U.PORTO

FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO

RUA DE JORGE VITERBO
FERREIRA N.º 228
4050-313 PORTO - PORTUGAL
WWW.FF.UP.PT

THE ROLE OF ADIPOCYTE IN THE MODULATION OF ERYTHROPOIESIS AND IRON METABOLISM

FERNANDO JORGE NETO MARTINS
MESTRADO EM ANÁLISES CLÍNICAS

U.PORTO

FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO

THE ROLE OF ADIPOCYTE IN THE MODULATION OF ERYTHROPOIESIS AND IRON METABOLISM

Fernando Jorge Neto Martins

MESTRADO EM ANÁLISES CLÍNICAS

PORTO 2014

U.PORTO

FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO

The role of adipocyte in the modulation of erythropoiesis and iron metabolism

O papel do adipócito na modulação do metabolismo do ferro e da eritropoiese

Dissertação do 2º Ciclo de Estudos Conducente ao Grau de Mestre em Análises Clínicas submetida à Faculdade de Farmácia da Universidade do Porto

Fernando Jorge Neto Martins

Orientadores:

Professora Doutora Alice Santos-Silva

Professora Doutora Susana Coimbra

Setembro, 2014

DE ACORDO COM A LEGISLAÇÃO EM VIGOR, NÃO É PERMITIDA A REPRODUÇÃO DE QUALQUER PARTE DESTA DISSERTAÇÃO

Acknowledgements

À Professora Doutora Alice Santos Silva quero manifestar a minha profunda gratidão, não só por ter tornado possível a realização desta dissertação de Mestrado, mas também pela sábia orientação científica e pelo rigor dos conhecimentos transmitidos.

À Professora Doutora Susana Coimbra quero transmitir um agradecimento muito especial, pela confiança depositada, pelos valiosos ensinamentos, pela prontidão e pelo apoio inesgotável.

À Professora Doutora Cristina Catarino, o meu muito obrigado, pela disponibilidade dispensada e pelo apoio dado na concretização da parte experimental do trabalho desenvolvido.

Ao Professor Doutor Elísio Costa e à Professora Doutora Elsa Bronze um agradecimento especial pelos valiosos conselhos.

A todas as pessoas do Laboratório de Bioquímica da Faculdade de Farmácia da Universidade do Porto, que me acolheram de forma sempre simpática e familiar, com quem aprendi e com quem me diverti ao longo destes meses, o meu agradecimento. Um especial muito obrigado, ao Doutor João Fernandes, à Doutora Susana Rocha e à Mestre Sandra Ribeiro que me ajudaram sempre que alguma dúvida surgia no decorrer de todo o trabalho desenvolvido.

À Ana Paula pela prontidão, paciência e simpatia com que sempre me ajudou, um muito obrigado.

À Dona Casimira um obrigado pelas conversas e risotas proporcionadas que ajudam a desanuviar nas pequenas pausas de um dia de trabalho.

A todos os meus amigos que sempre me apoiaram em todas as alturas e me incentivaram à conclusão deste trabalho, um sentido obrigado.

À minha namorada um especial agradecimento pela paciência de ouvir todas as lamúrias e por me apoiar incondicionalmente.

Aos meus pais, por me incentivarem no amor ao estudo e à realização profissional, entre outros valores que orientam a minha vida. Obrigada pela paciência e pelo apoio constante.

A todos os que, com os seus gestos ou palavras de incentivo e amizade, contribuíram de forma significativa para a execução desta dissertação.

Abstract:

A relationship between adipose tissue and erythropoiesis is believed to exist. The adipose tissue secretes several adipokines, some of which are known to interfere with erythropoiesis. Adipocytes are also capable of synthesizing hepcidin, the principal regulator of iron availability, but it is not completely clarified if this hepcidin synthesis is stimulated by inflammation, hypoxia or by BMP/SMAD pathway.

We aimed to understand how an increasing erythropoietic stimuli affects the expression of some inflammatory cytokines, of iron metabolism and erythropoiesis, in both visceral (VAT) and subcutaneous (SAT) adipose tissue.

We performed a 6 weeks follow-up study in lean, adult, normotensive rats submitted to erythropoietic stimuli, by administering two high doses of recombinant human erythropoietin (rHuEPO) during the last 3 weeks of the protocol. The rats were randomly divided in 3 groups – control, rHuEPO200 and rHuEPO600. Hematological and biochemical evaluations, including markers of renal function, inflammation, iron status and lipid profile, were done at starting and at the end of the protocol. The gene expression of erythropoietic and iron regulatory proteins, namely, hepcidin, interleukin (IL)-6, bone morphogenetic protein (BMP)6, EPO receptor (EPOR) and ferritin, by the VAT and SAT, were executed.

In both rHuEPO groups was observed an increase in several erythrocytes parameters that was higher for the rHuEPO600 group. Concerning SAT, both groups presented a significant overexpression of hepcidin and ferritin genes and an under expression of BMP6 and EPOR genes; IL-6 gene was only overexpressed in the rHuEPO600 group. For the VAT, in the rHuEPO200 group we found an overexpression of hepcidin, IL-6 and ferritin and under expression of BMP6 and EPOR genes; in the rHuEPO600 group, all the 5 studied genes were under expressed, presenting the lower expression of hepcidin and IL-6 genes a positive significant genes correlation.

In summary, the BMP/SMAD pathway does not seem to have an important role in the regulation of hepcidin expression in adipocyte. When submitted to erythropoietic stimuli, the VAT and SAT respond differently. In VAT, a higher erythropoietic stimuli associates with a lower expression of hepcidin and IL-6 that correlated positively with each other, suggesting that under high erythropoietic stimuli, IL-6 and hepcidin expression are tightly related.

Key-words: Erythropoiesis, iron metabolism, adipose tissue genetic expression.

Resumo:

O tecido adiposo e o processo eritropoiético parecem estar intimamente associados. O tecido adiposo secreta várias adipocinas, algumas das quais interferem com a eritropoiese. Os adipócitos são também capazes de sintetizar hepcidina, o principal regulador da biodisponibilidade do ferro, contudo não é totalmente claro se esta síntese é estimulada pelo processo inflamatório, hipoxia ou pela via BMP/SMAD.

O nosso objetivo foi tentar compreender de que forma um estímulo eritropoético afeta a expressão de citocinas inflamatórias, o metabolismo do ferro e da eritropoiese, no tecido adiposo visceral (TAV) e subcutâneo (TAS).

Assim, realizou-se um estudo longitudinal (6 semanas) com ratos adultos, de peso e tensão arterial normais, submetidos a um estímulo eritropoético por administração de doses elevadas de eritropoietina recombinante humana (rHuEPO) administradas durante as últimas 3 semanas. Os ratos foram aleatoriamente divididos em 3 grupos – controlo, rHuEPO200 e rHuEPO600. Foram efetuadas avaliações hematológicas e bioquímicas (hemograma, marcadores da função renal, inflamação, metabolismo do ferro e perfil lipídico) no início e no final do estudo. Foi determinada a expressão génica, no TAV e no TAS, de proteínas reguladoras do metabolismo do ferro e da eritropoiese (hepcidina, interleucina (IL)-6, proteína morfogénica óssea (BMP)6, recetor da EPO (EPOR) e ferritina).

Nos grupos rHuEPO observou-se um aumento no valor de eritrócitos, que foi maior no grupo rHuEPO600. No TAS, ambos os grupos apresentaram uma sobre-expressão dos genes da hepcidina e da ferritina e uma sub-expressão de BMP6 e EPOR; o gene da IL-6 encontrava-se sobre-expresso apenas no grupo rHuEPO600. No TAV observou-se, para o grupo rHuEPO200, uma sobre-expressão dos genes da hepcidina, IL-6 e ferritina, e uma sub-expressão dos genes BMP-6 e EPOR; no grupo rHuEPO600 foi observada uma sub-expressão de todos os 5 genes, apresentando a expressão significativamente mais baixa dos genes de hepcidina e de IL-6, uma correlação positiva significativa.

Resumindo, a via BMP/SMAD não parece ser importante na regulação da expressão de hepcidina no tecido adiposo. Quando submetidos a um estímulo eritropoético, o TAV e o TAS respondem de forma diferente. No TAV, um estímulo eritropoético maior associa-se a uma menor expressão dos genes da hepcidina e da IL-6, que se correlacionam positivamente, sugerindo que sobre um forte estímulo eritropoético, a expressão dos dois genes está fortemente relacionada.

Palavras-chave: Eritropoiese, metabolismo do ferro, expressão genética, tecido adiposo.

Index

Chapter 1 – Introduction 1

1.1 – Erythropoiesis 1

1.1.1 – Erythropoietin 3

1.2 – Iron metabolism 5

1.2.1 – Hepcidin 9

1.3 – Adipose tissue 12

1.4. – Modulation of erythropoiesis 17

Chapter 2 – Objectives and work plan 20

Chapter 3 – Materials and methods 21

3.1. – Animals and experimental protocol 21

3.1.1. – Sample collection 21

3.2. – Hematological studies 22

3.3. – Biochemical studies 22

3.4. – Molecular genetic studies 23

3.4.1. – RNA extraction by TriPure reagent 23

3.4.2. – RNA concentration and purity 23

3.4.3. – cDNA synthesis 24

3.4.4. – Gene expression analysis with qPCR 24

3.5. – Statistical analysis 25

Chapter 4 – Results 25

4.1. – Hematological studies 25

4.2. – Study of iron metabolism 28

4.3. – Biochemical studies 30

4.4. – Gene expression studies 32

Chapter 5 – Discussion 34

Bibliographic references 38

Index of figures

Figure 1 - Erythroid differentiation (3). 1
Figure 2 - Erythropoietin receptor signaling via Jak/Stat pathway (8). 4
Figure 3 - Iron homeostasis and major pathways of iron traffic (approximate values) (10). 5
Figure 4 - Absorption of iron through enterocytes (12). 6
Figure 5 - Incorporation of iron in developing red cells (17). 8
Figure 6 - Stimulatory and inhibitory signals of hepcidin regulation (17). 11
Figure 7 - White adipose tissue. (A) Optical microscopy of subcutaneous adipose tissue of human white adipose tissue (formaldehyde-fixed and paraffin-embedded). Bar = 30 µm. (B) Electron microscopy of murin white adipose tissue. Bar=2 µm (25). 12
Figure 8 - Brown adipose tissue. (A) Optical microscopy of human brown adipose tissue. Bar = 15 µm (B) Electron microscopy of mouse brown adipose tissue. Bar = 1 µm (25). .. 13
Figure 9 - The multiple adipokines produced in white adipose tissue in the different fat depots in the body (3). 14
Figure 10 - Contribution of adipose tissue to modulation of erythropoiesis. 18
Figure 11 - Regulation of hepcidin expression by the adipose tissue (37). 19
Figure 12 – rHuEPO effects on relative gene expression mRNA/alpha-tubulin, in subcutaneous adipose tissue, of hepcidin – A; IL-6 – B; BMP6 – C; EPOR – D; ferritin -- E. Results are presented as mean + standard error. * P< 0.05, ** P< 0.01 and *** P< 0.001 vs Control. $^{\#}$ P< 0.05 for rHuEPO200 vs rHuEPO600. 33
Figure 13 - rHuEPO effects on relative gene expression mRNA/alpha-tubulin, in visceral adipose tissue, of hepcidin – A; IL-6 – B; BMP6 – C; EPOR – D; ferritin -- E. Results are presented as mean + standard error. * P< 0.05, ** P< 0.01 and *** P< 0.001 vs Control. $^{\#}$ P< 0.05 for rHuEPO200 vs rHuEPO600. 33

Index of Tables

Table 1 - Primers used on qPCR for gene expression analysis. 24
Table 2 – Body weight and hematological study at the beginning (T0) and at the end (T1) of the protocol for the Control, rHuEPO200 and rHuEPO600 groups. 27
Table 3 – Erythropoietin and iron metabolism at the beginning of the study (T0) and at the end of the protocol (T1), for Control, rHuEPO200 and rHuEPO600 groups. 29
Table 4 - Biochemical studies at beginning of the protocol (T0) and at the end of the protocol (T1) for the Control, rHuEPO200 and rHuEPO600 groups. 31

Abbreviations

BFU-e: burst-forming unit – erythroid

BMI: Body mass index

BMP: Bone morphogenic protein

BP: blood pressure

BW: body weight

cDNA: complementary deoxyribonucleic acid

CFU-e: colony-forming unit – erythroid

CFU-GEMM: colony forming unit – granulocytes, erythrocytes, monocytes and megakaryocytes

CMP: common myeloid progenitor

CRP: C reactive protein

Dcytb: duodenal cytochrome b

DEPC: diethylpyrocarbonate

DMT1: divalent metal transporter 1

dNTP: deoxynucleotides triphosphate

ELISA: enzyme-linked immunosorbent assay

EPO: erythropoietin

EPOR: erythropoietin receptor

ERC: erythropoietin responsive cells

FFAs: free fatty acids

FPN1: ferroportin

GDF15: growth differentiation factor 15

GM-CSF: granulocyte monocyte colony-stimulating factor

HAMP: hepcidin antimicrobial peptide

HCP1: haem carrier protein 1

HCT: hematocrit

HFE: hemochromatosis

HGB: hemoglobin

HIF: hypoxia inducible factor

HJV: hemojuvelin

HO1: haem-oxygenase 1

HR: heart rate

HSC: hematopoietic stem cell

IFN- γ: interferon-gamma

IGF-1: insulin-like growth factor-1

IL: interleukin

IL-1Ra: interleukin 1 receptor antagonist

IP-10: interferon-inducible protein-10

IPRs: iron response proteins

IRE: iron responsive element

IREG1: iron-regulated transporter-1

IRP: iron regulatory protein

Jak-2: Janus kinase 2

K3EDTA: tri-potassium ethylenediaminetetraacetic acid

MAP: mitogen-activated protein

MCP-1: monocyte chemoattractant protein-1

MEP: megakaryocyte-erythroid progenitor

M-MLV RT: Molony - Murine Leukemia Virus Reverse Transcriptase

mRNA: messenger ribonucleic acid

NF-KB: nuclear factor kappa-light-chain-enhancer of activated B cells

NOS: nitric oxide-synthase

PAI-1: plasminogen activator inhibitor-1

PI3 kinase: phosphatidylinositol 3 kinase

PLCγ: phosphoinositide-specific phospholipase Cγ

qPCR: quantitative polymerase chain reaction

RANTES: Regulated on Activation, Normal T Expressed and Secreted

RBC: red blood cell

RBP-4: Retinol-binding protein-4

RES: reticuloendothelial system

rHuEpo: recombinant human erythropoietin

ROS: reactive oxygen-species

RT-PCR: reverse transcriptase polymerase chain reaction

SAA: serum amyloid A

SCAT: subcutaneous adipose tissue

SCF: stem cell factor

SPSS: Statistical Package for Social Sciences

STAT: signal transducer and activator of transcription

TF: Transferrin

TFR: transferrin receptor

TGF: transforming growth factor

TGs: triglycerides

Th: T-helper

TNF-α: tumor necrosis factor alpha

TWSG1: Twisted gastrulation protein homolog 1

UTR: untranslated region

VAT: visceral adipose tissue

WAT: white adipose tissue

Chapter 1 – Introduction

The purpose of this chapter is to explain the linkage between the adipose tissue, iron metabolism and erythropoiesis. We will focus the erythropoiesis process, the iron metabolism, the characteristics of the adipose tissue and its potential contribution to iron bioavailability and to erythropoiesis.

1.1 – Erythropoiesis

Erythropoiesis is the process of continuous production of erythrocytes, the cells responsible for the transport of oxygen from the lungs to oxygen dependent tissues and for the mediation of carbon dioxide removal from the tissues. Red blood cells (RBCs) represent 40% to 45% of total blood volume and the mean lifespan of a RBC is 120 days. Everyday about 1% of circulating senescent RBCs are removed and, to replace them, the body produces approximately 2.0×10^{11} cells every day. This is sustained by hematopoietic stem cells, which are characterized by their ability to self-renew, differentiate, proliferate and give rise to all types of mature blood cells (1, 2).

Erythropoiesis starts in the yolk sac, around the 21st day of gestation, and erythroid cells can be found in multilineage colonies, colony forming unit – granulocytes, erythrocytes, monocytes and megakaryocytes (CFU-GEMM). From this multilineage colonies appears the erythroid cell, the burst-forming unit – erythroid (BFU-e) that in a later mature stage gives rise to colony-forming unit – erythroid (CFU-e). This CFU-e differentiates into proerythroblasts and erythroblasts that will mature into reticulocytes and, finally, into erythrocytes. The reticulocytes extrude their nucleus and complete the synthesis of hemoglobin (HGB) giving rise to the mature cell, the erythrocyte (Figure 1) (1, 3).

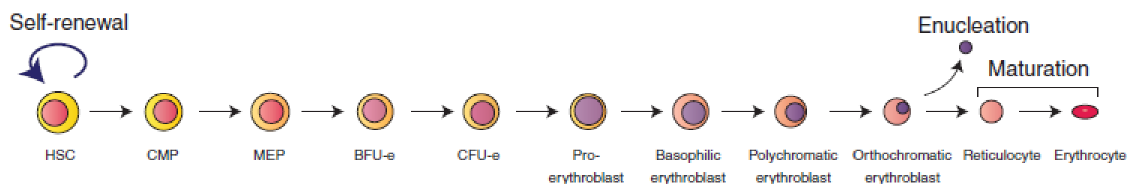

Figure 1 - Erythroid differentiation (3).

BFU-e: burst-forming unit erythroid; CFU-e: colony-forming unit erythroid; CMP: common myeloid progenitor; HSC: hematopoietic stem cell; MEP: megakaryocyte-erythroid progenitor.

The first recognizable erythroid precursor in the bone marrow is the pro-erythroblast. This is a large cell with dark blue cytoplasm, central nucleus with nucleoli and slightly clumped chromatin (Figure 1). By cell division, the pro-erythroblasts give rise to smaller cells called erythroblasts that will proliferate and differentiate from basophilic erythroblast to polychromatic erythroblast and finally to orthochromatic erythroblast. They contain progressively more hemoglobin in the cytoplasm, their size decreases and the nuclear chromatin becomes more condensed (Figure 1). In the orthochromatic erythroblasts the nucleus is extruded and the resulting cell is the reticulocyte that contains some ribosomal RNA and is still capable of hemoglobin synthesis. This cell stains pale blue, as some RNA is still present (Figure 1). It is larger than the erythrocyte and stays in the bone marrow before entering the peripheral blood, where it circulates 1-2 days before maturing into erythrocytes. These non-nucleated biconcave cells stain pink and RNA is completely lost (Figure 1). Mature erythrocytes have a diameter of 6-8 μm and their biconcave shape creates a large surface for gas exchange and allows them to circulate through the microcapillaries of the vascular network. One proerythroblast, usually, gives rise to 8-16 mature erythrocytes (2).

Erythropoiesis is regulated by the hormone erythropoietin (EPO), which stimulates the terminal proliferation and differentiation of BFU-e and CFU-e progenitors. EPO activates intracellular signaling by binding to its receptor (EPOR), expressed on the erythroid cell surface. EPO acts on both BFU-e and CFU-e cells, but the last one shows greater sensitivity to EPO (4). BFU-e cells respond to other factors besides EPO, including stem cell factor (SCF), insulin-like growth factor-1 (IGF-1), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and IL-9. Apparently, the first phase of CFU-e erythroid differentiation is highly EPO dependent, decreasing EPO sensitivity as erythroid cells mature (1).

For a normal erythropoiesis some erythropoietic nutrients are needed, like: iron (for the formation of hemoglobin), B12 vitamin and folic acid (for the synthesis of DNA), and aminoacids (for the synthesis of proteins) (2).

Inflammation may disturb erythropoiesis, by reducing erythropoiesis, the life span of erythrocytes and by altering iron metabolism through the iron-modulating peptide, hepcidin. In fact, inflammatory cytokines, particularly IL-6 and IL-1, that are increased in inflammatory states, lead to increased production of hepcidin that will reduce the absorption of iron from the gut and the release of iron from macrophages that is needed for hemoglobin synthesis

(4). Tumor necrosis factor alpha (TNF-alpha) and interferon-gamma (IFN-γ), also increased in inflammatory conditions, are known to inhibit erythropoiesis. Moreover, the activation of inflammatory cells by releasing reactive oxygen species, contribute to the development of oxidative stress which might induce oxidative damages to RBCs, favoring its removal.

1.1.1 – Erythropoietin

Erythropoietin is a 30.4 κDa glycoprotein with four carbohydrate residues. In fetal and neonatal life the hepatocyte is the prime source of EPO and this capacity continues in adult life, synthesizing, however, only a small amount of EPO; indeed, the major source of EPO in the adult comes from the kidney. In experimental animals, the site of EPO production is also the kidney. EPO is produced by specialized peritubular cells, in the proximal tubular region. The gene responsible for the expression of EPO has been demonstrated in the kidney and in various extrarenal locations (2, 4).

The regulation of erythropoiesis is achieved by an oxygen-sensing system. Actually, the EPO gene is under the regulation of the hypoxia inducible factor 1 alpha (HIF-1α), which is activated during hypoxia. When the oxygen tension is reduced in the kidney, the α-subunits of HIF-1 accumulate and link with HIF-1β (hypoxia inducible factor 1 beta) forming heterodimers that activate the hypoxia-response genes, namely the EPO gene. Androgenic steroids, anabolic steroids and cobaltous chloride also stimulate EPO production by an unknown mechanism (4).

Erythropoietin interacts with EPO receptor (EPOR), which is located on the surface of erythroid cells in the marrow, promoting the production of erythrocytes by different transduction pathways. The first erythroid cells presenting EPOR are the later BFU-e cells. The EPOR are not expressed in the last cells of the maturation process, the reticulocytes and mature erythrocytes. EPOR are expressed, therefore, on the erythropoietin responsive cells (ERC), which are: later BFU-e, the CFU-e and proerythroblasts (1, 4).

EPOR contains two polypeptide chains on its extracellular component. Binding of Epo to its receptor induces receptor homodimerization and autophosphorylation of the receptor-associated Janus kinase 2 (JAK2). Activated JAK2 mediates the phosphorylation of key tyrosine residues on the distal cytoplasmic region of EPOR, which then serve as docking sites for downstream effectors, including signal transducer and activation of transcription protein 5 (STAT5) and phosphatidylinositol 3 kinase (PI3 kinase). Activated STAT5 homodimerizes and translocates to the nucleus. This transmission of the signal to the nucleus prevents apoptosis and allows the cell maturation into erythrocytes. Another effect

of EPO is to increase the size of ERC population (1, 4). Other intracellular signal transduction pathways are the mitogen-activated protein (MAP) kinase, the phosphatidylinositol 3 (PI3) kinase and the phosphoinositide-specific phospholipase Cγ (PLCγ) pathways (Figure 2).

Accumulating evidence indicates that all the hematopoietic growth factors and cytokines are able to activate simultaneously all the major signal transduction pathways. The JAK/STAT pathway is implicated in IL-3, IL-6, EPO and GM-CSF signaling (5), the MAP kinase pathway in EPO, GM-CSF, G-CSF and IL-3 signaling (6), and the PI-3 kinase pathway in EPO, IL-6, GM-CSF, G-CSF and M-CSF signaling (7).

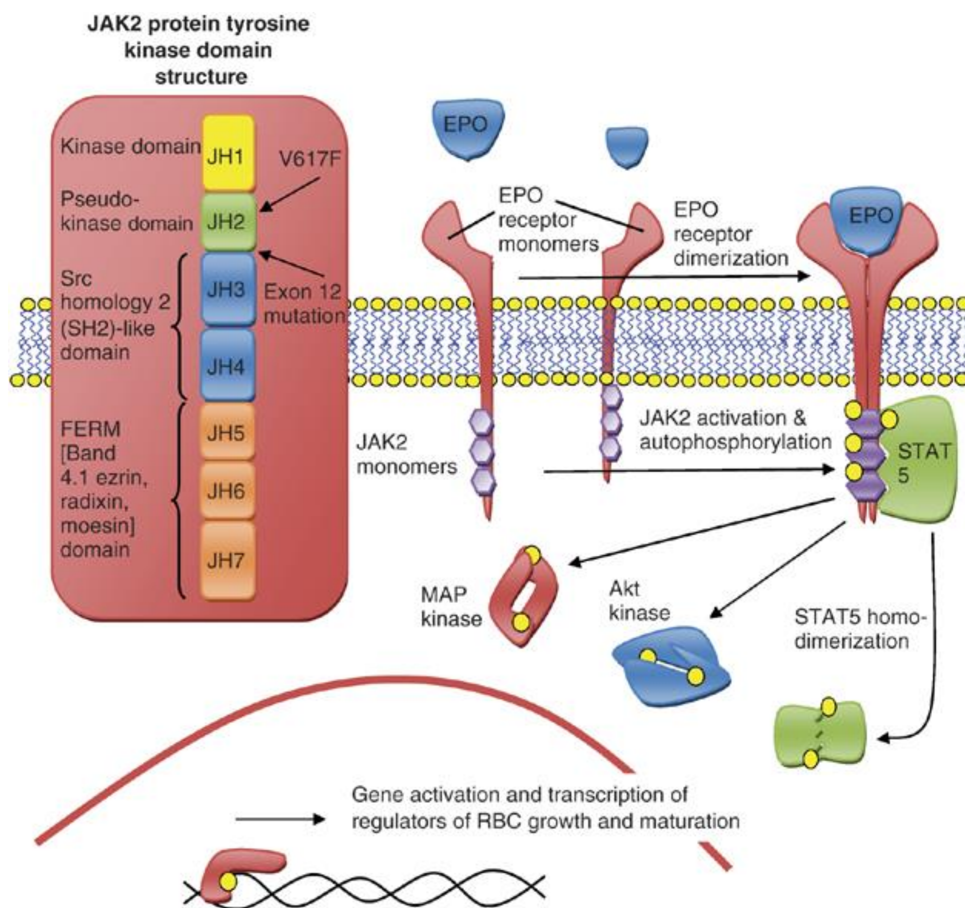

Figure 2 - Erythropoietin receptor signaling via Jak/Stat pathway (8).

Akt: V-akt murine thymoma viral oncogene homolog 1; EPO: erythropoietin; JAK-2: Janus kinase 2; JH: Jack homology; MAP: mitogen-activated protein; RBC – red blood cell; STAT - signal transducer and activation of transcription protein.

1.2 – Iron metabolism

Iron is needed for HGB production, at the later stages of erythroid differentiation and maturation. To produce 200 billion of new erythrocytes every day, 20-25mg of iron are required. From a normal diet providing 10mg of iron daily, only 1mg is absorbed (~5-10% of dietary iron); the major source of iron comes from recycling iron from senescent erythrocytes, which are phagocytized by reticuloendothelial macrophages (9). The iron from diet only covers the real loss of iron that represents about 1-2mg/day (Figure 3) (10).

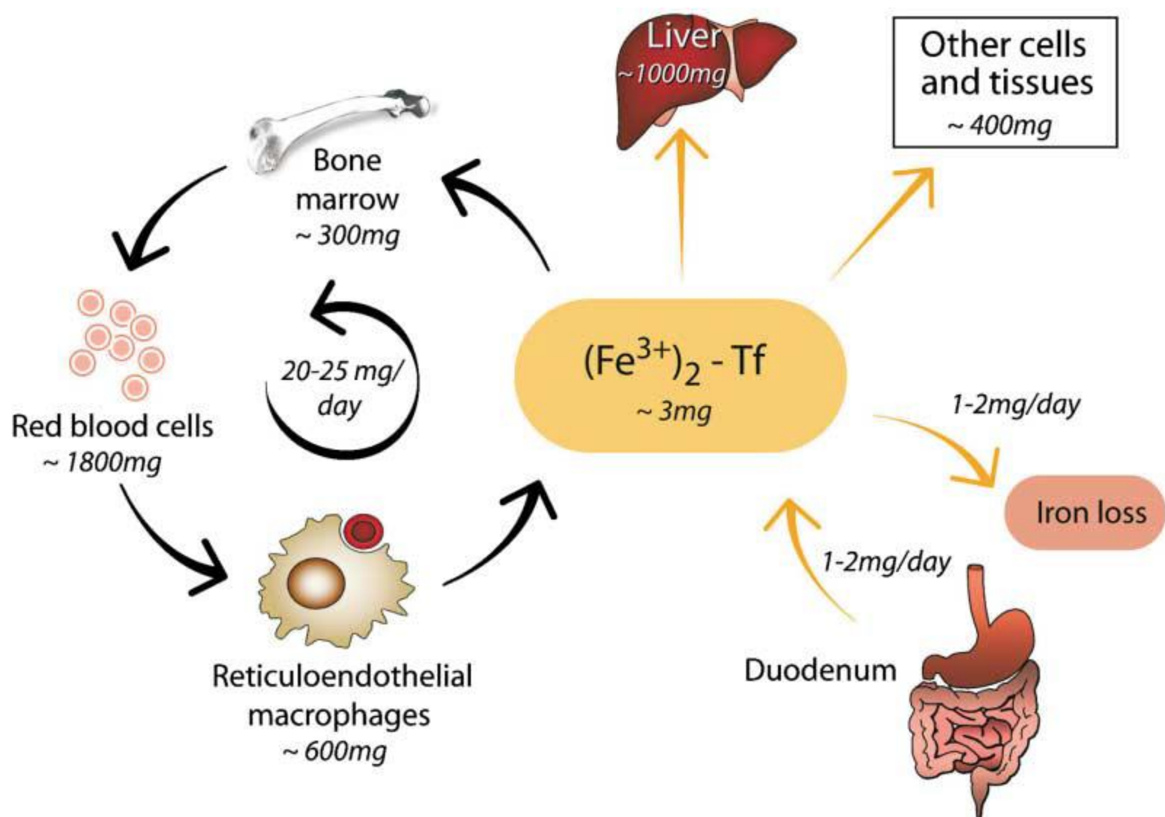

Figure 3 - Iron homeostasis and major pathways of iron traffic (approximate values) (10).

Fe^{3+}: ferric iron; TF: transferrin.

Iron enters the system in three forms: ferrous (Fe^{2+}), ferric (Fe^{3+}) and heme. It is absorbed in the proximal duodenum by enterocytes. Fe^{3+} is reduced to Fe^{2+} by the mucosal ferrireductase duodenal cytochrome b (Dcytb), which is present in the apical surface of the duodenum cells; Fe^{2+} enters the enterocytes by a facilitated transport mechanism, mediated by the divalent metal transporter 1 (DMT1) (Figure 4). Protein and mRNA levels of Dcytb are elevated in iron deficiency and hypoxia, favoring iron absorption, and are decreased in

iron overload. Protein and DMT1 mRNA levels are decreased when cellular iron is increased, and decrease when cells are iron deficient. This regulation is achieved by binding iron response proteins (IRPs) to the iron responsive element (IRE) within the 3 prime untranslated region (3'-UTR) of the C-terminal end of DMT1, protecting the mRNA from endocytic cleavage, increasing mRNA half-life and consequently increasing the amount of translated protein (10, 11).

Heme iron enters directly into enterocytes as an intact iron-protoporphyrin complex, possibly by endocytosis or by binding to a haem receptor on the brush border of enterocytes, the haem carrier protein 1 (HCP1) , that was recently described. Inside the cell, it is most likely to be cleaved by the intracellular microsomal enzyme, haem-oxygenase 1 (HO1), releasing ferrous iron and forming biliverdin (Figure 4) (10).

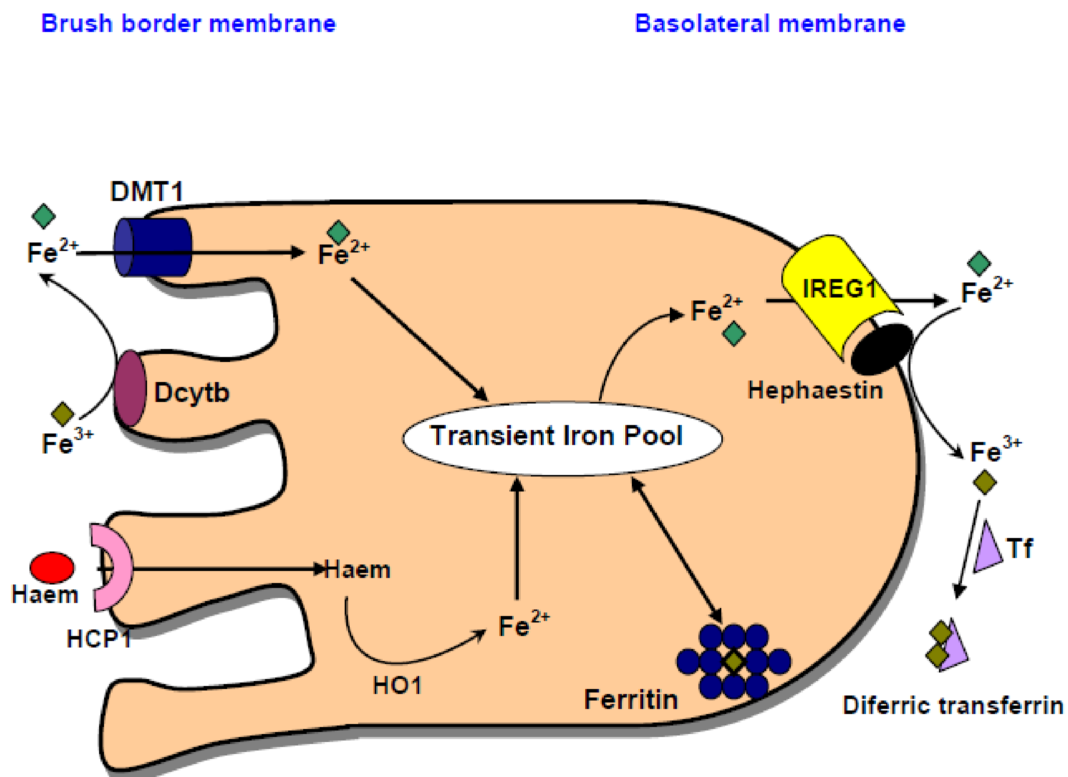

Figure 4 - Absorption of iron through enterocytes (12).

Dcytb - duodenal cytochrome b; DMT1 - divalent metal transporter 1; HCP1 - haem carrier protein 1; HO1 - haem oxygenase-1; IREG1 - Iron-regulated transporter-1; Tf – transferrin.

Inside the enterocytes the iron can be stored as ferritin or transferred across the basolateral membrane of the enterocyte to the bloodstream. Ferritin is a spherical protein ubiquitously expressed and its main function is to store and sequester excess iron in a soluble, non-toxic form. There are two forms of this shell form protein with 24 subunits, light (L) and heavy (H) chains with a central cavity able to store up to 4500 iron atoms in the form of hydrous ferric oxide phosphate. The two chains have different properties, while L-chains are important for core iron binding, H-chains confer ferroxidase activity needed for iron incorporation. Ferritin also have an IRE in the 5' UTR of its mRNA; in case of iron deficiency, IPRs bind to IRE and block translation, increasing cellular iron availability (10). Because of its capacity to sequester iron, ferritin has also an anti-oxidant function. It is regulated by cytokines, like IL-6, IL-1 and IFN- ɣ (13, 14).

Ferroportin (FPN1), also known as iron-regulated transporter-1 (IREG1), is a tubular transmembrane protein expressed in the basolateral membrane of the duodenal enterocyte that mediates iron efflux to the circulation. FPN1 is also found in the liver, spleen and reticuloendothelial macrophages. FPN1 mRNA also contains an IRE in its 5' UTR, and, it is also controlled by iron; duodenal FPN1 mRNA and its protein expression are decreased in iron overload and increased in iron deficiency. FPN1 is also influenced by inflammation and is post-transcriptionally regulated by hepcidin, which binds to FPN1 inducing its internalization and lysosomal degradation (10, 15, 16).

Transferrin (TF), a 80kDa monomeric glycoprotein mainly produced by the liver, transports iron between the locals where iron is absorbed and the local of storage and usage. It contains two globular domains, each one with a high affinity binding site to one molecule of Fe^{3+}. This affinity is dependent on pH; iron is released from TF at pH lower than 6.5, while at the physiological pH 7.4, iron is strongly bound to TF. This plays an important role on the mechanism of iron release from TF (10).

Transferrin has also the ability to bind to other divalent metals, but with less affinity (copper, cobalt, manganese and cadmium). A requisite for iron to bind to TF is a simultaneous binding of a bicarbonate or carbonate to arginine and threonine residues. In circulation TF can be free of iron (apotransferrin) or to be linked to two molecules of iron (diferric transferrin).

TF transporting iron interacts with transferrin receptor (TFR) to transport iron into the cell, where DMT1 facilitates the release of iron from TF and its reduction from Fe^{3+} to Fe^{2+} by STEAP3, a ferrireductase; then iron is transported by mitoferrin, to the mitochondria, where heme is synthetized. Iron can also be stored as ferritin (Figure 5) (17).

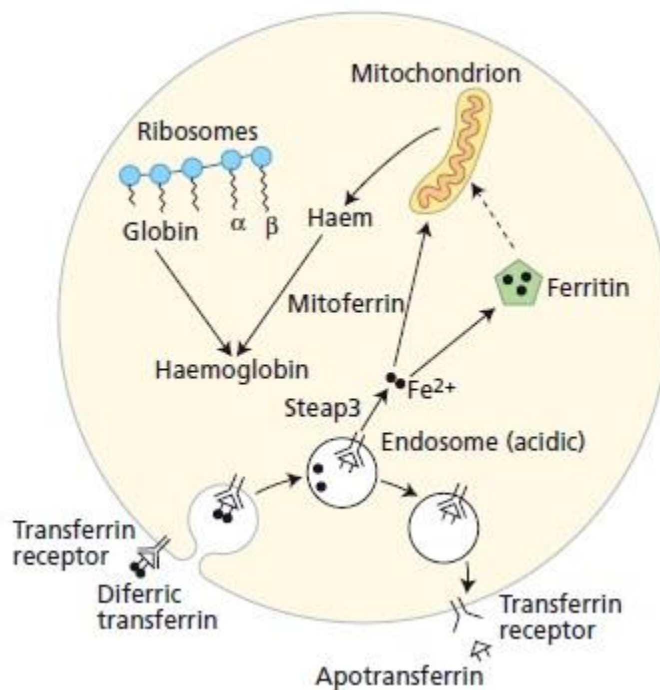

Figure 5 - Incorporation of iron in developing red cells (17).

TFR1 has its highest expression in placenta, erythroid marrow cells and liver, but is expressed in most cells. It is a disulfide-linked homodimeric glycoprotein of two 90 kDa subunits and is able to bind two TF molecules. Once again the pH dependent affinity of TFR1 to TF is an important key to the release of iron in the cells. At the physiological pH 7.4, the affinity of TFR1 to diferric TF is about 2000 times higher than the affinity of TFR1 to apotransferrin; once inside the cell, at the endosomal pH of 5.5, the affinity of TFR1 to apotransferrin is higher than the affinity to diferric TF, favoring the release of iron from TF (10).

Transferrin is up-regulated by iron deficiency and down-regulated by iron overload, and is also influenced by hydrogen peroxide and nitric oxide generated in oxidative stress conditions through iron regulatory protein (IRP).

Accumulation of iron in the body can cause serious damages. Considering that, in normal conditions, only 20 - 25 mg of iron is required for erythropoiesis, the regulation of iron that is transported from the enterocyte into the blood is very important. If too much iron is transported by TF1, its receptor, TFR1, becomes saturated and iron can be deposited in other tissues, like hepatocytes and cardiac myocytes, leading to serious problems (10).

HFE (hemochromatosis) gene located in chromosome 6 encodes for an atypical major histocompability class I protein that heterodimerizes with β-2 microglobulin. Mutations in this gene affect HFE function and can lead to iron overload. The normal function of HFE is not well established but it is known that it forms a high-affinity complex with TFR1 at the enterocytes. TFR1 sequesters HFE to silence its activity. Diferric transferrin competes with HFE for the binding to TFR1 and, in this way, when serum transferrin saturation increases occurs dissociation of HFE. (10).

Another receptor for transferrin can be found highly expressed in the liver, the TFR2. It is not regulated by iron since it does not have any IRE's in its UTR, but its expression is decreased in iron deficiency and increased in iron overload. It can also bind to iron bound to transferrin in a pH dependent manner but with a lower affinity than TFR1 (10, 18).

1.2.1 – Hepcidin

Hepcidin is a 25-amino acid peptide that derives from a precursor pre-prohepcidin of 84 amino acids that undergoes two enzymatic cleavages. The human hepcidin gene is called HAMP and is located in the chromosome 19 (19). Hepcidin was first identified in 2000, in urine and plasma.

It has an important role in the regulation of iron absorption in the gastrointestinal tract and in iron availability from macrophages of the reticuloendothelial system (RES). Its structure is a simple hairpin with 8 cysteines that form 4 disulfide bonds in a ladder-like configuration. It is predominantly expressed in the liver but it can be expressed also in other tissues, such as in the heart, kidney, adipose tissue, pancreas and hematopoietic cells (20). Hepcidin controls iron availability by binding to ferroportin, causing its degradation and removal from the surface of cells, thus preventing iron from leaving the cells to bloodstream. Besides the role on controlling iron absorption in the gastrointestinal tract it also controls iron availability from the reticuloendothelial system, binding to FPN1 present on the membranes of the macrophages (21).

Hepcidin is also a type II acute-phase protein, with some importance in immunity.

Hepcidin synthesis is increased in response to raised serum iron, iron overload and inflammation, and is decreased by iron deficiency, hypoxia and increased erythropoietic activity (Figure 6). In normal conditions the expression of hepcidin depends on signaling through the BMP/SMAD pathway. Hemojuvelin (HJV) bound to the cell membrane

participates as a BMP6 (Bone morphogenic protein 6) coreceptor in the pathway regulating hepcidin expression, whereas soluble HJV antagonizes BMP6, which is the master hepcidin activator *in vivo*. The binding of HJV/BMP6 phosphorylates SMADs to form an SMAD-1/-5/-8-SMAD-4 complex that translocates to the nucleus activating the promoter for hepcidin production. Mutations in the genes encoding for these molecules involved in hepcidin expression may lead to low serum hepcidin levels that, ultimately, will give rise to haemochromatosis (17).

It was shown, in mice, that oral administration of iron increases urinary hepcidin excretion, in contrast with induced iron deficiency by phlebotomy that reduces hepcidin expression (22).

Inflammation controls hepcidin expression through IL-6 and IL-1β by activating STAT3 and its binding to a regulatory element in the hepcidin promoter. In humans, recombinant IL-6 infusion triggered, in just two hours, a significant increase in urinary hepcidin concentration and a decrease in serum iron levels (Figure 6) (17).

In ineffective erythropoiesis, increased erythroid precursors secrete growth differentiation factor 15 (GDF-15) and twisted gastrulation protein homolog 1 (TWSG1) which inhibit hepcidin production (Figure 6). Hypoxia inducible factors, that stimulate erythropoietin production, control iron homeostasis by downregulation of hepcidin, repressing its promoter with the upregulation of ferroportin (Figure 6) (17).

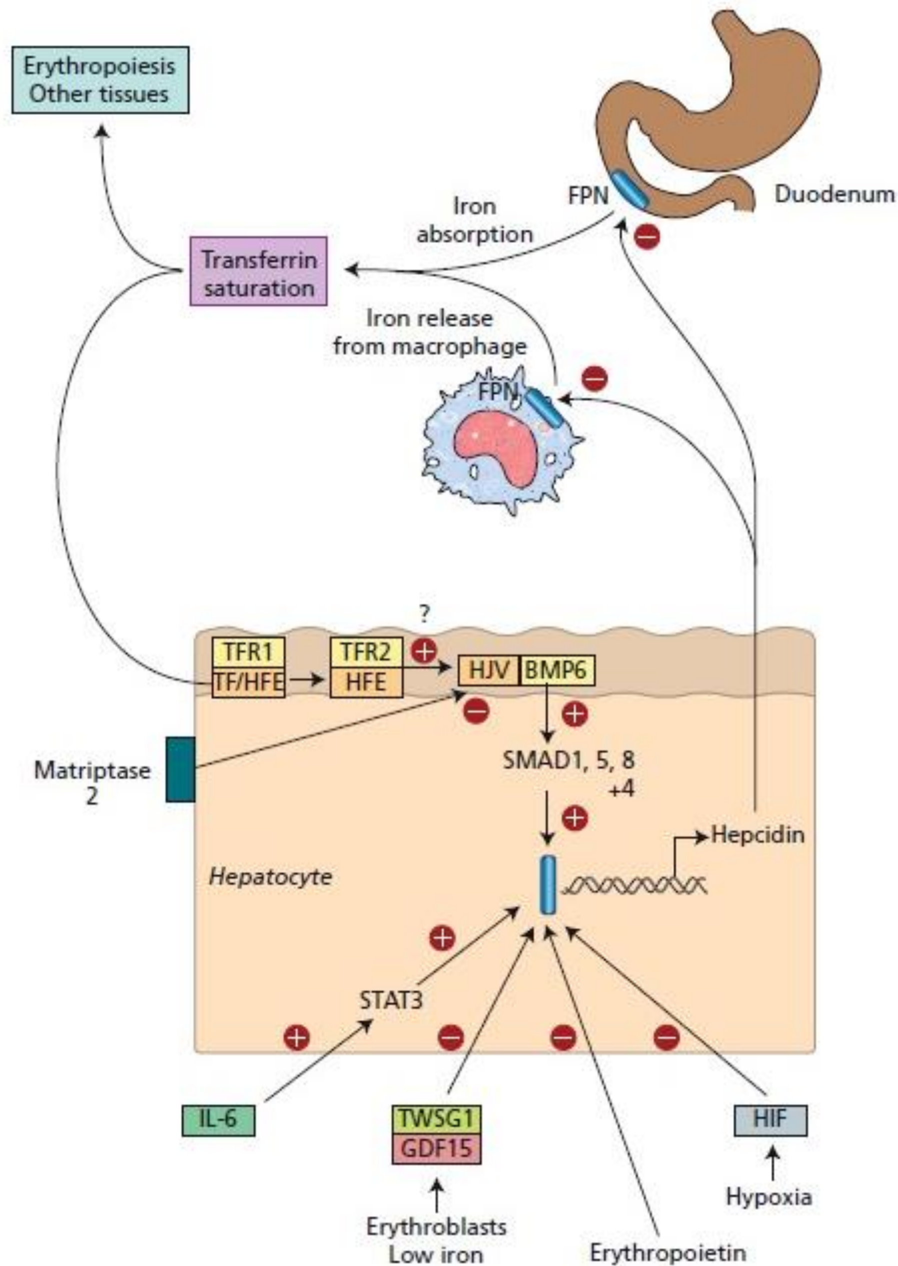

Figure 6 - Stimulatory and inhibitory signals of hepcidin regulation (17).

BMP: bone morphogenic protein; FPN: ferroportin; GDF: growth differentiation factor; HFE: hemochromatosis; HIF: hypoxia inducible factor; HJV: hemojuvelin; IL: interleukin; STAT: signal transducer and activator of transcription; TF: trasnferrin; TFR: transferrin receptor; TWSG: twisted gastrulation protein.

1.3 – Adipose tissue

The adipose tissue, previously considered as a fat storage only, is now recognized as an active endocrine organ that contributes to metabolic control and body homeostasis (23, 24). In fact, adipose tissue is the energetic storage of our body and is regulated by the nervous system, nutrients and hormones, either by autocrine or paracrine fashion that can influence other organs and metabolisms (25).

There are two kinds of adipose tissue, brown and white. Brown adipose tissue is principally found in neonates. There are some differences between cells from white adipose tissue and brown adipose tissue (24, 25).

White adipocytes are spherical cells with a diameter from a minimum of 30 to 40 μm to a maximum of 150 to 160 μm (lean - subcutaneous adipose tissue) and from a minimum of 20 to 30 μm to a maximum of 90 to 100 μm (lean - visceral adipose tissue). Most of the cytoplasm is occupied by the lipid droplet and only a thin rim of cytoplasm is visible, occupied by an elongated mitochondria, Golgi complex, rough and smooth endoplasmic reticulum, vesicles and other organelles (Figure 7) (25).

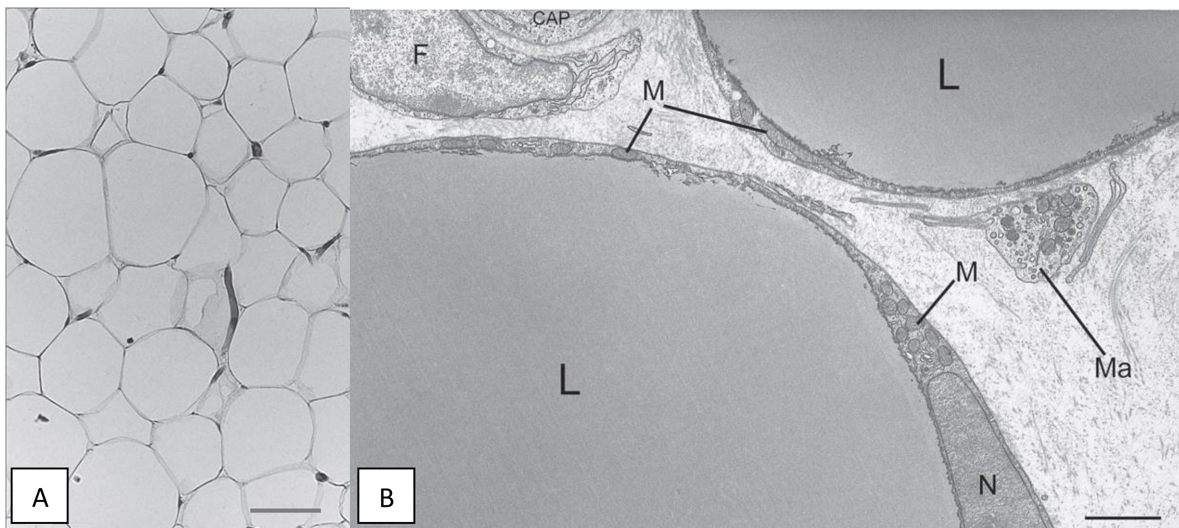

Figure 7 - White adipose tissue. (A) Optical microscopy of subcutaneous adipose tissue of human white adipose tissue (formaldehyde-fixed and paraffin-embedded). Bar = 30 μm. (B) Electron microscopy of murin white adipose tissue. Bar=2 μm (25).

CAP: capillary lumen; F: fibroblast; L: lipid droplet; M: mitochondria; Ma: macrophage; N: nucleus.

Brown adipocytes present numerous small vacuoles. They have a polygonal or ellipsoid shape with a diameter from a minimum of 15 to 20 μm to a maximum of 40 to 50 μm. Whereas white adipocytes only have one elongated mitochondria, brown adipocytes have numerous, big and rich mitochondria in transverse cristae. The other organelles are also present in these cells. Brown adipocytes are joined by gap junctions. (Figure 8) (23, 25).

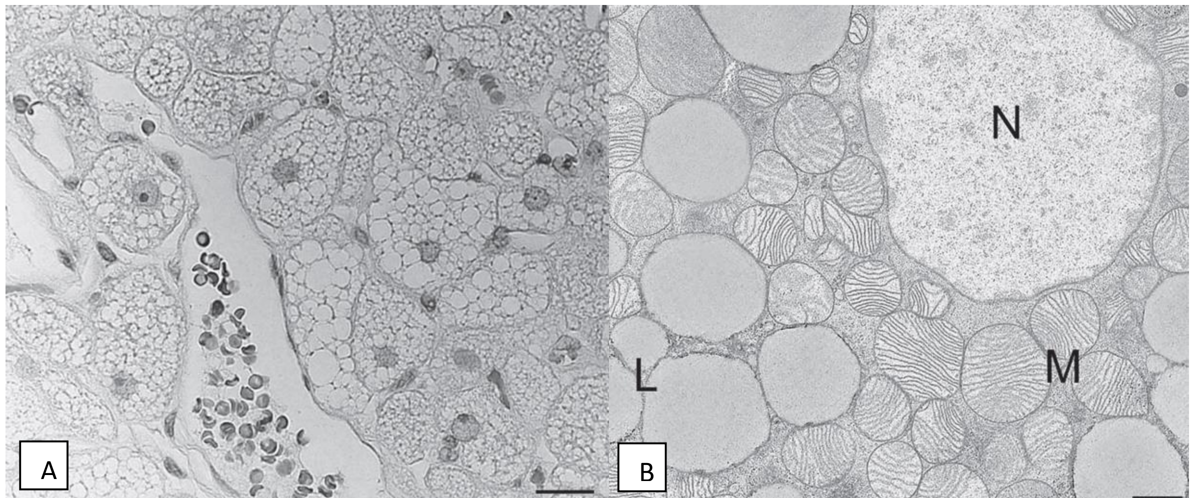

Figure 8 - Brown adipose tissue. (A) Optical microscopy of human brown adipose tissue. Bar = 15 μm (B) Electron microscopy of mouse brown adipose tissue. Bar = 1 μm (25).

L: lipid droplets; M: mitochondria; N: nucleus.

The predominant form of adipocytes found in adults is the white adipose tissue (WAT). Different types of cells are present in the WAT, namely, preadipocytes, adipocytes, macrophages, endothelial cells, fibroblasts and leukocytes. Due to this multifactorial composition, the WAT is an important mediator of several metabolic pathways and of inflammation. In the last years, the production of several adipokines by the adipose tissue has been reported, including: adiponectin, resistin, visfatin, apelin, vaspin, hepcidin, TNF-α, chemerin, omentin, monocyte chemoattractant protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1), among others. The different distribution of fat depots in the body give rise to different roles, by secreting different sets of adipokines (Figure 9) (24).

Adipose tissue depots are classified as visceral and subcutaneous according to its localization. In small mammals there are two principal subcutaneous depots, anterior and posterior, and several visceral depots, located inside the thorax (mediastinic) and abdomen (omental, mesenteric, perirenal, retroperitoneal, parametrial, periovaric, epididymal, perivescical). These two types of adipose tissue differ on the type of fat cells, endocrine function, lipolytic activity and response to insulin. The visceral adipose tissue is

metabolically more active than the subcutaneous adipose tissue, as it produces more adipokines and has more macrophages (25, 26).

Visceral fat venous blood is drained directly to the liver through the portal vein; the anatomical proximity provides a direct hepatic access to free fatty acids (FFAs) and adipokines. These adipokines activate hepatic immune mechanisms with production of inflammatory mediators. The subcutaneous fat venous drainage is performed through systemic veins. Visceral adipose tissue is also more vascularized and more heavily innervated than subcutaneous adipose tissue (26).

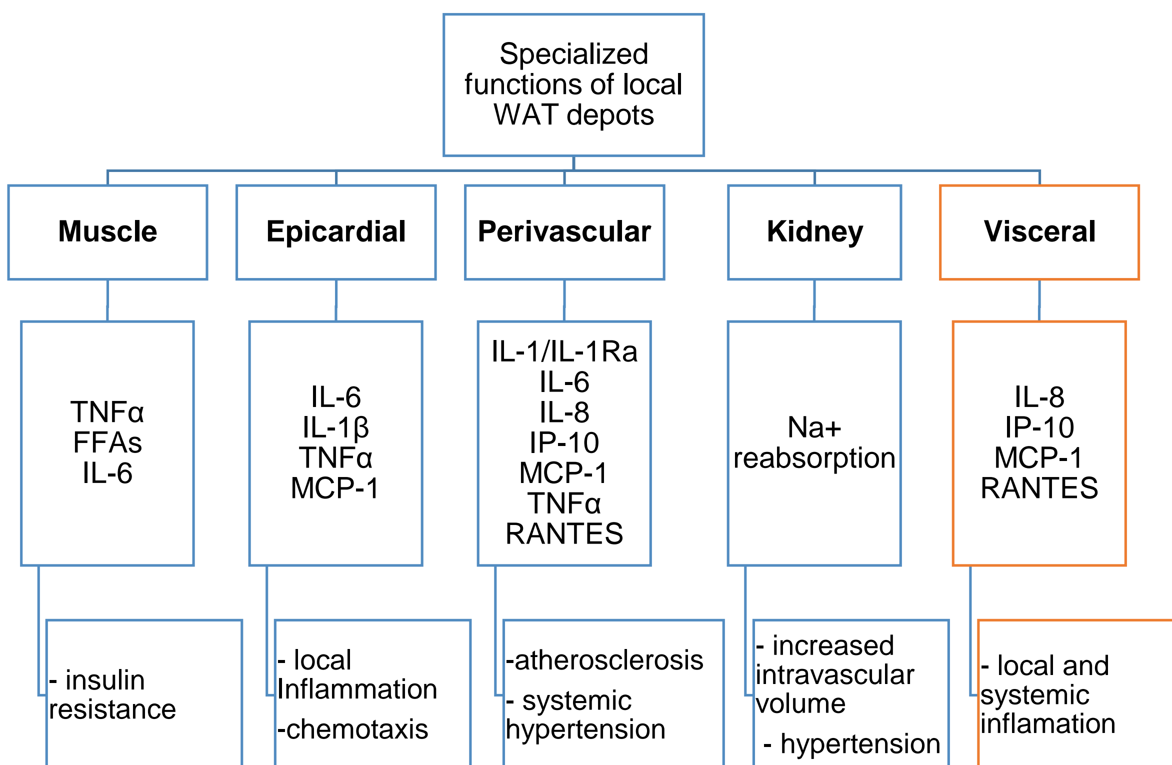

Figure 9 - The multiple adipokines produced in white adipose tissue in the different fat depots in the body (3).

FFAs: free fatty acids; IL: interleukin; IL-1Ra: interleukin 1 receptor antagonist; IP-10: interferon-inducible protein-10; MCP-1: monocyte chemoattractant protein-1; RANTES: regulated on activation, normal T expressed and secreted; TNFα: tumor necrosis factor alpha; WAT: white adipose tissue.

Adipocytes are storage depots of energy, in the form of triglycerides (TGs) droplets. New adipocytes avidly absorb FFAs and TGs in the postprandial period; however, once they grow larger they become dysfunctional. This larger dysfunctional adipocytes are more

abundant in visceral adipose tissue (VAT). The smaller adipocytes, which are more abundant in subcutaneous adipose tissue (SCAT), have more avidity for TGs and FFAs uptake, preventing their deposition in non-adipose tissue (26).

Adipocytes are provided with several receptors that can be activated by three types of signals: chemical signals from circulating hormones, chemical signals from adipokines and nervous signals in the form of nerve impulses, activating specific adrenergic receptors in fat tissue. They synthesize also, as referred before, a large number of adipokines under the form of peptides, proteins and cytokines and over 50 adipokines were already identified (26-28).

Mature adipocytes act like an endocrine organ and are able to influence the appetite, energy balance, immunity, insulin sensitivity, angiogenesis, blood pressure, lipid metabolism and homeostasis, by producing several adipokines (25).

Leptin is responsible for the control of adipose tissue growth. It controls food intake by reducing appetite, body weight and fat stores. It can influence immunity by enhancing T-helper (Th)1 immune responses, suppressing Th2 immune responses and by increasing macrophage activity, leading to production of different cytokines, like TNF-α and IL-6. Leptin can also interfere with the antioxidant balance, by increasing the production of reactive oxygen-species (ROS) and nitric oxide-synthase (NOS). A reduction in leptin levels leads to severe insulin resistance and vascular dysfunction. In obesity, leptin seems to be raised, but presents a reduced activity that could be explained by the development of resistance to its functions (23, 27).

Chemerin has a crucial role in adipocyte differentiation and development. It appears to act as modulator of the expression of genes involving glucose and lipid homeostasis in adipocytes. It has been also associated with several markers of metabolic syndrome, like increased body mass index (BMI), triglycerides, blood pressure and insulin resistance (27).

Visfatin contributes to leukocyte adhesion and to atherogenic plaque instability, and appears to have a direct role in vascular dysfunction and inflammation through NOS upregulation. Visfatin, mainly produced by visceral adipose tissue, is increased in subjects with obesity, type 2 diabetes mellitus, metabolic syndrome and cardiovascular disease (27).

Retinol-binding protein-4 (RBP-4) and lipocalin-2, two adipokines that belong to the lipocalin family, contribute to insulin resistance and atherosclerosis. RBP-4 is also increased in obesity and in individuals with type 2 diabetes mellitus. The gene and protein expression of lipocalin-2 in visceral adipose tissue is positively associated with inflammation (27).

Plasminogen activator inhibitor 1 (PAI-1) is considered a cardiovascular risk factor, as it decreases fibrinolytic capacity. PAI-1 and also serum amyloid A (SAA) are increased in obesity and associated with systemic inflammation and atherosclerosis (27).

Angiotensinogen plays a major role on the regulation of blood pressure, through salt reabsorption in renal tubules, and on vasoconstriction. It is produced in adipose tissue, and is thought to be responsible for systemic hypertension in obese individuals (27).

Apelin is an adipokine with a positive effect on glucose metabolism and seems to be linked to insulin secretion. However, its plasma concentration is raised in obese, hyperinsulinemic and type 2 diabetes mellitus individuals (23, 27).

Increased levels of resistin are associated with atherosclerosis and it has been reported that it increases the expression of several pro-inflammatory cytokines, like TNF-α, IL-1, IL-6 and IL-12 through a nuclear factor kappa-light-chain-enhancer of activated B cells-dependent pathway (NF-kB) (23, 27).

The production of IL-6 by the adipose tissue, in the absence of inflammation, is about 15-30% of circulating IL-6. It impairs insulin stimulation, associates with obesity-related hypertriglyceridemia, by stimulating hepatic secretion of very low-density lipoproteins and induces liver production of C reactive protein (CRP) (26, 27).

Adiponectin seems to have a protective role against metabolic diseases and against atherosclerosis. It has anti-inflammatory activity and a protective role against insulin resistance. It inhibits the expression of adhesion molecules and vascular smooth muscle cells proliferation and suppresses transformation of macrophages to foam cells. It is decreased in patients with abdominal obesity. It induces the production of important anti-inflammatory factors, such as IL-10 (23, 26, 27).

Omentin appears to have anti-inflammatory ability by inhibition of NF-kB, reducing endothelial dysfunction and atherosclerosis, and also seems to have a role in modulating insulin action. The levels of omentin are decreased in obese individuals (23, 27).

Increased expression of vaspin, a derived serine protease inhibitor from visceral adipose tissue, is associated with fat mass expansion and decreases insulin resistance (23, 27).

TNF-α, plays an important role in inflammatory cell activation and recruitment, and has been linked to insulin resistance in skeletal muscle and adipose tissue, and it seems to favor atherosclerosis through inducing adhesion molecules expression in endothelial and

vascular smooth muscle cells. It also induces activation of NF-kB factor that regulates an array of genes associated with inflammation. Obese individuals have higher circulating levels of TNF-α than lean individuals (23, 26, 27, 29).

1.4. – Modulation of erythropoiesis

A relationship is believed to exist between adipose tissue and erythropoiesis. An increase in body mass should trigger a stimulus of erythropoiesis, increasing the number of erythrocytes to face the higher oxygen requirements. However, apparently because obesity is associated with a low-grade inflammatory state, this is not always observed. This condition has been named as the “paradox of obesity”, and suggests an undergoing erythropoietic disturbance.

As a consequence of the underlying chronic inflammation in obesity, the production of pro-inflammatory adipokines that stimulate hepatic hepcidin production seems to influence erythropoiesis (Figure 10). The adipose tissue produces IL-6 that directly stimulates hepatic hepcidin production. Indeed, it was found that IL-6 is sufficient for the induction of hepcidin during inflammation and that the IL-6-hepcidin axis is responsible for the hypoferremia associated with inflammation (15). In a similar way, leptin is capable of inducing hepatic hepcidin expression via Jak2/STAT3 signaling pathway (30, 31). Moreover, TNF-α, a pro-inflammatory cytokine also produced by adipocytes, is known to inhibit erythropoiesis, through inhibition of CFU-e colony formation (Figure 10). Its blood levels are elevated in patients with anemia of chronic diseases, and it has been reported that exposure to TNF-α produces a similar anemia in either humans or mice, further suggesting that TNF-α is implicated in the pathogenesis of this anemia (32). Moreover, TNF-α stimulates the production of IL-6, triggering the acute phase response that leads to the up-regulation of several hepatic inflammatory mediators, such as hepcidin.

Nowadays, it is known that adipocytes are also capable of producing hepcidin (33). However, it remains the doubt if this production influences the circulating levels of hepcidin (Figure 10).

Hepcidin, by diminishing iron absorption and mobilization, will directly influence erythropoiesis. It is down-regulated in case of anemia, low iron, hypoxia and high levels of erythropoietin, showing a reduction in hepcidin serum and mRNA levels (34, 35).

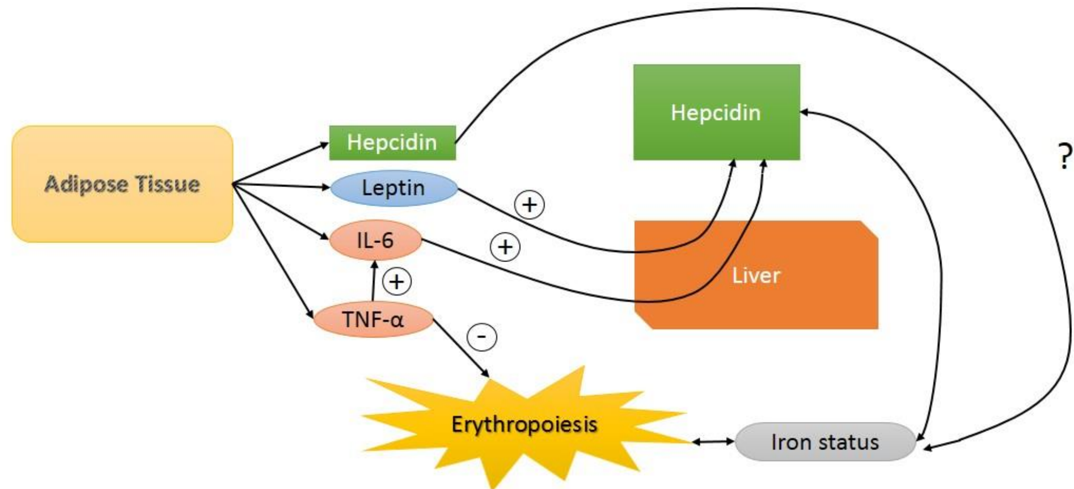

Figure 10 - Contribution of adipose tissue to modulation of erythropoiesis.

IL-6: interleukin 6; TNF: tumor necrosis factor alpha; “+”: induce; “-“: suppress; “?”: doubt.

An association between obesity and hypoferremia has been reported (36, 37), however, the mechanisms linking them are still poorly clarified. The most accepted hypothesis is that iron deficiency is a consequence of the chronic low-grade inflammation state in obesity (37). As already referred, besides the production of different regulators of hepatic hepcidin secretion, such as IL-6 and leptin (19), adipocytes are capable of producing hepcidin, affecting directly iron status and inhibiting erythropoiesis (36). If this production only becomes relevant in obesity is still a doubt. Thus, there are still controversies concerning the contribution of adipocytes to the circulating levels of hepcidin and its linkage to erythropoietic disturbances. Besides, the exact mechanism that regulates hepcidin expression by the adipose tissue is poorly clarified; it remains the question if hepcidin synthesis is stimulated by inflammation, hypoxia or by iron levels, and in the last case, which proteins are involved (Figure 11).

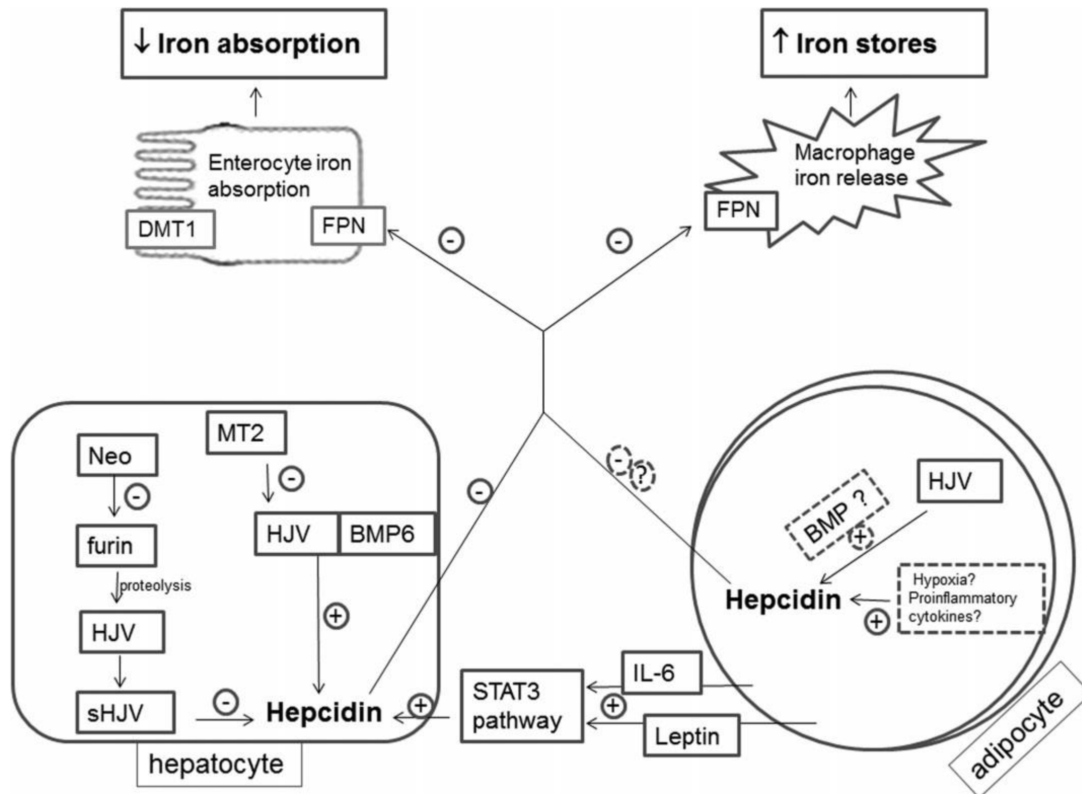

Figure 11 - Regulation of hepcidin expression by the adipose tissue (37).

BMP: bone morphogenic protein; DMT1: divalent metal transporter 1; FPN: ferroportin; HJV: hemojuvelin; IL: interleukin; MT2: matriptase 2; Neo: neogenin; sHJV: soluble hemojuvelin; STAT: signal transducer and activator of transcription; "+": induce; "-": suppress; "?": doubt.

Besides the hematopoietic effects, EPO has several pleiotropic actions, namely, cytoprotection, antiapoptotic, anti-inflammatory and angiogenic capacities, but high concentrations of EPO are needed to exert them (38). Chronic administration of EPO, in rats, in a dose that was insufficient to increase hematocrit, was shown to improve survival, repair endothelial damage and preserve renal function (39), suggesting that there is a different dose-response relationship for erythropoietic and for pleiotropic effects.

As reported by some authors, EPO supplementation can increase energy expenditure and reduce food intake and fat mass accumulation (40). This raises the question, if this reduction in fat mass, induced by EPO, influences the expression of hepcidin and of other proteins and adipokines produced by the adipocytes.

EPO seems to reduce the production of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β via STAT3/5 signaling. Its actions towards macrophages seems to inhibit

NF-kB that is a crucial activator for many pro-inflammatory cytokines, namely, TNF- α, IL-1β, IL-6, IL-12 and IL-23 (38, 41). This EPO action may, therefore, reduce the inflammation due to macrophage infiltration in the adipose tissue.

Chapter 2 – Objectives and work plan

The aim of this study was to clarify the role of subcutaneous and visceral adipose tissue in iron metabolism and in erythropoiesis, by using lean, adult, normotensive rats under an erythropoietic stimuli along 6 weeks. We induced an erythropoietic stimuli, by administering two high doses of rHuEPO (recombinant human erythropoietin) during the last 3 weeks of the protocol.

The rats were randomly divided in 3 groups – control, rHuEPO200 and rHuEPO600. Hematological and biochemical studies including markers of renal function, inflammation, iron status and lipid profile, were performed at starting (T0) and at the end of the protocol (T1). To perform genetic studies in the adipose tissue, we have carefully removed subcutaneous and visceral adipose tissue at the end of the study.

We studied the gene expression of erythropoietic and iron regulatory proteins, such as, hepcidin, IL-6, BMP6, EPOR and ferritin, by the visceral and subcutaneous adipose tissue.

We aim to understand the contribution of visceral and subcutaneous adipose tissue to the erythropoietic process.

Chapter 3 – Materials and methods

3.1. – Animals and experimental protocol

Male Wistar rats (Charles River Lab., Inc., Chatillon-sur-Chalaronne, France), with 320-350 g of body weight (BW), were maintained in ventilated cages, in an air conditioned room, subjected to 12 h dark/light cycles and given free access to rat laboratory chow (SAFE-A03, Augy, France) and tap water. Animal experiments were conducted according to the European Communities Council Directives on Animal Care. The experiments received the approval by the Ethics Committee of the Faculty of Medicine from the University of Coimbra.

The rats were randomly divided by groups: 1) control - receiving saline solution by subcutaneous injection; 2) rHuEPO200 – receiving 200 IU/BW/week of rHuEpo (NeoRecormon®, Roche) by subcutaneous injection; 3) rHuEPO600 – receiving 600 IU/BW/week of rHuEpo (NeoRecormon®, Roche) by subcutaneous injection, during 3 weeks. BW was monitored throughout the study and blood pressure (BP) and heart rate (HR) measures were obtained by the tail-cuff method, using a sphygmomanometer (LE5001 Pressure meter, Panlab Havard Apparatus, Barcelona, Spain).

3.1.1. – Sample collection

Blood collection was performed with rats under anesthesia (intraperitoneal) 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 was collected at the begging and at the end of the protocol, by venipuncuture, from the jugular vein, into Vacuette® tubes without anticoagulant (to obtain serum) and with K3EDTA (to obtain plasma) for hematological and biochemical studies. Plasma and serum aliquots were stored at -80ºC until assayed.

At the end of the protocol and after blood collection, the rats were sacrificed by cervical dislocation, and small portions (0.2 g) of subcutaneous and visceral adipose tissue were removed from each rat, immediately immersed in RNAlater® solution (Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA), and stored at 4 °C for 24h; afterwards, the samples were frozen at -20ºC.

3.2. – Hematological studies

The hematological determinations using whole blood K3EDTA were performed on an automatic blood cell counter (HORIBA ABX, Amadora, Portugal), including the concentration of red blood cells (RBC), white blood cell (WBC) and platelets (PLT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), red cell distribution width (RDW), HCT and HGB concentration. Reticulocyte count was measured by microscopic counting on blood smears after vital staining with New methylene blue (reticulocyte stain; Sigma-Aldrich Co. LLC. St Louis, Missouri, USA).

Serum levels of EPO were evaluated by rat specific ELISA kit (MyBioSource, San Diego, CA, USA). All assays were performed according to the manufacturer's recommendations.

3.3. – Biochemical studies

High-sensitive C-reactive protein (CRP) was determined by using a rat.specific Elisa kit from Alpha Diagnostic International (San Antonio, USA).

The following serum markers were analyzed on a Hitachi 717 analyser (Roche Diagnostics Inc., Massachuasetts, USA) using standard methods: creatinine, blood urea nitrogen (BUN) and uric acid, as renal function markers; glucose, serum total cholesterol, high-density lipoprotein cholesterol (chol-HDL), low-density lipoprotein cholesterol (chol-LDL), triglycerides as lipid profile markers. Atherogenic index was given by the equation: $\text{Atherogenic index} = \frac{\text{Total cholesterol (mg\textbackslash dL)}}{\text{Chol}-\text{HDL(mg\textbackslash dL)}}$. Creatine kinase was also analyzed on a Hitachi 717 analyser (Roche Diagnostics Inc., Massachuasetts, USA) using standard methods.

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). Transferrin saturation was determined by the equation: $\text{Transferrin saturation \%} = \frac{\text{Iron (µg\textbackslash dL)} \times 100}{\text{Transferrin (mg\textbackslash dL)} \times 2}$.

IL-6 was determined by rat specific ELISA kit (Invitrogen, Invitrogen Corporation, Camarillo CA).

3.4. – Molecular genetic studies

3.4.1. – RNA extraction by TriPure reagent

All the equipment and materials were prepared to avoid contamination of samples with RNAses. To ensure that, we used diethylpyrocarbonate (DEPC) to treat all the metalic objects and glassware that would be in touch with the samples. The material was cleaned with that detergent and immersed in it for two hours and, finally, heated at 120 ºC for one hour.

RNA extraction from adipose tissue was performed using a total volume of 1 ml TRI Reagent® (Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA) while using a mechanical homogenizer. Total RNA isolation was performed as described by the manufacturer. This process is based on the single step liquid-phase separation developed by Chomczynski and Sacchi (42), by which a Trizol based reagent is added to the tissue and homogenized. The reagent disrupts cells and denatures endogenous nucleases, thus preserving the integrity of RNA in the sample. The second step is to add chloroform to the extract that, after centrifugation, forms three phases: a colorless aqueous (upper) phase, a white interphase and the red organic (lower) phase. RNA is recovered from the upper colorless aqueous phase by precipitation with isopropanol. Afterwards, the RNA is washed with ethanol, resuspended in DEPC treated water, and stored at -80°C until required.

3.4.2. – RNA concentration and purity

Nucleic acids have maximum absorbance at 260 nm and proteins at 280 nm. The RNA concentration was quantified by measuring the absorbance of RNA in water treated with DEPC at 260nm using a spectrophotometer NanoDrop1000 (ThermoScientific, Wilmington, DE, USA). The concentration was calculated using the following formula:

RNA concentration (µg/mL) = A_{260} x dilution x 40.

The purity of the RNA was given by the ratio of A_{260}/A_{280}. A ratio between 1.8 and 2.00 indicates RNA free of contamination of proteins and is generally accepted as “pure”. The RNA samples were kept at -80°C until further analyses.

3.4.3. – cDNA synthesis

The synthesis of first-strand cDNA from the RNA extracted was performed according to the recommended protocol of the GRS RT-PCR Kit (from GRiSP Research Solutions, Porto, Portugal). This kit contains all the required components for the one step RT-PCR (reverse transcriptase polymerase chain reaction) and primer extension. This protocol uses an engineered M-MLV RT (Molony Murine Leukemia Virus Reverse Transcriptase) that is a RNA-dependent DNA polymerase with reduced RNAse H activity and increased thermal stability. This kit also includes an RNAse inhibitor to assure no RNA degradation by contamination. The protocol requires a first short incubation of the template RNA with random hexamer primers (included in the kit) at 70°C and then the final incubation for 60 minutes at 37°C with the necessary components for the reaction (First-strand Buffer, dNTP MIX, RNAse Inhibitor and M-MLV RT).

3.4.4. – Gene expression analysis with qPCR

The gene expression was analyzed by qPCR, performed in the Mini-Opticon Real-Time PCR System (Bio-Rad Laboratories), and data was analyzed with the software CFX Manager™. For the amplification we used the KAPA SYBR® FAST qPCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA) and specific primer pairs (Table 1). The qPCR reactions were performed using the following conditions: enzyme activation at 95ºC for 30 seconds; denaturation at 95ºC for 10 seconds; annealing for 20 seconds (for each pair of primers, the temperature is referred in Table 1). Gene expression was normalized to alpha-tubulin gene, and relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1 - Primers used on qPCR for gene expression analysis.

Primer	**Forward 5' – 3'**	**Reverse 5' – 3'**	**Mt**
Alpha-Tubulin	CACCCGTCTTCAGGGCTTCTTGGTTT	CATTTCACCATCTGGTTGGCTGGCTC	59°
HAMP	AAGGCAAGATGGCACTAAGC	GAAGTTGGTGTCTCGCTTCC	58°
IL-6	ATGTTGTTGACAGCCACTGC	TTTTCTGACAGTGCATCATCG	58°
BMP6	GGTGGAGTACGACAAGGAGTT	GTCACAACCCACAGATTGCTA	59°
EPOR	CTGCGACTATGGATGAAG	GGATGGTGTACTCAAAGC	59°
Ferritin	GCCCTGAAGAACTTTGCCAAAT	TGCAGGAAGATTCGTCCACCT	59°

BMP6: Bone morphogenic protein 6; EPOR: receptor for erythropoietin; IL-6: interleukin 6; HAMP: Hepcidin antimicrobial peptide; Mt: melting temperature.

3.5. – Statistical analysis

We used the Statistical Package for Social Sciences (SPSS, version 22 for windows, Chicago, IL, USA). A P value lower than 0.05 was considered as statistically significant. Comparison between groups were performed using Mann-Whitney U test. Measurements are expressed as mean ± standard error of the mean (SEM). The correlation analysis was performed by calculating the Spearman coefficient correlation.

Chapter 4 – Results

At starting, the hematological and biochemical values presented by the 3 groups were compared to assure that there were no differences between groups.

At the end, the control group was used to evaluate the physiological changes along the 6 weeks of the protocol and to evaluate the changes induced by the erythropoietic stimuli.

4.1. – Hematological studies

The hematological evaluation at starting (T0) and at the end of the protocol (T1) are presented in table 2.

There were no significant differences in body weight between the three groups of rats at the beginning of the study and at the end of 6 weeks, but, as expected, a gain of weight was observed, that was similar in the three groups.

The hematological evaluation, at T0, showed that there were no significant differences between the rats randomly included in the three groups of rats.

We found that after the 6 weeks of the protocol there was a significant physiological change to higher values of RBC and RDW and to lower values of MCV and MCH, as showed by the control group (control T0 *vs* control T1). WBC also showed a significant reduction, at the end of the protocol.

At the end of 6 weeks, the rats from the rHuEPO200 group, when compared with the control group, showed significantly higher RBC count, HGB concentration, HCT, RDW, MCV and lower MCHC values.

The rats from rHuEPO600 group, when compared with the control group, at the end of the protocol, showed significantly higher WBC, RBC and reticulocyte counts, as well as higher values of HGB, HCT, MCV and RDW; significantly lower PLT count and MCHC values were also found.

Comparing the rHuEPO600 group with the rHuEPO200 group, at the end of the protocol, we found that the higher dose showed significantly higher values of RBC, HGB, HCT, RDW and reticulocytes, and significantly lower platelet count.

Table 2 – Body weight and hematological study at the beginning (T0) and at the end (T1) of the protocol for the Control, rHuEPO200 and rHuEPO600 groups.

	T0			T1		
	Control (n=10)	rHuEPO200 (n=8)	rHuEPO600 (n=6)	Control	rHuEPO200	rHuEPO600
BW (g)	340.50±10.78	365.13±5.26	351.00±9.92	*423.60±13.87	440.63±9.46	418.00±14.55
RBC ($x10^{12}/L$)	7.46±0.08	7.54±0.10	7.57±0.13	*7.99±0.17	9.41±0.13 [aa]	10.84±0.29 [aa bb]
HGB (g/dL)	14.36±0.18	14.31±0.21	14.55±0.24	14.47±0.19	17.06±0.32 [aa]	20.05±0.47 [aa bb]
HCT (%)	42.19±0.52	42.58±0.81	42.73±0.91	43.57±0.85	53.21±1.16 [aa]	64.60±2.13 [aa bb]
MCV (fL)	56.70±0.37	56.63±0.68	56.33±1.20	** 54.50±0.48	56.38±0.92 [a]	59.50±1.15 [aa]
MCH (pg)	19.36±0.15	19.00±0.20	19.25±0.44	** 18.11±0.19	18.10±0.22	18.50±0.38
MCHC (g/dL)	34.18±0.21	33.68±0.18	34.07±0.32	33.27±0.35	32.10±0.21 [a]	31.08±0.42 [aa]
RDW (%)	12.25±0.11	12.69±0.34	12.40±0.26	** 13.34±0.06	15.56±0.17 [aa]	19.00±0.20 [aa bb]
Reticulocytes ($x10^9/L$)	228.01±13.21	243.03±17.41	251.47±22.43	205.68±16.16	179.34±26.07	429.87±54.92 [a b]
PLT ($x10^9/L$)	667.50±18.58	733.00±38.97	713.00±17.04	688.20±19.68	642.38±61.01	482.00±30.76[aab]
WBC ($x10^9/L$)	4.69±0.34	3.90±0.38	4.25±0.37	** 3.27±0.30	4.39±0.67	4.12±0.30 [a]

Results are presented as mean ± standard error mean. *P< 0.05 and **P< 0.01 for Time 0 *vs* Time 1. [a] P< 0.05 and [aa] P< 0.01 *vs* Control. [b] P< 0.05 and [bb] P< 0.01 for rHuEPO200 *vs* rHuEPO600. BW: body weight; HCT: haematocrit; HGB: haemoglobin; MCH: mean cell hemoglobin; MCHC: mean cell hemoglobin concentration; MCV: mean cell volume; PLT: platelets; RBC: Red blood cells: RDW: red cell distribution width: WBC: white blood cells.

4.2. – Study of iron metabolism

Table 3 presents the results of the study for EPO and iron metabolism.

No significant differences were observed at starting between the 3 groups, except for ferritin that was significantly higher in the rHuEPO600 group.

At the end of the protocol, in control group, we found a physiological significant increase in ferritin values, as showed by the control group (T0 *vs* T1).

The rHuEPO200 group showed, at the end of the protocol, significantly higher values of EPO, when compared with the control group and with the rHuEPO600 group.

A significant decrease in transferrin values was observed in both rHuEPO groups, when compared with the control group.

Table 3 – Erythropoietin and iron metabolism at the beginning of the study (T0) and at the end of the protocol (T1), for Control, rHuEPO200 and rHuEPO600 groups.

	T0			T1		
	Control (n=10)	rHuEPO200 (n=8)	rHuEPO600 (n=6)	Control	rHuEPO200	rHuEPO600
EPO (mUI/mL)	3.93±0.47	3.36±0.25	3.83±0.48	3.85±0.37	6.61±0.58 [aa]	3.16±0.16 [bb]
Iron (µg/dL)	192.78±7.66	192.25±16.12	203.33±4.97	183.56±5.83	196.88±16.85	228.00±36.49
Ferritin (ng/mL)	115.98±7.37	139.95±12.97	164.88±8.24[aa]	[*] 152.84±7.93	162.70±13.57	181.97±10.49
Transferrin (mg/dL)	365.03±16.00	346.88±17.38	376.55±16.48	370.32±13.86	316.56±15.4[a]	296.07±20.42[a]
Transferrin Saturation (%)	28.42±2.39	28.20±2.95	27.22±1.19	25.87±1.40	31.45±2.96	40.21±8.22

Results are presented as mean ± standard error mean. *P< 0.05 and **P< 0.01 for Time 0 *vs* Time 1. [a] P< 0.05 and [aa] P< 0.01 *vs* Control. [b] P< 0.05 and [bb] P< 0.01 for rHuEPO200 *vs* rHuEPO600. EPO: erythropoietin.

4.3. – Biochemical studies

The biochemical evaluation at starting (T0) and at the end of the protocol (T1) are presented in table 4.

At starting (T0) there were some significant differences between the groups, namely, urea concentration was higher in rats from groups rHuEPO200 and rHuEPO600, when compared with the control group. Significantly higher levels of urea were still observed for rHuEPO600 group, at T1, as compared to control.

Several differences in the lipid profile were observed at starting (T0), as compared to the control group; cholesterol and triglycerides were higher in rHuEPO200 group, HDL cholesterol levels were higher in both rHuEPO200 and rHuEPO600 groups. At the end of the protocol (T1), only HDL cholesterol levels were higher in the rHuEPO200 group when compared with the control.

At T1, rHuEPO200 group showed a creatine kinase (CK) concentration that was significantly higher than the control group and significantly lower than the rHuEPO600 group.

At the end of the protocol, CRP values were significantly lower in the rHuEPO600 group, when compared with the rHuEPO200 group. However, serum IL-6 levels, in all groups at both times of the study, were above the range of detection (0 – 5.54 pg/mL).

Table 4 - Biochemical studies at beginning of the protocol (T0) and at the end of the protocol (T1) for the Control, rHuEPO200 and rHuEPO600 groups.

	T0			T1		
	Control (n=10)	rHuEPO200 (n=8)	rHuEPO600 (n=6)	Control	rHuEPO200	rHuEPO600
CRP (μg/mL)	786.76±88.56	695.08±65.19	603.83±37.52	736.11±42.74	850.01±88.24	612.06±30.32 [b]
Urea (mg/dL)	20.15±0.56	22.35±0.47 [a]	22.73±0.57 [a]	21.02±0.33	21.70±0.42	23.33±0.76 [a]
Creatinine (mg/dL)	0.34±0.01	0.33±0.02	0.34±0.02	0.37±0.02	0.36±0.03	0.40±0.02
Uric acid (mg/dL)	0.98±0.05	1.14±0.10	1.08±0.19	0.81±0.06	1.18±0.18	1.30±0.22
CK (U/L)	456.29±49.70	570.67±85.13	523.40±125.15	446.86±71.56	90.67±16.72 [aa]	245.80±67.62 [b]
Glucose (mg/dL)	181.63±5.00	194.13±15.14	217.17±14.32	168.11±8.76	198.63±23.02	166.83±19.32
Chol-Total (mg/dL)	53.30±1.90	67.63±5.44 [a]	61.50±3.60	54.10±2.64	65.13±4.69	55.33±3.26
Chol-HDL (mg/dL)	28.10±1.29	38.00±4.07 [a]	34.83±2.73 [a]	28.00±1.20	35.50±3.56 [a]	30.50±3.08
Chol-LDL (mg/dL)	12.60±0.82	15.75±1.63	13.67±0.80	14.13±0.74	16.13±1.30	15.33±1.58
TGs (mg/dL)	140.10±23.11	189.13±10.85[a]	157.17±26.06	177.71±12.17	205.75±16.66	166.50±28.87
Atherogenic index	1.91±0.06	1.83±0.10	1.80±0.10	1.94±0.04	1.86±0.08	1.85±0.12

Results are presented as mean ± standard error mean. [a] $P < 0.05$ and [aa] $P < 0.01$ *vs* Control. [b] $P < 0.05$ and [bb] $P < 0.01$ for rHuEPO200 *vs* rHuEPO600. CRP – C reactive protein; Chol-HDL: high density lipoprotein cholesterol; Chol-LDL: low density lipoprotein cholesterol; Chol-Total: total cholesterol; CK: creatine kinase; TGs: triglycerides.

4.4. – Gene expression studies

To study the gene expression of several iron and erythropoietic regulatory factors, we used alpha-tubulin gene expression as a housekeeping gene.

Concerning the subcutaneous adipose tissue, we found that the rHuEPO200 group presented a significant overexpression of hepcidin (Figure 12 - A) and ferritin (Figure 12 - E) genes and an under expression of IL-6 (Figure 12 – B), BMP6 (Figure 12 – C) and EPOR (Figure 12 – D) genes.

The subcutaneous adipose tissue of the rHuEPO600 group, showed that hepcidin (Figure 12 - A), IL-6 (Figure 12 – B) and ferritin (Figure 12 – E) genes were significantly overexpressed, while BMP6 (Figure 12 – C) and EPOR (Figure 12 – D) genes were significantly under expressed.

Comparing the gene expression in the subcutaneous adipose tissue of these two rHuEPO groups, a significantly increased expression was found for IL-6 gene, in the rHuEPO600 group (Figure 12 - B).

In the visceral adipose tissue of the rHuEPO200 group we found a significant overexpression of hepcidin (Figure 13 – A), IL-6 (Figure 13 – B) and ferritin (Figure 13 – E) genes and a significant under expression of BMP6 (Figure 13 – C) and EPOR (Figure 13 – D) genes.

For the rHuEPO600 group, the visceral adipose tissue showed a significant under expression of all the 5 studied genes (Figure 13).

Comparing the gene expression of the visceral adipose tissue from the rHuEPO200 group with that from rHuEPO600 group, we found that the lower rHuEPO dose presented a significantly higher expression of hepcidin (Figure 13 – A), IL-6 (Figure 13 – B) and BMP6 (Figure 13 – C) genes and a trend towards a higher expression of ferritin gene ($P = 0.050$) (Figure 13 - E).

We also found that hepcidin expression in the visceral adipose tissue was significantly and positively correlated with CRP levels, in the rHuEPO200 rats, and with IL-6 expression ($P=0.037$; $r=0.900$), in the rHuEPO600 rats.

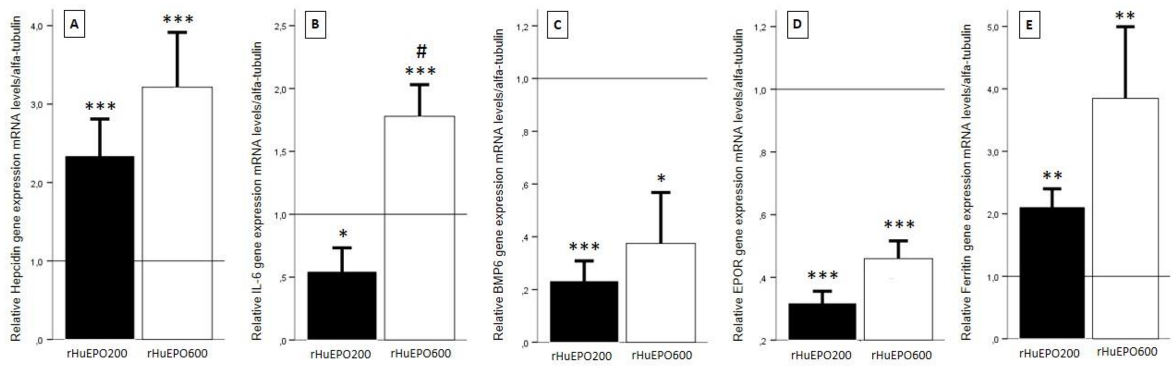

Figure 12 – rHuEPO effects on relative gene expression mRNA/alpha-tubulin, in subcutaneous adipose tissue, of hepcidin – A; IL-6 – B; BMP6 – C; EPOR – D; ferritin -- E. Results are presented as mean + standard error. * P< 0.05, ** P< 0.01 and *** P< 0.001 *vs* Control. # P< 0.05 for rHuEPO200 *vs* rHuEPO600.

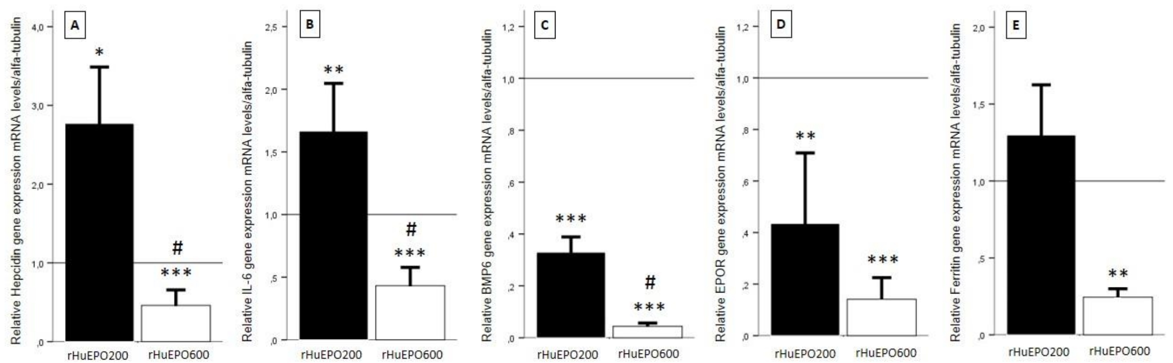

Figure 13 - rHuEPO effects on relative gene expression mRNA/alpha-tubulin, in visceral adipose tissue, of hepcidin – A; IL-6 – B; BMP6 – C; EPOR – D; ferritin -- E. Results are presented as mean + standard error. * P< 0.05, ** P< 0.01 and *** P< 0.001 vs Control. # P< 0.05 for rHuEPO200 vs rHuEPO600.

Chapter 5 – Discussion

It is known that the treatment with recombinant human erythropoietin stimulates erythropoiesis leading to an increase in RBC production, hemoglobin concentration and red cell volume (1, 43). Indeed, it was reported that rHuEPO has a potent erythropoietic action and that in rats is able to cure the anemia caused by renal failure (44).

\In this study we observed that the administration of rHuEPO in rats results in stimulation of erythropoiesis, as shown by the increase in the values of RBC and hemoglobin concentration, hematocrit, MCV and RDW. These changes were dose dependent, as the group that received a higher dose of rHuEPO presented a higher increase in those hematological parameters (e.g. RBC, HGB, HCT and RDW) and in reticulocytes, which is in agreement with a higher stimuli of erythropoiesis.

To maintain the erythropoietic process a correct amount of iron is required (4); therefore, a stimuli on erythropoiesis is, usually, associated with a raise in iron mobilization and absorption. We found an increase, although not significant, in circulating iron levels in both groups (rHuEPO200 and rHuEPO600), as compared to control (T1). This increase in iron mobilization was associated with a decrease in transferrin and a trend towards an increase in transferrin saturation and ferritin levels. The differences in iron metabolism parameters between the two rHuEPO groups were not statistical significant. In accordance, Adam et al. (45) reported that iron-loaded rats did not demonstrate an increase in intestinal iron absorption with rHuEPO therapy, despite the significant enhancement observed in erythropoiesis.

The erythropoietic stimuli, independently of the dose of recombinant erythropoietin administered, did not induce any significant alteration in lipid profile and in the markers of renal function.

Hepcidin is the main regulator of iron absorption and mobilization and its levels are dependent on iron status, inflammation, hypoxia and erythropoietic process (4, 21). A strong erythropoiesis stimuli triggers a decrease in hepcidin concentrations. The adipose tissue is capable of expressing hepcidin, both at mRNA and protein levels (33). Visceral and subcutaneous hepcidin mRNA expression is higher in adipose tissue of obese individuals, correlating with inflammatory parameters, such as IL-6 and CRP (33). Indeed, the expression within the adipose tissue is believed to be regulated by hypoxia and/or

inflammation and not by body iron stores (33, 46). Nemeth et al. (22) by performing studies in human liver cell cultures, in mice, and in human volunteers concluded that IL-6 is sufficient to induce hepcidin production during inflammation and that the IL-6-hepcidin axis is responsible for the hypoferremia associated with inflammation. Adipose tissue is known to express and release IL-6. Studies in obese (ob/ob line) and lean mice showed significantly higher IL-6 levels in obese mice, due to increased IL-6 expression in adipose tissue (47).

As far as we know, the effect of an erythropoietic stimuli, through rHuEPO administration, on the expression by the visceral and subcutaneous adipose tissue of erythropoietic and iron regulatory proteins gene, such as hepcidin and IL-6, had not been studied yet. For hepcidin gene expression, we found an overexpression by subcutaneous adipose tissue in both rHuEPO groups, and by visceral adipose tissue in the rHuEPO200 group. Considering IL-6 gene expression, an overexpression by the subcutaneous adipose tissue of the rHuEPO600 rats and by the visceral adipose tissue of the rHuEPO200 group was observed.

Considering ferritin gene expression, it was reported that differentiation of 3T3-L1 cells into adipocytes associates with a consistent increase of ferritin mRNA expression (48). In this study, it was observed that an erythropoietic stimuli associated with an overexpression of ferritin gene, except for the rats that received 600 IU/BW/week of rHuEPO.

Teng et al. disclosed, in wild type C57B6 mice, a high level of EPOR expression in white adipose tissue, comparatively to other non-hematopoietic tissues (40). We found that the expression of EPOR in visceral and subcutaneous adipose tissue of both rHuEPO groups was underexpressed. It seems that an erythropoietic stimuli, independently of the dose of recombinant erythropoietin administered, induces a lower expression of EPOR gene by both adipose tissues, suggesting that rHuEPO, in high doses, do not exercise its effects on adipose tissue in a direct way.

In normal conditions the expression of hepcidin depends on signaling through the BMP/SMAD pathway. HJV bounded to the cell membrane in hepatocyte participates as a BMP6 coreceptor in this pathway, regulating hepcidin expression, whereas soluble HJV antagonizes BMP6, which is the master hepcidin activator *in vivo* (17). We also studied the BMP6 gene expression to inquire if hepcidin expression in adipose tissue submitted to a erythropoietic stimuli could suffer a similar regulation, but the BMP6 gene was underexpressed by subcutaneous and visceral adipose tissues in both rHuEPO groups.

Our data suggests that hepcidin expression in adipose tissue is probably regulated by inflammation and/or hypoxia, which is in accordance with other reports (33, 46), and that the BMP/SMAD pathway do not seem to have an important role in the regulation of adipocyte hepcidin expression.

When comparing data of visceral adipose tissue from the two rHuEPO groups, we found that a higher erythropoietic stimuli associates with a significantly lower expression of both hepcidin and IL-6 genes. Apparently, the stimuli of erythropoiesis by a higher dose of recombinant erythropoietin affects not only the production of hepcidin by the liver (4, 21), but also the production of hepcidin by the visceral adipose tissue. Moreover, a significant positive correlation was observed between hepcidin gene and IL-6 gene expression in the visceral adipose tissue of rHuEPO600 group. A similar correlation between the gene expression of hepcidin and IL-6 was reported in a study performed in lean and obese mice by Gotardo et al. (49); they found also that the mRNA levels of both hepcidin and IL-6 were increased in visceral adipose tissue of obese mice. Our data suggest that under a high erythropoietic stimuli, IL-6 and hepcidin expression in visceral adipose tissue are tightly associated.

Comparing data from subcutaneous adipose tissue of the two rHuEPO groups, a significantly higher IL-6 gene expression and an increased hepcidin gene expression, although not significant, were detected in rHuEPO600 group, as compared to the rHuEPO200 group. It seems that the response to a higher erythropoietic stimuli, as the rHuEPO600 group underwent, is not the same in subcutaneous adipose tissue and in visceral adipose tissue.

Teng et al. (40) reported that erythropoietin administration decreases fat mass accumulation. In accordance, we found that the group of rats under a higher dose of rHuEPO showed at the end of the protocol less visceral adipose tissue accumulation than the group with lower dosage of rHuEPO (data not shown). Since no differences in the values of body weight were detected between the 2 rHuEPO groups, an increase in muscle mass may have occurred, which could be related with the higher CK circulating levels found for the rHuEPO600 group.

The different response observed in the two tissues after administration of 600 IU/BW/week of rHuEPO, may be related to the lower mass of visceral adipose tissue found for the rHuEPO600 group of rats. A decrease in visceral mass, accompanied by a reduction in its metabolic activity, may be associated with a lower IL-6 expression. Considering that inflammation may be the principal regulator of adipocyte hepcidin expression (33, 46), and

that our data showed that IL-6 and hepcidin expression are positively related, a reduction in IL-6 may induce a down-regulation in hepcidin expression in visceral adipose tissue.

As referred, a higher erythropoietic stimuli induced a significant increase in hematocrit, leading thus to higher blood viscosity. Visceral adipose tissue is metabolically more active and more vascularized than the subcutaneous adipose tissue. It is possible that the enhanced blood viscosity induces some stasis within the visceral adipose tissue leading to hypoxia. Hypoxia is known to reduce hepcidin expression (50); thus, the down-regulation of hepcidin found in visceral adipose tissue after a higher dose of recombinant erythropoietin might be a consequence of a hypoxic environment.

This study involves relatively small groups of rats, thus, further studies are warranted with a larger population in order to confirm our results. It was not possible to compare the gene expression between the two tissues, however it would be of interest in a future work to evaluate and compare the gene expression and the proteins produced by the visceral and subcutaneous adipose tissue. It is not well established if the production of hepcidin by the adipose tissue is reflected in alterations at circulating level and, therefore, if it is enough to influence iron absorption or even erythropoiesis. It has been speculated that in obese individuals, in whom the amount of adipose tissue is superior, the production of hepcidin by adipose tissue has an important impact in hepcidin circulating levels. It would be of interest to evaluate if a higher erythropoietic stimuli induces not only a decrease in the expression of IL-6 and, consequently, of hepcidin by the visceral adipose tissue.

In summary, the BMP/SMAD pathway does not seem to have an important role in the regulation of hepcidin expression, in adipocyte. When submitted to an erythropoietic stimuli the visceral and the subcutaneous adipose tissue respond differently. In the visceral adipose tissue, a higher erythropoietic stimuli associates with a lower expression of hepcidin and IL-6. Moreover, a positive correlation between hepcidin and IL-6 gene expression was found in the visceral adipose tissue of rats submitted to a higher dose of rHuEPO, suggesting that under a high erythropoietic stimuli, IL-6 and hepcidin expression are tightly related.

Bibliographic references

1. Elliott S, Pham E, Macdougall IC. Erythropoietins: a common mechanism of action. Experimental Hematology. 2008;36(12):1573-84.

2. Elliott SG, Foote MMG. Erythropoietins, erythropoietic factors, and erythropoiesis molecular, cellular, preclinical, and clinical biology Basel; Boston: Birkhäuser; 2009.

3. Dzierzak E, Philipsen S. Erythropoiesis: development and differentiation. Cold Spring Harbor Perspectives in Medicine. 2013;3(4):a011601.

4. Fried W. Erythropoietin and erythropoiesis. Experimental Hematology. 2009;37(9):1007-15.

5. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene. 2002;285(1-2):1-24.

6. Platanias LC. Map kinase signaling pathways and hematologic malignancies. Blood. 2003;101(12):4667-79.

7. Wymann MP, Pirola L. Structure and function of phosphoinositide 3-kinases. Biochimica et Biophysica Acta. 1998;1436(1-2):127-50.

8. Patnaik MM, Tefferi A. The complete evaluation of erythrocytosis: congenital and acquired. Leukemia. 2009;23(5):834-44.

9. Kyriazi VI. A holistic approach of erythropoiesis and iron in anemia. Health Science Journal. 2011;5(3):176-81.

10. Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. Cell. 2004;117(3):285-97.

11. McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, et al. An iron-regulated ferric reductase associated with the absorption of dietary iron. Science (New York, NY). 2001;291(5509):1755-9.

12. Chua AC, Graham RM, Trinder D, Olynyk JK. The regulation of cellular iron metabolism. Critical Reviews in Clinical Laboratory Sciences. 2007;44(5-6):413-59.

13. Wei Y, Miller SC, Tsuji Y, Torti SV, Torti FM. Interleukin 1 induces ferritin heavy chain in human muscle cells. Biochemical and Biophysical Research Communications. 1990;169(1):289-96.

14. Fahmy M, Young SP. Modulation of iron metabolism in monocyte cell line U937 by inflammatory cytokines: changes in transferrin uptake, iron handling and ferritin mRNA. The Biochemical Journal. 1993;296 (Pt 1):175-81.

15. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science (New York, NY). 2004;306(5704):2090-3.

16. Yang F, Liu XB, Quinones M, Melby PC, Ghio A, Haile DJ. Regulation of reticuloendothelial iron transporter MTP1 (Slc11a3) by inflammation. The Journal of Biological Chemistry. 2002;277(42):39786-91.

17. Hoffbrand V. Postgraduate haematology, 6th ed. Chichester UK: Wiley-Blackwell; 2011.

18. Kawabata H, Yang R, Hirama T, Vuong PT, Kawano S, Gombart AF, et al. Molecular cloning of transferrin receptor 2. A new member of the transferrin receptor-like family. The Journal of Biological Chemistry. 1999;274(30):20826-32.

19. Zekanowska E BJG-KPKJ. Obesity and iron metabolism. Biotechnologia Biotechnologia. 2011;92(2):147-52.

20. Piperno A, Mariani R, Trombini P, Girelli D. Hepcidin modulation in human diseases: from research to clinic. World Journal of Gastroenterology. 2009;15(5):538-51.

21. Palaneeswari MS, Ganesh M, Karthikeyan T, Devi AJ, Mythili SV. Hepcidin-minireview. Journal of Clinical and Diagnostic Research. 2013;7(8):1767-71.

22. Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. The Journal of Clinical Investigation. 2004;113(9):1271-6.

23. Wozniak SE, Gee LL, Wachtel MS, Frezza EE. Adipose tissue: the new endocrine organ? A review article. Digestive Diseases and Sciences. 2009;54(9):1847-56.

24. Fonseca-Alaniz MH, Takada J, Alonso-Vale MI, Lima FB. Adipose tissue as an endocrine organ: from theory to practice. Jornal de Pediatria. 2007;83(5 Suppl):S192-203.

25. Cinti S. The adipose organ at a glance. Disease models & Mechanisms. 2012;5(5):588-94.

26. Ibrahim MM. Subcutaneous and visceral adipose tissue: structural and functional differences. Obesity Reviews : an official journal of the International Association for the Study of Obesity. 2010;11(1):11-8.

27. Leal Vde O, Mafra D. Adipokines in obesity. Clinica Chimica Acta; International Journal of Clinical Chemistry. 2013;419:87-94.

28. Gimeno RE, Klaman LD. Adipose tissue as an active endocrine organ: recent advances. Current Opinion in Pharmacology. 2005;5(2):122-8.

29. Sethi JK, Hotamisligil GS. The role of TNF alpha in adipocyte metabolism. Seminars in Cell & Developmental Biology. 1999;10(1):19-29.

30. Chung B, Matak P, McKie AT, Sharp P. Leptin increases the expression of the iron regulatory hormone hepcidin in HuH7 human hepatoma cells. The Journal of Nutrition. 2007;137(11):2366-70.

31. del Giudice EM, Santoro N, Amato A, Brienza C, Calabro P, Wiegerinck ET, et al. Hepcidin in obese children as a potential mediator of the association between obesity and iron deficiency. The Journal of Clinical Endocrinology and Metabolism. 2009;94(12):5102-7.
32. Means RT, Jr., Krantz SB. Inhibition of human erythroid colony-forming units by tumor necrosis factor requires beta interferon. The Journal of Clinical Investigation. 1993;91(2):416-9.
33. Bekri S, Gual P, Anty R, Luciani N, Dahman M, Ramesh B, et al. Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH. Gastroenterology. 2006;131(3):788-96.
34. Ashby DR, Gale DP, Busbridge M, Murphy KG, Duncan ND, Cairns TD, et al. Erythropoietin administration in humans causes a marked and prolonged reduction in circulating hepcidin. Haematologica. 2010;95(3):505-8.
35. Huang H, Constante M, Layoun A, Santos MM. Contribution of STAT3 and SMAD4 pathways to the regulation of hepcidin by opposing stimuli. Blood. 2009;113(15):3593-9.
36. Zimmermann MB, Zeder C, Muthayya S, Winichagoon P, Chaouki N, Aeberli I, et al. Adiposity in women and children from transition countries predicts decreased iron absorption, iron deficiency and a reduced response to iron fortification. International Journal of Obesity (2005). 2008;32(7):1098-104.
37. Coimbra S, Catarino C, Santos-Silva A. The role of adipocytes in the modulation of iron metabolism in obesity. Obesity Reviews : an official journal of the International Association for the Study of Obesity. 2013;14(10):771-9.
38. Chateauvieux S, Grigorakaki C, Morceau F, Dicato M, Diederich M. Erythropoietin, erythropoiesis and beyond. Biochemical Pharmacology. 2011;82(10):1291-303.
39. Lipsic E, Westenbrink BD, van der Meer P, van der Harst P, Voors AA, van Veldhuisen DJ, et al. Low-dose erythropoietin improves cardiac function in experimental heart failure without increasing haematocrit. European Journal of Heart Failure. 2008;10(1):22-9.
40. Teng R, Gavrilova O, Suzuki N, Chanturiya T, Schimel D, Hugendubler L, et al. Disrupted erythropoietin signalling promotes obesity and alters hypothalamus proopiomelanocortin production. Nature Communications. 2011;2:520.
41. Nairz M, Sonnweber T, Schroll A, Theurl I, Weiss G. The pleiotropic effects of erythropoietin in infection and inflammation. Microbes and Infection / Institut Pasteur. 2012;14(3):238-46.

42. Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nature Protocols. 2006;1(2):581-5.
43. Lundby C, Thomsen JJ, Boushel R, Koskolou M, Warberg J, Calbet JA, et al. Erythropoietin treatment elevates haemoglobin concentration by increasing red cell volume and depressing plasma volume. The Journal of Physiology. 2007;578(Pt 1):309-14.
44. Kawamura A, Higuchi M, Imai N, Kawaguchi T, Ogura Y. Effect of purified recombinant human erythropoietin on anemia in rats with experimental renal failure induced by five-sixth nephrectomy. Biotherapy (Dordrecht, Netherlands). 1990;2(1):77-85.
45. Adams PC, Chau LA, Lin E, Muirhead N. The effect of human recombinant erythropoietin on iron absorption and hepatic iron in a rat model. Clinical and Investigative Medicine, Medecine clinique et experimentale. 1991;14(5):432-6.
46. Tussing-Humphreys L, Frayn KN, Smith SR, Westerman M, Dennis AL, Nemeth E, et al. Subcutaneous adipose tissue from obese and lean adults does not release hepcidin in vivo. The Scientific World Journal. 2011;11:2197-206.
47. Harkins JM, Moustaid-Moussa N, Chung YJ, Penner KM, Pestka JJ, North CM, et al. Expression of interleukin-6 is greater in preadipocytes than in adipocytes of 3T3-L1 cells and C57BL/6J and ob/ob mice. The Journal of Nutrition. 2004;134(10):2673-7.
48. Festa M, Ricciardelli G, Mele G, Pietropaolo C, Ruffo A, Colonna A. Overexpression of H ferritin and up-regulation of iron regulatory protein genes during differentiation of 3T3-L1 pre-adipocytes. The Journal of Biological Chemistry. 2000;275(47):36708-12.
49. Gotardo EM, dos Santos AN, Miyashiro RA, Gambero S, Rocha T, Ribeiro ML, et al. Mice that are fed a high-fat diet display increased hepcidin expression in adipose tissue. Journal of Nutritional Science and Vitaminology. 2013;59(5):454-61.
50. Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. The Journal of Clinical Investigation. 2002;110(7):1037-44.