid sequence, with slight differences in the sugar profile.[159] In clinical practice, rHuEPO is typically administered as a bolus injection, and the dose is titrated to give the desired effect. Attempts to improve or ‘‘reengineer’’ rHuEPO to meet the demands of patients and caregivers resulted in additional ESAs with increased serum half-lives (compared

with rHuEPO), as well as different receptor binding properties and *in vivo* biological potencies.[160-162] The characteristics and properties of these new ESAs allowed extension of the dosing intervals beyond the original thrice weekly.[163,164] All ESAs share the same mechanism of action, binding to and activating the EPO receptor (EPOR), but differences in pharmacokinetic, pharmacodynamic, and receptor-binding properties affect their clinical use.

4.1. Therapeutic uses of recombinant erythropoiesis-stimulating agents

The primary goal of the treatment with recombinant erythropoiesis-stimulating agents (ESAs) is to abolish the need for RBC transfusions. Hemoglobin concentrations [Hb] <10 g/dL are associated with an increased risk of morbidity and mortality in CKD patients.[165,166] The current NKF KDOQI guidelines recommend a target [Hb] of 11-12 g/dL on ESA treatment.[110] ESA associated changes in health-related quality of life (HRQOL) include improvements in physical symptoms (vitality, energy, performance), while domains of social functioning and mental health are little ameliorated.[167] ESAs are also administered to avoid RBC transfusions in cancer patients receiving myelosuppressive chemotherapy.[168]

4.1.1. Different types of ESAs

4.1.1.1. Epoetins

The epoetins currently available are produced using recombinant DNA technology in chinese hamster ovary (CHO) cell lines, which leads to a glycosylation structure that differs from endogenous human EPO.[159]

Epogen® , an epoetin alfa formulation produced by Amgen (Thousand Oaks, CA, USA), was approved by the Food and Drug Administration (FDA) for the treatment of anemic CKD patients on hemodialysis in 1989. The drug was originally derived from CHO cells maintained in roller bottles, which were later replaced by bioreactors. In addition, animal-derived additives have been replaced by synthetic molecules in modern recombinant manufacturing processes.[169] In 2000, Amgen received FDA approval for the manufacture of epoetin alfa at its new facilities in Longmont, CO, USA. Furthermore,

epoetin alfa has been distributed in the USA by Johnson & Johnson (J&J, New Brunswick, NJ, USA), under the name of Procrit®, for other indications through an agreement with Amgen. Outside the USA epoetin alfa has been marketed under the names of Eprex® or Erypo® (J&J subsidiary Ortho Biotech, Bridgewater, NJ, USA), which was approved in the European Union (EU) by the European Medicines Agency (EMA) in 1988.

NeoRecormon®, an epoetin beta originally manufactured by Boehringer Mannheim and subsequently by F. Hoffmann-LaRoche (Penzberg, Germany), received EU approval in 1990. Although epoetin beta differs slightly from epoetin alfa in glycosylation,[159,170] the two medicines are used for the same major indications (primarily anemias associated with CKD or myelosuppressive chemotherapy treated cancers) and are considered as interchangeable by healthcare professionals.

The native EPO has a half-life of 8.5 hours. After a subcutaneous injection, the half lives of rHuEPOs in plasma increase in the following manner: epoetin alfa to 20.5 hours and epoetin beta to 24 hours. Different sialic acid-containing carbohydrate components added to the 165 amino acids of epoetin alfa and epoetin beta confer longer half-lives to these compounds.[171] For epoetin alfa and epoetin beta, the recommended dosing to achieve and maintain hemoglobin levels between 10–12 g/dL is as follows: for anemia associated with CKD it is given subcutaneously (intravenous route is used for hemodialysis patients because of convenience), 50–100 units/kg three times/week; for anemia associated with chemotherapy it is given subcutaneously, 150 units/kg 3 times/week or 10,000 units 3 times/week or 40,000 units once weekly. Hemoglobin levels should not exceed 12 g/dL.[172]

In 2009 the EMA granted a marketing authorization for another CHO cell-derived rhEPO, epoetin theta (brand names: Biopoin®, CT Arzneimittel, Berlin, Germany; Eporatio®, Ratiopharm, Ulm, Germany), which was developed by Merckle Biotec (Ulm, Germany) using epoetin beta as a comparator. Epoetin theta is not a biosimilar but a stand-alone product.[173] It is approved for the treatment of symptomatic anemia in adult patients with CKD or myelosuppressive chemotherapy-treated cancer.

Differing from the other epoetins, epoetin omega (Repotin®, Bioclones, Cape Town, South Africa) is produced in epo cDNA-transfected Syrian baby hamster kidney (BHK) cells. Epoetin omega has an N-glycan with phosphorylated oligomannoside chains,

and it possesses less O-glycans than the CHO cell-derived rhEPOs.[159,174] There is little known about the clinical use of epoetin omega.

Epoetin delta (Dynepo®, Shire Pharmaceuticals, Wayne, PA, USA) is the first human cell line derived Epo and therefore has a human-type glycosylation profile. In a multicenter, open-label, uncontrolled study on predialysis CKD patients, epoetin delta was found to be an effective and well-tolerated agent for the management of anemia.[175,176] It is approved for clinical use in European countries but not in the United States.

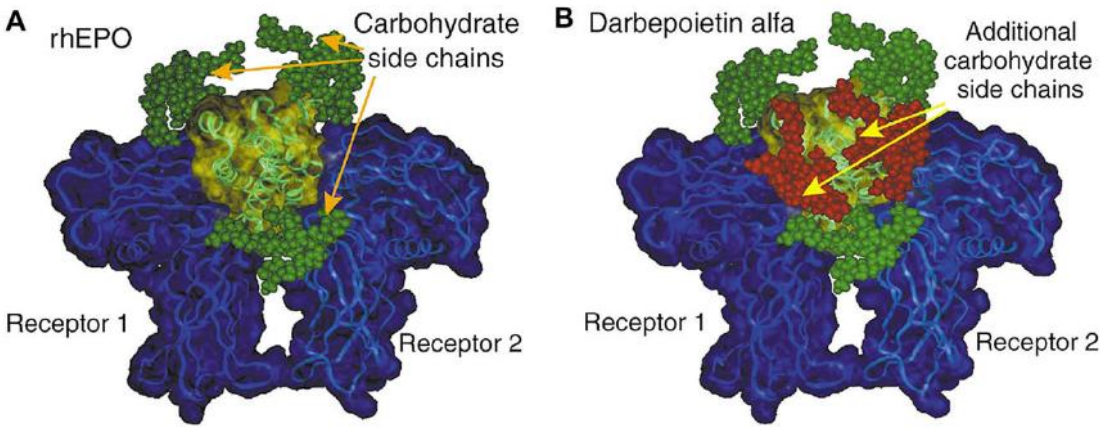

Figure I-13. Molecular structures of epoetin alfa (A) and darbepoetin alfa (B). (*Taken from* Elliott et al., 2008[57])

Furthermore, second-generation ESAs with a prolonged survival in circulation are available. Darbepoetin alfa (Aranesp®; Amgen), which was approved in the USA and the EU in 2001, possesses two additional N-glycans as a result of site-directed mutagenesis.[160,177] These additional N-linked oligosaccharide chains are accomplished by substitutions at five positions along the 165-aminoacid backbone without altering the tertiary structure. These additional carbohydrates confer a three times longer terminal half-life and a five times lower affinity for EPO receptors relative to erythropoietin alfa. After a subcutaneous injection, the half live of darbepoetin alfa in plasma is 49 hours.[171] Despite different pharmacodynamic and pharmacokinetic properties, all these products are considered to have similar clinical efficacy (Figure I-13).[161]

For darbepoetin alfa, the recommended dosing to achieve and maintain hemoglobin levels between 10–12 g/dL is as follows: for patients with anemia of CKD it is given intravenously or subcutaneously, 0.45 μg /kg once weekly; for anemia associated with chemotherapy it is given subcutaneously, 2.25 μg/kg once weekly or 200 μg every two weeks or 500 μg once every 3 weeks. Hemoglobin levels should not exceed 12 g/dL like reported previously.[172]

Continuous Erythropoietin Receptor Activator (CERA) (Mircera®, Hoffmann-La Roche Inc. (Roche), Nutley, N.J), is composed of a large methoxy-polyethylene glycol polymer chain integrated into the EPO molecule and linked primarily by amide bonds.[178] This results in a longer half-life of approximately 130 hours after both intravenous and subcutaneous administration. Compared with epoetin alfa, CERA has different binding characteristics at the EPO receptor, mainly because it associates with the receptor more slowly, but its better stability gives it a longer half-life.[179,180] In multiple, randomized phase III trials, CERA has been found to be as safe as currently-approved and inuse ESAs and has been shown to be efficacious in the management of anemia of CKD when given intravenously at 2-week or 4-week dosing intervals.[164,181]

CERA is another recently available ESAs obtained by adding a large water-soluble polyethylene glycol moiety to epoetin beta,[182] leading to a higher molecular weight ∼60 kDa) and a longer half-life. The total binding affinity of CERA for the EPO receptor is 50–100 times less than that of epoetin beta, mainly because of its much slower binding rate.[183] It has been suggested that the binding of CERA to the EPO receptor is too brief for internalization, thus leading to repeated cycles of receptor binding, stimulation, and dissociation, and a consequently greater erythropoietic activity.[178] Animal studies have shown that, despite its lower receptor affinity, CERA induces a greater erythropoietic response than epoetin alfa or epoetin beta,[162] thus indicating greater potency in vivo, although these differences may be partially influenced by the different doses of the drugs. It has been hypothesized that CERA elimination is mediated by hematopoietic and nonhematopoietic EPO receptor pathways. Unlike that of EPO, the nonerythropoietic pathway is saturable, which may mean that an increasing fraction of CERAs used for erythropoiesis for increasing concentrations because of greater bioavailability.

All ESAs are effective in correcting renal anemia and increasing hemoglobin levels, but the choice of which to use should also consider their pharmacokinetics and pharmacodynamics, their administration route, and economic issues.

Following the publication of a number of trials indicating no benefit (and even possible harm) when ESAs are used to achieve near-normal hemoglobin levels in CKD patients, the hemoglobin target has become a major subject of discussion. According to the position statement of the Anemia Group of the European Renal Best Practice, it should be about 11–12 g/dL; however, a risk– benefit evaluation is warranted in individual patients, and high ESAs doses driven by hyporesponsiveness should be avoided.[184]

4.1.1.2. Biosimilar epoetins in the European Union

Since developers of biosimilars usually do not have access to the originators' proprietary data they engineer their own manufacturing process to obtain a product as similar as possible to the original.[185] Because biosimilars are not fully identical to their reference product, the proof of bioequivalence is not sufficient for their approval. In this regard, they differ from generic drugs. The EMA committee for Medicinal Products for Human Use (CHMP), which is responsible for the scientific assessment of the products ("European Public Assessment Reports", EPARs), has exhibited guidelines on: i) the studies necessary to show "the similar nature, in terms of quality, safety and efficacy" in comparison to the originator's product, ii) the quality requirements regards the purity of the product and its components, iii) the non-clinical and clinical pharmacodynamic and toxicokinetic issues, and iv) the product-specific requirements for epoetins.[173] For their approval biosimilar epoetins must demonstrate therapeutic equivalence with the reference product in at least two randomized, parallel-group (and preferably double-blind) clinical trials, ideally in patients with anemia due to CKD. After all, the EMA has stated that "since the mechanism of action of epoetin is the same for all currently approved indications and there is only one known epoetin receptor, demonstration of efficacy and safety in renal anemia will allow extrapolation to other indications of the reference medicinal product with the same route of administration".[173]

Two biosimilar epoetins have been approved by the EMA, namely substance HX575 and substance SB309.[173] Both products exhibit minor differences in the structure of their glycans compared to the reference product (Eprex®/Erypo®). There is no evidence suggesting that these differences are clinically relevant. Different lots of recombinant glycoproteins from innovator companies may also vary in their glycosylation profiles [186]. The quality of the biosimilar epoetins proved to be high when studied by high-performance size-exclusion chromatography, SDS-PAGE, and capillary zone electrophoresis.[187] The two biosimilar epoetins differ in formulation, while substance HX575 is stabilized by polysorbate-80 and several amino acids, similar to the original epoetin alfa (Eprex®/Erypo®), substance SB309 is stabilized by polysorbate-20, similar to epoetin beta (NeoRecormon®). According to the European Pharmacopoeia, the activity (International Units, IU) of clinically used rHuEPOs must be assessed by *in vivo* bioassay in mice and confirm that "The estimated potency is not less that 80% and not more than 125% of the stated potency". Thus, the potency of clinically used epoetins can vary within this range.[188]

Substance HX575 (Rentschler Biotechnologie, Laupheim, Germany) was the first biosimilar epoetin with marketing authorization in the EU. HX575 has received the International Nonproprietary Name (INN) "epoetin alfa" despite its slightly different glycosylation pattern compared to the reference drug (Eprex®/Erypo®). HX575 has elevated levels of high-mannose structures and lower levels of N-glycolyl-neuraminic acid and diacetylated neuraminic acids. HX575 is traded as Binocrit® by Sandoz and as Epoetin alfa Hexal® by Hexal Biotech (Holzkirchen, Germany), both Novartis subsidiaries, and as Abseamed® by Medice Arzneimittel Putter (Iserlohn, Germany), a Sandoz licensing partner. HX575 has proved therapeutic equivalence with Eprex®/Erypo® on intravenous administration in an open, randomized, parallel-group study of 80 healthy males[189] and in a doubleblind randomized, parallel-group, multicenter Phase III trial involving 479 anemic CKD patients on hemodialysis. In the CKD patients, Hb concentrations and epoetin dosages remained stable throughout the study period of 56 weeks. The long-term safety profile of HX575 was found to be similar to that of the comparator epoetin alfa. No neutralizing anti-EPO antibodies (Abs) were detected.[190]

Substance SB309 (Norbitec, Uetersen, Germany) has received EU approval with the INN “epoetin zeta” (Silapo®, Stada, Bad Vilbel, Germany, and Retacrit®, Hospira, Lake Forest, IL, USA). SB309 has less O-glycans, and lower levels of N-glycolyl-neuraminic acid and O-acetyl neuraminic acid than the reference drug (Eprex®/Erypo®). According to the EPAR, SB309 was about 10% less potent than the reference product, when administered i.v. to CKD patients. However, the difference was apparently not intrinsic to the biosimilar but due to a relative high active substance content of the Eprex®/Erypo® batch used in the approval study.[191] In study with 609 patients with CKD and anemia ([Hb] <9 g/dL) that received either SB309 or Eprex®/Erypo® i.v., one to three times per week for 24 weeks the dosing was titrated individually to achieve a target [Hb] of 11 - 12 g/dL. The mean SB309 dose over the last 4 weeks of treatment was 182 ± 118 IU/kg/week, compared with 166 ± 110 IU/kg/week for the reference product. No unexpected adverse events were reported, and no patients developed neutralizing anti-EPO Abs.[192]

4.1.1.3. Other strategies for stimulating erythropoiesis

The inhibition or activation of transcription factors modulating EPO gene expression may be an alternative for stimulating erythropoiesis. The hypoxia-inducible transcription factors (HIFs) are central components in the cellular responses to hypoxia; under normoxic conditions, EPO gene expression is suppressed as a result of HIF inactivation because of $O(_2)$-dependent enzymatic hydroxylation and subsequent degradation of their alpha-subunit. 2-oxoglutarate analogs, which prevent HIF alpha hydroxylation, have emerged as promising tools for the stimulation of erythropoiesis and angiogenesis (the so called HIF stabilizers). Concern related to HIF stabilizers is related to the fact that the HIF system promotes the transcription of many other genes; careful evaluation of other effects than erythropoiesis stimulation seems mandatory. GATA genes are an evolutionarily conserved family, which encode a group of important transcription factors involved in the regulation of several processes, including the development of the heart, hematopoietic system, and sex gonads. Some of these factors also regulate EPO gene transcription.[193,194]

Another possible strategy to increase erythropoiesis is the inhibition of the hematopoietic cell phosphatase. This protein, also known as src homology domain 2-containing tyrosine phosphatase 1, binds and activates the negative regulatory domain of the EPO receptor, inhibiting transduction inside the cell.[195] Hematopoietic cell phosphatase inhibitors may be thus a novel target molecule to treat renal anemia and/or sensitize patients to EPO action.

Finally, EPO gene therapy has been proposed for the management of anemic patients with CKD. A number of different techniques have been tried, such as the delivering of naked plasmid DNA into skeletal muscles,[196,197] the subcutaneous implantation of autologous bone marrow stromal cells genetically engineered to secrete EPO,[198] viral transfection,[199] and the use of artificial human chromosome.[200] EPO gene therapy has the theoretical advantage of releasing small but continuous amount of EPO into the circulation, but has still a number of problems, such as immunogenicity and the difficulty to exactly tune the exact amount of EPO needed to correct anemia and maintaining a level of expression that sufficiently promotes erythropoiesis in the long term.[201]

4.2. Non-hematological beneficial effects of rHuEPO therapy

The pleiotropic effects of ESAs appear to result from the existence of two different, EPOR with different affinities for ESAs. In erythroid cells, ESAs bind to the EPOR homodimers in picomolar concentrations, whereas on the other cells and tissues, higher local ESAs concentrations are needed (nanomolar range) to trigger the activation of a heterodimer EPOR, constituted by EPOR and CD131 (beta common receptor – βcR), that is expressed in the different tissues, namely, brain, heart and kidney.[202]

Functional EPOR has been identified in non-erythroid cells such as endothelial, muscle, and neural cells, and there is increasing evidence that EPO can act to stimulate cell proliferation and cell-specific function, or promote cell survival in these tissues.[203] In contrast to erythropoiesis, which is mediated by an EPOR homodimer, EPO signaling in some non-erythroid tissue employs a heteromeric receptor consisting of EPOR and the βcR used by GM-CSF, IL-3, and IL-5.[202]

It has become apparent that the pleiotropic effects of ESAs in non-erythroid tissues are the result of EPO binding to EPOR, and, as in erythroid cells, the EPO-EPOR interaction initiates a signal transduction process that regulates the survival, growth, and differentiation of the involved tissue. EPO and EPOR have been shown to play a physiological role in many non-erythroid cells, including endothelial cells [204], megakaryocytes,[205] heart,[206] uterus,[207] breast[208] and testes,[209] however in some tissues, as brain (neurons, astrocytes, and microglia),[210,211] kidney,[212] female reproductive system,[213] cardiomyocytes,[214] lymphocytes and monocytes, [215] the signaling mechanism can be different since EPO can interact with EPOR and CD131 heterodimers.[216] EPO and EPOR exhibit a beneficial role in neural, cardiovascular, and retinal tissues and in immune function and tissue repair.

Higher doses of ESAs than those required to treat anemia are needed to exert pleiotropic effects in some tissues, which, inevitably, activate the hematopoietic pathway, and increase the risk of ESAs side effects. The use of ESAs as a tissue protective agent in patients without anemia, presents a risk to these patients as the activation of the hematopoietic system would be a side effect.

4.3. Adverse effects of rHuEPO therapy

All ESAs approved, until now, for the treatment of anemia present some disadvantages as immunogenicity, and the storage and stability, as well as the administration route have to be considered. ESAs are associated with some adverse effects, namely, cardiovascular effects, such as hypertension, retinopathy, neurotoxicity and thrombotic events; the impact of ESAs therapy on survival outcomes needs to be considered carefully in the design of future clinical studies.

Hypertension, which either develops or worsens in 20–30% of renal patients on rHuEPO therapy, is usually controlled without serious consequences.[217,218] Several mechanisms can explain the rise in blood pressure (BP) mediated by ESAs. Renal anemia is a factor predisposing to increased BP, due to the increased sympathetic activity and impaired nitric oxide (NO) availability.[219] ESAs impair the balance between vasodilating and vasoconstrictor factors, since it induces the production of vasoconstrictors as endothelin-1 (ET-1), thromboxane B2 (TXB2) and prostaglandin 2α (PGF2α), and reduces

the production of the vasodilatory prostacyclin 2 (PGI2). A chronic treatment with ESAs appears to impair the vasodilatory capacity of endothelial NO, through an increase in the asymmetrical dimethylarginine (ADMA), an inhibitor of eNOS. ESAs seem to induce hypersensitivity to angiotensin II, a recognized vasoconstrictor. An increase in the concentration and hypersensitivity of noradrenaline, a vasoactive substance, may contribute also to the hypertension associated to ESA therapy.[220,221]

ESAs have the capacity of stimulating thrombopoiesis, increasing platelet count; however, ESAs also increase platelet reactivity, promoting a prothrombotic effect.[222] In some preclinical studies, very high doses of rHuEPO in conjunction with hypoxia has been associated with a paradoxical neurotoxic effect, suggesting the need for further work to determine dose-response conditions, to optimize the neuroprotective effects of EPO.[223-225] Several clinical studies evaluating rHuEPO therapy in CKD patients have raised the possibility that higher target haemoglobin levels may be associated with increased cardiovascular events and all-cause mortality, as well as a potential increased risk of arteriovenous access thrombosis.[154,155,226] Some other hemostatic disturbances have been described, as an increased expression in E selectin, P selectin, von Willebrand factor and plasminogen activator inhibitor-1, which may favor bleeding episodes, and increase the risk of thrombosis and thromboembolism.[220]

A serious complication associated with ESAs administration is pure red cell aplasia (PRCA), an immunogenic side effect that results from the production of anti-EPO antibodies induced by ESAs administration.[227-229] Although this disorder occurs very rarely, the number of reported cases has increased in recent years, with the introduction os ESAs biosimilars to treat CKD patients in some countries. Patients with CRF-associated anemia receiving subcutaneous injections of one particular formulation of recombinant epoetin-α also developed more frequently PCRA. This rare condition develops suddenly and is caused by the production of EPO-neutralizing antibodies that eliminate the biological activity of the recombinant protein as well as of endogenous EPO.[230] Low reticulocyte counts, a marked decrease in bone marrow erythroblasts, resistance to EPO therapy, and presence of anti-EPO antibodies are key features for the diagnosis of PRCA. Peptides and protein drugs have the potential to elicit host immune responses that can limit efficacy or cause adverse reactions. [227,231]

Indeed, the methods used to produce ESAs may not eliminate completely impurities or aggregated proteins that may trigger the immune response in patients.[229] Immunoprecipitation assays have shown that anti-EPO antibodies are directed against the protein moiety of the molecule.[228]

5. Resistance to recombinant human erythropoietin therapy

5.1. Definition and etiology

The NKF KDOQI defined the hyporesponsiveness to erythropoietin as the presence of at least one of the following three conditions: i) a significant decrease in Hb level at a constant ESAs dose; ii) a significant increase in the ESAs dose requirement to preserve a target Hb level, or iii) a failure to raise the Hb level to values higher than 11 g/dL despite an ESAs dose equivalent to erythropoietin greater than 500 IU/kg/week.[22]

Similarly, European guidelines also recommend to consider ESAs resistance when a patient either fails to attain the target hemoglobin concentration while receiving more than 300 IU/kg/week (20,000 IU/week) of erythropoietin or 1.5 mg/kg of darbepoetin-alfa (100 mg/ week), or has a continued need for such high dosages to maintain the target Hb concentration.[232] The erythropoietin resistance index (ERI), calculated by the ratio between the rHuEPO dose/kg/week and the Hb concentration (g/dL), is an alternative method, considered by some authors, as a better way to measure the degree of ESAs resistance. An ERI value higher than 0.02 μg/kg/week/g Hb indicates resistance to ESAs; additionally the time course of ERI may be important to demonstrate the degree of response to these agents.[233]

The main cause of ESAs resistance is iron deficiency, but rHuEPO-resistant anemia persists in some HD patients even after sufficient iron supplementation.[234] Iron deficiency may be absolute – with a ferritin concentration less than 100 mg/L – or functional – when the ferritin concentration is greater than 100 mg/L and total saturation of transferrin (TSAT) less than 20%. Besides noncompliance (that should be checked when EPO is self-administrated), other recognized causes of ESAs resistance in patients with adequate iron stores include concomitant inflammation or infection, neoplasia, chronic hemolysis, hemoglobinopathies, severe hyperparathyroidism,

aluminum intoxication, vitamin B 12 or folate deficiencies, inadequate dialysis, myelosuppressive agents, myelodysplasia, antibody-mediated pure red cell aplasia, thyroid dysfunction, and some drugs, including RAAS blockers (Figure I-14).[235-238]

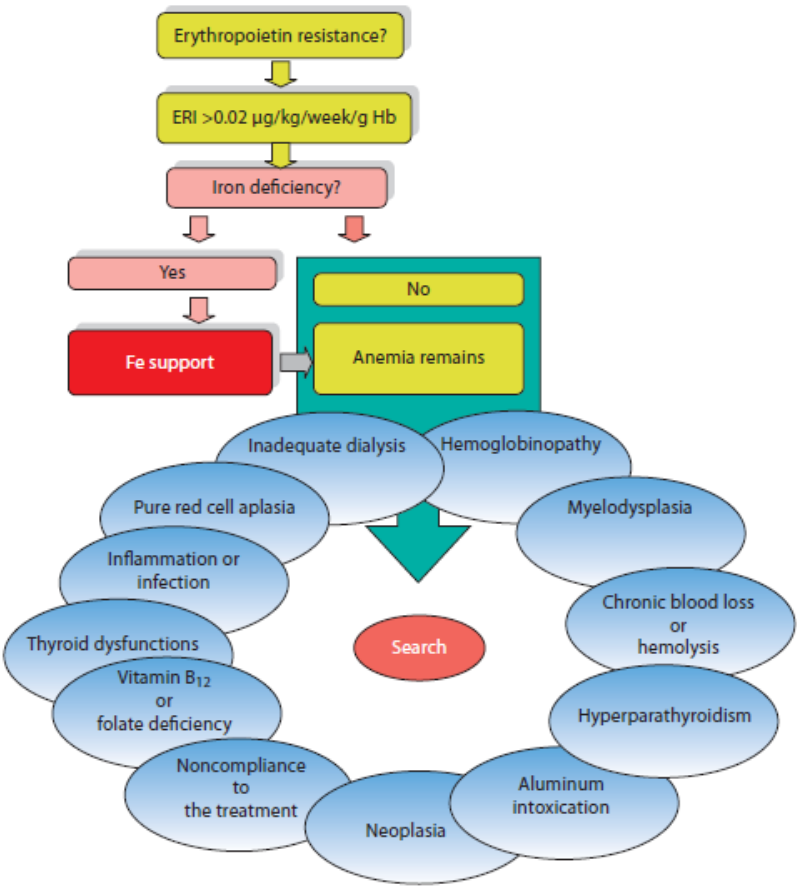

Figure I-14. Causes of ESAs-resistant anemia in CKD. (*Taken from* Kanbay et al., 2010[239])

5.2. Causes of resistance to recombinant human erythropoietin

5.2.1. Iron deficiency and hepcidin deregulation

The most common cause of an incomplete response to EPO is iron deficiency absolute or functional, as previously described. Iron deficiency was shown to be present in as many as 25–37.5% of CKD patients presenting with anemia. Enhanced iron utilization due to EPO-induced red blood cell formation can quickly deplete iron stores, especially if previously reduced by poor iron absorption, occult gastrointestinal bleeding, or dialysis-related blood losses. Because of ongoing iron losses,[240] the need for iron supplementation is ubiquitous in the ESRD population. The diagnosis of iron deficiency

in this setting can be somewhat challenging in that the ferritin level is often elevated due to systemic inflammation. Whereas a low serum ferritin level does imply iron deficiency, a high ferritin level cannot be used to rule out iron deficiency. Most workers in this area agree that the saturation of transferrin is a more helpful indicator of iron deficiency. It is estimated that a transferrin saturation of less than 20% has a sensitivity of 100% and a specificity of 80% for iron deficiency in the ESRD population, provided that hypoproteinemia is not present.[241]

Iron uptake from plasma to erythroid precursor cells is regulated by different proteins: transferrin receptor, hemochromatosis (HFE) protein and DMT1 (NRAMP2/DCT1). HFE gene mutations are associated with a reduction in the amount of rHuEPO necessary to support erythropoiesis in HD patients.[242] Conversely, some DMT1 gene mutations are associated with an inhibition of intestinal iron absorption and a decrease in erythroid cell precursor iron uptake, resulting in hypochromic and microcytic anemia.[243] Hepcidin may also be involved in the pathogenesis of anemia of inflammation. Growing evidence suggests that low-grade inflammation causes an increase in hepcidin production, limiting the availability of iron for erythropoiesis, thus, providing a direct link between inflammation and metabolism of iron.[244-246] Costa et al. (2008) demonstrated in 50 HD patients (25 nonresponders and 25 responders to rHuEPO therapy) and 25 healthy controls, that prohepcidin and soluble transferrin receptor, together with CRP, were good markers of resistance to rHuEPO therapy.[238] On the other hand, Kato et al. (2008) measured the peak intensity of serum hepcidin-25, the major form of mature hepcidin, in 24 HD patients and compared those between rHuEPO-hyporesponsive and rHuEPO-responsive patients in a cross-sectional study. This group found that serum hepcidin measurement, using currently available assays, was not valid in predicting rHuEPO responsiveness in chronic HD patients.[247] Clearly more work is required to provide a useful therapeutic biomarker and index for treatment monitoring.

5.2.2. Cytokines and inflammation

Uremic toxins are implicated in the propagation of proinflammatory cytokines, such as IL-1, IL-6, interferon-γ (IFN-γ), and tumor necrosis factor alfa (TNF-α). These cytokines downregulate the expression of EPO receptors on erythroid progenitors and

disrupt iron recycling by blocking its release from reticuloendothelial cells, and by inducing hepcidin synthesis.[248] Inhibition of erythropoiesis by cytokines, such as TNF-α and IFN-γ, is also important for erythropoietin resistance.[141] In CKD patients, elevated levels of IL-1, IL-6, TNF-α and CRP (suggestive of a chronic inflammatory status) have been frequently described.[249] Del Vecchio et al. (2005) reported that cytokine-induced inflammation suppresses bone marrow erythropoiesis in HD patients and is a possible cause of anemia.[250] Shinzato et al. (2008) found higher levels of ferritin, hs-CRP and IL-6 in 19 HD patients with rHuEPO-resistant anemia, compared to control HD patients without anemia and iron deficiency.[251] Kalantar-Zadeh et al. (2003) confirmed the strong association between indices of EPO hyporesponsiveness and high levels of inflammatory markers in a larger cohort of 339 HD patients. Primary and secondary neutrophil granules contain elastase and lactoferrin, respectively, which are commonly used as indirect markers of neutrophil activation in vivo.[252] Costa et al., (2008) evaluated the neutrophil activation state in CKD, in 63 HD patients under rHuEPO treatment (32 responders and 31 nonresponders to rHuEPO therapy), compared with controls, and fount that CKD patients presented with significantly higher CRP, and neutrophils and elastase levels; moreover, nonresponders to the rHuEPO therapy showed significantly higher elastase levels than responders to rHuEPO therapy, which could be related to the rise in neutrophils and be part of the enhanced inflammatory process found in these patients. On the other hand, plasma levels of lactoferrin and the lactoferrin/neutrophil ratio did not differ between groups. Therefore, elastase, but not lactoferrin may be a better marker of resistance to rHuEPO therapy in CKD patients under hemodialysis.[253]

5.2.3. Pure red cell aplasia

Resistance to biological effects of EPO may result from its neutralization by circulating antibodies or by its inactivation by binding to soluble plasma receptors which, usually, lead to pure red cell aplasia (PRCA). The immunogenicity of rHuEPO may be enhanced by subcutaneous administration and by certain host biological factors. Indeed, several cases of PCRA were associated to the subcutaneous administration of rHuEPO, particularly of Eprex® which was removed the European market.[254]

PRCA is a syndrome characterized by a severe normocytic anemia, reticulocytopenia, and absence of erythroblasts from an otherwise normal bone marrow. This is a very rare cause of ESA resistance in ESRD patients. For example, HD patients who were either hypo or normoresponsive to epoetin treatment were tested for anti-erythropoietin antibodies, and, the prevalence of anti-erythropoietin antibodies in HD patients without symptoms of PRCA was determined by screening sera of 536 patients, using ELISA.[255] Positive results were also verified by radioimmunoprecipitation assay and neutralizing activity was determined by bioassay. Anti-erythropoietin antibodies were detected in 3 hyporesponsive and 3 normoresponsive patients, suggesting that anti-erythropoietin antibodies are not a major cause of ESA hyporesponsiveness.[255] Similarly, in a multicenter, cohort study of 1677 patients with incident end-stage renal disease, 57 patients with inadequate EPO response were identified; however, only 2 of these 57 patients were positive for anti-EPO antibodies and one patient had clinical PRCA. In another study, with 1346 patients without evidence of inadequate EPO response, only one patient tested borderline positive for anti-EPO antibodies. The incidence of EPO-induced PRCA and EPO antibodies was very low in this study.[256]

5.2.4. Other potential causes of resistance to rHuEPO therapy

Erythropoiesis-stimulating agents hyporesponsiveness represents an important diagnostic and management challenge. As already referred, other recognized causes of resistance to rHuEPO therapy have been reported, such as malnutririon,[257] secondary hyperparathyroidism,[258-260] uremic toxins, inadequade dialysis [261,262], angiotensin-modelating agents,[263-266] oxidative stress,[267,268] vitamin B12, folate or L -carnitine deficiencies,[269-271] neoplasia, chronic hemolysis, hemoglobinopathies, aluminum intoxication, myelosuppressive agents, myelodysplasia, thyroid dysfunction, and some drugs, including RAAS.[235-238] Enhancing ESA responsiveness is critically important to minimizing the high cost of ESA therapy as well as improving clinical outcomes.

6. Animal models of chronic renal failure and erythropoietic disturbances

Surgical manipulations of experimental animals have been widely used to model CKD, in order to mimic the injury that causes renal disease or reproduce their consequences. The pre-requisite of developing a renal failure model is to define a target range of renal insufficiency, in order to achieve a stable uremic milieu allowing experimental manipulation. Animal models of chronic renal failure (CRF) have been mostly achieved by partial ablation of renal parenchyma, and the two most common techniques employed were surgical resection or infarction.[272] However, there are other animal models described in the literature to induce chronic renal failure, including microembolism by michrospheres [273], electrocoagulation of the renal cortex[274] and cryosurgery.[275]

6.1. Nephrectomy (or renal ablation): resection *versus* infarction model

In the resection model, a portion of one kidney is resected, followed by contralateral nephrectomy. With the animal in ventral recumbent position, a 2–3 cm skin incision is made, caudal to the rib cage, on the left side. After further muscle incision, the left kidney is gently dissected from the overlying fat and adrenal gland. A single silk thread is placed around the renal pedicle. The upper and lower kidney poles are excised after lifting the thread (in order to temporarily occlude the vascular flow during cutting). Meticulous resection is made under direct vision, to ensure that the remnant kidney represents the percentage wanted. With the remnant kidney wrapped in hemostatic gauze, it is returned into the abdominal cavity. Muscle wall is then sutured. One week after the first surgical procedure, right uninephrectomy is performed.[272]

The (5/6) surgical nephrectomy model has been the most used prototype, although different ways to achieve (5/6) reduction of nephron mass have been proposed and practiced.[276,277] This model involves substantial removal of renal mass or nephron number, followed by compensatory renal hypertrophy in the remnant kidney. The glomerular changes, in proportion to the amount of nephrons damaged, are characterized by hyperperfusion and hyperfiltration. Increasing workload by the

remaining pool of nephrons characterizes the progression of renal injury, namely chronic renal failure.[278-281] However, the 5/6 nephrectomy model has several limitations, including a substantial mortality rate, non-reversibility, and phenotypic alterations related to the surgical procedure rather than impaired kidney function.[282] Furthermore, the method is associated with relatively large inter-individual and inter-laboratory differences and its availability may be limited by lack of surgical expertise and the appropriate operating facilities.

Our group[283] (Garrido et al., 2009) has previously characterized a CRF model induced by (¾) nephrectomy which is consistent with a moderate degree of renal failure and a modest and quickly compensated anemia, though presenting a disturbance in iron metabolism. Despite the reasonable degree of functionality of the remnant kidney, as suggested by the correction of anemia, as well as by the kidney hypertrophy and the moderate structural modifications (lesions) observed, several important cardiovascular modifications were developed. This model of (3/4) nephrectomy have been established as a good tool to study the cellular/molecular pathophysiological mechanisms underlying moderate stages of chronic renal failure, as occurs in humans, and, even more relevant, to evaluate the efficacy of therapies used to prevent, treat or correct cardio-renal anemia syndromes and complications in early stages.[283]

Concerning the infarction model, it involves a large reduction of the blood flow through the renal artery from one kidney and contralateral nephrectomy. Briefly, the left kidney is exposed and decapsulated in a similar manner as above described. Instead of surgical ablation of renal tissue, the left renal arterial branches are isolated with blunt forceps and, by using sterile silk suture, the blood flow is largely reduced. Functional nephrectomy is, thus, achieved by renal tissue infarction. Discoloration of 75% of the kidney reflects ischemia that will progress to infarction. Right unilateral nephrectomy is then performed as in the resection model above mentioned. Both techniques are described in a variety of animal species. Ideally, these techniques should induce a reproducible degree of glomerular filtration rate. Decrease of renal mass by different methods might represent different degrees of renal injury and, thus, different stages of chronic renal failure.[272]

The reduction of renal mass is, thus, achieved by either infarction or surgical excision of both poles, with removal of the contralateral kidney. It enables to investigate

the influence of pharmacological, nutritional and other factors on functional and morphological renal parameters. The (5/6) nephrectomy produced by infarction is characterized by high plasma renin levels. By contrast, reduction of an equivalent amount of renal parenchyma by surgical excision does not result in the development of hypertension and plasma renin activity is normal to low. In this model of surgical nephrectomy, the initially normal remnant nephrons undergo compensatory functional and structural adaptations. Glomerular hypertension is one of the main factors responsible for the development of renal injury. Morphological studies show progressive focal segmental to global glomerulosclerosis, accompanied by increasing proteinuria and deteriorating renal function.[284]

In both models, the remnant kidney is histologically normal at the moment of chirurgical intervention, further undergoing a progressive impairment of renal function. In several cases of severe human renal diseases, the decrease in the number of functioning nephrons is associated with marked tubulointerstitial changes, and relatively undamaged nephrons coexist with those that are extensively damaged, until the intact nephrons spontaneously deteriorate. These features are common in both immunologically and non-immunologically mediated kidney diseases. Progressive CRF is histologically characterized by tubulointerstitial, glomerular and vascular lesions.

Few studies have shown that administration of low erythropoietic doses of EPO may retard progression of renal disease in rats with renal mass reduction[285] and in diabetic db/db mice.[286] High doses of EPO have been consistently shown to accelerate progression of renal disease in animals with renal mass reduction.[285,287,288] The contribution of high levels of EPO to progression of renal disease is strengthened by the recent demonstration of a strong link between development of severe diabetic proliferative retinopathy and nephropathy with a polymorphism of the EPO gene promoter that causes increased EPO production.[289] Thus, while physiologic levels of EPO seem to confer vital benefits through its numerous non-erythropoietic and erythropoietic actions, data derived from experimental animals, as well as from randomized clinical trials, suggest that high doses of EPO can accelerate progression of chronic renal disease. Further studies are required to elucidate the molecular mechanisms underlying these effects.

6.2. Models of erythropoietin-deficient or dysfunctional EPO

6.2.1. Pure red cell aplasia

As referred, antibody (Ab)-mediated pure red cell aplasia (PRCA) is an immunological pathology associated with the production of neutralizing Abs that inhibit the erythropoietic activity of endogenous EPO and recombinant erythropoiesis-stimulating agents (ESAs). Although very rare, the number of reported cases has, more recentetly, with the use of rHuEPO biosimilars to treat patients with CKD-associated anemia receiving subcutaneous injections of one particular formulation of recombinant epoetin-α have also shown a higher prevalence of PRCA. This disorder is differentiated from classic forms of PRCA that are caused by chemical toxemia (i.e. erythroblastopenia induced by chemical compounds), lymphoproliferative neoplasms, thymoma, human parvovirus B19 and certain autoimmune disorders.[290]

Genovese et al. (1975)[291] described two experimental models for PRCA. In the first model, the administration of serum IgG from a case of PRCA, to normal mice, induced a sustained inhibitory effect on erythropoiesis, with a progressive decline of the hematocrit values and an inverse rise of EPO levels in serum. Thus, the physiopathological pattern of PRCA type I was established. In the second model, a rabbit producing anti-EPO cross-reacting with endogenous EPO was subjected to a booster injection of EPO. The rise of the immune response was associated with disappearance of erythroid precursors from marrow smears, subsequent decline of circulating reticulocytes and worsening of the anemia, thus reproducing the physiopathological pattern of PRCA type II.[291] This model could be useful for studies of the effects of resistance to rHuEPO therapy in CKD.

6.2.2. Erythropoietin-deficient anemia

Few *in vivo* models exist that display a phenotype similar to the anemia of CRF, specifically, low RBC production in conjunction with depleted serum EPO levels. Models involving chemical injections are used to directly destroy RBCs, leading to the development of anemia. For instance, the injection of phenylhydrazine (PHZ) results in the destruction of RBCs by binding to hemoglobin proteins within these cells,[292]

triggering their destruction and, therefore, an anemia. However, regardless of the methods employed, these techniques lead to a rapid increase in serum EPO levels and are not representative of an EPO-deficient anemia. To overcome this limitation, nephrectomy has been used to create an EPO-limiting environment *in vivo*. However, as we observed in the surgical model already described, this model involves an invasive procedure and is therefore not appropriate for large-scale experiments. In addition, these animals experience a shortened lifespan, which prevents the study of long-term effects of low EPO production. Finally, nephrectomized animals seem to be still able to produce EPO in secondary tissues, such as the liver.[293]

The introduction of transgenic mice technology has also yielded some innovative mouse models for the study of EPO function; however, they also have limitations. As referrred, *EPO*-knockout mice are embryonic lethal, making it an ineffective model to study anemia and erythropoiesis in the adult system.[58] EPO is not necessary for erythroid lineage commitment; however, it is needed to assure the survival of erythroid progenitors (CFU-E) that would differentiate into RBCs.

In order to obtain a preclinical model for an EPO-deficient anemia, Zeigler et al., (2010)[294] developed a mouse in which *EPO* is silenced by Cre recombinase. After induction of Cre activity, $EPO^{KO/flox}$ mice experience a significant reduction of serum EPO levels and, consequently, develop a chronic, normocytic and normochromic anemia.[294] The authors showed that the conditional deletion of *EPO* in the adult animal results in an anemia of EPO deficiency. These transgenic animals have the ability to survive for long term studies, unlike the previously used models, including those using nephrectomy. Thus, $EPO^{\Delta/\Delta}$ mice will be a valuable tool for the development of new therapeutic approaches for the treatment of the anemia of CKD. In addition, this model will allow the study of erythropoiesis under EPO-limiting conditions as well as studies analyzing the contribution of extra-renal EPO production to erythropoiesis. Furthermore, analyzing the stress-induced erythropoietic response in these animals has the potential to discover new erythropoiesis regulating mechanisms that are masked by EPO in the currently used models. Elucidating these mechanisms has the potential to identify new target opportunities for the treatment of anemia.[294]

The erythropoietic agents have the potential to play a therapeutic role in several pathologies. Considering that the use of ESAs might have negative consequences (e.g.,

increased blood pressure and thromboembolism), the risks of EPO therapy should be weighed against the potential beneficial effects of improving anemia, quality of life, neurocognitive performance, and of decreasing the impact of ischemia in brain, heart, and other organs. Although an animal model can never be a perfect match for human disease, adequate animal models have provided valuable insights into the mechanisms in anemia/CKD. There is now clear experimental evidence from animal models that EPO can reduce the severity of renal injury induced by ischemia reperfusion, systemic shock, and nephrotoxic insults, and may also contribute to clarify the process of regeneration/amelioration of renal damage. Further studies, supported by the proper animal models, might improve the knowledge about some areas that remain to be elucidated, concerning the use of rHuEPO in kidney diseases/anemia, including the mechanisms behind resistance to therapy and the impact of high doses on hemodialysis patients, but also the putative therapeutic relevance of erythropoietin on cardiac and neuronal disorders, namely due to the cytoprotective actions previously documented.

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Chapter II

Rationale, aims and outline

1. Rationale, aims and outline

Anemia is a common complication in hemodialysis patients. This anemia is due to the inability of the failing kidneys to secrete erythropoietin (EPO) to stimulate erythropoiesis. The introduction of recombinant human EPO (rHuEPO) therapy in the early 1990s led to a significant reduction in anemia and improved patients' quality of life. However, there is a marked variability in the sensitivity to rHuEPO, with up to 10-fold variability in dose requirements to achieve correction of anemia. About 5-10% of the patients show a marked resistance to rHuEPO therapy, worsening patient's prognosis. To better clarify the mechanism underlying resistance to rHuEPO therapy, our team studied in previous work[1-9] the systemic changes associated with that resistance, in two groups of chronic kidney disease (CKD) patients, one responder and the other non-responder to rHuEPO therapy. This study was focused on inflammation, leukocyte activation, iron status, oxidative stress and erythrocyte damage. Non-responders presented a mild to moderate hypochromic anemia, even with the administration of higher rHuEPO doses. As all patients were under iron and folate prophylactic therapies, and iron and vitamin B12 serum levels were normal, these changes could not be attributable to a deficiency in these erythropoietic nutrients. Non-responders to rHuEPO therapy seem, therefore, to present a "functional" iron deficiency characterized by the presence of adequate iron stores, but apparently with an inability to sufficiently mobilize iron to adequately support erythropoiesis. Inflammation, a hallmark in these patients, was higher in non-responders; C-reactive protein (CRP) was inversely correlated with hypocromia, serum iron and transferrin saturation, suggesting that the enhanced inflammation, may lead to trapping of iron within the macrophages and to a reduction in serum iron levels.

Further studies are needed to understand the mechanisms explaining the marked variability in the response/resistance to rHuEPO therapy; however, to clarify these mechanisms we need to perform blood, cellular and tissue studies that cannot be performed in humans, due to ethical limitations. In accordance, we figured that the use of appropriate animal models could be useful to estimate the pathophysiology of CKD and the rHuEPO response/resistance.

With this perspective, we developed a rat model of chronic renal failure (CRF) induced by 3/4 surgical nephrectomy.[10] We found that this model was consistent with a moderate degree of renal failure, showing a modest and quickly compensated anemia, erythropoietic disturbances, hypertension, dyslipidemia and oxidative stress.[11] These preliminary studies showed us that we needed to perform a larger nephrectomy to achieve a model of renal failure similar to those found in patients with stage 5 of CKD.

The aims of the present work were:

1. To contribute to a better understanding of the anemia of chronic kidney disease.
   In that way, we evaluated, by using an experimental animal model of CRF induced by 5/6 surgical nephrectomy, the development of anemia and the evolution of renal damage, focusing on iron impairment, erythropoiesis and inflammation at blood, tissue and cellular levels. The results of this study are presented in **Chapter III**.

2. To understand the mechanisms explaining the marked variability in the response/resistance to rHuEPO therapy, by using an experimental animal model of CRF, induced by 5/6 surgical nephrectomy, and focusing on renal damage, iron impairment, erythropoiesis and inflammation, at blood, tissue and cellular levels; moreover, to explain the persistence of anemia in CRF rats, observed in the previous study (Chapter III). Indeed, we found that anemia persisted throughout the protocol, in spite of normal EPO levels. The results of these studies are presented in **Chapter IV**.

3. To study the effect of rHuEPO *per se* on some of the mechanisms previously described.
   By performing this study, besides the evaluation of the impact of rHuEPO on iron metabolism, erythropoiesis and inflammation at blood and molecular levels, we managed to develop a rat model of antibody mediated erythroid hypoplasia induced by rHuEPO. The results of these studies are presented in **Chapter V**.

4. Finally, as this study involved animal research, to develop experimental animal models that can be used in following studies of our team, to be applied in this area of research or in other is which the CKD associated-anemia and PCRA could be used.

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Chapter III

Iron-hepcidin dysmetabolism, anaemia and renal hypoxia, inflammation and fibrosis in the remnant kidney rat model

Manuscript submitted:

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1. Abstract

Anemia is a common complication of chronic kidney disease (CKD) that develops early and its severity increases as renal function declines. It is mainly due to a reduced production of erythropoietin (EPO) by the kidneys; however, there are evidences that iron metabolism disturbances increase as CKD progresses. Our aim was to study the mechanisms underlying the development of anemia of CKD, as well as renal damage, in the remnant kidney rat model of CKD induced by 5/6 nephrectomy. We found that this model of CKD presented a sustained degree of renal dysfunction, with mild and advanced glomerular and tubulointerstitial lesions. Anemia developed 3 weeks after nephrectomy and persisted throughout the protocol. The remnant kidney was still able to produce EPO and the liver showed an increased *EPO* gene expression. In spite of the increased EPO blood levels, anemia persisted and was linked to low serum iron and transferrin levels, although iron storage was normal, as showed by ferritin; serum IL-6 and CRP levels showed the absence of inflammation. The reduced expression of hepcidin favours iron absorption, as suggested by the increased expression of duodenal ferroportin. However, iron contents remain reduced which might be due to advanced kidney lesions. Our data suggest that the persistence of anemia may result from disturbances in iron metabolism and, eventually, by an altered activity/function of EPO that might result from kidney cell damage and from the developed local inflammatory milieu, as showed by the increased gene expression of different inflammatory proteins in the remnant kidney.

Key words: Iron dysmetabolism; anemia; chronic kidney disease, renal hypoxia, inflammation, fibrosis

2. Introduction

Chronic kidney disease (CKD) is a pathological condition that results from a gradual and permanent loss of kidney function over time, usually, months to years. CKD can result from primary diseases of the kidneys, however, diabetic nephropathy and hypertension are the main causes of CKD.[1] Anemia is a common complication of CKD that develops early in the course of the disease, increasing its frequency and severity with the decline of renal function. The incidence of anemia is less than 2% in CKD stages 1 and 2, about 5% in CKD stage 3, 44% in CKD stage 4 and more than 70% in end-stage renal disease (ESRD).[2] This condition is associated with a decreased quality of life[3], increased hospitalizations,[4,5] cardiovascular complications - angina, left ventricular hypertrophy (LVH) and chronic heart failure – and mortality.[6-9]

Anemia is mainly associated with a reduced production of erythropoietin (EPO) by the kidneys. However, there are also evidences that iron metabolism disturbances increase as the CKD progresses. The reasons for this high proportion of CKD patients with iron disturbances are not well clarified; however, inflammation has been proposed to play an important role. In fact, previous works reported that ESRD patients under hemodialysis present higher hepcidin serum levels, increased markers of inflammation (such as CRP and IL-6) and reduced iron absorption and mobilization, thus presenting lower levels of iron and transferrin.[10-12] Increased hepcidin production during inflammation and infection explains sequestration of iron in the macrophages and inhibition of intestinal iron absorption, the two hallmarks of the anemia of inflammatory diseases.

During the last few years the mechanisms underlying hepcidin and iron regulation have been largely studied. In addition, the impact of renal hypoxia, through hypoxia-inducible factors (HIFs), on iron metabolism, on kidney lesion or regeneration, as well as on hepcidin expression, have been extensively debated.[13-15] Experimental models using transgenic mice, knockout for some of the key mediators, have been crucial to reveal some of these new findings.[16,17] Uremic rat models have been characterized and used for long time by our group as well as by other authors as tools to study the pathophysiological events underlying kidney disease development, including those with renal failure induced by nephrectomy and infarction.[18-20] However, the information is still scarce concerning the characterization of iron dysfunction associated with hypoxic anemia

of chronic kidney disease, namely in the 5/6 nephrectomized rat, which is one of the most used rat model of CKD. In this sense, we intended to elucidate the mechanisms underlying the development of anemia and evolution of renal damage in the remnant kidney rat model of CKD induced by 5/6 nephrectomy, focusing on iron impairment and kidney hypoxia, inflammation and fibrosis.

3. Material and methods

3.1. Animals and experimental protocol

Male Wistar rats (Charles River Lab., Inc., Barcelona, Spain) weighing ±300g were maintained in an air conditioned room, subjected to 12 h dark/light cycles and given standard rat diet (IPM-R20, Letica, Barcelona, Spain) *ad libitum* and free access to tap water. Animal experiments were conducted according to the European Communities Council Directives on Animal Care and the National Authorities.

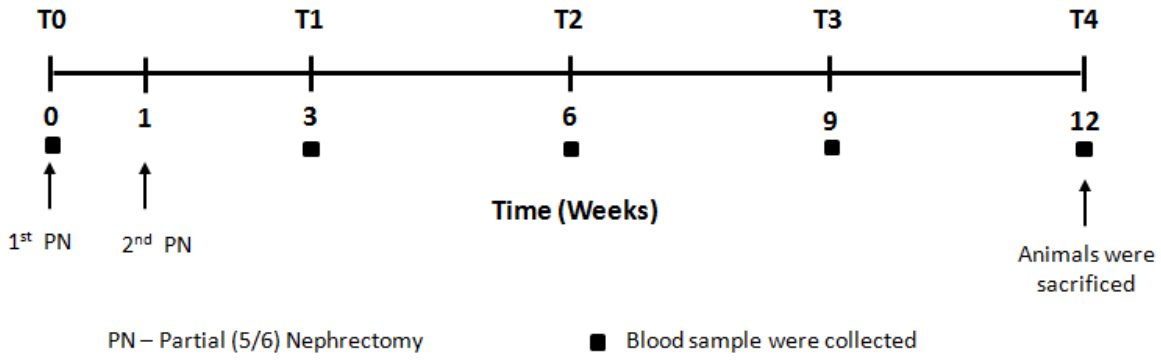

Figure III-1. Experimental protocol.

The rats were divided into two groups (7 rat each): Sham group – subjected to chirurgical process but without kidney mass reduction and chronic renal failure (CRF) group – induced by a two-stage (5/6) nephrectomy, with subtraction firstly of about 2/3 of the left kidney by left flank incision and, one week later, complete removal of the right kidney through identical incision/procedure. All the animals have completed 12

weeks of protocol (Figure III-1). Body weight (BW) was monitored throughout the study and blood pressure (BP) and heart rate (HR) measures were obtained using a tail-cuff sphygmomanometer LE 5001 (Letica, Barcelona, Spain).

3.2. Sample collection and preparation

At the beginning of the experiments (T0) and at 3 (T1), 6 (T2) 9 (T3) and 12 (T4) weeks after the surgical 5/6 nephrectomy, the rats were subjected to intraperitoneal anesthesia with a 2 mg/kg BW of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar®, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil®, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal), to collect blood by venipuncuture, from the jugular vein, into vacutainer tubes without anticoagulant (to obtain serum) or with K_3EDTA for hematological and biochemical studies; at T0, T1, T2 and T3 only 3 mL of blood were collected, to minimize interference with erythropoiesis mechanism, in order to monitor anemia and renal function; at the end of protocol (T4) 10 mL of blood were collected, to perform all the biochemical and hematological assays.

At the end of the protocol, after collection of blood, the rats were sacrificed by cervical dislocation; kidneys, duodenum, liver and heart were immediately removed, placed in ice-cold Krebs-Henseleit buffer and carefully cleaned. A bone marrow aspirate from the femur was also performed.

3.3. Biochemical and hematological assays

Serum creatinine and blood urea nitrogen (BUN) were used as renal function markers; glicose, total cholesterol (Total-c), triglycerides (TGs), creatine kinase (CK), aspartate transaminase (AST) and alanine transaminase (ALT) were analysed through automatic validated methods and equipments (Hitachi 717 analyser, Roche Diagnostics Inc., Massachusetts, USA).

Red blood cells (RBC) count, hematocrit (Hct), hemoglobin (Hb), reticulocyte count (Ret) mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), mean cell volume (MCV), platelet count (PLT), platelet distribution width (PDW), RBC

distribution width (RDW) and white blood cells count (WBC) were assessed in whole blood K_3EDTA (Coulter Counter®, Beckman Coulter, Inc., Fullerton, California, USA).

Serum iron concentration was determined using a colorimetric method (Iron, Randox Laboratories Ltd., North Ireland, UK), whereas serum ferritin and transferrin were measured by immunoturbidimetry (Laboratories Ltd., North Ireland, UK).

Serum levels of interleukin-6 (IL-6), interferon γ (IFN-γ), transforming growth factor beta 1 (TGF-β1) and vascular endothelial growth factor (VEGF) were all measured by rat-specific Quantikine ELISA kits from R&D Systems (Minneapolis, USA). High-sensitive C-reactive protein (hsCRP) was determined by using a rat-specific Elisa kit from Alpha Diagonostic International (San Antonio, USA). Serum levels of erythropoietin (EPO) were evaluated by rat specific ELISA kit (MyBioSource, USA).

Quantification of total bilirubin was performed by a colorimetric test of diazotized sulfanilic acid reaction (Roche Diagnostics Inc., Massachuasetts, USA); circulating levels of glucose and uric acid were determined by routine automated technology (ABX Diagnostics, CA, USA).

3.4. Flow cytometry

To study leukocyte activation, the mononuclear cells were then isolated from the other blood cells by density gradient centrifugation (700g, 20 min. at room temperature) (Histopaque-1077 and -1119, from Sigma-Aldrich, Sintra, Portugal) followed by 3 washing steps with phosphate buffered saline solution (PBS, pH =7.4), supplemented with 3% (v/v) fetal bovine serum (FBS). We used antibodies against CD3, CD4, CD8 and CD25 (all from BD Biosciences, San Diego, CA, USA), conjugated either to FITC, PE or PerCP. Fluorochrome-conjugated isotype-matched antibodies were used as negative controls. For surface staining, mononuclear cells (≈1 × 10^6 cells in 100 µl PBS containing 3% (v/v) fetal bovine serum and 0.1% NaN_3) were incubated with 1 µg antibody in the dark, at room temperature, for 30 minutes and then washed three times with PBS supplemented with FBS 3% (v/v); 400 µl of PBS supplemented with 3% (v/v) FBS was finally added to each tube. The treated samples and controls were analyzed by flow cytometry within a 1h period. Flow cytometric analysis was carried out in a FACS Calibur

(San Jose, CA, USA) based on the acquisition of 20000 events. Detectors for forward (FSC) and side (SSC) light scatter were set on a linear scale, whereas logarithmic detectors were used for all three fluorescence channels (FL-1, FL-2 and FL-3). Compensation for spectral overlap between FL channels was performed for each experiment using single-color-stained cell populations. For the experimental samples, a corresponding isotype control was used to set gates, or positive/negative cell populations. All data were analyzed using FlowJo software (TreeStar Inc, Ashland, OR, USA).

3.5. Gene expression analysis

In order to isolate total RNA, 0.2 g samples of liver, duodenum and kidney, from each rat, were immersed in RNA laterTM (Ambion, Austin, USA) upon collection and stored at 4°C for 24h; afterwards, samples were frozen at -80 ºC. Subsequently, tissue samples weighing 50±10 mg were homogenized in a total volume of 1 ml TRI® Reagent (Sigma, Sintra, Portugal) using a homogenizer, and total RNA was isolated as described in the TRI® Reagent Kit. To ensure inactivation of contaminating RNAses, all material used was cleaned and immersed in RNAse-free water (0.2% diethyl pyrocarbonate) for 2h and finally heated at 120ºC for 1h. RNA integrity (RIN, RNA Integrity Number) was analyzed using 6000 Nano ChipW kit, in Agilent 2100 bioanalyzer (Agilent Technologies, Walbronn, Germany) and 2100 expert software, following manufacturer instructions. The yield from isolation was from 0.5 to 1.5 µg; RIN values were 7.0–9.0 and purity (A260/A280) was 1.8–2.0. The concentration of the RNA preparations were confirmed with NanoDrop1000 (ThermoScientific,Wilmington, DE, USA). Possible contaminating remnants of genomic DNA were eliminated by treating these preparations with deoxyribonuclease I (amplification grade) prior to RT-qPCR amplification. Reverse transcription and relative quantification of gene expression were performed as previously described.[21]

Table III-1. List of primer sequences (F: forward; R: reverse).

Gene	Primer sequences
EPO	F: 5'-AGGGTCACGAAGCCATGAAG-3'
	R: 5'-GAT TTC GGC TGT TGC CAG TG-3'
EPOR	F: 5'-GCG ACT TGG ACC CTC TCA TC-3'
	R: 5'-AGT TAC CCT TGT GGG TGG TG-3'
Hamp	F: 5'-GAA GGC AAG ATG GCA CTA AGC-3'
	R: 5'-CAG AGC CGT AGT CTG TCT CG-3'
TfR2	F: 5'-CAA GCT TCG CCC AGA AGG TA-3'
	R: 5'-CGT GTA AGG GTC CCC AGT TC-3'
SLC40A1	F: 5'-CAG GCT TAG GGT CTA CTG CG-3'
	R: 5'-CCG AAA GAC CCC AAA GGA CA-3'
HJV	F: 5'-GCC TAC TTC CAA TCC TGC GT-3'
	R: 5'-GGT CAA GAA GAC TCG GGC AT-3'
TF	F: 5'-GGC ATC AGA CTC CAG CAT CA-3'
	R: 5'-GCA GGC CCA TAG GGA TGT T-3'
Hfe	F: 5'-CTG GAT CAG CCT CTC ACT GC-3'
	R: 5'-GTC ACC CAT GGT TCC TCC TG-3'
DMT1	F: 5'-CAA CTC TAC CCT GGC TGT GG-3'
	R: 5'-GTC ATG GTG GAG CTC TGT CC-3'
TfR1	F: 5'-GCT CGT GGA GAC TAC TTC CG-3'
	R: 5'-GCC CCA GAA GAT GTG TCG G-3'
TMPRSS6	F: 5'-CCG AAT ATG AGG TGG ACC CG-3'
	R: 5'-GGT TCA CGT AGC TGT AGC GG-3'
BMP6	F: 5'-GCT GCC AAC TAT TGT GAC GG-3'
	R: 5'-GGT TTG GGG ACG TAC TCG G-3'
IL-6	F: 5'-ATG TTG TTG ACA GCC ACT GC-3'
	R: 5'- TTT TCT GAC AGT GCA TCA TCG-3'
IL-1β	F: 5'-CTC TGT GAC TCG TGG GAT GAT G-3'
	R: 5'-CAC TTG TTG GCT TAT GTT CTG TCC-3'
CTGF	F: 5'-CGT AGA CGG TAA AGC AAT GG-3'
	R: 5'-AGT CAA AGA AGC AGC AAA CAC-3'
NF-kB	F: 5'-ACC TGA GTC TTC TGG ACC GCT G-3'
	R: 5'-CCA GCC TTC TCC CAA GAG TCG T-3'
VEGF	F: 5'- GAA GTT CAT GGA CGT CTA CCA G -3'
	R: 5'- CAT CTG CTA TGC TGC AGG AAG CT -3'
TNF-α	F: 5'- CCC AGA CCC TCA CAC TCA GAT CAT -3'
	R: 5' – GCA GCC TTG TCC CTT GAA GAG AA-3'
18S	F: 5'-CCA CTA AAG GGC ATC CTG GG-3'
	R: 5'-CAT TGA GAG CAA TGC CAG CC-3'
Actb	F: 5'-GAG ATT ACT GCC CTG GCT CC-3'
	R: 5'-CGG ACT CAT CGT ACT CCT GC-3'

Real-time qPCR reactions were performed for erythropoietin (*EPO)*, erythropoietin receptor *(EPOR)*, transferrin receptor 2 (*TfR2*), hepcidin (*Hamp*), ferroportin (*SLC40A1*), hemojuvelin (*HJV*), transferrin (*TF*), hemochromatosis (*Hfe*), divalent metal transporter 1 (*DMT1*), transferrin recptor 1 (*TfR1*), matriptase-2 (*TMPRSS6*), interleukin-6 (*IL-6*) and bone morphogenic protein 6 (*BMP6*); vascular endothelial growth factor *(VEGF),* interleukin-1 beta *(IL-1β)*, nuclear transcription factor kappa B (*NF-kB)*, connective tissue growth factor (*CTGF)* and tumor necrosis factor alpha *(TNF-α)* which were normalized in relation to the expression of beta-actin (*Actb*), and 18S ribosomal subunit (*18S*).Primer sequences are listed on Table III-1. Results were analyzed with SDS 2.1 software (Applied Biosystems, Foster City, CA, USA) and relative quantification calculated using the $2^{-\Delta\Delta Ct}$ method.[22] In liver tissue we studied the *EPO*, *EPOR*, *TfR1*, *TfR2*, *Hamp*, *Il-6*, *SLC40A1*, *HJV*, *TF*, *Hfe, BMP6* and *TMPRSS6* gene expression; in duodenum tissue the gene expression of *DMT1* and *SLC40A1* were studied, and in the kidney we evaluated the expression of *EPO, EPOR, Il-6, IL-1β, TNF-α, NF-kB, CTGF and VEGF* genes.

3.6. Histopathological analysis

Tissue samples were fixed in neutral formalin 10% and embedded in paraffin wax; afterwards, 4μm thick sections for routine histopathological studies were stained with hematoxylin and eosin (H&E). Periodic acid of Shiff (PAS) was used to evaluate and confirm the levels of mesangial expansion, thickening of basement membranes and sclerotic parameters. For PAS staining, the tissue samples were fixed in neutral formalin 10%, embedded in paraffin wax, and 4μm thick sections were immersed in water and subsequently treated with 1% aqueous solution of periodic acid (1%), then washed to remove any traces of the periodic acid, and finally treated with Schiff's reagent. All samples were examined by light microscopy using a Microscope Zeiss Mod. Axioplan 2. The degree of injury visible by light microscopy was scored in a double-blinded fashion by two pathologists. Lesions were evaluated on the total tissue on the slide. Glomerular and tubulointerstitial lesions were divided in mild and advanced. Mild glomerular damage was assessed by evaluating thickening of Bowman's capsule, hyalinosis of the

vascular pole, glomerular atrophy, hypercellularity and dilatation of Bowman´s space. Advanced glomerular damage was assessed by grading sequentially four main lesions, from the less to the worse one: 1 – thickening of glomerular basement membrane (GBM), 2 – mesangial expansion, 3 – nodular sclerosis and 4 – global glomerulosclerosis. When advanced lesions were presented at a given glomeruli, the analysis of mild lesions become unavailable. Mild tubulointerstitial lesions included tubular hyaline droplets, tubular basement membrane (TBM) irregularity, tubular dilatation, interstitial inflammatory infiltration and vacuolar tubular degeneration. Advanced tubulointerstitial lesions were assessed evaluating the presence of hyaline cylinders, tubular calcification, necrosis and the association of interstitial fibrosis and tubular atrophy (IFTA). The evaluation of vascular lesions was focused on arteriolar hyalinosis, arteriolosclerosis and arteriosclerosis.

A semiquantitative rating for each slide ranging from normal (or minimal) to severe (extensive damage) was assigned to each component. Severity of lesions was graded according to the extension occupied by the lesion (% area): 0 – absent/normal: 0 %; 1 – <25%; 2 – 25–50%; 3 – >50%. The final score for each sample was obtained by the average of scores observed in individual glomeruli in the analysed microscopic fields. Tubulointerstitial damage was evaluated and graded by the same semiquantitative method. When using PAS, the rating was set for intensity and extension of staining, ranging from 0 (no staining) to 3 (intense and extensive staining), referring tissue specificity scoring when adequate.

3.7. Immunohistochemistry analysis

Liver and renal cortex/medulla paraffin sections (4 μm) from each sample were dewaxed in xylene, rehydrated in a series of ethanol washes, and placed in distilled water before staining procedures. The samples were processed for indirect immune detection using a mousse and rabbit specific horseradish peroxidase (HRP)/ diaminobenzidine (DAB) detection IHC kit (ab80436, Abcam Inc, Cambridge, UK), according to the manufactory's protocol. Negative controls were included in each staining series, by omission of the primary antibodies. Antigen retrieval was performed

for 20 minutes for paraffin-embedded tissue in the preheated Citrate Buffer (10 mM Citric Acid, pH 6.0) using a pressure cooker. Between incubations with the antibodies, the specimens were washed two to four times in buffer PBS (pH 7.4). All incubations were performed overnight at 4ºC in a humidified chamber. In this study, we employed primary antibodies from Abcam Inc. (Cambridge, UK) for detection of hepcidin (dilution 1:150; ab81010) and CTGF (dilution 1:250, ab6992), and from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) from detection of NF-κB p50 (dilution 1:500, sc-114); HIF-2α (dilution 1:250, sc-28706) and HIF-2β (dilution 1:100, sc-5581). For immunohistochemical quantification, ten 400x microscopic views of liver and renal cortex and medulla per slide were selected randomly and photographed using a Leica DFC480 microscope (Leica Microsystems, Wetzlar, Germany). Intensity and area of positive staining, detected by brown staining were used as criteria: intensity was evaluated as weak (1), moderate (2) or strong (3); the percentage of area was quantified. A staining score (Quick Score) was then calculated according to previously described,[23] using the formula: Quick Score = intensity (1, 2 or 3) multiplied by area (percentage). The final score (out of maximum of 300) for each group was obtained averaging the individual scores of each animal.

3.8. Data analysis

For statistical analysis, we used the IBM Statistical Package for Social Sciences (SPSS), version 20 (2011). Significance level was accepted at *p* less than 0.05. Results are presented as means ± standard error of means (SEM). Comparisons between groups were performed using non-parametric tests (Mann-Whitney test).

4. Results

4.1. Body and tissue weights and blood pressure

At the end of experimental protocol (12 weeks), a significant decrease ($p<0.001$) of BW was observed in CRF rats, when compared to Sham. In addition, KW and KW/BW ($p<0.001$ for both) presented higher values in the CRF animals. While HW was

unchanged, HW/BW ratio was higher (p<0.01) in CRF rats. Despite the lower value (p<0.01) of LW in the CRF group, LW/BW was higher (p<0.05) when compared with the Sham group (Table III-2). CRF rats presented a significantly higher (p<0.01) systolic blood pressure at the final time, when compared with the Sham animals, but similar values were found for DBP, MBP and HR (Table III-2).

4.2. Biochemical and hematological data

The biochemical and hematological data for the Sham and CRF groups are presented in Tables III-2 and III-3 and Figure III-2. The CRF rats presented significantly (p<0.001) increased serum BUN and creatinine concentrations three weeks after the partial 5/6 nephrectomy.

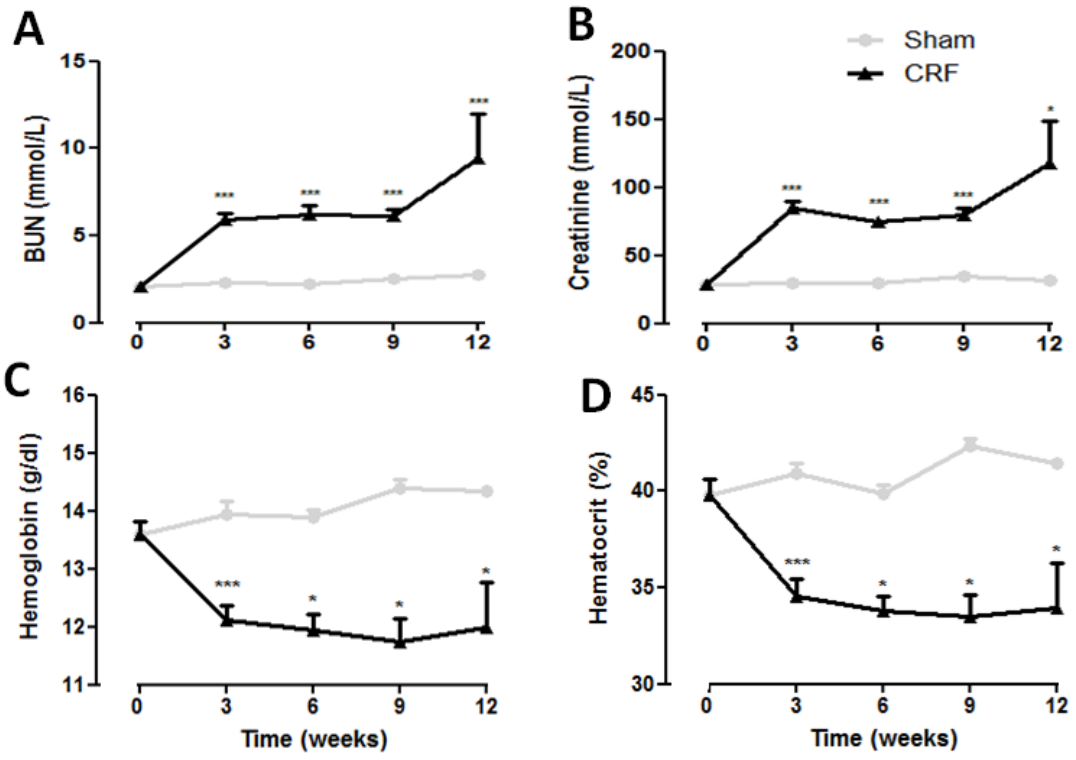

Figure III-2. Renal and hematological data throughout the follow-up period of 12 weeks. Evolution of BUN (A), creatinine (B), hemoglobin (C) and hematocrit (D) values throughout the experimental protocol. Results are presented as mean ± SEM: *- p < 0.05, **- p < 0.01, and ***- p < 0.001 *versus* Sham group. BUN, blood urea nitrogen.

The values remained elevated until the 9th week, after which a further increase was observed at the final time (p<0.001 and p<0.05, respectively), when compared with the Sham group (Figures III-2A and III-2B). Concerning the other biochemical parameters, similar values were observed, except for Total-c, ALT, hsCPR and VEGF, which were significantly higher in CRF rats (p<0.05) at the final time (Table III-2 and Figure III-8B).

Table III-2. Body and tissue weights, blood pressure and biochemical data at the final time.

Parameters	Final time (12 weeks)	
	Sham group	CRF group
Body and tissues weights		
BW (Kg)	0.45±0.02	0.36±0.01 ***
KW (g)	1.22±0.03	1.65±0.04 ***
KW/BW (g/Kg)	2.72±0.05	4.61±0.22 ***
HW (g)	1.16±0.03	1.24 ± 0.07
HW/BW (g/Kg)	2.58±0.08	3.48±0.25 **
LW (g/Kg)	13.33±0.48	11.32±0.34 **
LW/BW (g/Kg)	29.61±0.65	31.43±0.71 *
Blood pressure and heart rate		
SBP (mmHg)	117.7 ± 1.15	134.1 ± 4.6 **
DBP (mmHg)	112.0 ± 0.58	111.5 ± 4.5
MBP (mmHg)	114.3 ± 1.45	121.3 ± 4.5
HR (beats/min)	360.7± 1.20	373.0 ± 9.2
Biochemical data		
Glicose (mmol/L)	9.46±0.31	8.66±0.60
TGs (mmol/L)	1.05±0.14	1.58±0.32
Total-c (mmol/L))	1.25±0.06	2.44±0.54 *
CK (U/L)	540.57±58.94	473.00±85.57
ALT (U/L)	35.17±2.21	42.00±18.53*
AST (U/L)	80.57±7.84	139.43±70.70
Bilirubin (µmol/L)	8.04e-5±1.03e-5	1.03e-4±1.71e-5
hsCRP (µg/mL)	262.25±12.43	225.31±7.95 *
INF-γ (pg/mL)	23.30±3.10	25.51±2.26
TGF-β1 (ng/mL)	75.74±5.62	84.13±3.85

Results are presented as mean ± SEM: *- p < 0.05, **- p < 0.01, and ***- p < 0.001 *versus* Sham group. ALT, alanine transaminase; AST, aspartate transaminase; BW, body weight; CK, creatine kinase; DBP, diastolic blood pressure; HR, heart rate; hsCRP, high-sensitive C reactive protein; HW, heart weight; IFN-γ, interferon γ; KW, kidney weight; LW, liver weight; SBP, systolic blood pressure; TGs, triglycerides; TGF-β1, transforming growth factor beta 1; Total-c, total cholesterol; VEGF, vascular endothelial growth factor.

Three weeks after the 5/6 nephrectomy, the CRF rats developed anemia, as showed by the significant decline of Hb and HTC (p<0.001); the anemia persisted along the protocol. Analysing the results at the end of the protocol, we found that CRF animals showed significantly (p<0.01) decreased RBC count, and a trend towards a reduced reticulocyte count, when compared to the Sham rats. The MCV, MCH, RDW, PLT count and PDW values were similar and MCHC was significantly higher. Moreover, we found that WBC count, as well lymphocytes $CD3^+$, $CD3^+CD4^+$, $CD3^+CD4^+CD25^+$ and $CD3^+CD8^+$ counts were similar for both groups. However, the percentage of activated cytotoxic T cells ($CD3^+CD8^+CD25^+$) was significantly increased in the CRF group (p<0.01) *versus* Sham (Table III-3).

Table III-3. Hematological data at the final time.

Parameters	Final time (12 weeks)	
	Sham group	CRF group
Hematological data		
RBC (x 10^{12}/L)	7.94±0.08	6.53±0.43 **
Ret (x10^9/L)	181.22±6.82	168.14±17.32
MCV (fL)	52.52±0.53	51.93±0.69
MCH(pg)	18.08±0.18	18.36±0.24
MCHC (g/dL)	34.60±0.08	35.37±0.19 **
RDW (%)	11.48±2.53	18.34±3.23
PLT (x 10^9/L)	713.75±15.19	769.00±73.17
PDW (%)	16.34±0.18	16.44±0.20
WBC (x 10^9/L)	1.78±0.30	5.01±1.76
T Lymphocytes (%)	57.20±1.36	54.67±2.91
$CD3^+CD4^+$ T cells (%)	72.18±0.60	72.48±1.16
$CD3^+CD4^+$ $CD25^+$ T cells (%)	5.97±0.62	5.85±0.70
$CD3^+CD8^+$ T cells (%)	24.16±1.34	28.17±2.17
$CD3^+CD8^+$ $CD25^+$ T cells (%)	0.40±0.04	0.63±0.01**

Results are presented as mean ± SEM: *- p < 0.05, **- p < 0.01, and ***- p < 0.001 *versus* Sham group. Hb, hemoglobin; Hct, hematocrit; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; PDW, platelet distribution width ; PLT, platelets; RBC, red blood cells; RDW, RBC distribution width; Ret, reticulocytes; WBC, white blood cells.

4.3. Serum EPO and liver and kidney EPO and EPOR mRNA expression

At the final time, serum EPO was significantly higher (p<0.05) in the CRF group when compared with the Sham (Figure III-3A). In addition, there was a significant

(p<0.01) overexpression of EPO mRNA in the kidney and liver tissues of the CRF rats, when compared with the Sham animals (Figure III-3B and III-3C, respectively). Concerning EPOR mRNA, a significant (p<0.01) overexpression was found in the kidney tissue of the CRF rats, accompanied by a reduced (p<0.01) expression in the liver, when compared with the Sham group (Figures III-3B and III-3C, respectively).

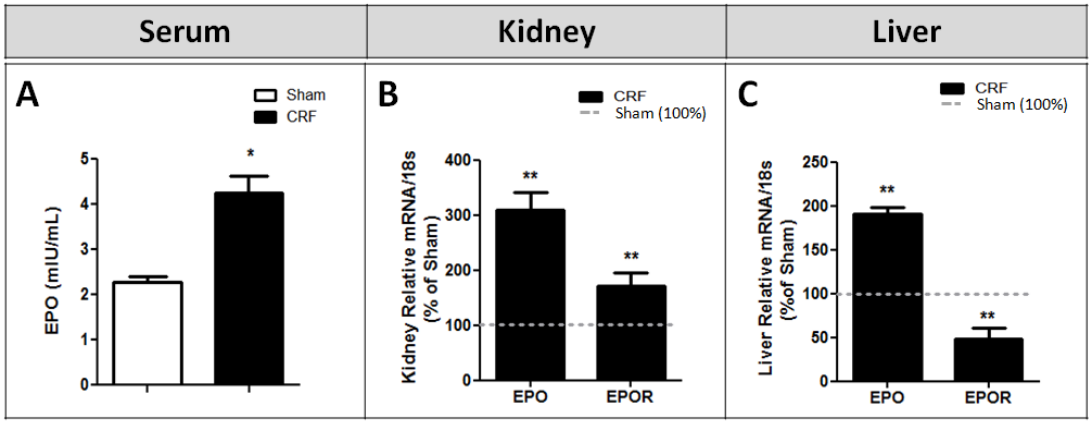

Figure III-3. Serum EPO and kidney and liver gene expression of EPO and EPO receptor. Serum EPO (A), EPO and EPOR mRNA levels/18s expression (% of Sham group) in kidney (B) and liver (C) tissues, at the end of the study (12 weeks). Results are presented as mean ± SEM: *- p < 0.05, and **- p < 0.01 *versus* Sham group. EPO, erythropoietin.

4.4. Iron metabolism

To study iron metabolism we evaluated several markers at blood and tissue (liver and duodenum) levels. When compared to sham group, we found a significant decrease in serum iron (p<0.001) and transferrin (p<0.001) in CRF rats, (Figures III-4A$_1$ and III-4A$_3$), and similar values for serum ferritin (Figure III-4A$_2$).

No significant changes between groups were found for liver mRNA expression of *IL-6*, *sTfR2, TMPRSS6* and *SLC40A1* (Figure 3B); however, there was a significantly lower liver mRNA expression of *Hamp, sTfR1, TF, BMP6, Hfe, HJV* (p<0.01 for all), *BMP6* (p<0.05) and *HIF-2*α (p<0.001) in the CRF rats when compared with the Sham ones (Figure III-4B); in the duodenum, a significant mRNA overexpression (p<0.05) of *SLC40A1* and a similar mRNA expression of *DMT1* (Figure III-4C), were observed in CRF rats.

The immunohistochemical expression of liver hepcidin (Figures III-5A$_1$- III-5A$_3$) and of HIF-2α (Figures III-5B$_1$- III-5B$_3$) presented similar values for CRF and Sham rats.

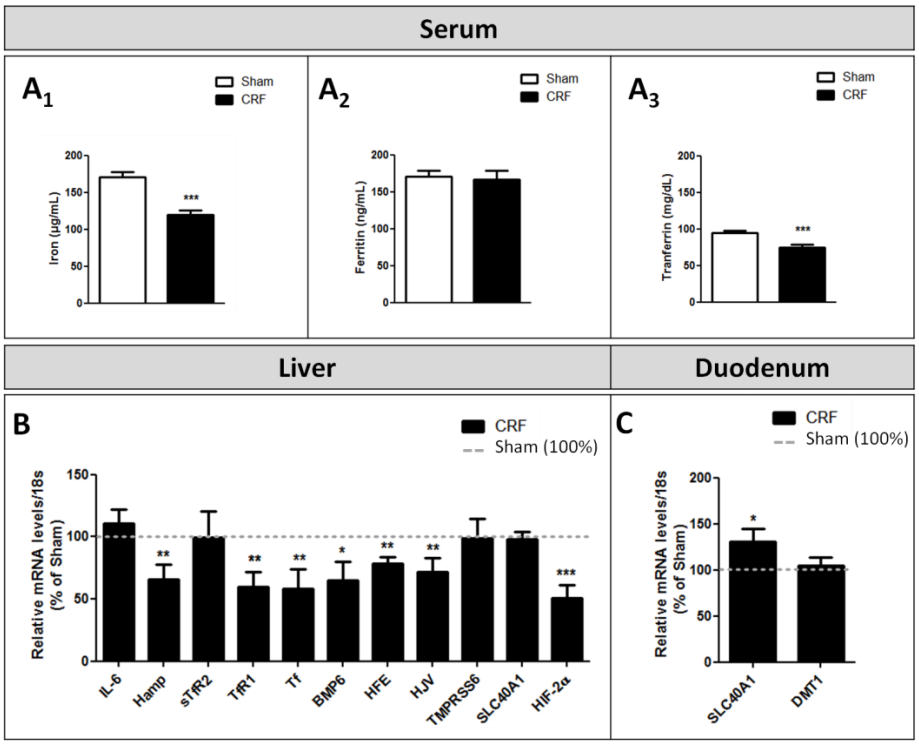

Figure III-4. Mediators of iron-hepcidin metabolism in serum, liver and duodenum. Serum iron (A_1), ferritin (A_2) and transferrin (A_3) levels; relative gene expression mRNA levels/18s (% of Sham group) in liver (B) and duodenum (C) tissues, at final time (12 weeks). Results are presented as mean ± SEM: *- $p < 0.05$, **- $p < 0.01$, and ***- $p < 0.001$ *versus* Sham group. BMP6, Bone morphogenic protein 6; DMT1, divalent metal transporter 1; HFE, Hemochromatosis; HJV, Hemojuvelin; IL-6, interleukin-6; SLC40A1, ferroportin; TMPRSS6, matriptase-2; sTfR2, soluble transferrin receptor 2; Tf, transferrin and TfR1, transferrin receptor 1.

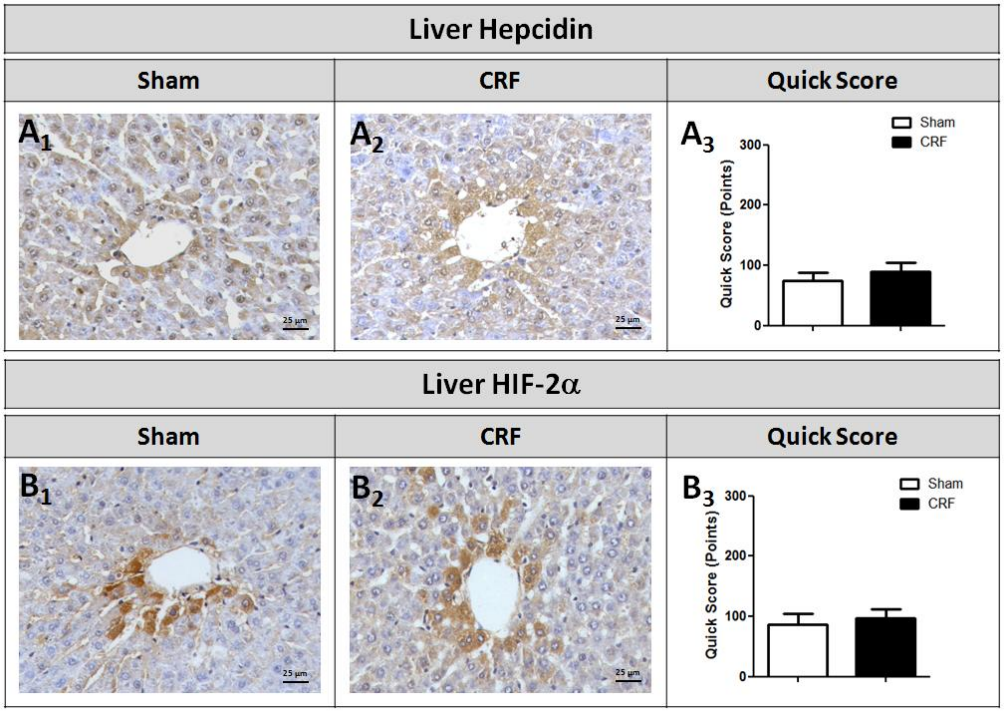

Figure III-5. Liver protein expression (by immunohistochemistry) of hepcidin (A_1-A_3) and HIF-2α (B_1-B_3). Results are presented as mean ± SEM: *- $p < 0.05$, **- $p < 0.01$, and ***- $p < 0.001$ *versus* Sham group. Hamp, hepcidin antimicrobial peptide; HIF-2α, Hypoxia inducible factor 2 alpha.

4.5. Kidney lesions

No significant histomorphological changes were found in the kidneys of Sham rats at the end of the experimental period (Figures III-6A_1 and III-6B_1, Tables III-4 and III-5). However, the CRF rats presented several glomerular (cortex) and tubulointerstitial (medulla) lesions. Concerning the mild glomerular lesions, most of the animals of the CRF group presented thickening of Bowman´s capsule (score: 1.57±0.43; p<0.01), hyalinosis of vascular pole (score: 0.86±0.14; p<0.01), glomerular atrophy (score: 0.86±0.14; p<0.01) and hypercellularity (score: 1.00±0.00; p<0.01), while in Sham rats the lesions were absent (Table III-4 and Figure III-6).

Table III-4. Scoring and distribution (%) of mild and advanced glomerular lesions.

Mild lesions		Score				
		0 Absent	1 <25%	2 25-50%	3 >50%	Total Score
Thickening of Bowman´s Capsule	Sham, n (%)	7 (100%)	0	0	0	0.00±0.00
	CRF, n (%)	1 (14.3%)	3 (42.9 %)	1 (14.3%)	2 (28.6 %)	1.57±0.43**
Hyalinosis of the vascular pole	Sham, n (%)	7 (100%)	0	0 (0 %)	0 (0 %)	0.00±0.00
	CRF, n (%)	1 (14.3%)	6 (85.7%)	0	0	0.86±0.14**
Glomerular atrophy	Sham, n (%)	7 (100%)	0	0	0	0.00±0.00
	CRF, n (%)	1 (14,3%)	6 (85.7%)	0	0	0.86±0.14**
Hypercellularity	Sham, n (%)	7 (100%)	0	0	0	0.00±0.00
	CRF, n (%)	0	7 (100%)	0	0	1.00±0.00**
Dilatation of the Bowman's Space	Sham, n (%)	7 (100%)	0	0	0	0.00±0.00
	CRF, n (%)	5 (71.4%)	2 (28.6%)	0	0	0.29±0.18
TOTAL GROUP SCORE	Sham					**0.00±0.00**
	CRF					**0.91±0.12*** **

Rat Groups	Advanced glomerular lesions					
	None of the previous lesions (0)	Thickening of GBM (1)	Mesangial expansion (2)	Nodular sclerosis (3)	Global Glomerulosclerosis (4)	TOTAL GROUP SCORE
Sham, n (%)	7 (100%)	0	0	0	0	**0.00±0.00**
CRF, n (%)	0	1 (14.3%)	5 (71.4%)	1 (14.3%)	0	**2.0±0.22****

Results are presented as mean ± SEM: *- p < 0.05, **- p < 0.01, and ***- p < 0.001 *versus* Sham group.

In addition, all CRF rats presented at least one of the advanced glomerular lesions, namely mesangial expansion, that was present in 5 out of 7 rats (Table III-4 and Figure III-6A$_3$). The total score of both mild (0.91±0.12; p<0.001) and advanced (2.00±0.22; p<0.01) glomerular lesions showed a significantly increased value in CRF rats (Table III-4 and Figure III-6A$_4$).

Table III-5. Scoring and distribuition (%) of mild and advanced tubulointerstitial lesions.

Mild Lesions		Score				
		0 Absent	1 <25%	2 25-50%	3 >50%	Total Score
Tubular Hyaline Droplets	Sham, n (%)	7 (100%)	0	0	0	0.00±0.00
	CRF, n (%)	0	7 (100%)	0	0	1.00±0.00 **
TBM Irregularity	Sham, n (%)	7 (100%)	0	0	0	0.00±0.00
	CRF, n (%)	0	3 (42.9%)	2 (28.5%)	2 (28.5%)	1.86±0.34**
Tubular Dilatation	Sham, n (%)	7 (100%)	0	0	0	0.00±0.00
	CRF, n (%)	2 (28.5%)	1 (14.3%)	2 (28.5%)	2 (28.5%)	1.57±0.48*
Interstitial Inflammatory Infiltrate	Sham, n (%)	4 (57.2 %)	3 (42.9%)	0	0	0.29±0.18
	CRF, n (%)	0	0	7 (100%)	0	2.00±0.00**
Vacuolar Tubular Degeneration	Sham, n (%)	0	7 (100%)	0	0	1.00±0.00
	CRF, n (%)	3 (42.9%)	3 (42.9%)	1 (14.3%)	0	0.71±0.29
TOTAL GROUP SCORE	**Sham**					**0.26±0.08**
	CRF					**1.43±0.15*** **

Advanced Lesions		Score				
		0 Absent	1 <25%	2 25-50%	3 >50%	Total Score
Hyaline cylinders	Sham, n (%)	6 (85.7%)	1 (14.3%)	0	0	0.14±0.14
	CRF, n (%)	0	1 (14.3%)	6 (85.7%)	0	1.86±0.14**
Tubular Calcification	Sham, n (%)	7 (100%)	0	0	0	0.00±0.00
	CRF, n (%)	7 (100%)	0	0	0	0.00±0.00
Necrosis	Sham, n (%)	7 (100%)	0	0	0	0.00±0.00
	CRF, n (%)	2 (28.6%)	5 (71.4%)	0	0	0.71±0.18*
IFTA	Sham, n (%)	7 (100%)	0	0	0	0.00±0.00
	CRF, n (%)	0	3 (42.9%)	4 (57.1%)	0	1.57±0.20**
TOTAL GROUP SCORE	**Sham**					**0.04±0.04**
	CRF					**1.04±0.16*** **

Results are presented as mean ± SEM: *- p < 0.05, **- p < 0.01, and ***- p < 0.001 *versus* the Sham group. TBM, tubular basement membrane; IFTA, interstitial fibrosis and tubular atrophy.

Concerning the mild tubulointerstitial lesions, most of the animals of the CRF group presented tubular hyaline droplets (score: 1.00±0.00; p<0.01), TBM irregularity (score: 1.86±0.34; p<0.01), tubular dilatation (score: 1.57±0.48; p<0.05) and interstitial inflammatory infiltration (score: 2.00±0.00; p<0.01) (Table III-5 and Figure III-6). Considering advanced tubulointerstitial lesions, the formation of hyaline cylinders (score: 1.86±0.14; p<0.01) and IFTA (score: 1.57±0.20; p<0.01) were the most relevant lesions observed in CRF rats, when compared with Sham rats (Table III-5 and Figures III-$6B_2$- III-$6B_3$). Once again, total score of both mild (1.43±0.15; p<0.001) and advanced (1.04±0.16; p<0.001) tubulointerstitial lesions showed a significantly increased value in the CRF rats (Figure III-$6B_4$).

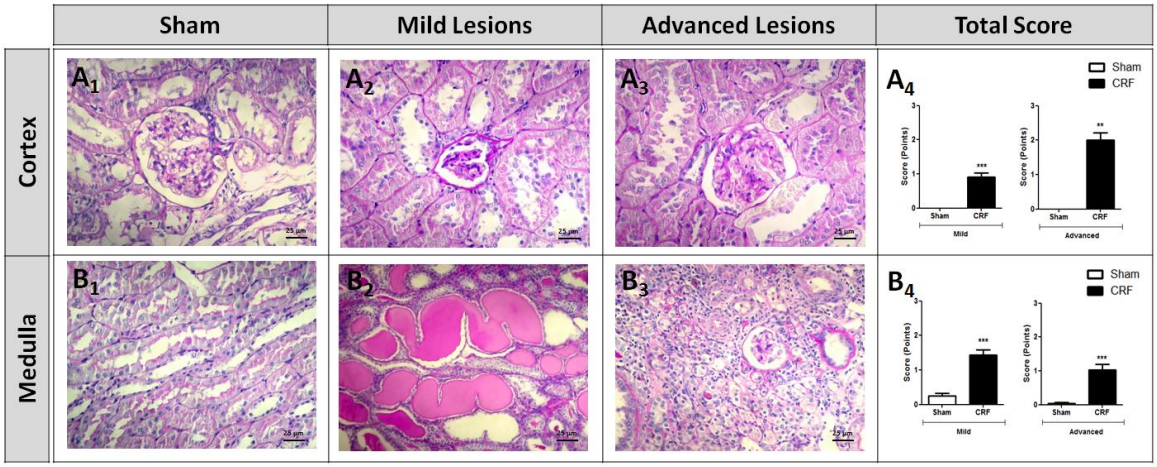

Figure III-6. Glomerular and tubulointerstitial lesions. Representative glomerular (cortex) and tubulointerstitial (medulla) lesions observed in kidneys of CRF and Sham groups, at the final time (PAS staining): A_1 – normal glomerulus histology in the Sham rats; A_2 – glomerular atrophy and thickening of glomerular basement membrane; A_3 – glomerulus presenting mesangial expansion; A_4 – total score of mild and advanced glomerular lesions in both rat groups; B_1 – normal tubulointerstitial histology in the Sham rats; B_2 – hyaline cylinders; B_3 – interstitial fibrosis and tubular atrophy (IFTA); B_4– Total score of mild and advanced tubulointerstitial lesions in both rat groups. Results are presented as mean ± SEM: **- p < 0.01 , and ***- p < 0.001 *versus* Sham group.

4.6. Renal expression of hypoxia inducible factors and other markers of kidney lesion

The immunohistochemical study showed a significant increase (p<0.01) in the expression of *HIF-2*α and *HIF-2*β in the kidney of CRF rats, as compared to Sham rats (Figures III-7A and III-7B, respectively). In addition, a significant overexpression of *IL-6*, *IL-1*β and *TNF-*α mRNA in the CRF rats was found, when compared with Sham rats

(p<0.001); contrarily, a significant downexpression of *NF-kB*, *CTGF* and *VEGF* (p<0.01) was found in the remnant kidney of CRF rats, when compared with Sham ones (p<0.001) (Figure III-8A). Serum values for IL-6 were similar for both groups, while a significantly higher concentration for VEGF was observed in the CRF rats (p<0.05) (Figure III-8B).

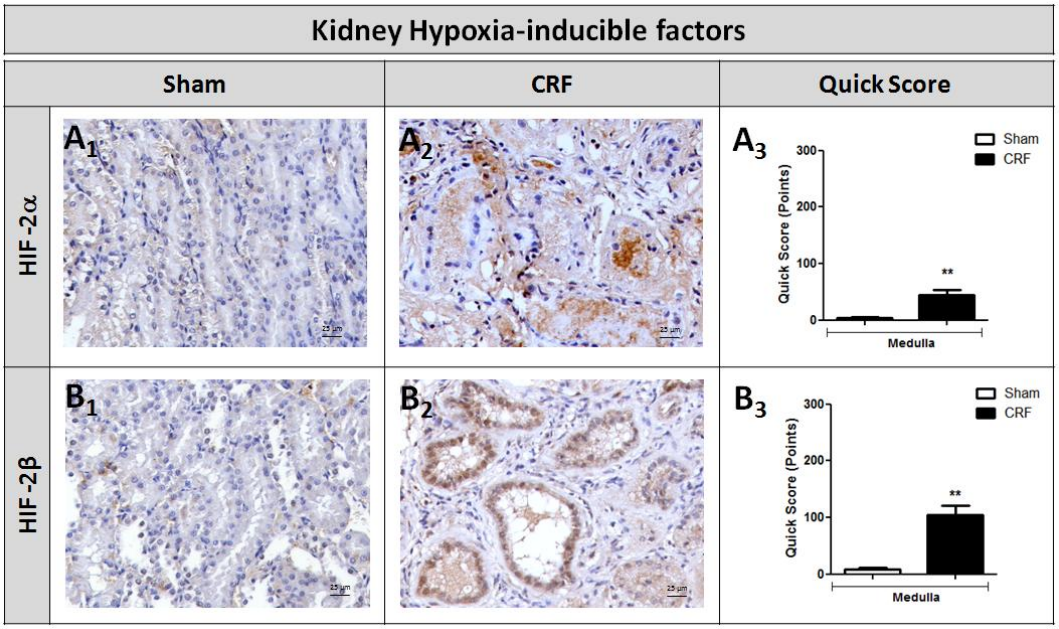

Figure III-7. HIF-2α and HIF-2β immunohistochemical expression in the kidney. Kidney immunohistochemical expression of HIF-2α (A) and HIF-2β (B) in the renal cortex of sham (1) and CRF (2) rats and corresponding Quick scores (3). Results are presented as mean ± SEM: ***- p < 0.001 *versus* Sham group. HIF-2α, hypoxia inducible factor 2 alpha, and HIF-2β, hypoxia inducible factor 2 beta.

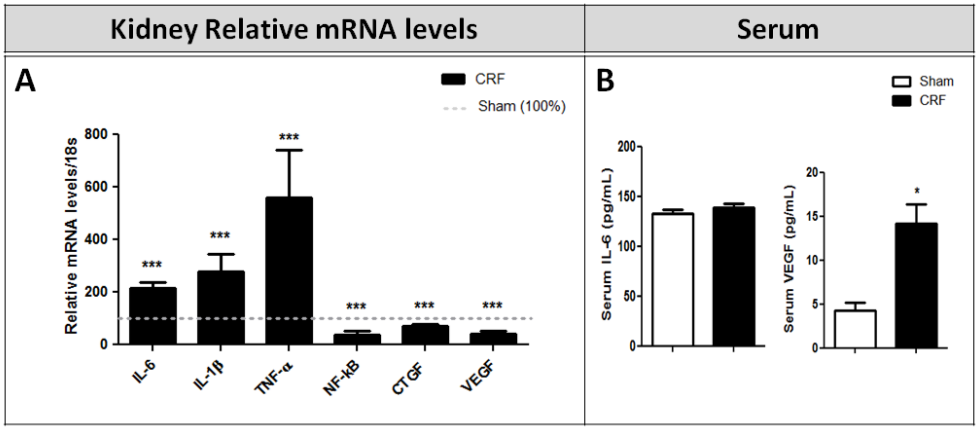

Figure III-8. Mediators of inflammation, fibrosis and angiogenesis. Relative gene expression mRNA levels/18s (% of Sham group) of mediators of kidney lesions (A) and serum IL-6 and VEGF-α levels (B). Results are presented as mean ± SEM: *- p < 0.05, and **- p < 0.01 *versus* Sham group. CTGF, connective tissue growth factor; IL-1β, interleukin-1 beta; IL-6, interleukin-6; NF-kB; nuclear transcription factor kappa B; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

Concerning protein expression of NF-kB in the kidney tissue, a significantly higher immunoreactivity in the cortex and in the tubular epithelial cells (medulla) of the CRF rats was found, when compared with the Sham ones (p<0.01) (Figures III-9A_2 and III-9B_2, respectively). CTGF was weakly expressed in the glomerular (cortex) and interstitial cells (medulla) of Sham rat's kidneys (Figures III-9C_1 and III-9D_1, respectively), while a significantly increased (p<0.01) expression was noted in the glomeruli and interstitial cells (medulla) of the CRF rats (Figures III-9C_2 and III-9D_2, respectively).

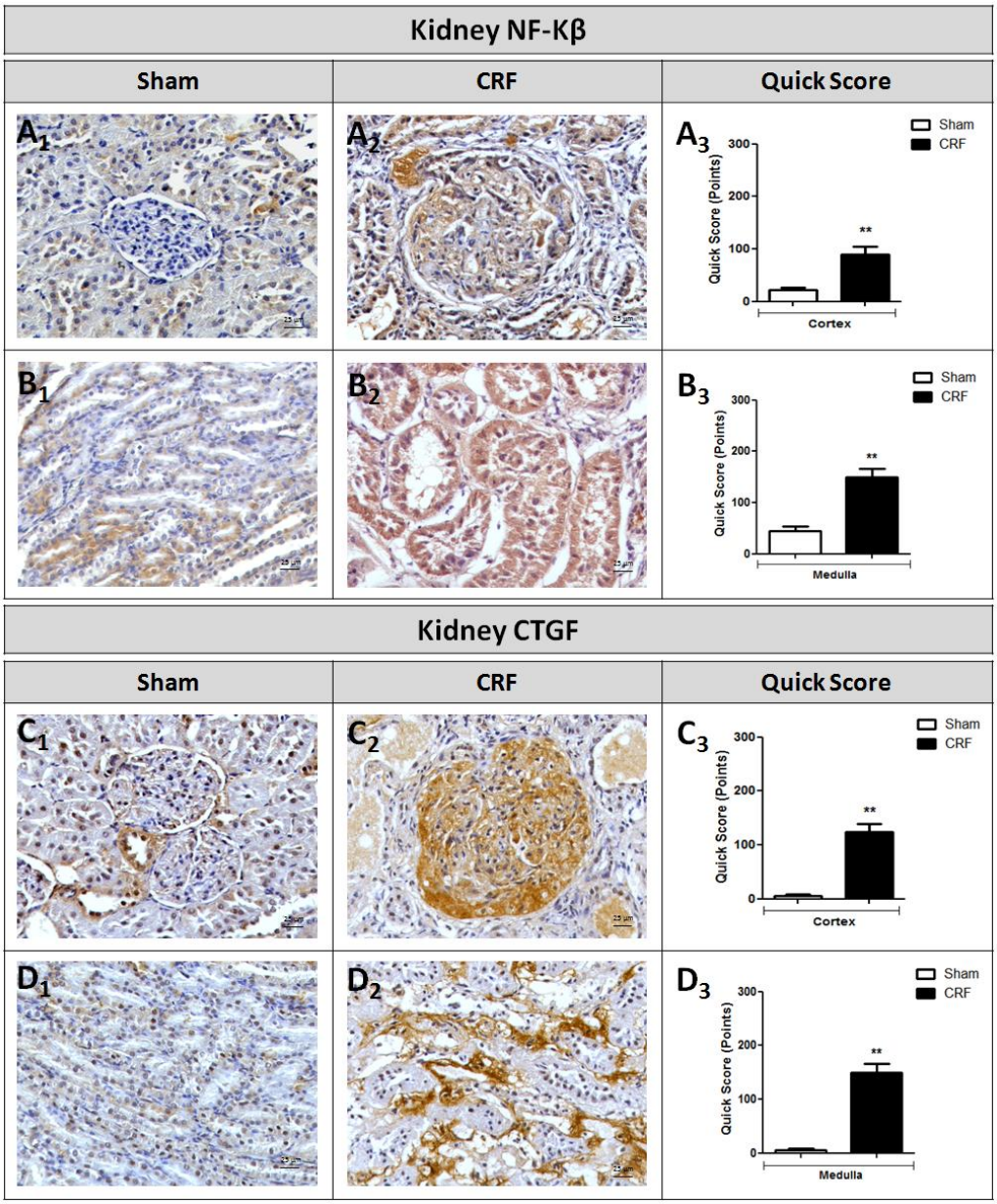

Figure III-9. Kidney protein expression (by immunohistochemistry) of NF-kB and CTGF in renal cortex (A_1-A_3 and C_1-C_3, respectively) and medulla (B_1-B_3 and D_1-D_3, respectively). Results are presented as mean ± SEM: *- p < 0.05, and **- p < 0.01 *versus* Sham group. CTGF, connective tissue growth factor; NF-kB; nuclear transcription factor kappa B.

5. Discussion

Chronic kidney disease (CKD) is a general term for heterogeneous disorders affecting the structure and function of the kidney. The variation in the clinical pattern of the disease has been associated to its etiology, severity and rate of progression. Since the introduction of the conceptual model for definition and staging of CKD and the establishment of the clinical practice guidelines to treat kidney disease patients, the disease evolved from a life-threatening disorder affecting few people who needed care by nephrologists, to a common disorder of varying severity that deserves the attention of a multidisciplinary team and needs a concerted public health approach for prevention, early detection and management.[24,25]

Early detection of renal failure and initiation of treatment contribute to prevent or delay some of these associated adverse effects.[26] Anemia, one of the most common complications of CKD, develops in the early phases of the disease, increasing its severity as the disease progresses, contributing to a patient's poor quality of life.[27] Anemia is mainly associated with a reduced production of EPO by the failing kidneys and with disturbances in iron metabolism. However, the clear relationship between renal and extra-renal EPO production, iron deficiency, hypoxia and evolution of kidney lesions remain to be elucidated.

Animal models of CKD have been used as a tool to study the pathophysiological mechanisms underlying different stages of renal disease and of the associated anemia, as well as to test the efficacy of different therapies. The (5/6) nephrectomy model is the most used model of CKD, although there are different ways to achieve (5/6) reduction of nephron mass.[26,28] This model involves substantial removal of nephrons, followed by compensatory renal hypertrophy of the remnant kidney. Increasing workload by the remaining nephrons leads to progression of renal injury, namely to CKD.[29-32]

The results of the present study confirmed that the surgical (5/6) nephrectomy model of CKD produced a sustained stage of renal insufficiency, as shown by the significantly increased BUN and creatinine concentrations, after three weeks of the surgical procedure (Figure III-2); these values persisted for more 6 weeks, after which a further increment was observed, at the 12th week, the end of protocol. In addition, a significant increase in KW and in KW/BW ratio were found in CRF rats (Table III-2),

showing a compensatory renal proliferation/hypertrophy of the remnant kidney, as previously described for this model.[33] A trend towards an increase in HW and a significant increase of HW/BW ratio was also found, suggesting the development of left ventricule hypertrophy, which is a cardiac complication found in the CKD patients. In fact, besides the anemia secondary to renal insufficiency, CKD patients usually develop cardiac failure that further aggravates renal disease. This triad of dysfunctions, known as cardio-renal anemia syndrome, is responsible for the serious complications observed in these patients.[34,35] The progression of kidney disease and its associated cardiac/cardiovascular complications are the major causes of morbidity and mortality in these patients. One of the most prevalent co-morbidities is hypertension, which is present at all stages of CKD.[36] Our results confirmed the development of systolic hypertension, which is a typical feature in CKD rat model (Table III-2). Usually, hypertension is inversely proportional to the residual functional renal mass, as occurs in human pathology.[37,38]

As widely occurs in human CKD, we also observed the development of anemia, secondary to renal mass reduction, as shown by the reduced Hb and HTC values in the CRF rats, three weeks after nephrectomy that persisted along the following 9 weeks. Moreover, the reticulocyte count showed a trend towards reduced values, suggesting a reduced erythropoietic response to overcome the anemia. However, we found that serum EPO concentration was increased in CRF rats at the final time (T4), as compared to sham animals (Figure III-3), suggesting that EPO production is not reduced in this model of CKD-associated anemia, and that the remnant kidney or even extra-renal tissues were able to compensate EPO production. When peritubular fibroblasts in the kidney sense reduced oxygen tension, the production of hypoxia inducible factor is induced, via oxygen sensitive prolyl hydroxylases (PHDs), triggering the activation of hypoxia response genes, leading to an increase in the production of EPO.[39] In the present work, we found a notable overexpression of EPO mRNA and EPOR mRNA in the kidney tissue of the CRF rats, as compared to Sham rats, as well as, an increased expression of HIF-2α in the kidney tissue (Figure III-7). These results suggest that the reduction of Hb levels in CRF rats, by leading to a reduced oxygen kidney perfusion, induced renal EPO production in response to low oxygen tension. The kidney is not the only organ able to adapt to EPO production, in response to low oxygen tension.[40,41]

Apparently, chronic hypoxia is also sensed by a HIF-dependent mechanism in which a nitric oxide (NO)-mediated redistribution of blood flow from inner organs to the skin is able to cause a secondary increase in renal EPO production.[42] Extra-renal EPO production has been also described in the liver, although its contribution to the circulating EPO concentration in adults is highly debatable, even in kidney disease states.[41] Actually, we found a significantly increased liver EPO mRNA expression in CRF rats (Figure III-3), accompanied by a reduced expression of EPOR mRNA. In spite of the increased serum EPO levels found in CRF rats, anemia persisted in these animals throughout the protocol, suggesting a blockade and/or a reduction in the activity of EPO. This hypothesis is supported by the fact that, when we administered rHuEPO (200 IU/Kg/week) to these CRF rats, a rise in hemoglobin level was observed, reaching similar or even higher values than those found in the sham group (data presented in next chapter). Following the translation of the *EPO* gene, three *N*-linked and one *O*-linked carbohydrate chains are added to erythropoietin; these chains normally exhibit heterogeneity in the type of carbohydrate moieties incorporated, chain length and branching configuration;[43,44] healthy individuals may present up to four residues on each *N*-linked carbohydrate chain, or up to two residues on the *O*-linked chain. Indeed, a variability in sialic acid composition may[45] affect the circulating half-life of erythropoietin and the interactions with its receptor; in general, increasing sialic acid content correlates with longer and greater potency of EPO.[46] Although the current understanding is, probably, incomplete, it is known that erythropoietin gene (*EPO*) expression is tightly regulated by several stimulators, namely, hypoxia-inducible transcription factors and hepatocyte nuclear factor 4α, and by several inhibitors, including nuclear factor kappa B and GATA2.[47]

Iron is essential for the production of mature red blood cells and a normal iron metabolism is crucial to maintain body iron levels.[10,48,49] A disturbance in iron homeostasis is a hallmark of the anemia of CKD, which, usually, presents as a functional iron deficient anemia, with low serum iron and transferrin alongside with normal or even high ferritin.[50] In accordance, we found that CRF rats, as compared to Sham rats, showed a significant decrease in serum iron and transferrin levels and similar values for ferritin (Figure III-4). Therefore, in spite of the reduced serum iron, the anemia is not iron deficient, as the iron storage is normal, as showed by ferritin levels. In CKD patients

the functional iron deficient anemia is explained by the underlying inflammatory process, with increased hepcidin levels.[51] Indeed, it has been reported that hepcidin plays pivotal role in the development of the anemia associated with CKD.[10] Hepatocytes play a dual role in iron metabolism, acting as the major site of iron storage and of secretion of the iron regulatory hormone hepcidin (codified by the gene *Hamp*).[51] Hepcidin orchestrates systemic iron fluxes by controlling iron absorption through enterocytes and iron mobilization from macrophages. Hepcidin binds to the iron exporter ferroportin (SLC40A1, Solute carrier family 40, member 1) on the surface of iron-releasing cells, triggering its degradation and, therefore, reducing the iron absorption through the linkage of iron to transferrin.[51] *Hamp* expression is elevated in inflammatory conditions (through IL-6 dependent pathway), in increased erythropoiesis and iron overload, and down-regulated during hypoxia or iron deprivation.[52] In this animal model, a systemic inflammatory state cannot be recognized, as showed by CRP and IL-6 serum values that are similar for the two groups (Table III-2). However, a local renal inflammation, as suggested by the increased expression of *IL-6*, *IL-1*β and *TNF-*α mRNA in the kidney tissue (Figure III-8), might contribute to alter EPO renal production/function and, therefore, erythropoiesis.

The evidence for hypoxic regulation of *Hamp* remains controversial. Some studies show that *Hamp* is suppressed by hypoxia through HIF-1- and (possibly) HIF-2-dependent pathways.[13,53-55] Indeed, *Hamp* contains some HREs in its promoter region and its expression might be reduced directly by hypoxia.[13] The evidence for this, however, is conflicting, as a recent study showed that HIF-1α and HIF-2α knockdown failed to reverse human *Hamp* repression by hypoxia; in addition, inducers of HIF (CO, hypoxia, oxalylglycine) also showed controversial results.[56] Furthermore, deletion of putative HREs in the human *Hamp* promoter did not alter its response to hypoxia. Choi et al. (2007)[57] also showed that the hypoxic downregulation of *Hamp* was independent of HIF-1α overexpression or knockdown; however, they suggested that the suppression of *Hamp* during hypoxia may involve HIF-2α. Our results are in accordance with this, as we observed a markedly increased kidney expression of HIF-2α in renal medulla of CRF rats (Figure III-7). It is known that the expression of *Hamp* is modulated through several hepatocyte cell-surface proteins including Hfe, TfR2, HJV, TMPRSS6 and IL-6. Regardless

of the underlying molecular mechanism of reduction of *Hamp* expression during hypoxia, a decrease in hepcidin leads to an increased iron uptake and absorption at the duodenum, as well as to an increased iron release from the macrophages, favoring Hb synthesis and erythropoiesis.[14] Actually, we found a significant downregulation in liver mRNA expression of *Hamp*, *sTfR1*, *TF, Hfe, HJV* and *BMP6* in CRF rats, as compared with the Sham rats (Figure III-4), together with a significant duodenal overexpression of *SLC40A1* gene in CRF rats and similar values for the expression of *DMT1* gene (Figure III-4), suggesting that iron absorption in the enterocytes was normal or even enhanced, as the increase in duodenal ferroportin expression may occur to counteract the low iron levels observed in CRF rats; in addition, hypoxia might contribute to the significantly decreased liver *Hamp* mRNA expression in CRF rats. Likewise, the absence of inflammation in CRF rats, as showed by normal values of serum and liver IL-6 expression, as well as of reduced serum hs-CRP, are in agreement with lower liver *Hamp* mRNA expression in these animals.

This model of CKD has been associated to glomerulosclerosis and progressive tubulointerstitial damage. Although the mechanisms of tubular injury are poorly clarified in this and in other models of renal disease, proteinuria has a crucial role.[58] It has been proposed that filtered iron may have also a role in tubular injury, when associated with proteinuria. Actually, in proteinuric states, as a result of the glomerular leak of transferrin, iron might be released from transferrin in the acid milieu of the tubular lumen.[58] In fact, iron accumulation is observed in the proximal tubule in human CKD,[59] as well as in rat models with nephropathy[58,60,61] and seems to be associated with the progression of CKD. The glomerular leakage of iron may explain the reduced serum iron and transferrin observed in CRF rats. Indeed, considering the anemia in the absence of inflammation, a rise in serum iron would be expected to face the needs for erythropoiesis. Naito et al. (2012)[62] studied the effect of dietary iron restriction on the renal damage developed in a rat model of CKD, presenting nephron hyperfiltration, glomerulosclerosis and tubulointerstitial injury, and found that iron restriction attenuated these changes in CKD rats. This beneficial effect of iron restriction on renal damage is consistent with the results previously reported in the different models of renal disease.[63,64]

It is widely accepted that, regardless of the initial cause of renal failure, tubulointerstitial fibrosis is the major cause of disease progression in CKD.[65,66] Typically, the functional impairment in CKD correlates with tubulointerstitial fibrosis, and with glomerulosclerosis. Tubulointerstitial damage is closely correlated with reduced creatinine clearance and is currently the best predictor of disease progression.[66] Hypoxia and altered O_2 perfusion are also potential players in the development of renal injury.[66] As referred, in response to low oxygen supply, HIFs are produced triggering the expression of the hypoxia response genes, increasing the production of EPO, VEGF and of glycolytic enzymes. It is unclear if the increase in HIF has a renoprotective role, or if it contributes to interstitial fibrosis and/or tubular atrophy. This duality of effects has also been described for VEGF, another target gene of HIFs.[67] We found significantly high serum VEGF levels (almost three fold the control value) (Figure III-8), probably explaining the repression in VEGF gene expression in the remnant kidney of CRF. Under hypoxic conditions in renal injury, the HIF system is activated, even before any histological evidence of tubulointerstitial damage,[68,69] and the degree of HIF expression seems to correlate with the extent of tubular injury. However, whether this increased activity is beneficial or harmful is unclear and may well depend on the context, the cell type affected and/or the duration of HIF expression. Another major target gene for HIF is the pro-fibrotic connective tissue growth factor (CTGF).[70] In our study, CRF rats presented several glomerular and tubulointerstitial lesions (Figure III-6). Mild glomerular lesions were observed in most of the CRF rats, presenting thickening of Bowman capsule, hyalinosis of vascular pole, glomerular atrophy and hypercellularity. All CRF rats presented at least one advanced glomerular lesion; mesangial expansion was the more frequent lesion (Table III-3). Regarding the mild tubulointerstitial lesions, most of the CRF rats presented tubular hyaline droplets, TBM irregularity, tubular dilatation and interstitial inflammatory infiltration. Concerning advanced tubulointerstitial lesions, hyaline cylinders and IFTA were the most relevant lesions observed in CRF rats (Table III-5). As referred, besides hypoxia, CRF rats showed local inflammation in the remnant kidney, as suggested by overexpression of *IL-6, IL-1*β and *TNF-*α genes, as well as NF-kB, a key mediator of inflammation. In addition, we found an overexpression of CTGF in the glomeruli and in interstitial cells (medulla), in agreement with the existence of tubulointerstitial lesions and fibrosis.

This model of CKD induced by 5/6 nephrectomy presented a sustained degree of renal dysfunction with mild and advanced glomerular and tubulointerstitial lesions. An anemia developed early after nephrectomy and persisted throughout the study. The remnant kidney was still able to produce EPO and the liver seems to increase EPO production. In spite of the increased EPO blood levels, the anemia persisted and was linked to low serum iron and transferrin levels, although iron storage was normal, and to absence of inflammation. The development of glomerular and tubular kidney damage may underlie iron leakage, explaining the reduction in iron. Actually, the reduced expression of hepcidin, probably induced by hypoxia, should favour iron absorption; indeed, iron absorption seems to be increased, as suggested by the increased expression of duodenal ferroportin. The persistence of anemia may result from the disturbance in iron metabolism and, eventually, by an altered activity/function of EPO that might result from hypoxic kidney cell damage and from the developed local inflammatory milieu.

These findings might contribute to elucidate the mechanisms behind anemia persistence in chronic kidney disease patients, even when erythropoietin levels are not reduced, and open new windows to identify putative therapeutic targets for this condition, as well as for rHuEPO resistance, which occurs in a worrying percentage of CKD patients.

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Chapter IV

Impact of rHuEPO dose on iron metabolism and kidney hypoxia, inflammation and fibrosis in the remnant kidney rat model associated anemia

Manuscript submitted:

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1. Abstract

Despite of the multifactorial nature of anemia of CKD, the most well-known cause is inadequate erythropoietin production. Recent studies from our laboratory using the remnant kidney rat model of CKD showed that anemia persisted in spite of normal EPO serum levels, suggesting that anemia may result from disturbances in iron metabolism and from an altered activity/function of EPO that might result from kidney cell damage and from the developed kidney local inflammatory milieu. Our aim was to elucidate the mechanisms explaining the persistence of anemia in this model induced by 5/6 nephrectomy, by using two rHuEPO doses (50 and 200 UI/kg/week) and focusing on iron metabolism, kidney hypoxia, inflammation and fibrosis.

CRF rats showed a persistent anemia, a significant increase in BUN and creatinine levels, kidney hypertrophy, heart hypertrophy, arterial hypertension and glomerular and tubulointersititial kidney lesions. The lowest dose of rHuEPO (50IU) was unable to correct anemia and CRF-induced iron reduction; kidney hypertrophy and angiogenesis were enhanced. The highest dose (200IU) reduced kidney hypertrophy and angiogenesis and corrected anemia; serum iron increased and an overexpression of *Tf, TfR2, BMP6, Hfe*, *HJV* and *Hamp* mRNA was found in the liver; in addition, while tubular kidney lesions were aggravated, there was a more unfavorable environment for a normal EPO synthesis as well as for iron availability, which might be due to improved glomerular function.

Our data suggest that the anemia developed in rats with long-term CRF is due to reduced erythropoietic activity, as the CRF rats were able to overcome the anemia when treated with rHuEPO. The local kidney inflammation and the type of kidney lesions seem to be crucial to the outcome of the anemia. A long-term treatment of CRF rats with rHuEPO leads to the development of rHuEPO antibodies and, therefore, to anemia, after about 6 weeks of treatment. In addition, the impact of this treatment depends on the rHuEPO doses, which should be enough to correct anemia, preventing kidney and heart hypertrophy, but not too high that will lead to worsening of glomerular and tubulointerstitial kidney lesions.

Keywords: Anemia; chronic renal failure; rHuEPO therapy; iron metabolism, kidney hypoxia, fibrosis, inflammation, kidney damage.

2. Introduction

Chronic kidney disease (CKD) is a debilitating disease affecting about 7% of people with more than 30 years and older, which translates to more than 70 million people in developed countries worldwide.[1] This number is likely to be much higher given the unknown prevalence in underdeveloped countries. The increased prevalence of diabetes, hypertension and obesity, as well as the aging of the population, will perpetuate the rise of CKD.[2,3] Several studies have documented that patients with CKD are at higher risk of cardiovascular disease than the general population, and show a higher rate of cardiovascular mortality.[4-6] In particular, end-stage renal disease (ESRD) patients have a 500-fold greater rate of cardiovascular mortality than age-matched controls with normal renal function.[7] Due to the asymptomatic nature of this disease, CKD is not frequently detected until its later progress, resulting in lost opportunities for prevention. Progress to kidney failure or other adverse outcomes could be prevented or delayed through early detection and treatment of CKD.[8,9]

Anemia, very common in patients with CKD, is already observed in early stages of CKD (stage 3) and its prevalence and severity increases as renal failure progresses to more advanced stages.[10] Despite the multifactorial nature of anemia, the most well-known cause is inadequate erythropoietin (EPO) production. Indeed, a complex chain of events associated to the disease, including chronic inflammation, blood loss, vitamin deficiencies, decreased iron absorption and utilization, might contribute also to the anemia of CKD. As renal failure progresses, the contribution of EPO deficiency to anemia increases.[10,11]

Recombinant human erythropoietin (rHuEPO) treatment has been used to correct anemia, particularly in end stage renal disease (ESRD) patients[11-13], reducing the requirements for transfusion, improving quality of life, reducing left ventricular hypertrophy and improving morbidity and mortality in these patients.[14-16] However, the target hemoglobin value has been subject of debate in recent years; in fact, randomized studies and meta-analyses showed that a total correction of the anemia (hemoglobin concentration > 12 g/dL) with ESA treatment is not associated with increased survival or with a significant improvement in the quality of life of the patients. Instead, it has been

associated with a potential increase in adverse cardiovascular effects, including increased risk of stroke, venous thromboembolism and mortality. [17-19]

In addition, there is a marked variability in the sensitivity to rHuEPO, with up to 10-fold variability in dose requirements to achieve correction of the anemia. Up to 5–10% of CKD patients show weak responses to ESA,[20] this ESA hyporesponsiveness is associated with higher morbidity and mortality in end-stage renal disease (ESRD) patients.[21,22] Although the mechanisms underlying this variability in ESA response are unclear,[23,24] ESA resistance has been associated with inflammation, oxidative stress and iron deficiency, as major causes, and blood loss, hyperparathyroidism, aluminium toxicity and vitamin B12 or folate deficiencies, as minor causes.[15,23,24] In addition, a serious adverse effect of the long-term administration of rHuEPO is pure red cell aplasia (PRCA), although rare. RHuEPO is weakly immunogenic, however, it may induce the production of immunoglobulin G antibodies against the recombinant molecules and the residual endogenous EPO.[25-30] This adverse effect is more common since the introduction of biosimilar products, which could allow more patients to have access to these high-cost therapies.

Despite a profusion of mechanistic and therapeutic insights, the treatment of anemia may be the most controversial aspect of CKD management. Recent evidences suggest that CKD anemia might be due to defective hypoxic signaling rather than an inability of the EPO-producing cells to synthesize EPO.[31] A disturbance in iron homeostasis is also a hallmark of the anemia of CKD, which usually presents as a functional iron deficient anemia, with low serum iron and transferrin alongside with normal or even high ferritin.[32] This functional iron deficient anemia has been explained by the underlying inflammatory process in CKD patients, presenting increased hepcidin levels.[33] Hepcidin controls enterocyte iron absorption and macrophage iron mobilization by linking to the iron exporter ferroportin present on the surface of those iron-releasing cells, triggering its degradation.[33] A recent study of our group[34], using the remnant kidney rat model of CKD induced by 5/6 nephrectomy, showed that along 9 weeks after nephrectomy the rats presented a sustained degree of renal dysfunction and an anemia developed 3 weeks after nephrectomy that persisted along the protocol, in spite of the high levels of serum EPO. This anemia was associated with low serum iron and transferrin levels, although the iron storage was normal, in the absence of systemic

inflammation; advanced kidney lesions were observed that might allow the leakage of iron-transferrin complexes and, therefore, the persistence of anemia may result from iron disturbances due to kidney lesions; another hypothesis is that an altered activity/function of EPO results from hypoxic kidney cell damage and/or from the local inflammatory milieu developed in the remnant kidney. It would be important to clarify the mechanisms underlying this hyporesponse to endogenous EPO, as it could give new insights into the mechanisms of development of resistance to rHuEPO therapy in CKD patients. Moreover, it would be also important to further clarify the impact of the treatment of anemia with high doses of rHuEPO that are usually used in non-responders patients.

The aim of our study was to elucidate the mechanisms explaining the persistence of the anemia in the remnant kidney rat model of CKD induced by 5/6 nephrectomy, by treating the rats with rHuEPO and focusing our studies on iron metabolism, kidney hypoxia, inflammation and fibrosis.

3. Material and methods

3.1. Animals and experimental protocol

Male Wistar rats (Charles River Lab., Inc., Barcelona, Spain) weighing ±300 g were maintained in an air conditioned room, subjected to 12 h dark/light cycles and given standard rat chow (IPM-R20, Letica, Barcelona, Spain) *ad libitum* and free access to tap water. Animal experiments were conducted according to the European Communities Council Directives on Animal Care and the National Authorities.

The rats were divided into four groups (7 rats each): Sham group – submitted to surgical process but without kidney mass reduction and rHuEPO treatment; chronic renal failure (CRF) group - induced by a two-stage (5/6) nephrectomy (about 2/3 of the left kidney was removed by left flank incision and, one week later, the right kidney was removed through identical procedure); CRF+50IU rHuEPO group - treated with rHuEPO (beta epoetin) - 50 IU/Kg/week s.c., Recormon® (Roche Pharmaceuticals – Roche Farmacêutica Química, Lda., Amadora, Portugal) after the third week of surgery; and

CRF+200 IU rHuEPO group - treated with rHuEPO (beta epoetin) - 200 IU/Kg/week s.c, after the third week of surgery (Figure IV-1). All animals completed the 12 weeks of protocol. Body weight (BW) was monitored throughout the study and blood pressure (BP) and heart rate (HR) measures were obtained using a tail-cuff sphygmomanometer LE 5001 (Letica, Barcelona, Spain).

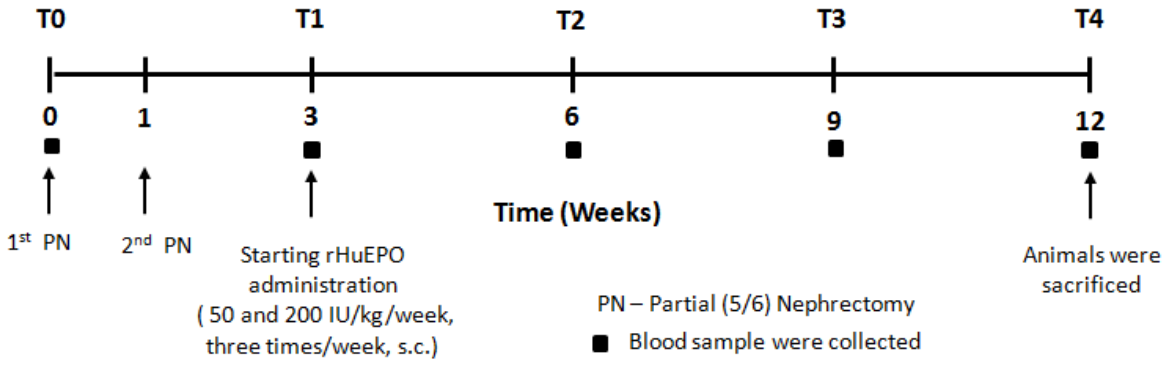

Figure IV-1. Experimental protocol.

3.2. Sample collection and preparation

At the beginning of the experiments (T0) and at 3 (T1), 6 (T2), 9 (T3) and 12 (T4) weeks after the surgical (5/6) nephrectomy, the rats were subjected to intraperitoneal anesthesia with a 2 mg/kg BW of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar®, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil®, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal). Blood samples were collected by venipuncuture, from the jugular vein, into vacutainer tubes without anticoagulant (to obtain serum) or with K_3EDTA for hematological and biochemical studies; only 3 mL of blood were collected at T0, T1, T2 and T3, to minimize interference with erythropoiesis mechanism and to monitor anemia and renal function; at the end of protocol (T4), 10 mL of blood were collected to perform all the biochemical and hematological assays.

At the end of the protocol, after collection of blood, the rats were sacrificed by cervical dislocation and the kidneys, heart, liver and duodenum were removed, placed in ice-cold Krebs-Henseleit buffer. The body weight (BW) and the weight of kidney (KW) or of the 1/6 remnant kidney; the heart (HW) and the liver (LW) were measured to calculate the trophism indexes (KW/BW, HW/BW and LW/BW).

3.3. Biochemical and hematological assays

The following serum markers were analyzed on a Hitachi 717 analyser (Roche Diagnostics Inc., Massachuasetts, USA) using standard methods: creatinine, blood urea nitrogen (BUN), as renal function markers; glycose, serum total cholesterol (Total-c), triglycerides (TGs), creatine kinase (CK), aspartate transaminase (AST), and alanine transaminase (ALT). Quantification of total bilirubin was performed by the colorimetric test of diazotized sulfanilic acid reaction (Roche Diagnostics Inc., Massachusetts, USA). Serum levels of interleukin-6 (IL-6), interferon-γ (IFN-γ), transforming growth factor beta 1 (TGF-β1) and vascular endothelial growth factor (VEGF) were all measured by rat-specific Quantikine ELISA kits from R&D Systems (Minneapolis, USA). High-sensitive C-reactive protein (hsCRP) was determined by using a rat-specific Elisa kit from Alpha Diagonostic International (San Antonio, USA). Serum levels of erythropoietin (EPO) were evaluated by rat specific ELISA kit (MyBioSource, USA). All assays were performed according to the manufacturers' recommendations. Red blood cell (RBC) count, hematocrit (Hct), hemoglobin concentration (Hb), mean corpuscular hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), mean corpuscular volume (MCV), RBC distribution width (RDW), platelet distribution width (PDW), platelets (PLT) and white blood cell (WBC) count were assessed in whole blood K_3EDTA (Coulter Counter®, Beckman Coulter, Inc., Fullerton, California, USA); reticulocyte (RET) count was performed by microscopic counting on blood smears after vital staining with New methylene blue (Reticulocyte stain, Sigma-Aldrich, St Louis, MO, USA).

Serum iron concentration was determined using a colorimetric method (Iron, Randox Laboratories Ltd., North Ireland, UK), whereas serum ferritin and transferrin were measured by immunoturbidimetry (Laboratories Ltd., North Ireland, UK).

3.4. Detection of anti-EPO antibodies

To detect anti-EPO antibodies we used an ELISA technique, according to Urra et al. (1997), using rHuEPO (Recormon®, Roche Pharmaceuticals) as antigen and, as secondary antibody, goat anti-rat IgG conjugated with horseradish peroxidase (Sigma; 100 ng/ml for 1h, at room temperature).[35] The substrate tetramethylbenzidine (TMB) (Sigma) was added and the reaction was stopped by the addition of sulphuric acid

1.25mol/l. The optical density at 450 nm (OD450) was determined with an automatic plate reader.

3.5. Gene expression analysis

In order to isolate total RNA, 0.2 g samples of liver, duodenum and kidney, from each rat, were immersed in RNA laterTM (Ambion, Austin, USA) upon collection and stored at 4°C for 24h; afterwards, samples were frozen at -80ºC. Subsequently, tissue samples weighing 50±10 mg were homogenized in a total volume of 1 mL TRI® Reagent (Sigma, Sintra, Portugal) using a homogenizer, and total RNA was isolated according to TRI® Reagent Kit recomendations. To ensure inactivation of contaminating RNAses, all material used was cleaned and immersed in RNAse-free water (0.2% diethyl pyrocarbonate) for 2h and finally heated at 120ºC for 1h. RNA integrity (RIN, RNA Integrity Number) was analyzed using 6000 Nano ChipW kit, in Agilent 2100 bioanalyzer (Agilent Technologies, Walbronn, Germany) and 2100 expert software, following manufacturer instructions. The yield from isolation was from 0.5 to 1.5 µg; RIN values were 7.0–9.0 and purity (A260/A280) was 1.8–2.0. The concentration of the RNA preparations were confirmed with NanoDrop1000 (ThermoScientific,Wilmington, DE, USA). Possible contaminating remnants of genomic DNA were eliminated by treating these preparations with deoxyribonuclease I (amplification grade) prior to RT-qPCR amplification. Reverse transcription and relative quantification of gene expression were performed as previously described.[36] Real-time qPCR reactions were performed using the following primer sequences for erythropoietin (*EPO)*, erythropoietin receptor *(EPOR)*, transferrin receptor 2 (*TfR2*), hepcidin (*Hamp*), ferroportin (*SLC40A1*), hemojuvelin (*HJV*), transferrin (*TF*), hemochromatosis (*Hfe*), divalent metal transporter 1 (*DMT1*), transferrin receptor 1 (*TfR1*), matriptase-2 (*TMPRSS6*), Interleukin-6 (*Il-6*) and bone morphogenic protein 6 (*BMP6*), transforming growth factor beta 1 (*TGF-β1)*; vascular endothelial growth factor *(VEGF),* interleukin-1 beta *(IL-1β)*, nuclear transcription factor kappa B (*NF-kB)*, connective tissue growth factor (*CTGF),* thrombospondin-1 *(TSP-1)*, pro (III) collagen *(Pro (III) collagen)*, cytocrome C *(CytC)*, hypoxia inducible factor 2 alpha *(HIF-2α)* and hypoxia inducible factor 2 beta *(HIF-2β)*,

which were normalized in relation to the expression of beta-actin (*Actb*), and 18S ribosomal subunit (*18S*). Primer sequences are listed on Table IV-1.

Table IV-1. List of primer sequences (F: forward; R: reverse).

Gene	Primer sequences
EPO	F: 5'-AGGGTCACGAAGCCATGAAG-3'
	R: 5'-GAT TTC GGC TGT TGC CAG TG-3'
EPOR	F: 5'-GCG ACT TGG ACC CTC TCA TC-3'
	R: 5'-AGT TAC CCT TGT GGG TGG TG-3'
Hamp	F: 5'-GAA GGC AAG ATG GCA CTA AGC-3'
	R: 5'-CAG AGC CGT AGT CTG TCT CG-3'
TfR2	F: 5'-CAA GCT TCG CCC AGA AGG TA-3'
	R: 5'-CGT GTA AGG GTC CCC AGT TC-3'
SLC40A1	F: 5'-CAG GCT TAG GGT CTA CTG CG-3'
	R: 5'-CCG AAA GAC CCC AAA GGA CA-3'
HJV	F: 5'-GCC TAC TTC CAA TCC TGC GT-3'
	R: 5'-GGT CAA GAA GAC TCG GGC AT-3'
TF	F: 5'-GGC ATC AGA CTC CAG CAT CA-3'
	R: 5'-GCA GGC CCA TAG GGA TGT T-3'
Hfe	F: 5'-CTG GAT CAG CCT CTC ACT GC-3'
	R: 5'-GTC ACC CAT GGT TCC TCC TG-3'
DMT1	F: 5'-CAA CTC TAC CCT GGC TGT GG-3'
	R: 5'-GTC ATG GTG GAG CTC TGT CC-3'
TfR1	F: 5'-GCT CGT GGA GAC TAC TTC CG-3'
	R: 5'-GCC CCA GAA GAT GTG TCG G-3'
TMPRSS6	F: 5'-CCG AAT ATG AGG TGG ACC CG-3'
	R: 5'-GGT TCA CGT AGC TGT AGC GG-3'
BMP6	F: 5'-GCT GCC AAC TAT TGT GAC GG-3'
	R: 5'-GGT TTG GGG ACG TAC TCG G-3'
Il-6	F: 5'- CGA GCC CAC CAG GAA CGA AAG TC-3'
	R: 5'- CTG GCT GGA AGT CTC TTG CGG AG-3'
HIF-2β	F: TGA AAG AAG GAG AAG CCC AAT A-3'
	R: CAT CAG AGT TAT GCC GAG ACA G-3'
18S	F: 5'-CCA CTA AAG GGC ATC CTG GG-3'
	R: 5'-CAT TGA GAG CAA TGC CAG CC-3'
Actb	F: 5'-GAG ATT ACT GCC CTG GCT CC-3'
	R: 5'-CGG ACT CAT CGT ACT CCT GC-3'
Il-1β	F: 5'-CTC TGT GAC TCG TGG GAT GAT G-3'
	R: 5'-CAC TTG TTG GCT TAT GTT CTG TCC-3'
CTGF	F: 5'-CGT AGA CGG TAA AGC AAT GG-3'
	R: 5'-AGT CAA AGA AGC AGC AAA CAC-3'
NF-kB	F: 5'-ACC TGA GTC TTC TGG ACC GCT G-3'
	R: 5'-CCA GCC TTC TCC CAA GAG TCG T-3'

Table IV-1 (Continuation). List of primer sequences (F: forward; R: reverse).

Gene	Primer sequences
VEGF	F: 5' -GAA GTT CAT GGA CGT CTA CCA G -3' R: 5' -CAT CTG CTA TGC TGC AGG AAG CT -3'
Pro (III) Collagen	F: 5'- CCA CCC TGA ACT CAA GAG TGG-3' R: 5' -CCA TCC AGA ACT GTG TAA GTG-3'
TSP-1	F: 5'- CCG GTT TGA TCA GAG TGG T-3' R: 5' GGT TTC GGA AGG TGC AAT-3'
Cyt C	F: 5'- CTT GTC ATA AAG TGG ATA TGA TC-3' R: 5' CAA TAG GTT TGA GGC GAC ACC CTC-3'
HIF-2α	F: 5'- TGA CTT CAC TCA TCC TTG CGA CCA-3' R: 5'- ATT CAT AGG CAG AGC GGC CAA GTA-3'

Results were analyzed with SDS 2.1 software (Applied Biosystems, Foster City, CA, USA) and relative quantification calculated using the $2^{\Delta\Delta Ct}$ method.[37] In liver tissue we studied the *EPO*, *EPOR*, *TfR1*, *TfR2*, *Hamp*, *Il-6*, *SLC40A1*, *HJV*, *TF*, *Hfe, BMP6* and *TMPRSS6* gene expression; in duodenum tissue the gene expression of *DMT1* and *SLC40A1* were studied, and in the kidney we evaluated the expression of *EPO, EPOR, IL-1β, TSP-1, Pro (III) collagen, CytC, NF-kB, CTGF, VEGF , HIF-2α* and *HIF-2β* genes.

3.6. Histopathological analysis

Tissue samples were fixed in Bock's fixative and embedded in paraffin wax; 4μm thick sections were stained for routine histopathological studies with hematoxylin and eosin (H&E). Periodic acid of Shiff (PAS) was used to evaluate and confirm the levels of mesangial expansion, thickening of basement membranes and sclerotic parameters. For PAS staining, the samples were fixed in neutral formalin 10%, embedded in paraffin wax, and 4μm thick sections were immersed in water and subsequently treated with an aqueous solution of periodic acid (1%), then washed to remove any traces of the periodic acid, and finally treated with Schiff's reagent. All samples were examined by light microscopy using a Microscope Zeiss Mod. Axioplan 2. The degree of injury visible by light microscopy was scored in a double-blinded fashion by two pathologists. Lesions were evaluated on the total tissue on the slide. Glomerular and tubulointerstitial kidney lesions were classified as mild and advanced. Mild glomerular damage was assessed by evaluating thickening of Bowman capsule, hyalinosis of the vascular pole, glomerular atrophy, hypercellularity and dilatation of Bowman´s space. Advanced glomerular

damage was assessed by grading sequentially four main lesions, from the less to the worse one: 1 – thickening of glomerular basement membrane (GBM), 2 – mesangial expansion, 3 – nodular sclerosis and 4 – global glomerulosclerosis. When advanced lesions were presented at a given glomeruli, the analysis of mild lesions become unavailable. Mild tubulointerstitial lesions included tubular hyaline droplets, tubular basement membrane (TBM) irregularity, tubular dilatation, interstitial inflammatory infiltration and vacuolar tubular degeneration. Advanced tubulointerstitial lesions were assessed by the presence of hyaline cylinders, tubular calcification, necrosis and the association of interstitial fibrosis and tubular atrophy (IFTA). The evaluation of vascular lesions was focused on arteriolar hyalinosis, arteriolosclerosis and arteriosclerosis. A semiquantitative rating for each slide, ranging from normal (or minimal) to severe (extensive damage), was assigned to each component. Severity of lesions was graded according to the extension occupied by the lesion (% area): 0 – absent/normal: 0 %; 1 – <25%; 2 – 25–50%; 3 – >50%. The final score of each sample was obtained by the average of scores observed in individual glomeruli in the observed microscopic fields. Tubulointerstitial damage was evaluated and graded by the same semiquantitative method. When using PAS, the rating was set for intensity and extension of staining, ranging from 0 (no staining) to 3 (intense and extensive staining), referring tissue specificity scoring when adequate. We performed Perl's staining of kidney slides to search for iron accumulation within rat kidney tubules.

3.7. Immunohistochemistry analysis

Liver and renal cortex/medulla paraffin sections (4 µm) were dewaxed in xylene, rehydrated in a series of ethanol washes, and placed in distilled water before staining procedures. The samples were processed by indirect immune detection technique with mousse and rabbit specific horseradish peroxidase (HRP)/ diaminobenzidine (DAB) detection IHC kit (ab80436, Abcam Inc, Cambridge, UK), according to the manufactory's protocol. Negative controls were included in each staining series, by omission of the primary antibodies. Antigen retrieval was performed for 20 minutes for paraffin-embedded tissue in the preheated citrate buffer (10mM citric acid, pH 6.0) using a pressure cooker. Between incubations, specimens were washed two to four times in PBS

(pH 7.4). All incubations were performed in a humidified chamber. In this study, we employed primary antibodies from Abcam Inc. (Cambridge, UK) for detection of hepcidin (dilution 1:150; ab81010) and CTGF (dilution 1:250, ab6992) and from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) from detection of NF-κB p50 (dilution 1:500, sc-114), HIF-2α (dilution 1:250, sc-28706), and HIF-2β (dilution 1:100, sc-5581). For immunohistochemical quantification, five 400x microscopic views of liver and renal cortex and medulla per slide were selected randomly and photographed using a Leica DFC480 microscope (Leica Microsystems, Wetzlar, Germany). The area of positive staining was detected by brown staining in the liver and renal cortex, and measured. The mean average staining was defined as the percentage of positive area in the total area of livers and renal cortex and medulla. Then the percentage of weakly (1), moderately (2), and strongly (3) staining cells was determined. A staining score (Quick Score) was then calculated according to previously described,[38] using the formula: Quick Score = intensity (1, 2 or 3) multiplied by area (percentage). The final score (out of maximum of 300) for each group was obtained averaging the individual scores of each animal.

3.8. Data analysis

For statistical analysis, we used the IBM SPSS Statistics 20 (2011). Significance level was accepted at *p* less than 0.05. Results are presented as means ± standard error of means (SEM). Comparisons between groups were performed using ANOVA and the *Post hoc* Tukey test.

4. Results

4.1. Body and tissue weights and blood pressure

The CRF rats presented a reduced ($p<0.05$) BW when compared with the Sham group. In addition, significantly higher ($p<0.01$) values of KW/BW and HW/BW ratios were found *versus* those of the Sham animals, although, KW and HW were similar. Despite the lowest LW in the CRF group, LW/BW showed a trend to increased values when compared with the Sham group (Table IV-2). The CRF rats under 50IU rHuEPO treatment presented similar body and tissue weights when compared with the CRF rats without rHuEPO therapy, excepting for the significantly ($p<0.05$) increased KW. The CRF

rats treated with 200IU rHuEPO exhibited values similar to those found for CRF animals, excepting a significantly lower (p<0.05) KW/BW.

Table IV-2. Effects of rHuEPO treatment on body and tissue weights and blood pressure at the end of protocol.

Parameters/Groups	Sham (n=7)	CRF (n=7)	CRF+50IU rHuEPO (n=7)	CRF+200IU rHuEPO(n=7)
Body and tissues weights				
BW (Kg)	0.45±0.02	0.36±0.01 a	0.37±0.01^{aa}	0.39±0.01
KW (g)	1.22±0.03	1.65±0.04	2.14±0.27 $^{aaa\ b}$	1.69±0.08 ^{a}
KW/BW (g/Kg)	2.72±0.05	4.61±0.22 aa	5.83±0.74 ^{aaa}	4.34±0.20 $^{aa\ b}$
HW (g)	1.16±0.03	1.24 ± 0.07	1.39±0.07	1.25±0.04
HW/BW (g/Kg)	2.58±0.08	3.48±0.25 aa	3.73±0.19 ^{aaa}	3.21±0.14 ^{a}
LW (g/Kg)	13.33±0.48	11.32±0.34	13.45±0.91	13.44±0.42
LW/BW (g/Kg)	29.61±0.65	31.43±0.71	36.06±2.19a	34.41±1.14 ^{a}
Blood pressures				
SBP (mmHg)	117.7 ± 1.15	134.1 ± 4.6aa	176.1±0.9$^{aaa\ bbb}$	169.1±1.5$^{aaa\ bbb}$
DBP (mmHg)	110.1 ± 0.59	113.2 ± 2.08	124.,8±2.7 ^{a}	133.3±7.2$^{aaa\ bb}$
MBP (mmHg)	115.0 ± 0.97	117.5 ± 1.21	144.8±5.6$^{aaa\ bbb}$	143.9±4.8$^{aaa\ bbb}$
HR (beats/min)	357.7± 2.74	367.6 ± 5.19	383.7±7.2 ^{a}	376.4±3.2

Results are presented as mean ± SEM (7 rats per group): a- p < 0.05, aa- p < 0.01, and aaa- p < 0.001 vs Sham; b- p < 0.05, bb- p < 0.01, and bbb- p < 0.001 vs CRF; c- p < 0.05, cc- p < 0.01, and ccc- p < 0.001 vs CRF+50IU rHuEPO group. BW, body weight; DBP, diastolic blood pressure; HR, heart rate; HW, heart weight; KW, kidney weight; LW, liver weight; MBP, mean blood pressure; SBP, systolic blood pressure.

The CRF rats presented significantly increased (p<0.01) SBP, and similar DBP, MBP and HR, when compared with the normotensive animals of the Sham rats. The CRF rats treated with 50 IU rHuEPO showed a significant increase in SBP (p<0.001) and MBP (p<0.001) when compared with the CRF group. The CRF rats treated with 200 IU rHuEPO, presented significantly higher values of SBP (p<0.001), DBP (p<0.01) and MBP (p<0.001) when compared with CRF rats (Table IV-2). No significant changes were observed between CRF rats treated with 50 IU rHuEPO or with 200 IU rHuEPO.

4.2. Biochemical and hematological data

The hematological and biochemical data for the different groups are presented in Figure IV-2 and Table IV-3. The Sham rats showed normal sustained hematologic values throughout the entire study (Figure IV-2A – IV-2D, respectively).

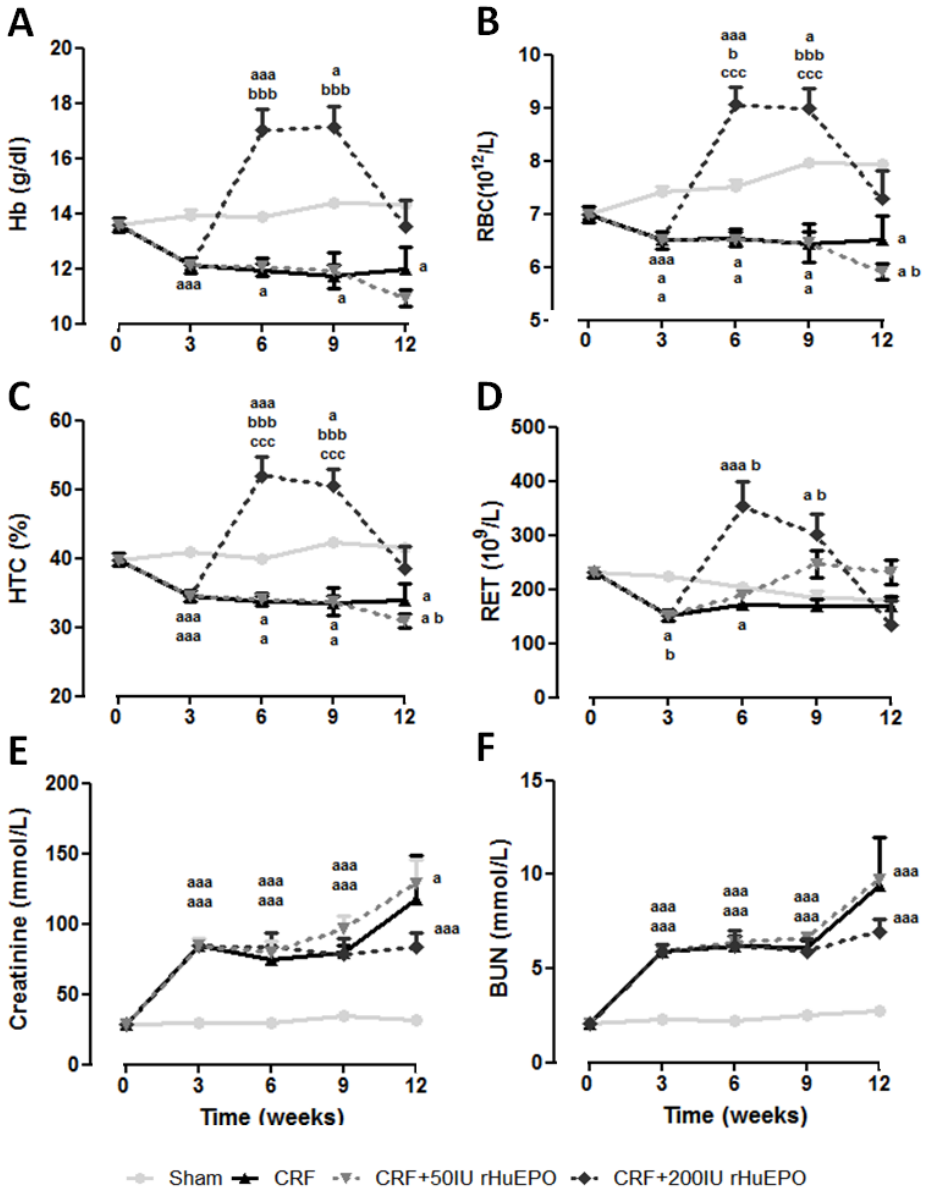

Figure IV-2. Hematological and renal data throughout the follow-up period of 12 weeks. Hemoglobin concentration (A), red blood cell count (B), hematocrit (C), reticulocyte count (D), creatinine (E) and BUN concentrations (F). Results are presented as mean ± SEM (7 rats per group): a- $p < 0.05$, aa- $p < 0.01$, and aaa- $p < 0.001$ vs Sham; b- $p < 0.05$, bb- $p < 0.01$, and bbb- $p < 0.001$ vs CRF; c- $p < 0.05$, cc- $p < 0.01$, and ccc- $p < 0.001$ vs CRF+50IU rHuEPO group. BUN, blood urea nitrogen.

Three weeks after the 5/6 nephrectomy, the CRF rats developed anemia, as showed by the reduced Hb concentration, RBC count and HTC (p<0.001 for all), when compared to Sham group; RET count presented also a decrease (p<0.05). The anemia persisted until the end of the protocol. In the CRF rats were treated with 50 IU of rHuEPO, the anemic profile remained until the 9th week, after which there was a further reduction (p <0.05) of RBC count and HTC, as compared to CRF rats. In opposition, 3 weeks after starting 200IU rHuEPO therapy in CRF rats (the 6th week of the protocol), the anemia was corrected and the rats presented significantly increased Hb concentration, HTC, RBC and RET counts, when compared to CRF rats. This erythropoietic response remained until the 9th week, after which the values returned to basal levels (Figure IV-2A – IV-2D).

Concerning the other hematological measures, similar values (WBC, MCV, MCH, PLT and PDW) were found between CRF and Sham groups, excepting an increased (p<0.01) MCHC in the CRF rats. No significant differences were obtained between CRF rats and CRF rats under rHuEPO treatment, excepting for a reduced (p<0.05) RDW in the rats treated with the highest rHuEPO dose, when compared with the other groups (Table IV-3). In CRF rats, significantly (p<0.001) increased serum creatinine and BUN concentrations were found three weeks after 5/6 nephrectomy, as compared to Sham (Figures IV-2E and IV-2F, respectively). These values remained high until the 9th week, after which a further increment was observed at the final time (p<0.001 and p<0.05, respectively), when compared with the Sham group. Similar values of serum creatinine and BUN were observed between the CRF rats treated with rHuEPO (both doses) and those without treatment, throughout the entire protocol. Regarding the other biochemical data, the CRF rats presented increased (p<0.05) total-c, ALT and VEGF, when compared with Sham rats (Table IV-3). All the analysed parameters were similar between untreated and rHuEPO treated CRF rats, excepting for a reduced AST in the group under 200IU rHuEPO treatment. The 200IU rHuEPO group showed significantly higher levels of VEGF, as compared to Sham, and significantly lower levels of VEGF when compared with 200IU rHuEPO group.

Serum samples from all animals were also analyzed for anti-rHuEPO antibodies. These antibodies were detected in 6 CRF rats under 50 IU rHuEPO treatment (85.7%), as well as in 7 CRF rats treated with 200 IU rHuEPO (100%).

Table IV-3. Effects of rHuEPO treatment on hematological and biochemical parameters at the end of protocol.

Parameters/Groups	Sham (n=7)	CRF (n=7)	CRF+50IU rHuEPO (n=7)	CRF+200IU rHuEPO (n=7)
Hematological data				
WBC (x 10^3/μL)	1.78±0.30	5.01±1.76	3.74±0.95	3.16±0.70
MCV (fL)	52.52±0.53	51.93±0.69	52.36±0.67	52.83±0.81
MCH(pg)	18.08±0.18	18.36±0.24	18.46±0.24	18.60±0.19
MCHC (g/dL)	34.60±0.08	35.37±0.19 [aa]	35.30±0.07a	35.26±0.30
RDW (%)	11.48±2.53	18.34±3.23	15.03±0.48	17.88±0.67[abc]
PLT (x 10^3/μL)	713.75±15.19	769.00±73.17	914.71±77.05	786.00±50.83
PDW (%)	16.34±0.18	16.44±0.20	16.00±0.11	16.44±0.24
Biochemical parameters				
Glicose (mmol/L)	9.46±0.31	8.66±0.60	7.50±0.69	8.54±0.21
TGs(mmol/L)	1.05±0.14	1.58±0.32	1.88±0.37	1.58±0.29
Total-c (mmol/L)	1.25±0.06	2.44±0.54 [a]	2.98±0.47	2.74± 0.25
CK (U/L)	540.57±58.94	473.00±85.57	237.14±74.26 [a]	294.86±35.86[ab]
ALT (U/L)	35.17±2.21	42.00±18.53a	21.86±2.09 [a]	26.14±1.18 [a]
AST (U/L)	80.57±7.84	139.43±70.70	57.57±6.23	54.57±3.02[ab]
Bilirubin (μmol/L)	8.04e-5±1.03e-5	1.03e-4±1.71e-5	8.55e-5±1.71e-5	1.20e-4±1.71e-5
IL-6 (pg/mL)	132.29±4.28	138.33±4.22	130.16±2.20	138.73±1.70
hsCRP (μg/mL)	262.25±12.43	225.31±7.95 [a]	219.98±9.85 [a]	244.23±7.99
INF-γ (pg/mL)	23.30±3.10	25.51±2.26	28.98±1.89	25.51±1.22
TGF-β1 (ng/mL)	75.74±5.62	84.13±3.85	87.82±8.03	72.37±4.55
VEGF (pg/mL)	4.23±0.94	14.16±2.24 [a]	19.08±1.95[a]	10.03±1.00[ac]

Results are presented as mean ± SEM (7 rats per group): a- $p < 0.05$, aa- $p < 0.01$, and aaa- $p < 0.001$ vs Sham; b- $p < 0.05$, bb- $p < 0.01$, and bbb- $p < 0.001$ vs CRF; c- $p < 0.05$, cc- $p < 0.01$, and ccc- $p < 0.001$ vs CRF+50IU rHuEPO group. ALT, alanine transaminase, AST, aspartate transaminase; CK, creatine kinase; hsCRP - high-sensitive C reactive protein; MCH, mean corpuscular hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean corpuscular volume; PDW, platelet distribution width ; PLT, platelets; RDW, RBC distribution width; Total-c, total cholesterol; TGs, triglycerides; WBC, white blood cells

4.3. Serum EPO, liver and kidney EPO and EPOR mRNA expression

The CRF rats and both groups of CRF rats treated with 50IU and 200IU rHuEPO showed significantly higher (p<0.001) serum EPO concentrations, when compared with Sham group (Figure IV-3A). A trend towards increased serum EPO concentration was observed with increasing rHuEPO dose. There was a notable (p<0.05) overexpression of *EPO* and *EPOR* mRNA in the kidney tissue of CRF rats, when compared with Sham rats. The *EPO* and *EPOR* mRNA expressions in CRF rats treated with rHuEPO (both doses) (Figure IV-3B and IV-3D, respectively) was similar, and significantly lower than their expression in CRF rats. In the liver tissue, a significant overexpression (p<0.001) of *EPO*

mRNA was found in CRF rats, when compared with Sham rats, which was not observed in rats under rHuEPO treatment (both doses) (Figure IV-3C). *EPOR* mRNA levels were similar for all groups, excepting for a significant (p<0.001) overexpression in CRF rats treated with 200IU rHuEPO (Figure IV-3E).

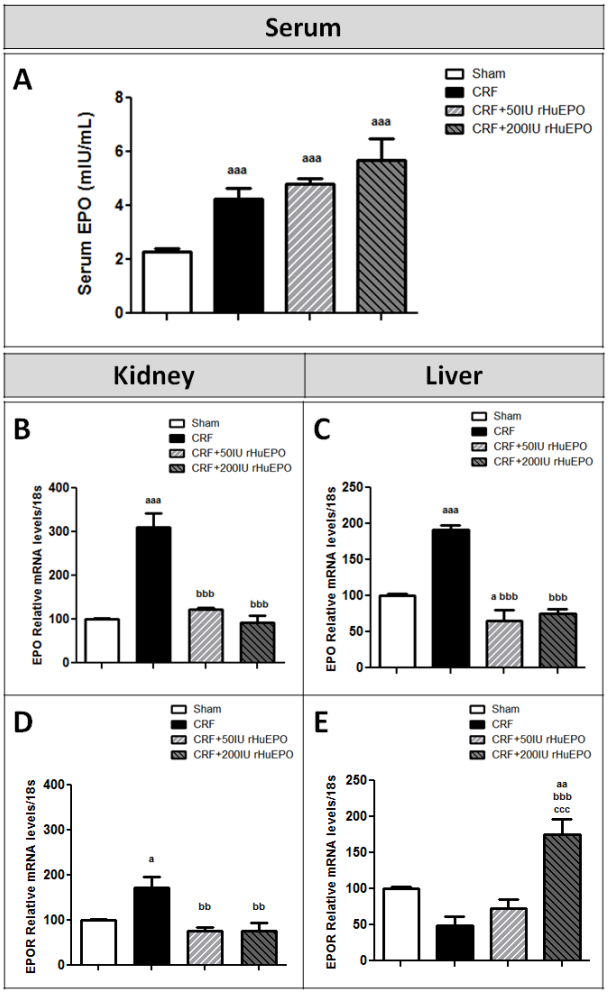

Figure IV-3. Serum EPO (A), *EPO* mRNA levels/18S expression (% of Sham group) in kidney (B) and liver (C) tissues at the end of the study (12 weeks); and *EPOR* mRNA levels/18S expression (% of Sham group) in kidney (D) and liver (E) tissues. Results are presented as mean ± SEM (7 rats per group): a- $p < 0.05$, aa- $p < 0.01$, and aaa- $p < 0.001$ vs Sham; b- $p < 0.05$, bb- $p < 0.01$, and bbb- $p < 0.001$ vs CRF; c- $p < 0.05$, cc- $p < 0.01$, and ccc- $p < 0.001$ vs CRF+50IU rHuEPO group.

4.4. Iron metabolism

At the end of the experimental protocol, there was a significant (p<0.001) decrease in serum iron and transferrin levels, and similar ferritin contents in CRF and in 50IU rHuEPO treated CRF rats, when compared to those found in Sham animals (Figure

IV-4A). The 200IU rHuEPO CRF rats presented serum iron levels similar to those of the Sham rats; a trend towards a reduction in ferritin, *versus* CRF rats, was also found (Figure IV-4A). In the duodenum, no significant differences were observed for *SLC40A1* and *DMT1* mRNA expression in CRF rats and in the CRF+50IU rHuEPO group, when compared with Sham. However, in the CRF+200IU rHuEPO rats, there was a significant (p<0.001) overexpression of *DMT1* and a trend to increased expression of *SLC40A1*, *versus* the CRF rats (Figure IV-4B).

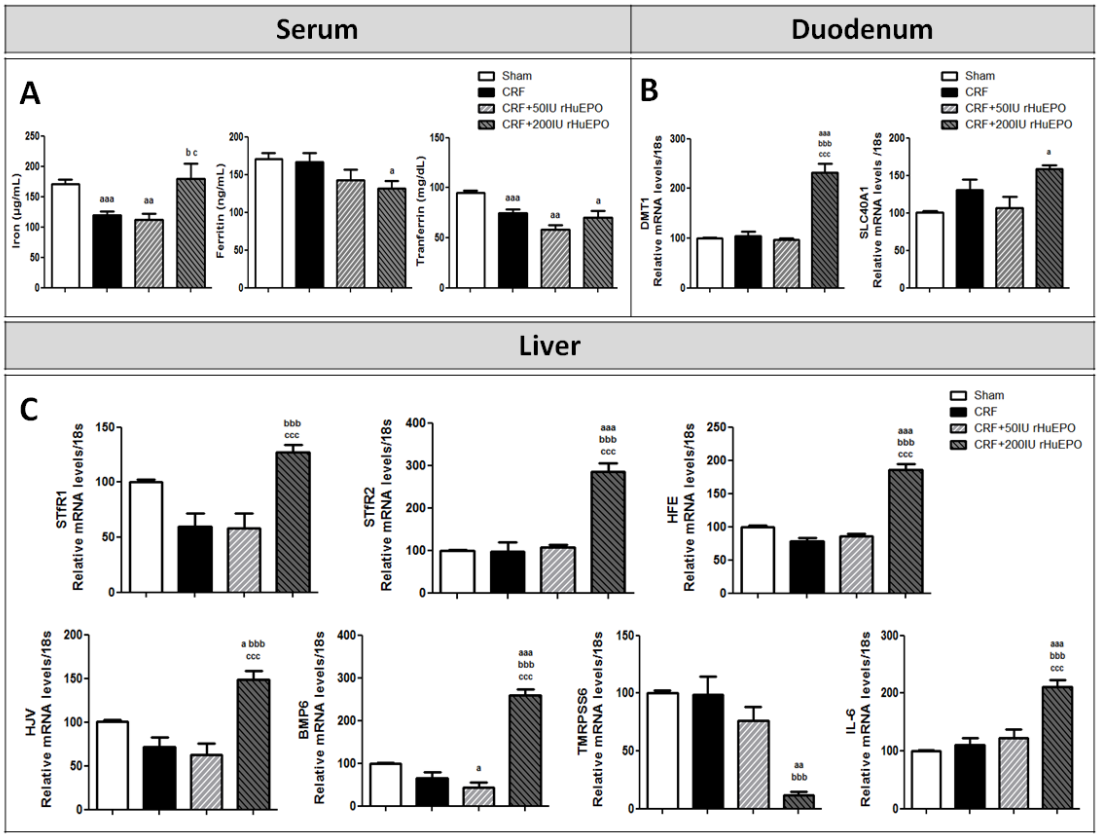

Figure IV-4. Mediators of iron metabolism in serum, liver and duodenum. Serum iron, ferritin and transferrin (A); relative gene expression mRNA levels /18S in the duodenum (B) and liver (C) at the end of protocol. Results are presented as mean ± SEM (7 rats per group): a- $p < 0.05$, aa- $p < 0.01$, and aaa- $p < 0.001$ vs Sham; b- $p < 0.05$, bb- $p < 0.01$, and bbb- $p < 0.001$ vs CRF; c- $p < 0.05$, cc- $p < 0.01$, and ccc- $p < 0.001$ vs CRF+50IU rHuEPO group. BMP6, bone morphogenic protein 6; DMT1, divalent metal transporter 1; Hamp, hepcidin antimicrobial peptide; HFE, hemochromatosis; HJV, hemojuvelin; IL-6, interleukin-6; SLC40A1, ferroportin-1; sTfR2, soluble transferrin receptor 2; Tf, transferrin; TfR1, transferrin receptor 1 and TMRPSS6, matriptase-2.

In the liver tissue, no significant changes were found for mRNA expression of several iron regulatory proteins (*sTfR1*, *sTfR2, Hfe, HJV, BMP6, TMPRSS6*, *IL-6* and *HIF-2*α) in the CRF group *versus* the Sham (Figures IV-4C and IV-5B$_1$), and the same occurred when comparing CRF rats with CRF+50 IU rHuEPO group. However, there was a

significantly reduced (p<0.05) expression of *Hamp* mRNA in CRF rats and in CRF+50 IU rHuEPO rats, when compared with Sham animals (Figure $3D_1$).The CRF rats under 200 IU rHuEPO, as compared to CRF, showed significant changes in the liver expression of most of those mediators of iron metabolism, namely, a significant (p<0.001) overexpression of *sTfR1*, *sTfR2, Hfe, HJV, BMP6 and IL-6*, and a significantly reduced expression of *TMPRSS6*; *Hamp* and *HIF-2*α presented similar values *versus* CRF rats (Figures IV-4B, IV-$5A_1$ and IV-$5B_1$). In addition, no significant differences were observed between groups for hepcidin and HIF-2α protein immunostaining in liver tissue (Figures IV-$5A_2$ – IV-$5A_6$; IV-$5B_2$ – IV-$5B_6$).

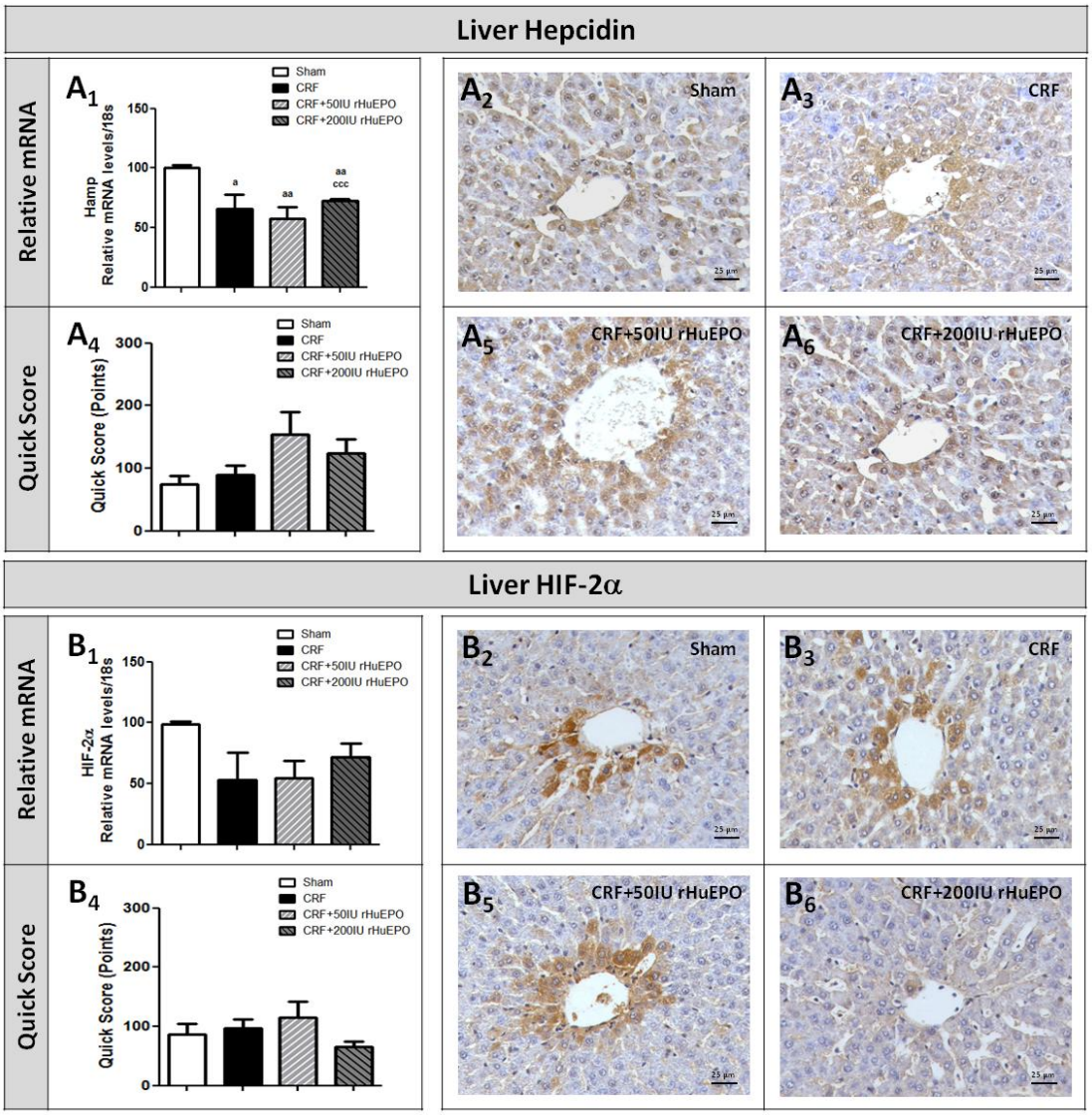

Figure IV-5. Liver protein expression (by immunohistochemistry) of hepcidin (A_1-A_6) and HIF-2α (B_1-B_6). Results are presented as mean ± SEM (7 rats per group): a- p < 0.05, aa- p < 0.01, and aaa- p < 0.001 vs Sham; b- p < 0.05, bb- p < 0.01, and bbb- p < 0.001 vs CRF; c- p < 0.05, cc- p < 0.01, and ccc- p < 0.001 vs CRF+50IU rHuEPO group. HIF-2α, hypoxia inducible factor 2 alpha.

4.5. Kidney lesions

No significant changes were found in kidneys histomorphology of Sham rats after the experimental period (Tables IV-4, IV-5 and IV-6; Figures IV-6A_1 and IV-6B_1).

Table IV-4. Scoring and distribution (%) of mild and advanced glomerular kidneys lesions of rat groups under study.

Mild Lesions	Rat Group	Score (n, %)				Total Score
		0 Absent	1 <25%	2 25-50%	3 >50%	
Thickening of Bowman´s Capsule	Sham	7 (100%)	0	0	0	0.00±0.00
	CRF	1 (14.3%)	3 (42.9 %)	1 (14.3%)	2 (28.6 %)	1.57±0.43[a]
	CRF+50IU rHuEPO	3 (42,9 %)	4 (57,1%)	0	0	2.14±0.40[aaa]
	CRF+200IU rHuEPO	3 (42,9 %)	3 (42,9 %)	1 (14,3%)	0	0.71 ±0.28
Hyalinosis of the vascular pole	Sham	7 (100%)	0	0 (0 %)	0 (0 %)	0.00±0.00
	CRF	1 (14.3%)	6 (85.7%)	0	0	0.86±0.14
	CRF+50IU rHuEPO	0	5 (71,4%)	0	2 (28,6%)	1.57±0.37[aa]
	CRF+200IU rHuEPO	5 (71,4%)	1 (14,3%)	0	1 (14,3%)	0.57±0.43
Glomerular atrophy	Sham	7 (100%)	0	0	0	0.00±0.00
	CRF	1 (14,3%)	6 (85.7%)	0	0	0.86±0.14[a]
	CRF+50IU rHuEPO	1 (14,3%)	4 (57,1%)	2(28,6%)	0	1.14±0.26[aaa]
	CRF+200IU rHuEPO	5 (71,4%)	2 (28,6%)	0	0	0.29±0.18
Hypercellularity	Sham	7 (100%)	0	0	0	0.00±0.00
	CRF	0	7 (100%)	0	0	1.00±0.00[aa]
	CRF+50IU rHuEPO	4 (57,1%)	1 (14,3%)	2 (28,6%)	0	0.71 ± 0.36[a]
	CRF+200IU rHuEPO	7 (100%)	0	0	0	0.00 ±0.00[bb]
Dilatation of the Bowman's Space	Sham	7 (100%)	0	0	0	0.00±0.00
	CRF	5 (71.4%)	2 (28.6%)	0	0	0.29±0.18
	CRF+50IU rHuEPO	6 (85,7%)	1(14,3%)	0	0	0.14 ± 0.14
	CRF+200IU rHuEPO	5 (71,4%)	2 (28,6%)	0	0	0.28 ± 0.18
TOTAL GROUP SCORE	**Sham**					**0.00±0.00**
	CRF					**0.91±0.12[aaa]**
	CRF+50IU rHuEPO					**1.14±0.18[aaa]**
	CRF+200IU rHuEPO					**0.37± 0.12[b]**

Rat Groups	Advanced glomerular lesions (n, %)					
	None of the previous lesions (0)	Thickening of GBM (1)	Mesangial expansion (2)	Nodular sclerosis (3)	Global Glomerulosclerosis (4)	TOTAL GROUP SCORE
Sham	7 (100%)	0	0	0	0	**0.00±0.00**
CRF	0	1 (14.3%)	5 (71.4%)	1 (14.3%)	0	**2.00±0.22[aaa]**
CRF+50IU rHuEPO	0	0	0	1 (14,3%)	6 (85,7%)	**3.85 ±0.14[aaa bbb]**
CRF+200IU rHuEPO	0	0	2 (28,6%)	0	5 (71,4%)	**3.43 ±0.37[aaa bbb]**

Results are presented as mean ± SEM (7 rats per group): a- $p < 0.05$, aa- $p < 0.01$, and aaa- $p < 0.001$ vs Sham; b- $p < 0.05$, bb- $p < 0.01$, and bbb- $p < 0.001$ vs CRF; c- $p < 0.05$, cc- $p < 0.01$, and ccc- $p < 0.001$ vs CRF+ 50IU rHuEPO group.

However, CRF rats presented several glomerular (cortex) and tubulointerstitial (medulla) lesions. Concerning mild glomerular lesions, most of the CRF rats presented thickening of Bowman's capsule, hyalinosis of vascular pole, glomerular atrophy and hypercellularity (Table IV-4; Figure IV-6). In addition, all CRF rats presented at least one of the advanced glomerular lesions, and mesangial expansion was present in 5 out of 7 rats (Table IV-4; Figure IV-6A_5).

Table IV-5. Scoring and distribution (%) of mild tubulointerstitial kidney lesions of rat groups under study.

Mild Lesions		Score (n, %)				
		0 Absent	1 <25%	2 25-50%	3 >50%	Total Score
Tubular Hyaline Droplets	Sham	7 (100%)	0	0	0	0.00±0.00
	CRF	0	7 (100%)	0	0	1.00±0.00 ^{aaa}
	CRF+50IU rHuEPO	6 (85,4%)	1 (14,3%)	0	0	0.14 ± 0.14
	CRF+200 IU rHuEPO	0	7 (100%)	0	0	1.00± 0.00 ^{aaa}
TBM Irregularity	Sham	7 (100%)	0	0	0	0.00±0.00
	CRF	0	3 (42.9%)	2 (28.5%)	2 (28.5%)	1.86±0.34 ^{aaa}
	CRF+50IU rHuEPO	0	3 (42,9%)	1 (14,3%)	3 (42,9%)	2.00±0.38 ^{aaa}
	CRF+200 IU rHuEPO	0	5 (71,4 %)	2 (28,5%)	0	1.29 ± 0.18 ^{a}
Tubular Dilatation	Sham	7 (100%)	0	0	0	0.00±0.00
	CRF	2 (28.5%)	1 (14.3%)	2 (28.5%)	2 (28.5%)	1.57±0.48 ^{a}
	CRF+50IU rHuEPO	5 (71,4%)	1 (14,3%)	1 (14,3%)	0	0.43 ± 0.30 ^{b}
	CRF+200 IU rHuEPO	7 (100%)	0	0	0	0.00 ± 0.00 ^{bb}
Interstitial Inflammatory Infiltrate	Sham	4 (57.2 %)	3 (42.9%)	0	0	0.29±0.18
	CRF	0	0	7 (100%)	0	2.00±0.00 ^{aaa}
	CRF+50IU rHuEPO	0	0	7 (100%)		2.00±0.00 ^{aaa}
	CRF+200 IU rHuEPO	0	0	7 (100%)		2.00±0.00 ^{aaa}
Vacuolar Tubular Degeneration	Sham	0	7 (100%)	0	0	1.00±0.00
	CRF	3 (42.9%)	3 (42.9%)	1 (14.3%)	0	0.71 ± 0.29 ^{aaa}
	CRF+50IU rHuEPO	3 (42,9%)	4 (57,1%)	0	0	0.57 ± 0.20 $^{aa\ bb}$
	CRF+200 IU rHuEPO	0	7 (100%)	0	0	1.00 ± 0.00 ^{bb}
TOTAL GROUP SCORE	**Sham**					**0.26±0.08**
	CRF					**1.43±0.15** ^{aaa}
	CRF+50IU rHuEPO					**1.03 ± 0.17** ^{aaa}
	CRF+200 IU rHuEPO					**1.06 ± 0.12** ^{aaa}

Results are presented as mean ± SEM (7 rats per group): a- $p < 0.05$, aa- $p < 0.01$, and aaa- $p < 0.001$ vs Sham; b- $p < 0.05$, bb- $p < 0.01$, and bbb- $p < 0.001$ vs CRF; c- $p < 0.05$, cc- $p < 0.01$, and ccc- $p < 0.001$ vs CRF+50IU rHuEPO group. TBM, tubular basement membrane.

The CRF animals treated with 50IU rHuEPO, when compared with the CRF rats, presented only a slight worsening (though without statistical significance) aggravation of mild glomerular lesions; however, a significant aggravation of advanced glomerular lesions was observed, due to an increase in global glomerulosclesosis that was present in 6 out of 7 rats (Table IV-4 and Figure IV-6A). The CRF+200IU rHuEPO rats, as compared to CRF ones, displayed an improvement in mild glomerular lesions; however, the advanced lesions were unchanged, with a predominance of global glomerulosclesosis (5 out of 7 rats). The changes reported are viewed by the total scores of mild and advanced glomerular lesions (Table IV-4 and Figure IV-6A).

Table IV-6. Scoring and distribution (%) of advanced tubulointerstitial kidney lesions of rat groups under study.

Advanced Lesions		Socre (n ,%)				
		0 Absent	1 <25%	2 25-50%	3 >50%	Total Score
Hyaline cylinders	Sham	6 (85.7%)	1 (14.3%)	0	0	0.14±0.14
	CRF	0	1 (14.3%)	6 (85.7%)	0	1.86±0.14^{aa}
	CRF+50IU rHuEPO	0	0	2 (28,6%)	5 (71,4%)	2.71 ± 0.18 $^{aaa\ bbb}$
	CRF+200 IU rHuEPO	0	0	4 (57,1%)	3 (42,9%)	2.43± 0.20^{aaa}
Tubular Calcification	Sham	7 (100%)	0	0	0	0.00±0.00
	CRF	7 (100%)	0	0	0	0.00±0.00
	CRF+50IU rHuEPO	6 (85,7%)	1 (14,3%)	0	0	0.14 ± 0.14
	CRF+200 IU rHuEPO	1 (14,3%)	6 (85,7%)	0	0	0.85 ± 0.14$^{aaa\ bbb}$
Necrosis	Sham	7 (100%)	0	0	0	0.00±0.00
	CRF	2 (28.6%)	5 (71.4%)	0	0	0.71±0.18
	CRF+50IU rHuEPO	3 (42,9%)	2 (28,5%)	2 (28,5%)	0	0.86 ± 0.34 ^{a}
	CRF+200 IU rHuEPO	0	0	4 (57,1%)	3 (42,9%)	2.43± 0.20$^{aaa\ bbb}$
IFTA	Sham	7 (100%)	0	0	0	0.00±0.00
	CRF	0	3 (42.9%)	4 (57.1%)	0	1.57±0.20^{aaa}
	CRF+50IU rHuEPO	0	0	2 (28,5%)	5 (71,4%)	2.71 ± 0.18$^{aaa\ bbb}$
	CRF+200 IU rHuEPO	0	0	0	7 (100%)	3.00 ± 0.00$^{aaa\ bbb}$
TOTAL GROUP SCORE	**Sham**					**0.04±0.04**
	CRF					**1.04±0.16**^{aaa}
	CRF+50IU rHuEPO					**1.61 ± 0.24**^{aaa}
	CRF+200 IU rHuEPO					**2.18 ± 0.17**$^{aaa\ bbb}$

Results are presented as mean ± SEM (7 rats per group): a- $p < 0.05$, aa- $p < 0.01$, and aaa- $p < 0.001$ vs Sham; b- $p < 0.05$, bb- $p < 0.01$, and bbb- $p < 0.001$ vs CRF; c- $p < 0.05$, cc- $p < 0.01$, and ccc- $p < 0.001$ vs CRF+50IU rHuEPO group. IFTA, interstitial fibrosis and tubular atrophy.

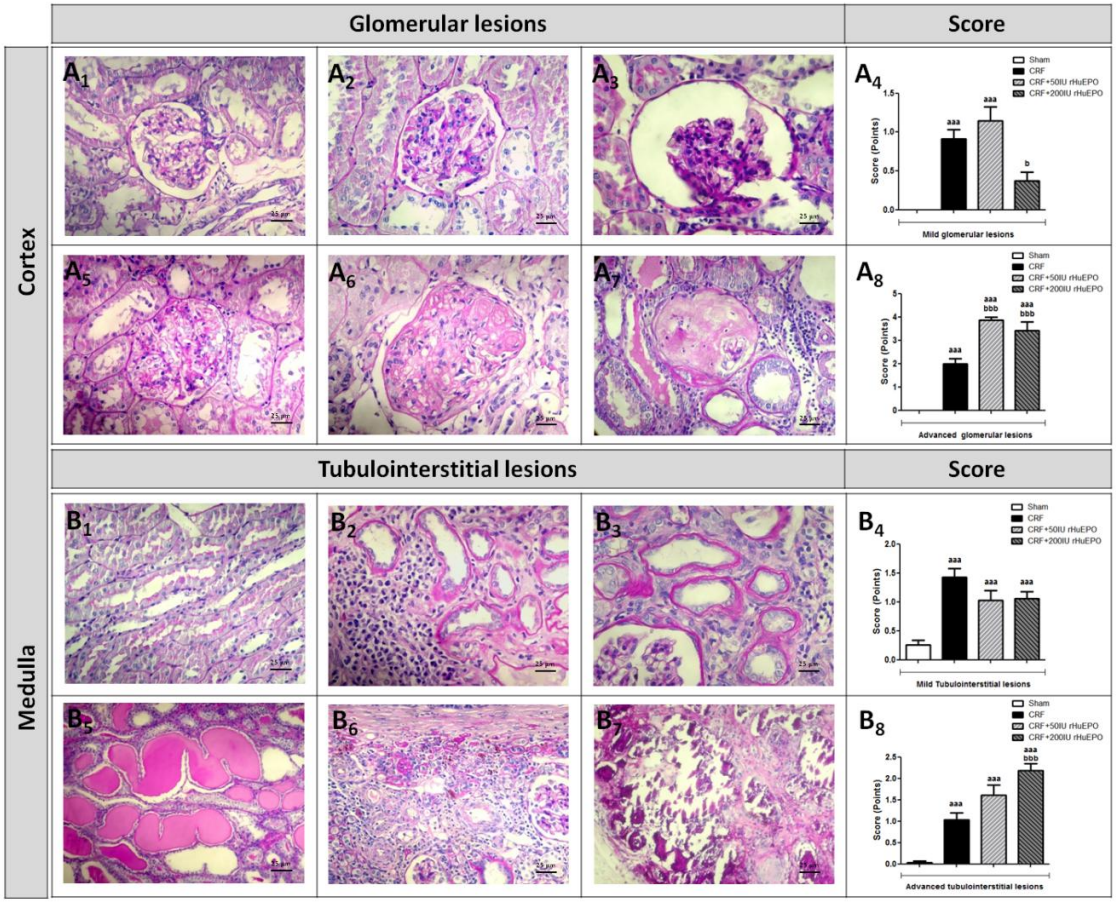

Figure IV-6. Glomerular and tubulointerstitial lesions. Representative glomerular (cortex) and tubulointerstitial (medulla) lesions observed in kidneys of rat groups under study, at the final time (PAS staining): A_1 – normal glomerulus histology in a Sham rat; A_2 – glomerular hypercellularity; A_3 - dilatation of the Bowman's Space and glomerular atrophy; A_4 – Total score of mild glomerular lesions for each rat group; A_5 - mesangial expansion; A_6- nodular sclerosis; A_7- global glomerulosclerosis; A_8- total score of advanced glomerular lesions for each rat group; B_1- normal tubulointerstitial histology in Sham group of rats; B_2- interstitial inflammatory infiltration; B_3 – tubular basements membrane irregularity; B_4- total score of mild tubulointerstitial lesions in lesions for each rat group. B_5-hyaline cylinders; B_6- interstitial fibrosis and tubular atrophy (IFTA); B_7- tubular calcification; B_8- Total score of advanced tubulointerstitial lesions for each rat group. Results are presented as mean ± SEM (7 rats per group): a- $p < 0.05$, aa- $p < 0.01$, and aaa- $p < 0.001$ vs Sham; b- $p < 0.05$, bb- $p < 0.01$, and bbb- $p < 0.001$ vs CRF; c- $p < 0.05$, cc- $p < 0.01$, and ccc- $p < 0.001$ vs CRF+50IU rHuEPO group.

Comparing CRF rHuEPO treated groups it seems there is a trend towards a protection of glomerular kidney tissue by increasing rHuEPO dose. Concerning the mild tubulointerstitial lesions, all animals of the CRF group presented tubular hyaline droplets, TBM irregularity, interstitial inflammatory infiltration, and most of them presented tubular dilatation, as compared to Sham rats (Table IV-5 and Figure IV-6B).

The two groups of CRF rats treated with rHuEPO, as compared to the CRF animals showed a trend towards a reduction in mild tubulointerstitial lesions; however, a trend to worsening of advanced lesions, namely the formation of hyaline cylinders and IFTA, was observed in CRF+50 IU rHuEPO rats. The CRF rats treated with 200rHuEPO presented a significant increase in advanced lesions, namely in hyaline cylinders, calcification, necrosis and IFTA (Table IV-6 and Figure IV-6B).

As the changes in serum iron presented by CRF rats and CRF rats treated with rHuEPO could be due to iron leakage due to kidney lesions, we performed iron staining according to Perls method. We found that iron deposits were almost undetectable in Sham rats, increased in CRF+200 IU rHuEPO rats and were even more enhanced in CRF rats and in CRF+50 IU rHuEPO rats (Figure IV-7).

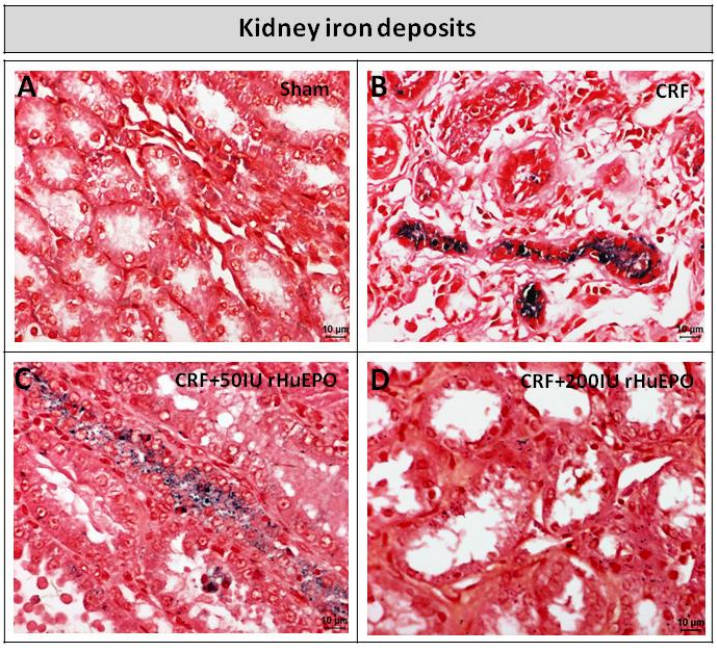

Figure IV-7. Illustration of iron deposits (Perls staining) in kidney tubules of rats, at the final time. Sham group (A), CRF group (B), CRF+50 IU rHuEPO group (C), and CRF+200 IU rHuEPO group (D).

4.6. Mediators of kidney lesions

No significant changes were observed in kidney mRNA expression of *IL-1β*, *TSP-1*, *Pro(III) collagen* and *CTGF* in CRF rats, when compared with Sham, however a down-regulation ($p<0.05$ and $p<0.01$, respectively) of *CytC* and *NF-kB* expression was observed (Figure IV-8).

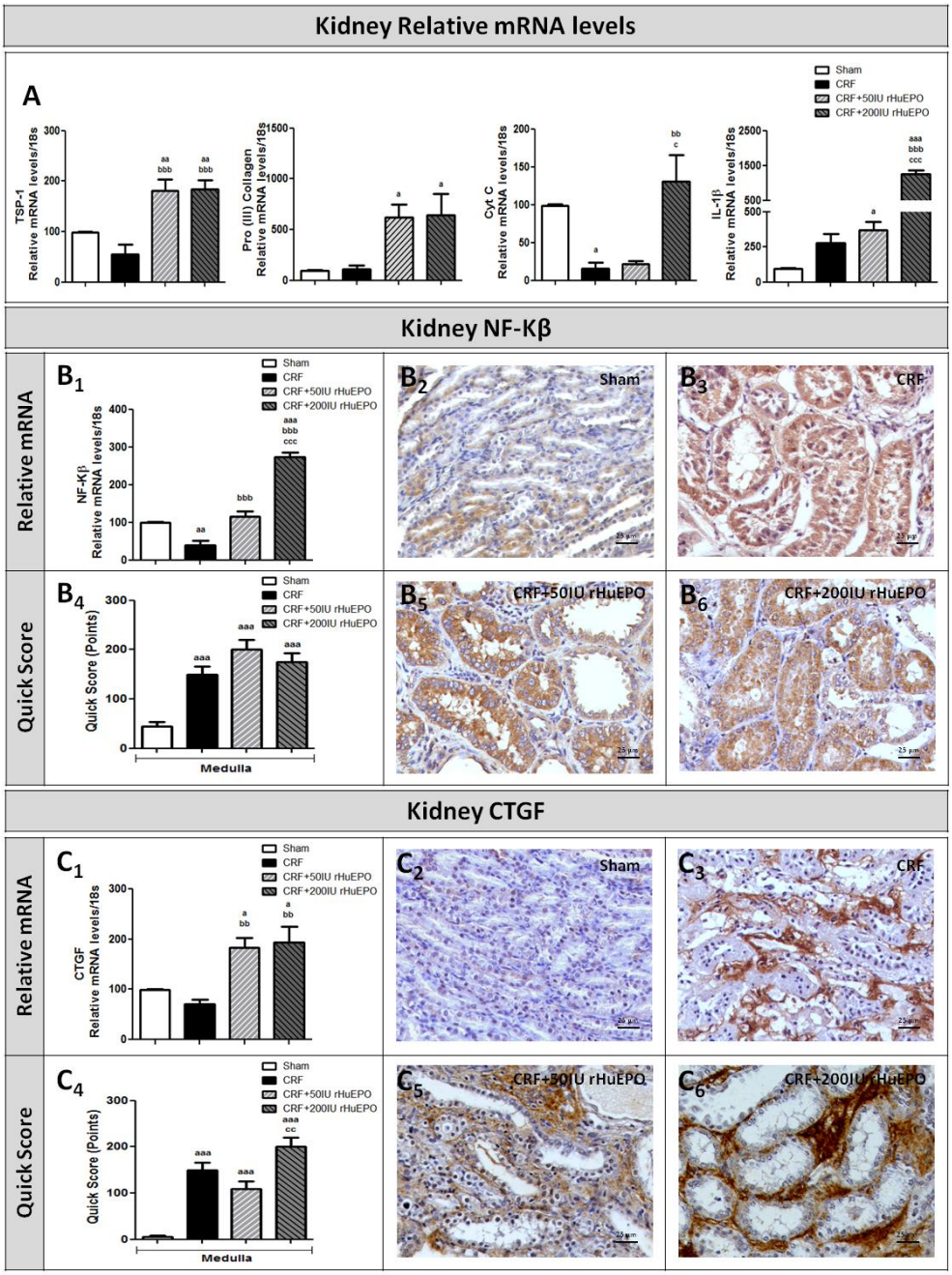

Figure IV-8. Mediators of kidney lesions. RHuEPO effects on relative gene expression mRNA levels/18S (% of Sham group) of some mediators of kidney lesions (A) and kidney protein expression (by immunohistochemistry) of NF-kB (B_1-B_6) and CTGF (C_1-C_6) in renal medulla. Results are presented as mean ± SEM (7 rats per group): a- $p < 0.05$, aa- $p < 0.01$, and aaa- $p < 0.001$ vs Sham; b- $p < 0.05$, bb- $p < 0.01$, and bbb- $p < 0.001$ vs CRF; c- $p < 0.05$, cc- $p < 0.01$, and ccc- $p < 0.001$ vs CRF+50IU rHuEPO group. CytC, cytocrome C; CTGF, connective tissue growth factor; IL-1β, interleukin-1 beta; IL-6, interleukin-6; NF-Kb, nuclear transcription factor kappa B; TNF-α, tumor necrosis factor alpha; TSP-1, thrombospondin-1.

In the CRF rats treated with 50IU of rHuEPO, revealed an overexpression of *TSP-1, NF-kB* and *CTGF* was found, without significant changes in the expression of *IL-1β*, *Pro(III) collagen*, and *CytC*, *versus* CRF rats. Major changes on kidney mRNA expression

were observed in the CRF+200 IU rHuEPO rats *versus* CRF animals; in fact, a significant overexpression of *IL-1β, TSP-1, CytC, NF-kB* and CTGF were encountered; no significant changes were observed for the expression of *Pro(III) collagen* (Figure IV-8). Kidney immunostaining for NF-kB and CTGF showed increased protein expression in CRF rats when compared with Sham animals, as well as in CRF rats treated with rHuEPO (both doses).

4.7. Kidney mRNA and protein expression of hypoxia inducible factors

No significant changes were observed in kidney mRNA *HIF-2α* expression in the CRF rats, when compared to Sham animals, although a trend towards an increased protein expression (immunostaining) was found.

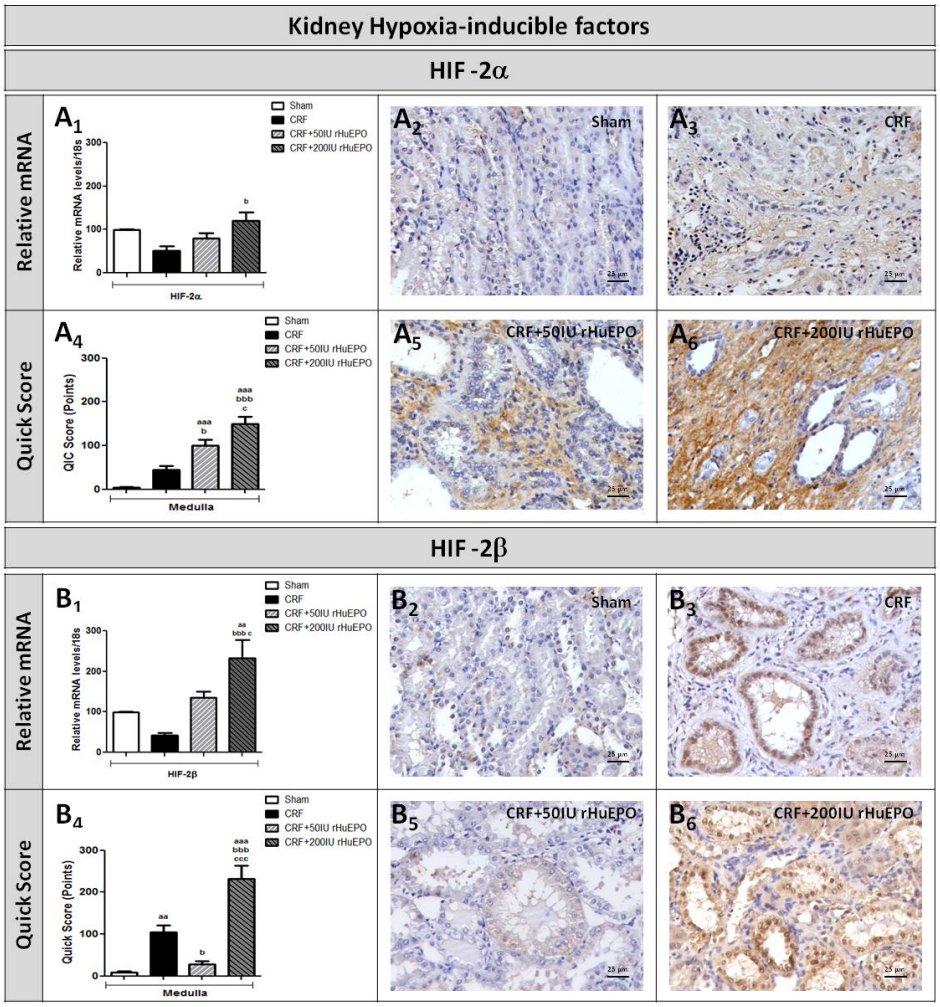

Figure IV-9. RHuEPO effects on relative gene expression mRNA levels/18s (% of Sham group) and kidney protein expression (by immunohistochemistry) of HIF-2α (A) and HIF-2β (B). Results are presented as mean ± SEM (7 rats per group): a- $p < 0.05$, aa- $p < 0.01$, and aaa- $p < 0.001$ vs Sham; b- $p < 0.05$, bb- $p < 0.01$, and bbb- $p < 0.001$ vs CRF; c- $p < 0.05$, cc- $p < 0.01$, and ccc- $p < 0.001$ vs CRF+50IU rHuEPO group. HIF-2α, hypoxia-inducible factor 2 alpha; HIF-2β, hypoxia-inducible factor 2 beta.

The CRF+50IU rHuEPO rats showed a trend to increased *HIF-2α* mRNA expression and significant protein immunoreactivity, when compared with CRF rats. The same was observed for the CRF+200IU rHuEPO rats, presenting an overexpression of both mRNA and protein in the kidney tissue. In addition, increased mRNA and protein HIF-2β expression was found in CRF+200IU rHuEPO rats, when compared with CRF rats (Figure IV-9).

5. Discussion

Anemia is prevalent in patients with CKD and contributes to a lower quality of life and numerous adverse outcomes, including cardiovascular disease events. Inadequate production of erythropoietin is commonly thought to be the most important factor in the pathogenesis of anemia in these patients. RHuEPO is, therefore, usually used in the treatment of CKD anemia, however, some patients are poorly responsive to ESAs, requiring higher doses,[39] being important to understand the impact of increased rHuEPO doses on key aspects of CKD associated-anemia, including erythropoiesis and iron metabolism, inflammation and kidney damage. Animal models have been important tools to study the cellular and molecular changes in CKD, and the remnant kidney model is one of the most used models.[40-42]

By using the remnant kidney model (5/6 nephrectomy) in the present study, we found that the rats developed a sustained renal failure (Figure IV-2), as showed by the significantly increased values of creatinine and BUN from 3 to 9 weeks after the second nephrectomy, worsening afterwards.

As referred, the anemia secondary to CKD development is one of the most severe complications of CKD patients.[43] In accordance with previously described in Chapter III, the CRF rats developed anemia 3 weeks after nephrectomy, which persisted throughout the protocol (Figure IV-2); however, the CRF rats were still able to produce EPO, almost doubling serum EPO levels, and, in accordance, the kidney and liver showed an increased *EPO* gene expression (Figure IV-3). The persistence of the anemia was associated with low serum iron and transferrin levels, although iron storage was normal, as showed by ferritin levels (Figure IV-4). This kind of disturbance in iron metabolism is

common in inflammatory conditions, however, serum IL-6 and CRP levels showed the absence of systemic inflammation (Table IV-3). Actually, we observed a reduced expression of hepcidin that favours iron absorption, as suggested by the increased mRNA expression of duodenal ferroportin (Figure IV-4); however, serum iron was reduced. This reduced might be due to iron loss through damaged kidney glomerulus (Tables IV-4, IV-5 and IV-6). It was reported that in proteinuric conditions, due to glomerular leakage of transferrin, iron might be released from transferrin in the acid milieu of the tubular lumen,[44] leading to iron accumulation in the proximal tubule [44-47] and worsening of CKD. Actually, we found that iron deposits were almost undetectable in Sham rats, increased in CRF+200 IU rHuEPO rats and were even more increased in CRF rats and in 50 IU rHuEPO rats (Figure IV-7), suggesting that the leakage of iron through damaged glomerulus may explain the reduced serum iron and transferrin that we observed in CRF rats. Another hypothesis for the persistence of the anemia in CRF rats is that an altered activity/function of EPO has occurred, resulting from kidney cell damage. It was reported that in healthy individuals erythropoietin may present up to four residues on each *N*-linked carbohydrate chain, or up to two residues on the *O*-linked chain. A variability in sialic acid composition may,[48] therefore, affect the circulating half-life of erythropoietin and the interactions with its receptor; in general, increasing sialic acid correlates with longer and greater potency of EPO.[49] Thus, the persistence of the anemia, in spite of the serum EPO levels, may be explained by iron disturbances and/or by a reduced (altered) EPO activity, and both changes may result from the glomerular kidney lesions and from the developed local inflammatory milieu, as showed by the increased gene expression of different protein mediators of inflammation and fibrosis in the remnant kidney (Figure IV-8). Extra-renal EPO production has been also described in the liver, although its contribution to the circulating EPO concentration in adults is highly debatable, even in kidney disease states.[50]

We treated CRF rats with two therapeutic doses of rHuEPO - 50 and 200 IU - to clarify the mechanisms explaining the persistence of anemia, in spite of the normal EPO bood levels.

The CRF rats treated with the lowest dose of rHuEPO (50 IU) presented anemia, though a slight rise in Hb and in reticulocyte count was observed, until the ninth week; afterwards Hb, HTC and RBC count showed a decrease. In opposition, the anemia of CRF

rats under treatment with the highest rHuEPO dose (200IU) was rapidly corrected and the Hb concentration reached values significantly higher, as compared to Sham and CRF rats, until the ninth week, after which the hematological values declined to values similar to those of the Sham group. This decrease, observed in both CRF groups treated with rHuEPO was due to the formation of EPO-neutralizing antibodies (Abs). This condition, although rare, has been also reported in humans,[51,52] and, more recently, has increased due to the introduction of EPO biosimilars to treat the anemia, in some countries.[29,30] At the final time, the anemia presented by both CRF treated groups was more severe in CRF rats treated with the lowest rHuEPO dose (50IU) (Figure IV-2); both groups (50 and 200 IU) presented similar levels of serum EPO, although slightly higher in the CRF+200 IU rHuEPO group (Figure IV-3). This slight increase in EPO serum levels might be explained by a compensatory production of EPO by the liver, given the significant overexpression of *EPOR* mRNA in liver tissue, and considering that *EPO* mRNA expression was similar for the remnant kidney of both CRF treated rats. While the liver has a role in EPO production in the fetal age, in the adulthood the main producer of EPO is the kidney.[53-55] However, in renal disease conditions the extra-renal tissues, such as the liver, might assume a higher part on the compensatory synthesis of EPO. In spite of the anemia, both CRF treated groups presented a similar down-regulation of kidney and liver *EPO* gene expression, suggesting a deregulation of the kidney hypoxia sensing mechanisms.

We must remind that the anemia developed in CRF rats was not due to deficient EPO production, but, instead, it seems to result from a reduced (altered) erythropoietic activity of endogenous EPO, once the treatment of CRF rats with rHuEPO was able to improve the anemia until the ninth week.

Renal insufficiency was accompanied by a compensatory renal hypertrophy and angiogenesis, as revealed by the increase in KW/BW and serum VEGF levels in CRF rats; a high HW/BW ratio was also found in CRF group, which might be caused by the supplementary effort of the left ventricle muscle to pump blood in this condition of anemia secondary to renal failure development, was also found in CRF group (Table IV-2). CKD patients, usually present a concomitant rise in systolic blood pressure,[56-61] which was also observed in CRF rats. The correction of anemia along 6 weeks in CRF treated with 200IU rHuEPO seems to explain, the improvement in KW/BW, serum VEGF levels,

HW/BW and in hypertension, as compared to CRF rats and CRF+50IU rHuEPO treated rats (Table IV-2). In fact, while the lowest dose (50 IU) was unable to correct the anemia (only a slight improvement) leading to kidney hypertrophy and angiogenesis, as compared to CRF rats, while the highest (200 IU) reduced kidney hypertrophy and angiogenesis, as it was able to correct anemia until the 9th week.

Worsening of hypertension associated to rHuEPO therapy have been assigned to several possible mechanisms, apart from the rHuEPO - hyperviscosity due to increasing RBC concentration, such as, several vascular mechanisms, including changes in the production or sensitivity to endogenous vasopressors, alterations in vascular smooth-muscle ionic milieu, deregulation of production or response to endogenous vasoactive factors, a direct vasopressor action of EPO, and, finally, arterial remodeling through stimulation of vascular cell growth.[60,62,63] Blood hyperviscosity and hypertension among other cardiovascular risk factors may contribute to the high morbidity and mortality in CKD patients.[31] In the present study, we found that the highest rHuEPO dose attenuated the rHuEPO associated increase in SBP, MBP and HR, when compared to the lowest rHuEPO dose, as occurred with KW/BW and HW/BW.

By evaluating the changes/damages occurring in the remnant kidney we found that the treatment with rHuEPO was associated with increased glomerular and tubulointerstitial lesions (Tables IV-4, IV-5 and IV-6, Figure IV-6), as compared to CRF rats; however, the glomerular lesions were significantly higher in CRF+50IU rHuEPO rats, as compared to CRF+200IU rHuEPO rats, while the tubulointerstitial lesions were significantly higher in the latter group. Moreover, CRF rats treated with 200IU rHuEPO also presented significantly higher values in protein and/or gene expression of several mediators/markers of kidney inflammation and fibrosis, namely, *Cyt C*, *IL-1β, NF-kB* and *CTGF* (Figure IV-8).

Under hypoxic conditions HIFs promote the transcription of regenerative factors, such as EPO, GLUT receptor, VEGF and CTGF, among others.[64-66] However, some of them (namely, VEGF and CTGF) might contribute to worsening of kidney disease by promoting inflammation and fibrosis.[66,67] It is becoming widely accepted that, regardless of the initial cause of renal failure, tubulointerstitial fibrosis is the major cause of disease progression,[68,69] although glomerulosclerosis is one of the major signs of ESRD. Tubulointerstitial damage is typically associated with accumulation of extracellular

matrix (ECM), infiltration of inflammatory cells, increased number of interstitial fibroblasts, tubular atrophy and finally loss of peritubular capillaries.[70] Given the close association between hypoxia, EPO, fibrosis and inflammation, it is of major importance to elucidate how rHuEPO therapy affects the evolution of kidney lesions. In fact, EPO interaction with its receptor leads to activation of several important pathways related with proliferation, angiogenesis, inflammation and oxidative stress, which might deeply affect influence the renal tissue.[71-73]

Lower impact of the treatment with the highest rHuEPO dose (200IU) on the glomerulus, as compared to the lowest dose, may explain the improvement in iron metabolism, with an increase in serum iron and transferrin and a slight decrease in ferritin levels, as well as an increase in duodenal ferroportin and *DMT1* mRNA expression suggesting that mild glomerular lesions were able to reduce the loss of iron and transferrin levels.

Hepcidin has been described as a major regulator of body iron homeostasis, acting directly on a number of cell types, including iron recycling macrophages, intestinal epithelial cells and hepatocytes. Hepcidin, synthesized by the liver, acts by inhibiting cellular iron efflux in hepatocytes, enterocytes and macrophages, through binding to ferroportin, inducing its degradation.[74] Hepatic *Hamp* expression is regulated by several proteins, namely, Hfe, TfR1, TfR2, HJV, BMP6, matriptase-2 and transferrin.[75-77] In the liver, diferric transferrin competes with TfR1 for binding to Hfe, and, when iron is increased, more Hfe is available to bind to TfR2; this complex, TfR2-Hfe, promotes HJV binding to BMP6, increasing hepcidin synthesis.[78,79] Moreover, hypoxia and EPO, as well as twisted gastrulation protein 1 (TWSG1) and growth differentiation factor 15 (GDF15),[80] both produced by erythroblasts, are also important modulators of hepcidin synthesis, by inducing a downregulation of hepcidin synthesis.[80]

We found that increased serum iron in the CRF+200 IU rHuEPO group, as compared to CRF+50IU rHuEPO group, was associated with an overexpression of *Tf, TfR2, BMP6, Hfe* and *HJV* in the liver, and, in accordance, with an overexpression of *Hamp* in the CRF+50IU rHuEPO group; moreover, a downregulation in *matriptase-2* mRNA expression was observed in the liver that might further contribute to the overexpression of hepcidin (Figure IV-5). However, this hepcidin overexpression in CRF+200IU rHuEPO group was not traduced in a higher protein expression, as showed by

the immunochemical studies. The same was observed for *HIF-2α,* which presented a slightly increased overexpression that was associated with a slight decrease in the protein expression. Thus, it seems that the less severe anemia/hypoxia, as well as the reduced glomerular kidney lesions, at the final time, in the CRF+200IU rHuEPO rats, as compared to CRF+50IU rHuEPO rats, might explain the decrease in hepcidin and HIF-2α protein expression; however, we must notice that the decrease in Hb concentration, almost thrice in the CRF+200IU rHuEPO group, as compared to CRF+50IU rHuEPO group, might result, apart from the action of the anti-rHuEPO antibodies, from the synthesis of EPO with a reduced (altered) erythropoietic activity, due to the high enhancement in the inflammatory and fibrotic kidney milieu, as shown by the increased gene and/or protein expression of *Cyt C, IL-1β, NF-kB* and *CTGF*. Actually, the kidney tubules, where EPO is synthesized, of the CRF+200IU rHuEPO rats presented significantly higher advanced kidney lesions, as compared with rats treated with the lowest dose of rHuEPO, showing a more unfavorable environment for a normal EPO synthesis.

In summary, our data suggest that the anemia developed in rats with long term of CRF is due to a reduced (altered) erythropoietic activity, as the CRF rats were able to overcome the anemia when treated with rHuEPO; the local kidney inflammation and the type of kidney lesions seem to be crucial for the outcome of the anemia. A long-term treatment of CRF rats with rHuEPO leads to the development of rHuEPO antibodies and, therefore, to anemia, after about 6 weeks of treatment; the impact of rHuEPO treatment depends on the rHuEPO dose, which should be enough to correct anemia, preventing kidney and heart hypertrophy, but not too high as it will lead to worsening of advanced glomerular and tubulointerstitial kidney lesions. Further studies are needed to understand how hypoxia and/or kidney lesions induce a reduced (altered) EPO activity and how kidney glomerular lesions/iron leakage may contribute to the functional iron deficient anemia, usually observed in hemodialysis CKD patients and enhanced in case they develop resistance to rHuEPO treatment.

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Chapter V

Iron as the key modulator of hepcidin expression in antibody-mediated erythroid hypoplasia

Manuscript submitted

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1. Abstract

Erythroid hypoplasia (EH) is a rare complication associated with recombinant human erythropoietin (rHuEPO) therapies, due to development of anti-rHuEPO antibodies. The use of EPO biosimilars has increased the number of cases; however, the underlying mechanism and its treatment remain poorly clarified. Our aim was to manage a rat model of antibody-mediated EH induced by rHuEPO, and study the impact on iron metabolism and erythropoiesis at blood and molecular levels, by evaluating the expression of mRNA-encoding erythropoiesis and iron metabolism regulatory proteins, on duodenum, liver and kidney tissues.

The model was developed by long-term treatment of Wistar male rats with a high rHuEPO dose (200IU epoietin beta/Kg bw/week during 9 weeks), as showed by the presence of anemia, reduced erythroblasts, reticulocytopenia and the formation of plasmatic anti-rHuEPO antibodies. Serum iron was increased and associated with significant overexpression of hepatic hepcidin, hemochromatosis protein, hemojuvelin, transferrin receptor 2 and bone morphogenic protein 6 mRNAs, and up-regulation of matriptase-2; overexpression of divalent metal transporter 1 and ferroportin was observed in duodenum and liver. Decreased *EPO* expression was observed in kidney and liver, while EPO receptor was overexpressed in liver. Endogenous EPO levels were normal, suggesting that anti-rHuEPO antibodies blunted EPO function.

In our model, anti-rHuEPO antibodies inhibited both rHuEPO and endogenous EPO leading to anemia, iron was increased, triggering hepatic hepcidin expression, despite no evidence of inflammation, and it is the key modulator of hepcidin synthesis. These findings might contribute to improve new therapeutic strategies against rHuEPO resistance and/or development of antibody-mediated EH in patients under rHuEPO therapy.

Key words: Erythroid hypoplasia; Recombinant human erythropoietin; Hepcidin; Anti-EPO antibodies; Iron

2. Introduction

Erythropoietin (EPO), a 30.4 kDa glycoprotein, is a key hormone in the regulation of erythropoiesis, supporting proliferation, survival and terminal differentiation of erythroid progenitor cells in the bone marrow.[1,2] These effects are mediated through the interaction of EPO with its specific transmembrane receptor – erythropoietin receptor (EPOR).[3] Acquired EPO deficiency has been associated with chronic kidney disease (CKD) and with chronic inflammatory diseases. Treatment with recombinant human erythropoietin (rHuEPO) achieves correction of these types of anemia; however, 5-10% of CKD patients develop resistance to the erythropoietic stimuli of rHuEPO. Several factors have been proposed to play a role in the development of this resistance, but the etiology of the impaired erythropoiesis, leading to worsening of anemia or even to pure red cell aplasia (PRCA) is still unknown.[4] Casadevall et al. (1996) [5] reported the presence of anti-EPO antibodies in a patient with transient PRCA, which functionally blocked the interaction between EPO and EPOR, thus resulting in impaired erythropoiesis.[5] Hara et al. (2008)[6] also reported that serum from a patient with PRCA inhibited EPO-dependent cell proliferation, suggesting that the anemia was mediated by anti-EPO antibodies.[6] In several other cases, antibodies have been described in PRCA patients' sera that were selectively cytotoxic for marrow erythroid cells or were directed against EPO.[7-9]

The current therapeutic use of rHuEPO to correct anemia in CKD patients has been associated to some cases of PRCA, due to the development of cross-reactive anti-EPO antibodies.[10,11] Although this complication is very rare, the mechanisms underlying the break in immune tolerance to rHuEPO remain poorly clarified. The development of PRCA should be suspected in patients who have been under rHuEPO therapy for more than 3 months and develop a sudden severe unexplained fall in hemoglobin levels, reticulocytopenia and a marked decrease in bone marrow erythroblasts, despite continuing with rHuEPO treatment.[12] The diagnosis is confirmed by low serum EPO levels and detection of anti-EPO antibodies.[13] The production of anti-EPO antibodies and the inhibition of EPO-dependent erythroid cells are important mechanisms that might lead to erythroid hypoplasia (EH)/PRCA. The reduction in functional EPO affects the proliferative state of bone marrow erythroid cells, which will trigger several changes in iron metabolism, including in serum iron, transferrin and ferritin levels, among others.[14-

[16] Considering the scarce data in literature about how, facing a PRCA condition mediated by anti-EPO antibodies, the depression of marrow erythroid activity affects iron metabolism, our goal was to assess in male Wistar rats, the effects of this condition upon iron metabolism (iron absorption, iron traffic and storage), as well as upon erythropoiesis. For that purpose, we performed hematological and biochemical studies to evaluate the erythropoietic and iron status, and measured the expression of several mRNA-encoding erythropoiesis and iron metabolism regulating proteins, on duodenum, liver and kidney tissues.

3. Material and methods

3.1. Animals and experimental protocol

Male Wistar rats (Charles River Lab. Inc., Barcelona, Spain), weighing 200-250g, were maintained in an air conditioned room, subjected to 12h dark/light cycles and given standard laboratory rat diet (IPM-R20, Letica, Barcelona, Spain) and free access to tap water. Animal experiments were conducted according to the European Communities Council Directives on Animal Care and the National Authorities.

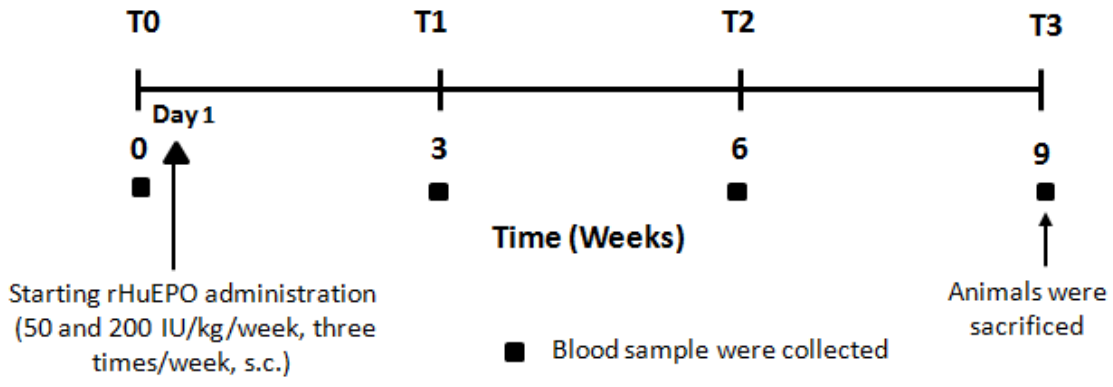

Figure V-1. Experimental protocol.

The rats were divided into 3 groups (8 rats each): control - treated with saline solution; 50IU rHuEPO - a therapeutic dose of 50 IU/Kg body weight (bw)/week of epoietin beta (Recormon® - Roche Pharmaceuticals); 200 IU rHuEPO – treated with 200 IU/Kg bw/week of rHuEPO, a high dose, usually used on rHuEPO resistant patients. All the animals have completed the 9-week protocol (Figure V-1).

3.2. Sample collection and preparation

At the beginning of the experiments (T0) and at 3 (T1), 6 (T2) and 9 (T3) weeks after the first rHuEpo dose, the rats were subjected to intraperitoneal anesthesia with a 2 mg/kg bw of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar®, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil®, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal). Blood samples were immediately collected by venipuncture, from the jugular vein, into vacutainer tubes without anticoagulant (to obtain serum) and with EDTA (to obtain whole blood and plasma) for hematological and biochemical studies; 3 mL blood samples were collected at T0, T1, T2, to minimize interference with erythropoiesis mechanism; at the end of protocol (T3) a 10 mL sample was collected in order to perform all the biochemical and hematological assays.

At the end of the protocol, blood was collected and, afterwards, the rats were sacrificed by cervical dislocation; duodenum, liver and kidneys were immediately removed, placed in ice-cold Krebs-Henseleit buffer and carefully cleaned. A bone marrow aspirate from the femur was also performed.

3.3. Biochemical and hematological assays

Serum creatinine and urea concentrations were used as renal function indexes through automatic validated methods and equipments (Hitachi 717 analyser, Roche Diagnostics Inc., Massachusetts, USA). Red blood cell (RBC), hematocrit and hemoglobin (Hb) concentration were assessed in whole blood EDTA by using an automatic Coulter Counter® (Beckman Coulter Inc., USA, CA). Serum levels of erythropoietin were evaluated by rat specific ELISA kit (MyBioSource, USA). Serum iron concentration was

determined using a colorimetric method (Iron, Randox Laboratories Ltd., North Ireland, UK), whereas serum ferritin and transferrin were measured by immunoturbidimetry (Laboratories Ltd., North Ireland, UK). Quantification of total bilirubin was performed by a colorimetric test (diazotized sulfanilic acid reaction, Roche Diagnostics). Circulating levels of glucose and uric acid were determined by routine automated technology (ABX Diagnostics). Serum levels of interleukin-6 (IL-6), interferon-γ (IFN-γ), transforming growth factor beta 1 (TGF-β1) and vascular endothelial growth factor (VEGF) were measured by rat-specific Quantikine ELISA kits from R&D Systems (Minneapolis, USA). High-sensitive C-reactive protein (hsCRP) was determined by using a rat-specific Elisa kit from Alpha Diagnostics International (San Antonio, USA).

3.4. Detection of anti-EPO antibodies

The detection of anti-EPO antibodies was carried out by ELISA, according to Urra et al. (1997) using rHuEPO (Recormon®, Roche Pharmaceuticals) as antigen and, as secondary antibody, goat anti-rat IgG conjugated with horseradish peroxidase (Sigma; 100 ng/mL for 1h, at room temperature).[17] The substrate tetramethylbenzidine (TMB) (Sigma) was added and the reaction was stopped by the addition of sulphuric acid 1.25 mol/L. The optical density at 450 nm (OD450) was determined with an automatic plate reader.

3.5. Gene expression analysis

In order to isolate total RNA, 0.2 g of liver, duodenum and kidney samples, from each rat, were immersed in RNA later™ (Ambion, Austin, USA) upon collection and stored at 4°C, for 24h; afterwards, samples were frozen at -80ºC. Subsequently, tissue samples weighing 50±10 mg were homogenized in a total volume of 1 mL TRI® Reagent using a homogenizer, and total RNA was isolated according to manufacturer instructions (Sigma, Sintra, Portugal). To ensure inactivation of contaminating RNAses, all material used was cleaned and immersed in RNAse-free water (0.2% diethyl pyrocarbonate) for 2h and finally heated at 120ºC for 1h. RNA integrity (RIN, RNA Integrity Number) was analyzed using 6000 Nano ChipW kit, in Agilent 2100 bioanalyzer (Agilent Technologies,

Walbronn, Germany) and 2100 expert software, following manufacturer instructions. The yield from isolation was from 0.5 to 1.5 µg; RIN values were 7.8–9.0 and purity (A260/A280) was 1.8–2.0.

Table V-1. List of primer sequences (F: forward; R: reverse).

Gene	Primer sequences
EPO	F: 5'-AGGGTCACGAAGCCATGAAG-3'
	R: 5'-GAT TTC GGC TGT TGC CAG TG-3'
EPOR	F: 5'-GCG ACT TGG ACC CTC TCA TC-3'
	R: 5'-AGT TAC CCT TGT GGG TGG TG-3'
Hamp	F: 5'-GAA GGC AAG ATG GCA CTA AGC-3'
	R: 5'-CAG AGC CGT AGT CTG TCT CG-3'
TfR2	F: 5'-CAA GCT TCG CCC AGA AGG TA-3'
	R: 5'-CGT GTA AGG GTC CCC AGT TC-3'
SLC40A1	F: 5'-CAG GCT TAG GGT CTA CTG CG-3'
	R: 5'-CCG AAA GAC CCC AAA GGA CA-3'
HJV	F: 5'-GCC TAC TTC CAA TCC TGC GT-3'
	R: 5'-GGT CAA GAA GAC TCG GGC AT-3'
TF	F: 5'-GGC ATC AGA CTC CAG CAT CA-3'
	R: 5'-GCA GGC CCA TAG GGA TGT T-3'
Hfe	F: 5'-CTG GAT CAG CCT CTC ACT GC-3'
	R: 5'-GTC ACC CAT GGT TCC TCC TG-3'
DMT1	F: 5'-CAA CTC TAC CCT GGC TGT GG-3'
	R: 5'-GTC ATG GTG GAG CTC TGT CC-3'
TfR1	F: 5'-GCT CGT GGA GAC TAC TTC CG-3'
	R: 5'-GCC CCA GAA GAT GTG TCG G-3'
TMPRSS6	F: 5'-CCG AAT ATG AGG TGG ACC CG-3'
	R: 5'-GGT TCA CGT AGC TGT AGC GG-3'
BMP6	F: 5'-GCT GCC AAC TAT TGT GAC GG-3'
	R: 5'-GGT TTG GGG ACG TAC TCG G-3'
18S	F: 5'-CCA CTA AAG GGC ATC CTG GG-3'
	R: 5'-CAT TGA GAG CAA TGC CAG CC-3'
Actb	F: 5'-GAG ATT ACT GCC CTG GCT CC-3'
	R: 5'-CGG ACT CAT CGT ACT CCT GC-3'

The concentration of the RNA preparations were confirmed with NanoDrop1000 (ThermoScientific, Wilmington, DE, USA). Possible contaminating remnants of genomic DNA were eliminated by treating these preparations with deoxyribonuclease I (amplification grade) prior to RT-qPCR amplification. Reverse transcription and relative

quantification of gene expression were performed as previously described.[18] Real-time qPCR reactions were performed for erythropoietin (*EPO)*, erythropoietin receptor *(EPOR)*, transferrin receptor 2 (*TfR2*), hepcidin (*Hamp*), ferroportin (*SLC40A1*), hemojuvelin (*HJV*), transferrin (*TF*), hemochromatosis (*Hfe*), divalent metal transporter 1 (*DMT1*), transferrin receptor 1 (*TfR1*), matriptase-2 (*TMPRSS6*), and bone morphogenic protein 6 (*BMP6*), which were normalized in relation to the expression of beta-actin (*Actb*), and 18S ribosomal subunit (*18S*). Primer sequences are listed on Table II-1. Results were analyzed with SDS 2.1 software (Applied Biosystems, Foster City, CA, USA) and relative quantification calculated using the $2^{-\Delta\Delta Ct}$ method.[19] In liver tissue we studied the *EPO*, *EPOR*, *TfR2*, *Hamp*, *SLC40A1*, *HJV*, *TF*, *Hfe, BMP6* and *TMPRSS6* gene expression; in duodenum tissue the gene expression of *DMT1* and *SLC40A1* were studied, and in the kidney we evaluated the expression of *EPO* gene.

3.6. Data analysis

For statistical analysis, we used the Statistical Package for Social Sciences (SPSS), version 22.0. Results are presented as mean ± standard error of means (SEM). Multiple comparisons between groups were performed by one-way ANOVA supplemented with Turkey's HSD post-hoc test. For single comparisons, we used the Mann–Whitney U-test. Significance was accepted for a *p* minor than 0.05.

4. Results

4.1. Biochemical and hematological data

The changes in RBC count, Hb concentration, hematocrit and in the number of reticulocytes for the three groups are presented in Figure V-2. During the 9 week experimental protocol, the 50 IU rHuEPO group, when compared to the control group, showed similar values for RBC count, Hb concentration, hematocrit and reticulocytes, though a significant increase in reticulocyte count was observed at T2 and T3. Concerning the rats treated with 200 IU rHuEPO, in the first 3 weeks, we found a significant increase (> 30% the basal value) in hemoglobin levels, RBC count, hematocrit

and reticulocyte count, as compared to control and 50 IU rHuEPO groups; after this period, the hemoglobin concentration, hematocrit and RBC count, as well as the number of reticulocytes decreased, reaching significantly lower values at T2, as compared to T1; this trend towards decreasing values was maintained till the end of the experiment (9 weeks).

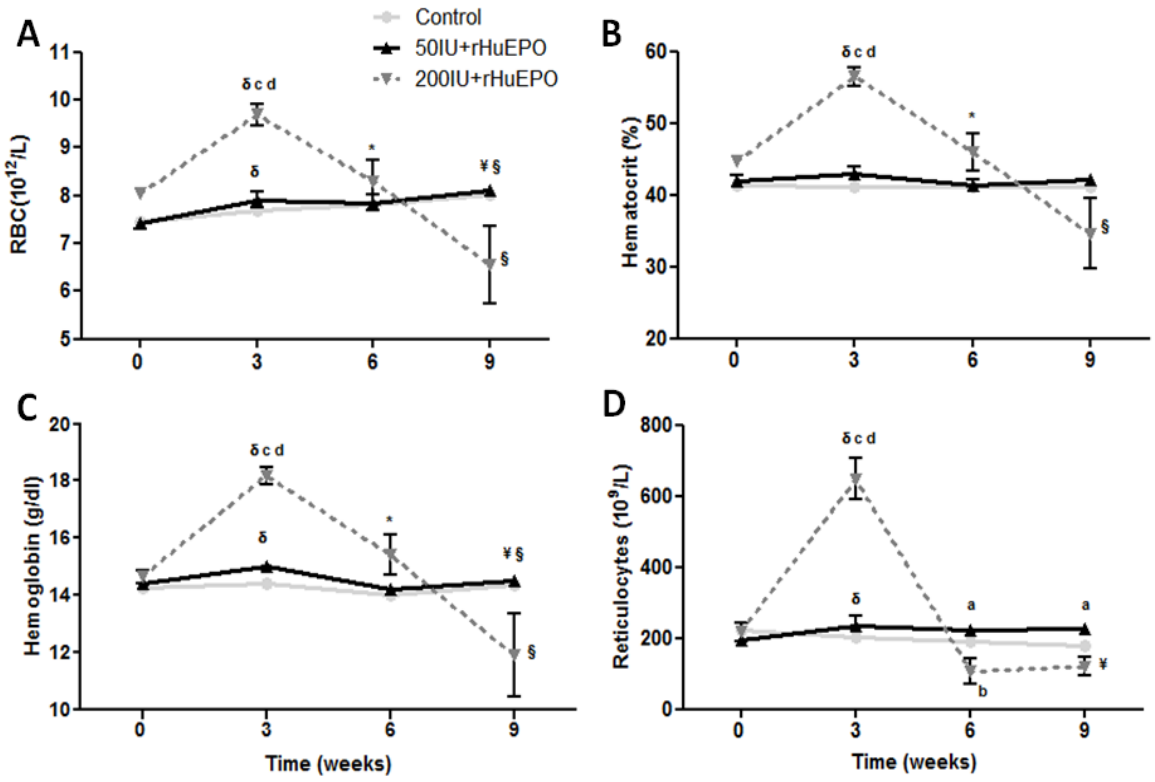

Figure V-2. Evolution of RBC count (A), hematocrit (B) hemoglobin (C) and reticulocyte count (D) during the follow-up period of 9 weeks under rHuEPO treatment. Results are expressed as mean ± SEM. [a]*p* <0.05 *vs* control; [b]*p* <0.05 *vs* 50 IU rHuEPO; [c]*p* <0.001 *vs* control; [d]*p* <0.001 *vs* 50 IU rHuEPO; [δ]*p* < 0.005 T0 *vs* T1; [*]*p* < 0.05 T1 *vs* T2; [§]*p* < 0.05 T2 *vs* T3.

At the end of the protocol, serum erythropoietin levels were similar for the three groups (Table II-2). No changes were observed in glucose, creatinine, urea, uric acid and bilirubin levels for both rHuEPO groups, when compared to the control. The inflammatory marker, IL-6, presented a significant decrease in the 200 IU rHuEPO group when compared with the control and 50 IU rHuEPO groups; hsCRP, IFN-γ, TGF-β1 and VEGF showed no significant differences between the three studied groups (Table II-2).

Table V-2. Hematological and biochemical data at the end of protocol (12 weeks).

Parameters	Groups		
	Control	50 IU rHuEPO	200 IU rHuEPO
EPO (mIU/ml)	1.87±0.11	1.65±0.09	1.80±0.11
WBC (x 10^9/L)	1.49±0.04	2.70±0.46[a]	1.47±0.18[b]
PLT (x 10^9/L)	767.88±19.68	803.25±35.72	751.67±38.70
Glucose (mg/dL)	160.88±10.27	174.62±7.14	187.71±14.15
Creatinine (mg/dL)	0.40±0.02	0.37±0.01	0.35±0.02
Urea (mg/dL)	45.10±1.16	45.05±1.26	42.74±1.92
Uric Acid (mg/dL)	0.94±0.46	0.70±0.13	1.03±0.52
Bilirubin (ng/mL)	0.043±0.006	0.054±0.005	0.064±0.0005
IL-6 (pg/mL)	138.10±1.89	139.98±2.16	130.16±1.91[a,b]
hsCRP (μg/mL)	229.31±8.68	237.49±10.23	249.46±12.66
IFN-γ (pg/mL)	24.44±8.72	21.45±2.40	20.82±3.83
TGF-β1 (ng/mL)	80.21±1.77	80.03±6.09	78.10±2.10
VEGF (pg/mL)	5.36±1.74	5.84±1.46	6.04±1.33

Results are expressed as mean ± SEM. [a]$p < 0.05$ *vs* control; [b]$p < 0.05$ *vs* 50 IU rHuEPO. WBC, white blood cells; PLT, platelets; IL-6, interleukin-6; hsCRP, high-sensitive C-reactive protein; IFN-γ, interferon-γ; TGF-β1, transforming growth factor beta 1; VEGF, vascular endothelial growth factor.

4.2. Anti-rHuEPO antibodies

Serum samples from all animals were also analyzed for anti-rHuEPO antibodies. These antibodies were detected in 5 rats under 50 IU rHuEPO treatment, as well as in 5 rats treated with 200 IU rHuEPO (62.5% for both groups); the antibody titer was fourfold higher in the group under 200 IU rHuEPO; the later presented a titer of 1:16, and the group under the lower rHuEPO dose presented a titer of 1:4. Within each rHuEPO group, no other significant differences were found between the rats with or without anti-rHuEPO antibodies.

Bone marrow examination of rats treated with 200 IU rHuEPO showed a proportion of red-cell precursors significantly lower than those without rHuEPO treatment. The myeloid:erythroid ratio was 2.1:1 for the control group, while for the 200 IU rHuEPO group the ratio was 6.8:1.; the 50 IU rHuEPO group presented a myeloid:erythroid ratio similar to that found for the control (2.5:1).

4.3. Iron metabolism

Concerning iron metabolism, we found that serum iron was significantly higher for the 200 IU rHuEPO group, when compared with the control and 50 IU rHuEPO groups; no significant differences were found in ferritin and transferrin serum levels between the three studied groups (Figure V-3).

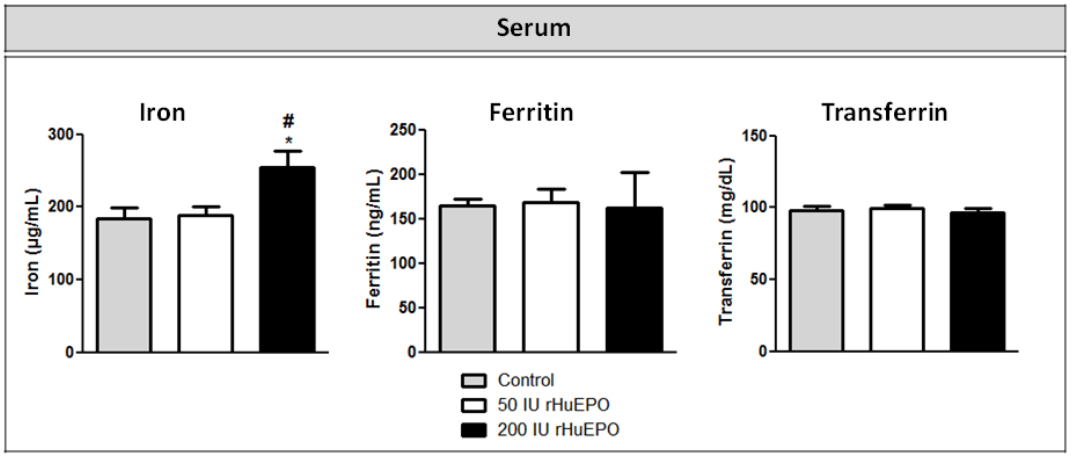

Figure V-3. Serum iron, ferritin and transferrin at final the time. Results are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ *vs* control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ *vs* 50 IU rHuEPO.

Major changes were observed in the gene expression of iron and erythropoietic regulatory proteins in liver tissue (Figure V-4), particularly in the 200 IU rHuEPO group. Indeed, the 50 IU rHuEPO group showed significant overexpression of *HJV* and *SLC40A1* genes, as compared to control, while the 200 IU rHuEPO group presented significant overexpression of *Hamp, Hfe, HJV, EPOR*, *SLC40A1, Tf , TfR2,* and *BMP6* genes, as compared to control and 50 IU rHuEPO groups. *EPO* gene was significantly down-regulated in both groups, and *TMPRSS6* gene expression was significantly down-regulated only in the 200 IU rHuEPO group, as compared to the control and 50 IU rHuEPO groups.

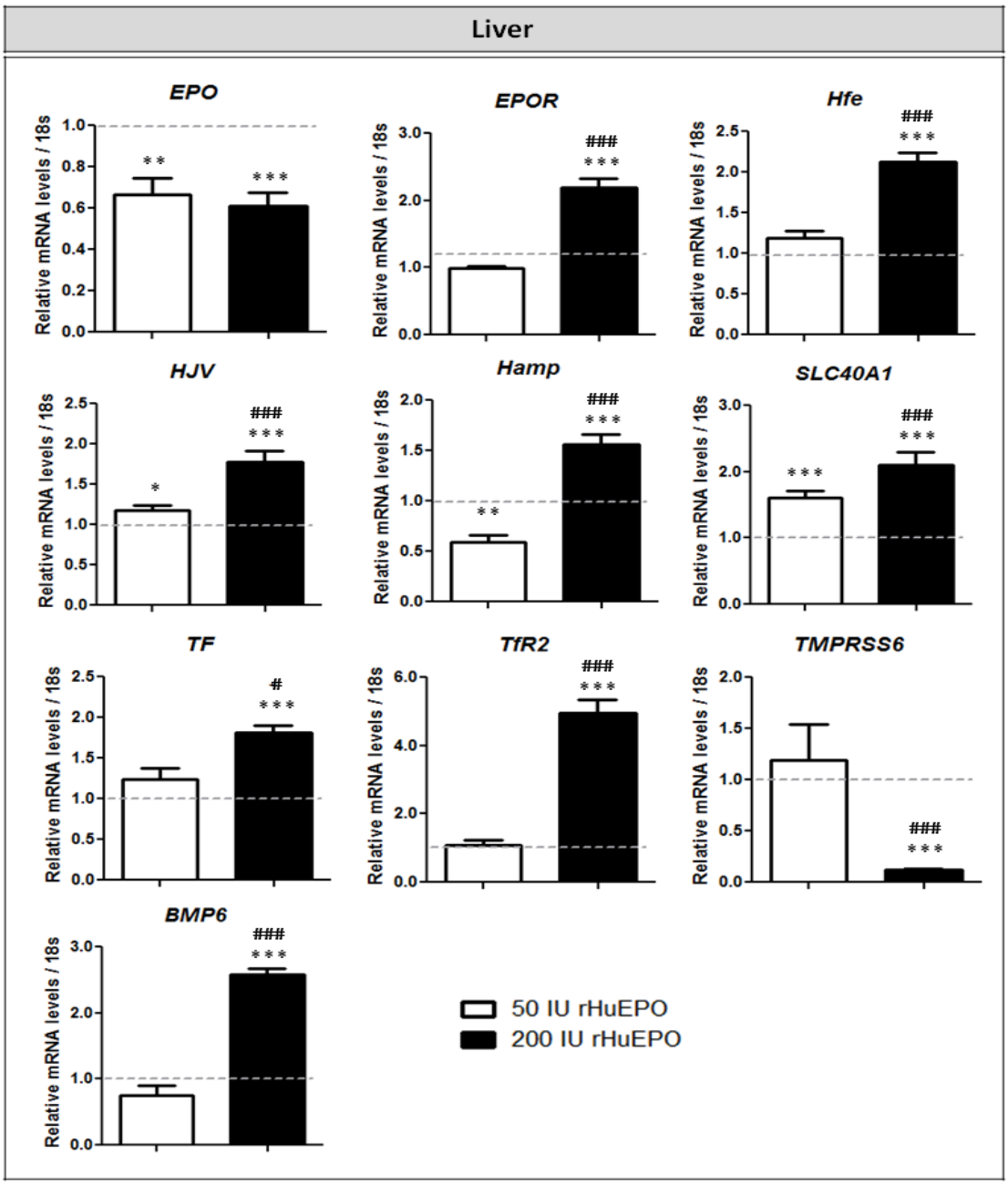

Figure V-4. Relative mRNA expression of erythropoietin and iron regulatory proteins in the liver, at the end of the protocol (9 weeks). 18S rRNA was used as reference gene. Results are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ *vs* control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ *vs* 50 IU rHuEPO. BMP6, Bone morphogenic protein 6; EPO, erythropoietin; EPOR, erythropoietin receptor; Hamp, hepcidin; HFE, Hemochromatosis; HJV, Hemojuvelin; SLC40A1, ferroportin; TMPRSS6, matriptase-2; sTfR2, soluble transferrin receptor 2; Tf, transferrin.

The evaluation of the expression of *DMT1* and *SLC40A1* genes in duodenum showed that only the 200 IU rHuEPO group presented a significantly up-regulation for both genes (Figure V-5), as compared to the control. Furthermore, as observed in liver,

the expression of *EPO* in kidney tissue presented a trend towards down-regulation in both rHuEPO groups, when compared to the control (Figure V-6).

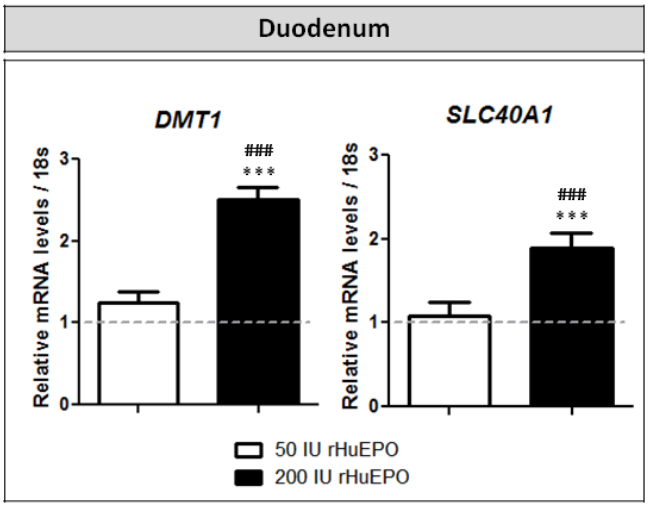

Figure V-5. Relative mRNA expression of DMT1 and SLC40A1 in the duodenum, at the end of the protocol (9 weeks). 18S rRNA was used as reference gene. Results are expressed as mean ± SEM. $^{***}p < 0.001$ *vs* control group; $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ *vs* 50 IU rHuEPO. DMT1, divalent metal transporter 1; SLC40A1, ferroportin.

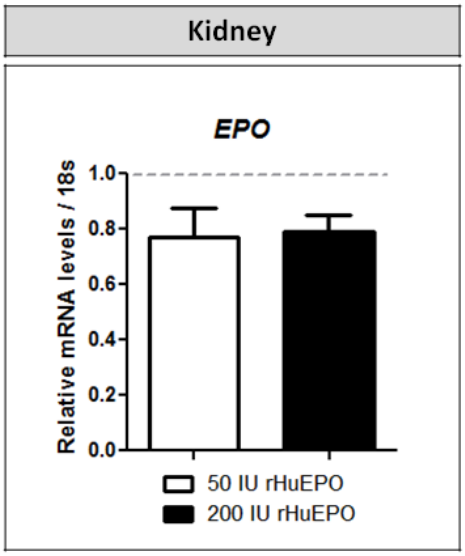

Figure V-6. Relative mRNA expression of erythropoietin in the kidney, at the end of the protocol (9 weeks). 18S rRNA was used as reference gene. Results are expressed as mean ± SEM. EPO, erythropoietin.

5. Discussion

Antibody-mediated EH/PRCA is a rare pathological complication associated with the use of recombinant human erythropoietins and development of anti-EPO antibodies. However, the use of EPO biosimilars has increased the number of cases in the last years.[11,20,21] Clinically, that condition not only abrogates the effect of the rHuEPO, but may also neutralize endogenous EPO, leading to severe EH and transfusion-dependent anemia.

In this work we developed an animal model of antibody-mediated EH, by long-term treatment with a high dose of rHuEPO (200 IU/Kg bw/week during 9 weeks) in Wistar rats. In order to confirm that we were in fact in the presence of an animal model of this hematological disorder, we assessed the parameters typically used as diagnostic criteria for EH/PRCA, which include normocytic anemia with sudden onset, normocellular bone marrow with a selective reduction in red blood cell precursors beyond proerythroblasts, reticulocytopenia, normal or slightly decreased leucocyte counts and increased serum iron.[22] We found that the group treated with 200 IU/Kg bw/week of rHuEPO along 9 weeks fits all these criteria and the majority (65%) of the rats presented detectable anti-rHuEPO antibodies, suggesting that we achieved an antibody-mediated EH. The group under 50 IU/Kg bw/week rHuEPO treatment presented only slight changes, as compared to control; however, most of the rats (65%) presented anti-rHuEPO antibodies at a lower title (four times less), suggesting that a longer experimental protocol could also lead to the changes associated with EH. Indeed, Casadevall et al. (2005) reported that the detection of anti-erythropoietin antibodies in circulation is not immediately associated with PRCA.[23] Moreover, no significant changes were observed in hsCRP, IL-6 and IFN-γ, for both rHuEPO treated groups, showing that no inflammatory changes could interfere with erythropoiesis and iron metabolism.

Our data showed that the development of EH was due to the antibodies directed to rHuEPO that inhibited the erythropoietic stimuli of both rHuEPO and endogenous EPO. Indeed, in spite of the normal endogenous EPO levels that were similar to those of the control group, the 200 IU rHuEPO group developed anemia, suggesting that the anti-rHuEPO antibodies also neutralized the action of endogenous EPO. It has been described that the presence of rHuEPO in circulation may increase several fold the expression of

EPOR.[4] Indeed, we found an overexpression of *EPOR* in the liver of the 200 IU rHuEPO group. The decreased *EPO* expression observed in kidney and liver tissues, for both rHuEPO groups, seems to be in accordance with a physiological response to the increased circulating levels of rHuEPO, used in the treatment.

Anemia, usually, triggers erythropoiesis by increasing EPO production through the HIF pathway, by mobilizing iron from the iron storage pool and by increasing iron absorption, in order to face the increased iron needs for erythropoiesis. Considering that in antibody-mediated EH, the erythropoietic stimuli fails, due to the inhibition of rHuEPO by anti-rHuEPO antibodies, the serum iron should increase, as its absorption and mobilization would be triggered by the anemic condition, and hepcidin should be repressed to favour iron absorption and mobilization. Actually, we found a significant increase in serum iron in the 200 IU rHuEPO group. It is known that increasing serum iron induces hepcidin synthesis by a complex pathway. Indeed, hepcidin, encoded by the *Hamp* gene*,* is believed to play a key regulatory role in iron absorption. It controls plasma iron concentration and tissue distribution of iron by inhibiting intestinal iron absorption, iron recycling by macrophages, and iron mobilization from stores.[24] Hepcidin acts by inhibiting cellular iron efflux in hepatocytes, enterocytes and macrophages, through binding to ferroportin, inducing its degradation.[25] Hepatic *Hamp* expression is regulated by a cohort of proteins, including Hfe, TfR2, HJV, BMP6, matriptase-2 and transferrin.[26-28] Studies in both human and animal models indicate that *Hfe* and *TfR2* are mild inducers of hepcidin expression,[26] as compared to *HJV* and *Tmprss6*, which are robust modifiers of hepcidin expression.[29-31] TfR2 has been hypothesized to act as a sensor for iron levels in the body because of its largely hepatocyte-specific expression and its ability to mediate cellular iron transport.[32] Although TfR2 is a minor contributor to the uptake of transferrin-bound iron by the liver, experiments with mice have shown that its major role is to modulate the signalling pathway that controls *Hamp* induction.[32] *Hfe* has also been implicated in the iron signalling complex that modulates hepcidin transcription by sensing changes in iron levels.[33] Increasing expression of *HJV* enhances *Hamp* expression by interacting with BMPs[34] possibly as a co-receptor. Matriptase-2 has been shown to negatively regulate *Hamp* gene and, therefore, to decrease hepcidin expression and promote iron uptake.[35] It is highly expressed in the liver and participates in a transmembrane signaling pathway triggered by iron deficiency and suppresses

hepcidin expression by cleaving membrane-bound hemojuvelin (mHJV) to increase iron absorption.[36]

In the liver, diferric transferrin competes with TfR for binding to Hfe, and, when iron is increased, more Hfe is available to bind to TfR2; this complex, TfR2-Hfe, promotes HJV binding to BMP6, increasing hepcidin synthesis.[32,34] Indeed, we found that increased serum iron in the 200 IU rHuEPO group was associated with an overexpression of *Tf, TfR2, BMP6, Hfe, HJV* in the liver, and, in accordance, with an overexpression of *Hamp*. Moreover, a downregulation in matriptase mRNA was observed in the liver that might further contribute to the overexpression of hepcidin. In accordance with the overexpression of hepcidin, a reduction in serum iron would be expected, due to the degradation of ferroportin by hecpcidin, instead of the increase that we found. It is known that regulation of iron absorption is mediated by signals reflecting oxygen tension in enterocytes, intracellular iron levels and systemic iron needs. Enterocyte oxygen tension regulates iron absorption through its effects on the transcription of *HIF*s and subsequent changes in transcription of *DMT1*.[37] Iron exits the enterocyte through the efflux transporter ferroportin 1 (FPN), the only member of the SLC40 family of transporters and the first reported protein that mediates the exit of iron from cells.[38] Actually, we found an overexpression of DMT1 and ferroportin in the duodenum and liver from the 200 IU rHuEPO group, in response to the anemic state. However, as the overexpression of hepcidin compromises iron absorption and mobilization, the observed increase in serum iron might result mainly from the decreased use of iron for the inhibited erythropoiesis.

Hypoxia, EPO, as well as twisted gastrulation protein 1 (TWSG1) and growth differentiation factor 15 (GDF15),[39] both produced by ertyhroblasts, are known to downregulate hepcidin synthesis. Considering the inhibition of erythropoiesis through the anti-rHuEPO antibodies, the production of TWSG1 and GDF15, might be reduced and, therefore, unable to downregulate hepcidin synthesis. We also found that rHuEPO treatment did not alter renal function, glucose levels and was not associated with an inflammatory process, when compared to the control.

Considering the four functionally hepcidin regulatory pathways described,[40-43] erythropoiesis, iron status, oxygen tension, and inflammation, we found that in our model, erythropoiesis was blunted leading to anemia, iron was increased and there was

no inflammation. It seems, therefore, that the increased iron concentration is the key modulator of hepcidin synthesis in this type of erythroid hypoplasia. In fact, it has been recently reported that the reduction of serum iron by iron chelation therapy promoted an improvement in erythropoiesis in PRCA, though the mechanism whereby this is achieved is still unclear.[44]

In conclusion, our data suggest that in the case of erythroid antibody-mediated hypoplasia/PRCA induced by a high rHuEPO dose (200 IU), as erythropoiesis is blunted through anti-rHuEPO antibodies, iron concentration becomes the key modulator for hepcidin synthesis, which will, probably, contribute to further aggravate the anemia. These findings might be important to improve new therapeutic strategies against rHuEPO resistance and/or development of antibody-mediated EH/PRCA in patients under rHuEPO therapy.

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Chapter VI

Final remarks and conclusions

1. Final remarks

CKD is a pathological condition that results from a gradual and permanent loss of kidney function over time, usually, months to years. CKD can result from primary diseases of the kidneys, however, diabetic nephropathy and hypertension are the main causes of CKD.[1] Anemia is a common complication of CKD that develops early in the course of the disease, increasing its frequency and severity with the decline of renal function. Anemia has been associated to a reduced production of erythropoietin by failing kidneys, to disturbances in iron metabolism and to the undelying inflammatory process.

Since the introduction of rHuEPO, the treatment of anemia with ESAs reduced the requirements for transfusion, improved the quality of life of the patients and reduced some associated complications of anemia.[2-4] However, the management of the anemia is still under debate, as a worrying percentage of patients develop hypo responsiveness to rHuEPO therapy, worsening their prognosis. Actually, the mechanisms underlying the development of resistance to rHuEPO therapy remain unsolved, although several hypotheses have been raised and investigated during the last years. Until the precise cellular and molecular mechanism explaining the development of anemia in CKD patients remain to be clarified, the clinical/therapeutic managing of this serious condition will remain difficult and unsatisfactory, and the mortality rate will remain too high, when compared with the normal population. Studies on animal models, taking advantage of the availability of tissues and the ability to modulate experimental conditions, are crucial to elucidate the cellular and molecular mechanisms responsible for these pathological circumstances. Indeed, although an animal model can never be a perfect match for human diseases, adequate animal models have been used, providing valuable insights into the mechanisms of anemia/CKD. Molecular studies, supported by proper animal models, might improve the knowledge about some areas that remain to be elucidated, concerning the use of rHuEPO in kidney disease/anemia, including the mechanisms behind resistance to therapy and the impact of high doses.

Uremic rat models have been characterized and used for long time by our group as well as by other authors as tools to study the pathophysiological events underlying kidney disease and the associated anemia, including those with renal failure induced by

nephrectomy and infarction.[5-7] However, the information is still scarce concerning the disturbances in iron metabolism associated with the hypoxic anemia of chronic kidney disease, namely in the 5/6 nephrectomized rat, which is one of the most used rat model of CKD.[7-9]

In this study, we opted to use the remnant kidney rat model of CKD induced by 5/6 nephrectomy, in order to, firstly, elucidate the mechanisms underlying the development of anemia and the evolution of renal damage, focusing on the quantification of endogenous EPO levels and kidney and liver EPO and EPOR mRNA expression, on iron metabolism, as well as, on kidney lesion, namely hypoxia, inflammation and fibrosis; and, secondly, to analyse the impact of rHuEPO therapy, by focusing on the same analytical studies.

Our CKD animal model produced a sustained degree of renal dysfunction, as shown by the significantly increased BUN and creatinine concentrations, together with presence of structural lesions, namely mild and advanced glomerular and tubulointerstitial lesions. In addition, we found a significant kidney hypertrophy (increased KW/BW index) showing a compensatory renal proliferation/hypertrophy of the remnant kidney, as previously described for this model.[10] A significant increase of HW/BW ratio was also found, suggesting the development of left ventricule hypertrophy, accompanied with hypertension, which is a cardiac complication found in CKD patients (Figure VI-1).

The progression of kidney disease and its associated cardiac/cardiovascular complications are the major causes of morbidity and mortality in these patients. One of the most prevalent co-morbidities is hypertension, which is present at all stages of CKD.[36] The results confirmed the development of systolic hypertension, which is a typical feature in the CKD rat model. Usually, hypertension is inversely proportional to the residual functional renal mass, as occurs in human pathology.[37,38]

In addition, and as expected, our CKD model developed anemia early after nephrectomy, which persisted throughout the study. The persistent anemia present in CRF rats was not due to EPO deficiency because the remnant kidney of CRF was still able to produce EPO (increased serum EPO levels), and the liver seems to increase *EPO* gene expression, suggesting that the remnant kidney or even extra-renal tissues were able to

compensate EPO production. Moreover, the reticulocyte count showed a trend towards reduced values, suggesting a reduced erythropoietic response to overcome the anemia.

Iron is essential for the production of mature red blood cells and a normal iron metabolism is crucial to maintain body iron levels.[10,48,49] Disturbances in iron homeostasis is a hallmark of the anemia of CKD, which, usually, presents as a functional iron deficient anemia, with low serum iron and transferrin alongside with normal or even high ferritin.[50] In spite of the increased EPO blood levels, anemia persisted and was associated to low serum iron and transferrin levels. However, the reduced iron content cannot be due to deficiency in iron stores or absorption because normal serum ferritin levels were found in CRF animals, together with reduced liver *Hamp* mRNA expression, and overexpression of duodenal ferroportin mRNA, that altogether would contribute to iron availability. In addition, the absence of systemic inflammation (normal serum IL-6 and hsCRP levels), could also not explain the presence of persistent anemia (Figure VI-1).

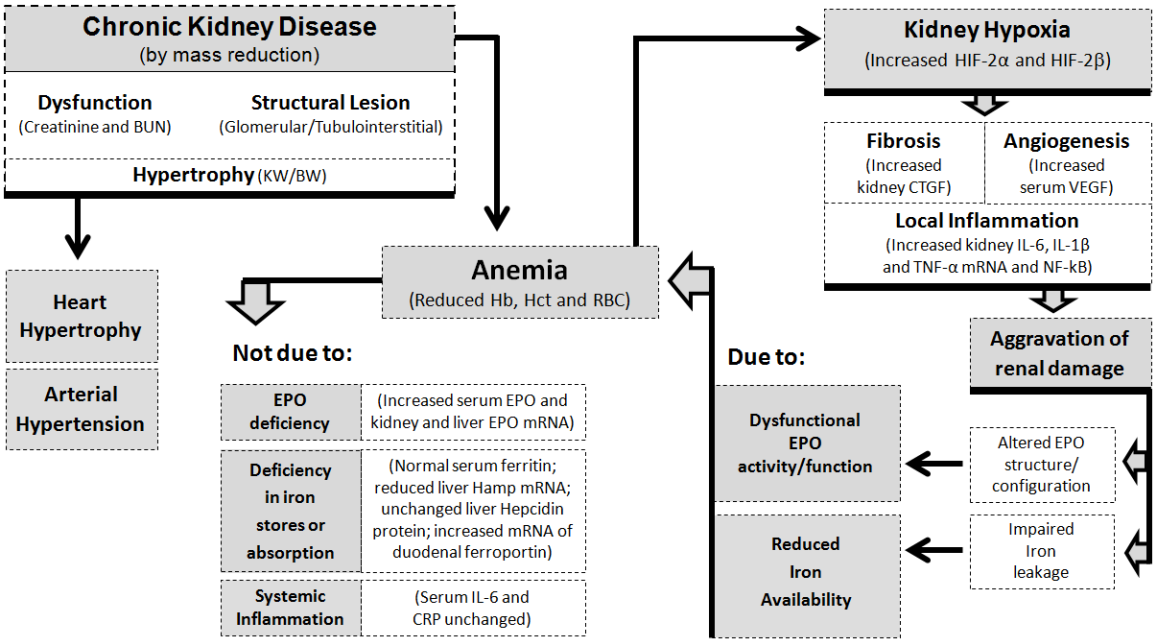

Figure VI-1. Schematic diagram representing a putative mechanistic model of the relationship between CKD, anemia, EPO production, iron/hepcidin metabolism, inflammation, hypoxia and renal damage/fibrosis in the remnant kidney rat model.

Regardless of the underlying molecular mechanism of reduction of *Hamp* expression during hypoxia, a decrease in hepcidin leads to an increased iron uptake and

absorption at the duodenum, as well as to an increased iron release from the macrophages, favoring Hb synthesis and erythropoiesis.[14] Actually, we found a significant downregulation in liver mRNA expression of *Hamp*, *sTfR1*, *TF, Hfe, HJV* and *BMP6* in CRF rats, together with a significant duodenal overexpression of *SLC40A1* gene in CRF rats and similar values for the expression of *DMT1* gene, suggesting that iron absorption in the enterocytes was normal or even enhanced, as the increase in duodenal ferroportin expression may occur to counteract the low iron levels observed in CRF rats; in addition, hypoxia might contribute to the significantly decreased liver *Hamp* mRNA expression in CRF rats (Figure VI-1).

These results suggest that the reduction of Hb levels in CRF rats, by leading to a reduced oxygen kidney perfusion, induced renal EPO production in response to low oxygen tension. Under hypoxic conditions in renal injury, the HIF system is activated, even before any histological evidence of tubulointerstitial damage,[68,69] and the degree of HIF expression seems to correlate with the extent of tubular injury. This persistent anemia in CRF rats, by leading to kidney hypoxia, activates the oxygen sensing pathway, increasing kidney protein expression of HIF-2α and HIF-2β and EPO. Some controversies exists about the role of HIF; some authors reported that HIF has a renoprotective role,[11,12] while others proposed that HIF contributes to interstitial fibrosis and/or tubular atrophy.[13,14] This duality of effects has also been described for vascular endothelial growth factor (VEGF), another target gene of HIFs.[15] We found significantly high serum VEGF levels, probably explaining the repression in VEGF gene expression in the remnant kidney of CRF.

All these conditions promoted the aggravation of renal damage, with mild and advanced glomerular and tubulointerstitial lesions, as well as the development of a local inflammatory milieu, as suggested by the increased expression of IL-6, IL-1β and TNF-α mRNA in the kidney tissue. The damages and the inflammatory milieu in the remnant kidney may cause iron leakage, explaining the reduced iron availability and, eventually, an altered structure/configuration of EPO that compromise its activity/function. The persistence of anemia may result from the disturbance in iron metabolism and, eventually, by an altered activity/function of EPO that might result from hypoxic kidney cell damage and from the developed local inflammatory milieu (Figure VI-1).

As already referred, there is a large variability in the erythropoietic response to ESA treatment, with up to 10-fold variability in dose requirements to achieve correction of the anemia. Hyporesponsiveness to ESA has been associated with higher morbidity and mortality in end-stage renal disease (ESRD) patients;[16,17] however, the target hemoglobin values are also crucial for the outcome of CKD patients; in fact, randomized studies and meta-analyses have demonstrated that correction of anemia for hemoglobin values higher than 12 g/dL is not associated with a better survival or a significant improvement in quality of life; instead, it has been associated with a potential increase in adverse cardiovascular effects, such as increased risk of stroke, venous thromboembolism and possibly death.[18-20]

Therefore, we figured as important to study the impact of increasing rHuEPO doses on CKD associated-anemia, using the same animal model and focusing again on erythropoiesis, iron metabolism, inflammation and kidney damage. Moreover, this study could provide some insights for the persistence of anemia observed in the CRF rats and to the mechanisms of development of resistance to rHuEPO therapy in CKD patients.

As previously commented, during the last few years the mechanisms underlying hepcidin and iron regulation have been largely studied. In addition, the impact of renal hypoxia, through hypoxia-inducible factors, on iron metabolism, on kidney lesion or regeneration, as well as on hepcidin expression has been increasingly debated.[21-23] Recent evidences suggest that CKD anemia might be due to defective hypoxic signaling rather than an inability of the EPO-producing cells to synthesize EPO. The long-term safety of therapeutic activation of the HIF system requires further study because of the broad biological potential of HIF in mediating hypoxia responses.[24] Meanwhile, it is of great clinical important to understand the impact of rHuEPO, namely at high doses, on some key aspects of CKD anemia, including on iron metabolism and the pre-existing kidney damage.

We used the animal model of CKD induced by 5/6 nephrectomy to evaluate the impact of two doses of rHuEPO - 50 and 200 IU - on the same analytical studies. The lower dose is used in responsive CKD patients, while the higher dose is used in patients presenting a poor response to rHuEPO therapy.

The CRF rats treated with the lowest dose of rHuEPO (50 IU) presented anemia, though a slight rise in Hb and in reticulocyte count was observed, until the ninth week;

afterwards Hb, HTC and RBC count showed a decrease. In opposition, the anemia of CRF rats under treatment with the highest rHuEPO dose (200 IU) was rapidly corrected and the Hb concentration reached values significantly higher, as compared to Sham and CRF rats, until the ninth week, after which the hematological values declined to values similar to those of the Sham group. In fact, while the lowest dose (50 IU) was unable to correct the anemia (only a slight improvement) leading to kidney hypertrophy and angiogenesis, as compared to CRF rats, the highest dose (200 IU) reduced kidney hypertrophy and angiogenesis, as it was able to correct anemia until the 9th week.

The decrease in hemoglobin, observed in both CRF groups treated with rHuEPO was due to the formation of EPO-neutralizing antibodies (Abs). This condition has been also reported in humans,[25,26] and, more recently, has increased due to the introduction of EPO biosimilars to treat the anemia, in some countries.[27,28] Although this complication is very rare, the mechanisms underlying the break in immune tolerance to rHuEPO remain poorly clarified.

Actually, when studying the effect of rHuEPO treatment, *per se*, in Wistar rats, we managed to develop a rat model of antibody-mediated EH, by long-term treatment with a high dose of rHuEPO (200 IU/Kg bw/week during 9 weeks). To confirm that we were, in fact, in the presence of an animal model of this hematological disorder, we assessed the parameters typically used as diagnostic criteria for EH/PRCA.

In this model, anti-rHuEPO antibodies inhibited both rHuEPO and endogenous EPO leading to anemia, iron was increased, triggering hepatic hepcidin expression, despite no evidence of inflammation. These results suggest that in erythroid antibody-mediated hypoplasia/PRCA induced by a high rHuEPO dose (200 IU), as erythropoiesis is blunted through anti-rHuEPO antibodies, the increased iron concentration becomes the major key modulator of hepcidin synthesis, increasing its expression, which will, probably, contribute to further aggravate the anemia.

The persistence of the anemia developed in the CRF model is not due to deficient EPO production, as viewed by unchanged serum EPO levels, normalization of kidney EPO and EPOR mRNA expression and an overexpression of liver EPOR mRNA, which might be explained by a compensatory production of EPO by the remnant kidney, as well as, most probably, by the liver tissue (Figure VI-2).

While the liver has a role in EPO production in the fetal age, in the adulthood the main producer of EPO is the kidney.[29-31] However, in renal disease conditions the extra-renal tissues, such as the liver, might assume a higher part on the compensatory synthesis of EPO.

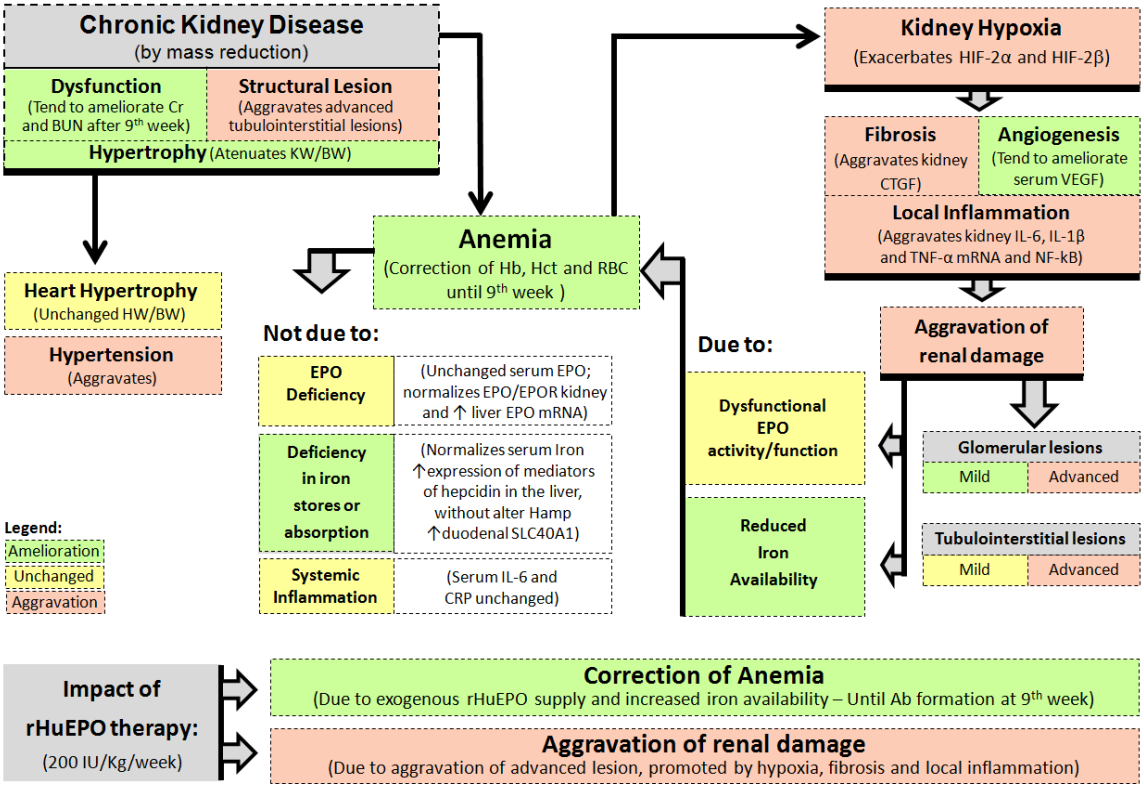

Figure VI-2. General synthesis of impact of rHuEPO therapy on key aspects of CKD associated-anemia.

In spite of the anemia, both CRF treated groups presented a similar down-regulation of kidney and liver *EPO* gene expression, suggesting a deregulation of the kidney hypoxia sensing mechanisms. As just referred, the anemia developed in CRF rats was not due to deficient EPO production, but, instead, it seems to result from a reduced (altered) erythropoietic activity of endogenous EPO, once the treatment of CRF rats with rHuEPO was able to improve the anemia until the ninth week.

A lower impact of the treatment with the highest rHuEPO dose (200IU) on the glomerulus, as compared to the lowest dose, may explain the improvement observed in iron metabolism, with an increase in serum iron and transferrin and a slight decrease in ferritin levels, as well as an increase in duodenal ferroportin and *DMT1* mRNA

expression, suggesting that less damaged glomerulus were able to reduce the loss of iron and transferrin levels. This was strengthened by the observation of increased iron deposits on the tubular structures of CRF rats and of CRF animals treated with 50 IU rHuEPO, as compared to CRF rats under 200IU rHuEPO therapy, in whom iron deposits were almost absent. Furthermore, the improvement in iron metabolism achieved with the highest dose (200 IU), promoted also a profound impact on liver and duodenal gene expression profile of iron-hepcidin mediators: increased mRNA expression of *sTfR1, sTfR2, Hfe, HJV, BMP-6 and IL-6* and reduced of *TMRPSS6* (matriptase-2) in the liver and, as expected, an overexpression of hepcidin gene (*Hamp*), that was not traduced in a higher protein expression, as showed by the immunochemical studies in the liver. The same was observed for HIF-2α, which presented a slightly increased overexpression that was associated with a slight decrease in the protein expression. An increased mRNA expression of *DMT1* and *SLC40A1* was also observed in the duodenum. Thus, it seems that for the highest rHuEPO dose, the less severe anemia/hypoxia is, probably, due to reduced glomerular kidney lesions associated to the improvement in iron metabolism, and might explain the decrease in hepcidin and HIF-2α protein expression. No changes were observed in systemic inflammation with the highest dose of rHuEPO (Figure VI-2).

Both rHuEPO doses were unable to prevent kidney damage. The highest dose, indeed, aggravated tubulointerstitial lesions. The deleterious effect of the highest rHuEPO dose on the kidney tissue might be explained by the increase in mediators of kidney hypoxia, fibrosis, angiogenesis and local inflammation, as this dose promoted an overexpression of *HIF-2* (α and β), *IL-6, IL-1β*, *NF-kB* and *CTGF*, accompanied by increased kidney protein expression (immunorreactivity) of NF-kB and CTGF, showing the development of a fibrotic and inflammatory milieu. However, we must notice that the decrease in Hb concentration, in the last three weeks, almost thrice in the CRF+200IU rHuEPO group, as compared to CRF+50IU rHuEPO group, might result, besides the action of anti-rHuEPO antibodies, from the synthesis of EPO with a reduced (altered) erythropoietic activity, due to the high enhancement in the inflammatory and fibrotic kidney milieu.

In summary, our data suggest that the anemia developed in rats with long term CRF is not due to reduced EPO synthesis, but to reduced (altered) erythropoietic activity, as the CRF rats were able to overcome the anemia when treated with rHuEPO; the local

kidney inflammation and the type of kidney lesions seems to be crucial for the outcome of the anemia. A long term treatment of CRF rats with rHuEPO leads to the development of rHuEPO antibodies and, therefore, to anemia, after about 6 weeks of treatment; the impact of rHuEPO treatment depends on the rHuEPO dose, which should be enough to correct anemia, preventing kidney and heart hypertrophy, but not too high as it will lead to worsening of glomerular and tubulointerstitial kidney lesions. Further studies are needed to understand how hypoxia and/or kidney lesions induce a reduced (altered) EPO activity and how kidney glomerular lesions/iron leakage may contribute to the functional iron deficient anemia, usually observed in hemodialysis CKD patients and enhanced in case they develop resistance to rHuEPO treatment.

2. Conclusions

The main goal of this thesis was to contribute to elucidate the cellular/molecular mechanisms underlying the development of resistance to endogenous EPO and to rHuEPO therapy, using experimental animal models of anemia and CKD. The main conclusions obtained were:

I) The model of CKD induced by 5/6 nephrectomy presented a sustained degree of renal dysfunction with mild and advanced glomerular and tubulointerstitial lesions. This model developed anemia early after nephrectomy and persisted throughout the study, in spite of augmented EPO levels.

II) The development of glomerular and tubular kidney damage in CKD model may underlie iron leakage, explaining the reduced iron availability. Actually, the reduced expression of hepcidin, probably induced by hypoxia, should favour iron absorption; indeed, iron absorption seems to be increased, as suggested by the increased expression of duodenal ferroportin.

III) The persistence of anemia may result from the disturbance in iron metabolism and, eventually, by an altered activity/function of EPO that might result from hypoxic kidney cell damage and from the developed local inflammatory milieu.

IV) The anemia developed in rats with long term CRF is not due to reduced EPO synthesis, but probably to a limited EPO functionality (erythropoietic activity), as the CRF rats were able to overcome the anemia when treated with rHuEPO.

V) Anemia correction by the high rHuEPO dose seems to result from an improved iron availability, which might result from an improved renal function given the amelioration of mild glomerular lesions.

VI) However, despite correcting anemia, a long-term treatment of CRF rats with rHuEPO might cause formation of rHuEPO antibodies, becoming resistant to exogenous EPO (rHuEPO) therapy.

VII) The impact of the treatment CRF of rats with rHuEPO depends on the dose, which should be enough to correct anemia, preventing kidney and heart hypertrophy, but not too high, as it will lead to worsening of glomerular and tubulointerstitial kidney lesions.

VIII) The development of EH was due to antibodies directed to rHuEPO that inhibited the erythropoietic activity of both rHuEPO and endogenous EPO.

IX) In erythroid antibody-mediated hypoplasia/PRCA model induced by a high rHuEPO dose, as erythropoiesis is blunted through anti-rHuEPO antibodies, iron concentration becomes the key modulator for hepcidin synthesis, which will contribute to further aggravate the anemia.

In conclusion, the findings of this thesis give further insights into the mechanisms behind the different response to rHuEPO treatment observed in chronic kidney disease patients, as well as to the development of resistance to rHuEPO; moreover, it might open new windows to identify putative therapeutic targets for this condition, as well as for rHuEPO resistance, which occurs in about 10 % of CKD patients. We managed also to develop two experimental models, of CKD and of PRCA, which are useful tools for further studies, namely pharmacological, in this research area.

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