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FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO

Morbid obesity: the role of the adiposity from visceral and subcutaneous adipose tissue in iron metabolism

Obesidade mórbida: o papel do adipócito do tecido adiposo visceral e subcutâneo no metabolismo do ferro

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

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Julho, 2016

Este trabalho foi realizado no Laboratório de Bioquímica da Faculdade de Farmácia da Universidade do Porto, na unidade de investigação Unit on Applied Molecular Biosciences (UCIBIO)/REQUIMTE, com a colaboração do Hospital da Prelada-Dr. Domingos Braga da Cruz (Ref: DC/2013DC).

O trabalho realizado recebeu financiamento de UCIBIO (PT2020_UCBIO [UID/MULTI/04378/2013 – POCI/01/0145/FEDER/007728].

Este estudo foi realizado sob a orientação de:

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“Iron rusts from disuse, stagnant water loses its purity, and in cold weather becomes frozen; even so does inaction sap the vigors of the mind.”

Leonardo da Vinci

Declaration

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

Acknowledgments

Quero aproveitar esta oportunidade para manifestar a minha gratidão à Professora Doutora Alice Santos-Silva, não só por ter tornado possível a realização desta dissertação, mas também por todo o tempo, dedicação e conhecimentos disponibilizados.

Agradeço também à Professora Doutora Cristina Catarino por toda a disponibilidade, atenção e apoio prestados na concretização deste trabalho.

Um agradecimento especial à Professora Doutora Susana Coimbra pela prontidão, apoio e paciência inesgotáveis numa fase crucial deste trabalho.

Estendo os meus agradecimentos a todas as pessoas do Laboratório de Bioquímica da Faculdade de Farmácia da Universidade do Porto, pelo acolhimento e auxílio ao longo de todos estes meses. Um especial muito obrigado, à Doutora Susana Rocha e à Doutora Sandra Ribeiro por toda a ajuda disponibilizada sempre que surgiam dúvidas.

Agradeço, com todo o carinho, aos meus colegas de mestrado, pelo ambiente que se criou, pelo espírito de equipa, apoio e entreajuda que tornou estes dois anos mais leves e divertidos e por todos os conhecimentos partilhados. Às meninas do MAC dedico um agradecimento ainda mais especial, por toda a amizade e auxílio durante este período.

Aos meus pais, avós, madrinha e prima, pelo apoio nas horas difíceis, pela compreensão, pelo incentivo, por me mostrarem sempre o valor do estudo e da realização pessoal, por me ensinarem que o caminho mais fácil nunca é o melhor, pelos valores transmitidos, pela confiança e pela paciência nos momentos de desespero, um sentido obrigado.

Agradeço aos meus amigos pelo apoio em mais uma fase da minha vida, pelas palavras de apoio e incentivo e por compreenderem a minha falta de disponibilidade e memória.

Por último, mas não menos importante, um agradecimento especial à Daniela e à Marta pela amizade e extrema paciência nos piores momentos, por me ouvirem, apoiarem e guiarem, pelos conselhos, por não me deixarem desistir e por estarem sempre disponíveis e presentes.

Abstract

Obesity is a serious health public problem and its prevalence has been increasing worldwide in the last decades. Obesity is often associated with iron deficiency and with a chronic low-grade inflammation state. It is known that the adipose tissue may secrete several adipokines that are usually increased in obese patients and that are able to induce disturbances in iron metabolism.

We aim to understand the role of subcutaneous (SAT) and visceral adipose tissue (VAT) in obesity-related iron disturbances by comparing severe obese patients with normal weighted healthy individuals, at blood and adipose tissue levels.

We studied a group of severe or morbid obese patients (n=30) and a group of 17 normal weight patients (as Control group).

Subcutaneous and visceral adipose tissues biopsies were collected during surgeries. Gene expression studies, including iron metabolism, erythropoietic and inflammatory markers were performed in both visceral and subcutaneous adipose tissue. Anthropological measurements, haematological, biochemical and iron metabolism studies were also performed.

We found that in SAT only TfR gene was overexpressed in Obese group. The gene expression of the inflammatory markers showed values similar to the Control. In VAT the gene expression of hepcidin, ferritin and CRP were significantly increased, in Obese group when compared to Control group. At circulating levels, we found that IL-6, CRP, hepcidin and sTfR were significantly increased in Obese group when compared with Control group.

In summary, concerning gene expression of inflammatory, erythropoietic and iron metabolism markers, in VAT and SAT showed a different profile, presenting VAT a more active metabolic role, with a high gene expression of pro-inflammatory adipokines that are able to induce disturbances in iron metabolism. Moreover, VAT presented an overexpression of IL-6 that is positively correlated with BMI, suggesting an increasing inflammation with adiposity.

Keywords: adipose tissue gene expression; inflammation; iron metabolism; obesity

Resumo

A obesidade é um problema de saúde pública e sua prevalência tem aumentado em todo o mundo, nas últimas décadas. A obesidade é frequentemente associada à deficiência de ferro e com um estado de inflamação crónica de baixo grau. Sabe-se que o tecido adiposo é capaz de secretar várias adipocinas que geralmente estão aumentadas em pacientes obesos e que são capazes de induzir distúrbios no metabolismo do ferro.

O nosso objetivo é compreender o papel do tecido adiposo subcutâneo (SAT) e visceral (VAT) em distúrbios do metabolismo do ferro relacionados com a obesidade comparando pacientes obesos severos com indivíduos saudáveis de peso normal, ao nível circulatório e do tecido adiposo.

Nós estudamos um grupo de pacientes obesos severos ou mórbidos (n = 30) e um grupo de 17 pacientes de peso normal (como grupo de controlo).

Biópsias de tecidos de adiposo subcutâneo e visceral foram recolhidas durante cirurgias. Estudos de expressão génica, incluindo o metabolismo do ferro, marcadores inflamatórios e eritropoiéticos foram realizados no tecido adiposo visceral e subcutâneo.

Também foram realizadas medições antropológicas, bioquímicas, hematológicas e estudos de metabolismo de ferro.

No SAT observou-se que só o gene TfR estava sobre expresso no grupo dos obesos. A expressão génica dos marcadores inflamatórios apresentou valores semelhantes aos do grupo de controlo. No VAT a expressão génica da hepcidina, ferritina e CRP estavam significativamente aumentados, no grupo dos obesos quando comparado ao grupo de controlo. Ao nível circulante, observou-se que a IL-6, CRP, hepcidina e sTfR estavam significativamente aumentadas no grupo dos obesos quando comparado com grupo de controlo.

Em resumo, no que se refere à expressão génica de marcadores inflamatórios, do metabolismo do ferro e eritropoiéticos, o VAT e o SAT mostraram um perfil diferente, apresentando o VAT um papel mais ativo e metabólico, com uma expressão génica elevada de adipocinas pro-inflamatórias que são capazes de induzir distúrbios no metabolismo do ferro. Além disso, o VAT apresentou uma sobre expressão de IL-6 que estava positivamente correlacionada com o BMI, sugerindo que a inflamação aumenta com o aumento da adiposidade.

Palavras-Chave: expressão génica no tecido adiposo; inflamação, metabolismo do ferro; obesidade

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Abbreviations

AT – Adipose Tissue
BAT – Brown Adipose Tissue
BeAT – Beige Adipose Tissue
BMI – Body Mass Index
BMP – Bone Morphogenic Protein
CRP – C-reactive Protein
CVD – Cardiovascular Disease
DBP – Diastolic Blood Pressure
Dcytb – Duodenal Cytochrome b
DGS – Direção Geral de Saúde
DMT1 – Divalent Metal Transporter 1
DNA - Deoxyribonucleic Acid
EDTA – Ethylenediaminetetraacetic Acid
ELISA - Enzyme-linked Immunosorbent Assay
EPO – Erythropoietin
EPOR – Erytropoietin Receptor
ERK/MAPK - extracellular signal–regulated kinases/mitogen-activated protein kinase
FIH – Factor Inhibiting HIF-1
FPN1 - Ferroportin
GLP-1 – Glutan-Like Peptide 1
HAMP - Hepcidin
HCP – Heme Carrier Protein 1
HFE - Human Hemochromatosis Protein
HIF – Hypoxia-inducible Factor
HJV - Hemojuveli
HO1 – Heme-oxidase
HRE – Hypoxia-response Element
IFN-γ – Interferon γ
IL – Interleukin
INE – Instituto Nacional de Estatística
INSA – Instituto Nacional de Saúde
LAGB – Laparoscopic Adjustable Gastric Banding
MCH – Mean Cell Haemoglobin
MCHC – Mean Cell Haemoglobin Concentrartion
MCV – Mean Cell Volume
mHJV – Membrane Anchored Hemojuvelin
MT – Matriptase
mRNA – Messenger Ribonucleic Acid
Neo - Neogenin
p300-CBP – p300-cAMP Response Element Binding Protein
PAI-1 – Plasminogen Activator Inhibitor-1
PCR - Polymerase Chain Reaction
PHD – Prolyl Hydroxylase
PYY – Peptide YY
RBC – Red Blood Cells
RDW – Red Cell Distribution Width
RNA - Ribonucleic Acid
ROS – Reactive Oxigen Species
RPI – Reticulocyte Production Index
SAT – Subcutaneous Adipose Tissue
SBP – Systolic Blood Pressure
sHJV – Soluble Hemojuvelin
STAT – Signal Transducer and Activator Transcription
sTfR – Soluble Transferrin Receptor
TBI – Transferrin-bound Iron
Tf – Transferrin
tfR – Transferrin Receptor
TG – Triglycerides
TGF-β – Transforming Growth Factor β
Th – T-helper cell
TNF-α – Tumour Necrosis Factor
Treg – T regulator cell
Ub - Ubiquitin
VAT – Visceral Adipose Tissue
vHL- von Hippel-Lindau Tumour Supressor Protein
WAT – White Adipose Tissue
WBC – White Blood Cells
WC – Waist Circunference
W:H – Waist:Height
WHO – World Health Organization

Chapter I – Introduction

This chapter will focus on obesity, the metabolic role of adipose tissue and their role in iron metabolism.

Obesity is a serious health public problem and its prevalence has been increasing worldwide in the last decades. Obesity is often associated with iron deficiency in children, adolescents, adults and post-menopausal women, in both developed and underdeveloped countries. Iron deficiency is one of the most prevalent worldwide nutritional problem, associating with poor cognitive function and exercise performance. Therefore, the association of obesity with hypoferremia may have critical physical and psychological development implications.

1. Obesity

1.1. Definition and Prevalence

Obesity is a pathology characterized by abnormal or excessive body fat accumulation. It seems to be a consequence of a positive energy balance, when energy intake is greater than energy expenditure.(1-3)

The amount and location of adipose tissue differ from individual to individual and can affect obesity associated complications. In fact, it has been reported that excess abdominal fat represents a higher risk factor for complications like cardiovascular diseases (CVD), diabetes, hypertension and dislipidemia, than the overall excess body fat. (3)

Obesity is a multifactorial disease, involving genetic, endocrine/regulatory, behavioral, psychosocial, and environmental factors. The regulation of adiposity is a complex process, involving the interaction of all these factors. (4)

The prevalence of obesity has increased significantly, being more relevant in developed countries, where the social and economic characteristics provide a favourable environment to this condition. However, the prevalence of obesity in the underdeveloped countries seems to be also increasing rapidly.(3-5)

According to Marie *et al.*, in 2013 obesity affected 36.9% of men and 38.0% of women worldwide. (6)

In Portugal, DGS (Direcção Geral de Saúde) data, from INE (Instituto Nacional de Estatística), in collaboration with INSA (Instituto Nacional de Saúde) estimated that 16.4% of Portuguese population, in 2014, was obese and over 50% was overweight. (7)

1.2. Obesity Classification

Classification of obese individuals is assessed in a graded manner. It allows the identification of the risk of morbidity and mortality and the intervention priorities for a certain individual or community. It also provides a comparison of the weight status within and between populations. (3, 8)

A few anthropometric indicators can be used to define obesity, namely body mass index (BMI) and waist circumference (WC) are two of the most used ones. However, additional methods can be used, such as magnetic resonance imaging and measuring energy intake and expenditure. (3, 8)

BMI is a simple index, commonly used to classify underweight, overweight and obesity in adults, and is calculated by dividing the weight value (kg), by the square of the height (m^2). Normal weight is defined by a BMI between 18.5 and 24.9 kg/m^2. Overweight is defined by a BMI 25.0-29.9 $kg/m^{2,}$ and obesity by a BMI equal or higher than 30.0 kg/m^2 (**Table 1**). (2, 3)

Table 1 – Obesity classification in adults based on body mass index (BMI)

Classification	**BMI (kg/m^2)**
Normal	18.50-24.99
Overweight	≥25
Obese Class I	30-34.99
Obese Class II	35-39.99
Obese Class III	≥40

Adapted from WHO Report. (3)

Besides WC, waist:hip ratio and waist:height (W:H) ratio are measurements that reflect abdominal adiposity, assessing in a more accurate manner the abdominal adipose tissue accumulation. The evaluation of abdominal adiposity is important due to its association with several metabolic dysfunctions, such as, glucose intolerance, reduced insulin

sensitivity and adverse lipid profiles, all known as risk factors for cardiovascular diseases (CVD) and diabetes *mellitus.* (3)

WC is accessible, simple to measure, unrelated to height and seems to be more accurate to access intra-abdominal adiposity than BMI, which is a measure of general obesity The cut-off points for WC suggested by WHO for caucasian men and women are presented at **Table 2**. (2, 3)

Table 2 – Cut-offs values for waist circumference

	Waist Circumference (cm)	
Metabolic Complications Risk	**Men**	**Women**
High	≥94	≥80
Very High	≥102	≥88

Adapted from WHO report. (3)

Waist:hip ratio is an anthropometric measure that assess the bone structured and muscle mass in the gluteofemoral area. A ratio above 0.90 in women and 0.94 in men has been associated with a higher risk of metabolic complications. (2, 3)

WC and W:H allows the classification of the morphologic type of obesity in android (when adipose tissue accumulates in abdomen and upper half of the body) or ginoid (when adipose tissue accumulates in the lower half of the body). The second type is more prevalent in women than men. This classification is important because there is scientific evidence that abdominal fat accumulation is related with a higher risk of comorbidities. (2, 3)

1.3. Adipose Tissue

Adipose tissue (AT) is no longer considered only as an energy storage organ, being accepted nowadays as an active endocrine organ with important metabolic functions. Indeed, adipose tissue participates in the regulation of lipid metabolism, glucose homeostasis, blood pressure, inflammation and angiogenesis, and has an important role in controlling appetite and food intake. (9-15)

The adipose tissue produces several adipokines that are enrolled in many metabolic and inflammatory pathways.

There are two types of adipose tissue, brown (BAT) and white adipose tissue (WAT) (**Figure 1**). BAT is abundant in small mammals and in newborns and is specialized in the dissipation of energy through the production of heat. WAT stores excess energy as triglycerides (TG), it increases with age and large amounts are associated with increased risk of obesity-related comorbidities. More recently, a third and intermediate “shade” of adipose tissue was reported: beige adipose tissue (BeAT) with characteristics from both BAT and WAT. (9-19)

According to studies in rodents, WAT and BAT have different origin lineages. BAT seems to derive from the same myocyte cell lineages and shares characteristics with muscle cells. WAT origin is less clear. BeAT can derive from WAT depending on external stimulation. (9, 15, 16)

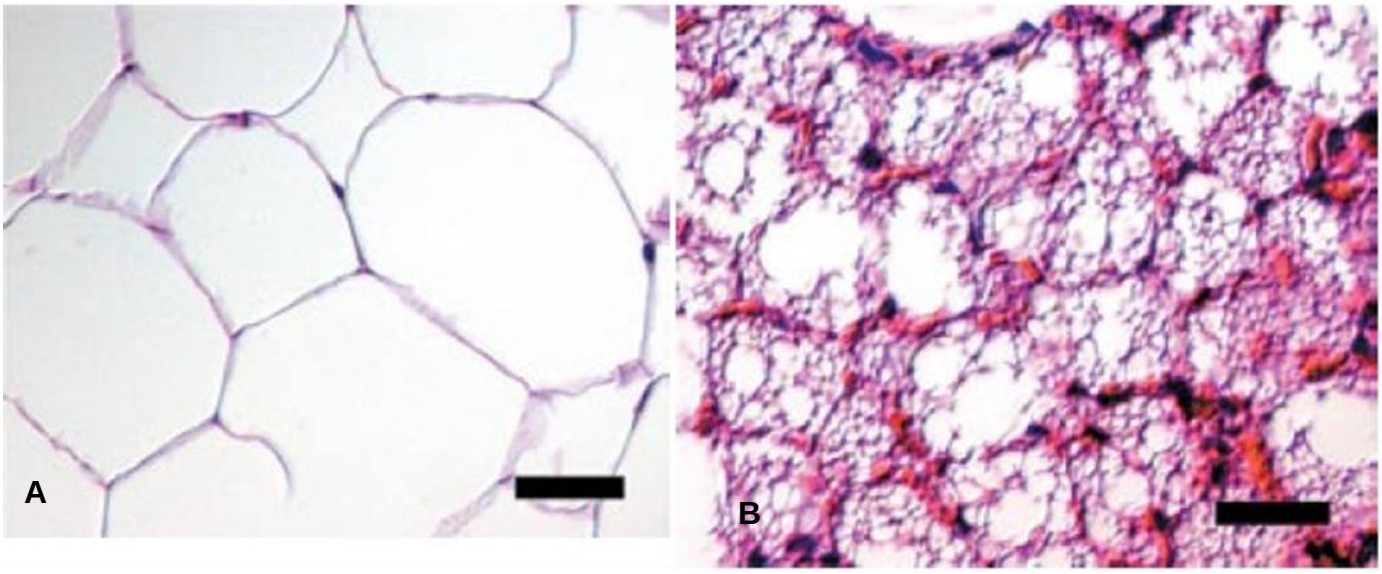


Figure 1 - Sections of (A) white adipose tissue and (B) brown adipose tissue in humans. Scale bars represent 30 µm. Figure adapted from Virtanen *et al*. (20)

Brown adipocytes are polygonal, with normal nucleus:cytoplasm ratio and several mitochondria. Recent findings showed that brown adipocytes exist in small fat depots in human adults, localized in cervical, supraclavicular, axillar and paravertebral areas. Besides, BAT seems to play a role in glucose homeostasis in obesity, and it was pointed as a possible target for the treatment of diabetes and obesity. (10, 11, 15)

White adipocytes are round, large (from 25 to 200 µm), with a large lipid droplet, thin cytoplasm and few mitochondria. White adipocytes are the majority of adipose cells in

human adults and can be found in fat depots in abdominal cavity and retroperineal, inguinal and gonadal areas. (15, 20)

BeAT are considered brown-like cells and can be found within WAT.(10, 12, 20)

The adipose tissue is a complex organ, composed not only by adipocytes, but also pre-adipocytes, leukocytes, macrophages, endothelial cells, fibroblasts, among other cell types, presenting nervous tissue, collagen fibres and lymph nodes. (21)

Energy is stored in the form of TG droplets in recently differentiated adipocytes. These cells are smaller and capable of absorbsing TG and free fatty acids, especially in postprandial period. These smaller adipocytes are more abundant in SAT. As adipocytes grow larger, they become dysfunctional, insulin resistant, hyperlipolytic and resistant to the anti-lipolytic effect of insulin. Larger adipocytes are more common in VAT (21)
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 and 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. (21)

According to data in literature, the location of fat tissue is closely associated with higher risk of metabolic syndrome, diabetes and cardiovascular disease; abdominal deposition of adipose tissue increases the risk of obesity-related complications. (22-27)
Thus, the correct assessment of quantity and location of adipose tissue provides important information concerning health risks. (21, 22, 25-29)
Adipokines have crucial metabolic functions, being involved in adipogenesis, energy homeostasis, control of feeding behaviour, immunity and inflammation, glucose and lipids metabolism and insulin sensitivity. (21, 22, 28-30)

1.4. Treatment of Obesity

Treating obesity includes non-surgical approaches and bariatric surgery. Non-surgical treatments are multicomponent approaches, comprising diet changes to reduce calorie intake, physical exercise, behavioural modification, pharmacological therapy and psychological intervention.(31)

However, bariatric surgery is the only long-term effective weight loss method in morbidly obese patients (BMI>40 kg/m^2) and in severe obese patients (BMI>35-40 kg/m^2) with comorbidities, such as diabetes, hypertension, dyslipidemia and CVD. (31-33)

There are different surgical procedures to treat morbid obesity: laparoscopic adjustable gastric banding or gastric balloons, sleeve gastrectomy, Roux-en-Y gastric bypass and biliopancreatic diversion (Figure 2) (31, 33)

Laparoscopic adjustable gastric banding or gastric balloons are pure restrictive surgical interventions, decreasing the volume capacity of the stomach, causing nutrient malabsorption, by either an adjustable external compressive device (gastric band) or by taking up space within the stomach (gastric balloon). These methods seem to be relatively ineffective in long-term weight lost and comorbidities resolution, when compared with hormonal and metabolic approaches. (33)

Sleeve gastrectomy is a surgical procedure that reduces the size (in approximately 70%) and curvature of stomach by excision. This kind of approach is not only restrictive, but seems to interfere with metabolism, decreasing circulating ghrelin concentrations and increasing secretion of the distal intestinal hormones, glutan-like peptide 1 (GLP-1) and peptide YY (PYY), even without intestinal rearrangement. However, these mechanisms are still unclear. (33)

Roux-en-Y gastric bypass and biliopancreatic diversion combine significant intestinal rearrangement with gastric restriction. These surgeries combine reduction of stomach volume with diversion of nutrient flow to various distal segments of intestine. Although these approaches seem to be highly effective in long-term weight loss and comorbidities resolution comparatively with restrictive procedures, there is also a higher risk of micronutrient and macronutrient deficiencies caused by the intestinal malabsorption component.(33)

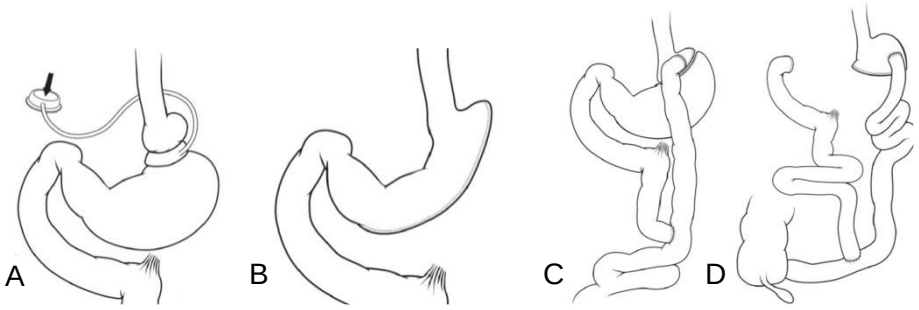


Figure 2 - Types of bariatric surgery. A: Laparoscopic adjustable gastric banding; B: Vertical sleeve, C: Roux-en-Y; D: Biliopancreatic Diversion. Figure adapted from Albaugh *et al.* (33)

2. Obesity-related comorbidities

Overweight and obesity are associated with a higher risk of several comorbidities, including CVD, insulin resistance, metabolic syndrome, diabetes, hypertension, dyslipidemia, gallbladder, pulmonary pathologies (dyspnoea and sleep apnoea), anemia and cancer (**Table 3**). (2, 3, 30)

The disregulation in adipokines secretion, inflammation and hypoxia within the adipose tissue, may favour some of those obesity associated comorbidities. (30)

Table 3 – The most common obesity associated complications.

Obesity-related comorbidities		
High Risk	**Moderated Risk**	**Low Risk**
Type 2 Diabetes	Coronary Pathology	Cancer
Insulin Resistance	Hypertension	Polycystic Ovary Syndrome
Dyslipidemia	Osteoarthrosis	Decrease in Fertility
Gallbladder Pathology	Hyperuricemia	Lumbar and Articular Pain
Dyspnoea		Aesthetic Risk
Sleep Apnoea		Foetal Malformation (Maternal Obesity)

Adapted from DGS *"Programa Nacional de Combate à Obesidade"* (2)

2.1. Inflammation

Adipose tissue secretes pro-inflammatory adipokines, such as leptin, resistin, chemerin, tumour necrosis factor alpha (TNF-α) and interleukin (IL)-6, as well as anti-inflammatory adipokines, namely adiponectin and omentin-1. In obesity, the distribution and function of adipose tissue and of the adipokine profile are altered. The unbalanced production of pro- and anti-inflammatory adipokines, the hypoxic milieu, the adipocyte hypertrophy and the infiltration of immune cells into the adipose tissue, contributes to the creation of a state of chronic low-grade inflammation in obesity. In accordance, data reported an increase in circulating levels of inflammatory markers, such as TNF-α, IL-6 and C-reactive protein (CRP), in obese individuals. This chronic low-grade inflammation has a crucial role in the development of obesity-related comorbidities. (30)

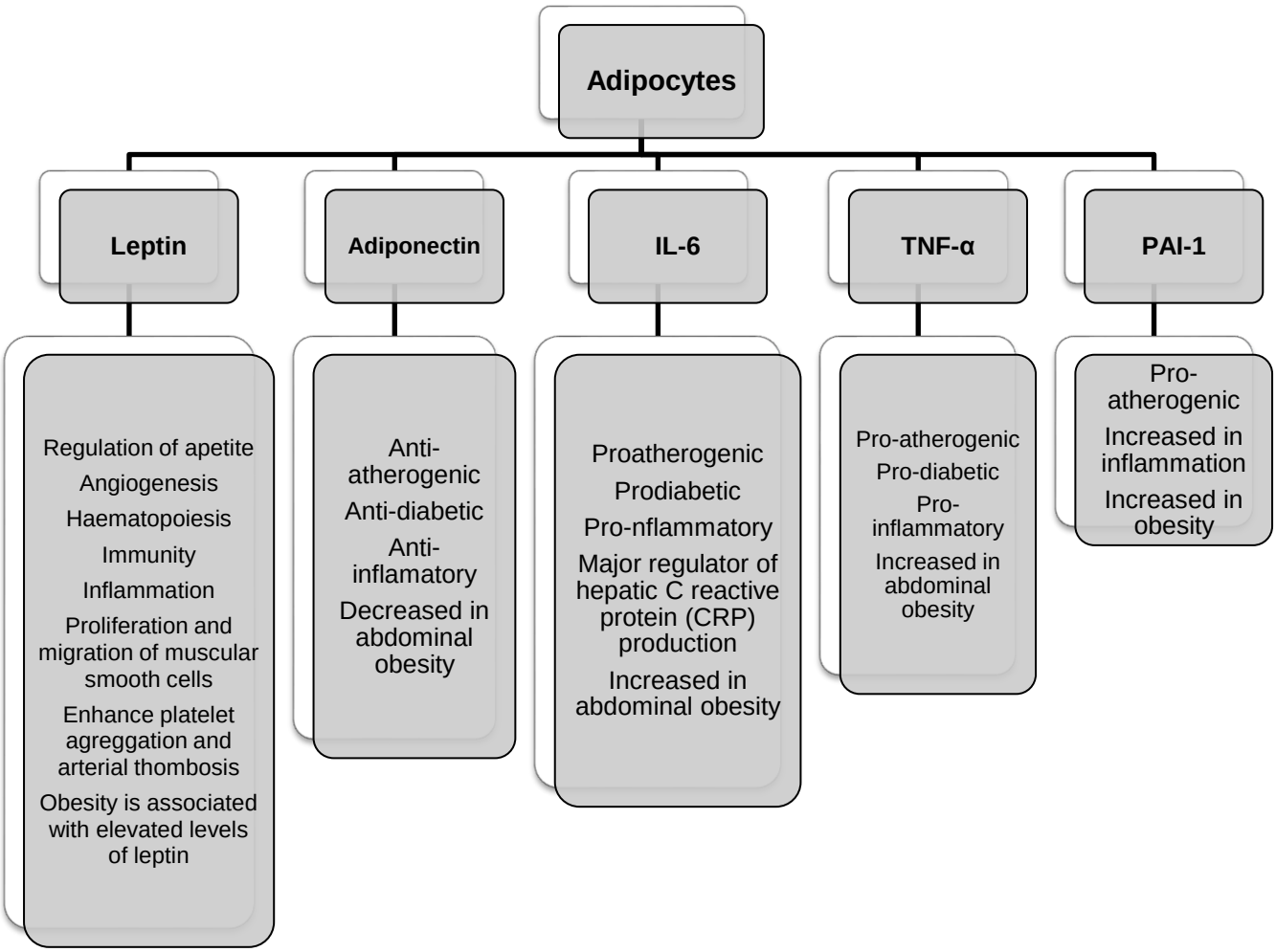


Figure 3 Summary on the functions of some important adipokines.
CRP: C-Reactive Protein; IL-6: Interleukin-6; PAI-1: Plamsminogen Activator Inhibitor-1; TNF-α: Tumour Necrosis Factor-α. Adapted from Nicklas *et al*. (24)

Leptin is a protein hormone secreted primarily by adipose tissue (AT) that participates in the processes of growth regulation and metabolism, and regulates food intake. Plasma levels of leptin, which correlate positively with body adiposity, are higher in obese patients when compared with healthy subjects. In obesity, a resistance to leptin may develop, explaining its reduced activity and its enhanced levels; the reduction in leptin activity leads to severe insulin resistance and vascular dysfunction.
Leptin regulates T-cell proliferation and activation, protecting these cells against apoptosis. Leptin also influences cytokine production from T lymphocytes, generally switching the phenotype toward a T-helper (Th) 1 immune response. Leptin stimulates the production of IL 1, 6 and 12 (IL-1, IL-6, IL-12), interferon (IFN)-γ and TNFα, by macrophages; the generation of reactive oxygen species (ROS); and it seems to increase T cell proliferation, Th1 polarization and to inhibit T regulator (Treg) cell proliferation. The increase in leptin levels in obesity seems to be a key event in the induction of adipose tissue inflammation, leading to a macrophage shift from M2 phenotype to M1 and to the development of a Th1 inflammatory response. (34-36)

TNF-α is a Th1 pro-inflammatory cytokine that presents both metabolic and immunological activities. It regulates the function and development of WAT by stimulating lipolysis and inhibiting lipogenesis and adipogenesis. TNF-α can lead to insulin resistance by inducing serine phosphorylation of the insulin receptor, which inhibits insulin signalling, being, therefore, considered a mediator of obesity-related type II diabetes. Furthermore, TNF-α increases the expression of leptin and reduces adiponectin secretion. Expression of TNF-α is increased in the WAT of obese subjects.(30, 36)

IL-6 is involved in the regulation of inflammation, inducing liver production of acute phase reactants, such as CRP, and increasing vascular inflammation. It also regulates lipid metabolism and haematopoiesis, and decreases insulin signalling, contributing to obesity-related diabetes. WAT contributes approximately with 35% of the circulating levels of IL-6 in non-inflammatory conditions. (36) Expression of IL-6 is increased in the WAT of obese subjects; visceral WAT secrets higher levels of IL-6 when compared with subcutaneous WAT. (30, 33, 36)

CRP is an acute phase protein of which hepatic production is triggered by IL-6 and TNF-α. Elevated levels of this inflammatory marker have been reported in diabetes and metabolic

syndrome, and associated with vascular inflammation. (34) Since IL-6 is an adipokine and is the major regulator of hepatic CRP production, adipose tissue has an indirect role in the regulation of circulating CRP levels.(33, 37) Some studies reported that CRP is expressed in adipose tissue, and that CRP mRNA levels correlated inversely with adiponectin. (37) Nevertheless, it is not clear if this production contributes significantly to circulating CRP levels. (33, 37)

Adiponectin has a protective role against insulin resistance and has an important anti-inflammatory activity. Adiponectin reduces the production of TNF-α, IL-6 and IFN-γ and induces the expression of anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonist. (30) As a result of this anti-inflammatory effect, high adiponectin concentrations have been associated with lower risk for cardiovascular and metabolic disorders. (36) Obese patients, especially those with visceral obesity, present decreased levels of adiponectin. (30, 36)

2.2. Hypoxia

Hypoxia seems to have a central role in obesity-related complications. It was proposed that the increased production of pro-inflammatory- adipokines in obesity, which favours the development of metabolic syndrome and other obesity-associated disorders, as a result from a response to adipose tissue hypoxia.(38)

The proportion of the cardiac output and blood flow that goes to WAT is not increased in the obese patients, despite adipocytes hypertrophy.(1, 38)

The most important protein associated with the body's response to low oxygen concentrations is hypoxia-inducible factor (HIF)-1. HIF-1 is a heterodimer composed by two units, α and β. The subunit HIF-1β is constitutively expressed and not regulated directly by O_2. HIF-α has three known subunits: HIF-1α, HIF-2α and HIF-3α and the combination of any of these subunits with HIF-1β forms the functional transcription factor. (1, 38)

In normoxic conditions, the subunit HIF-α is consistently synthesized and degraded. However, in obese patients its expression seems to be up-regulated, both in adipocytes and in infiltrating macrophages. There are several genes that are hypoxia sensitive through the hypoxia response elements for HIF, including genes that encode adipokines and cytokines involved in angiogenesis, cell proliferation and survival, apoptosis, vascular tone, and in metabolism of glucose and energy (**Figure 4**). Therefore, increase in HIF, leads to an enhanced production of adipokines and cytokines in hypoxic adipocytes. This hypoxic and inflammatory environment favour the development of comorbidities like type II

diabetes, dyslipidemia, hypertension, metabolic syndrome and CVD, among others. (1, 38, 39)

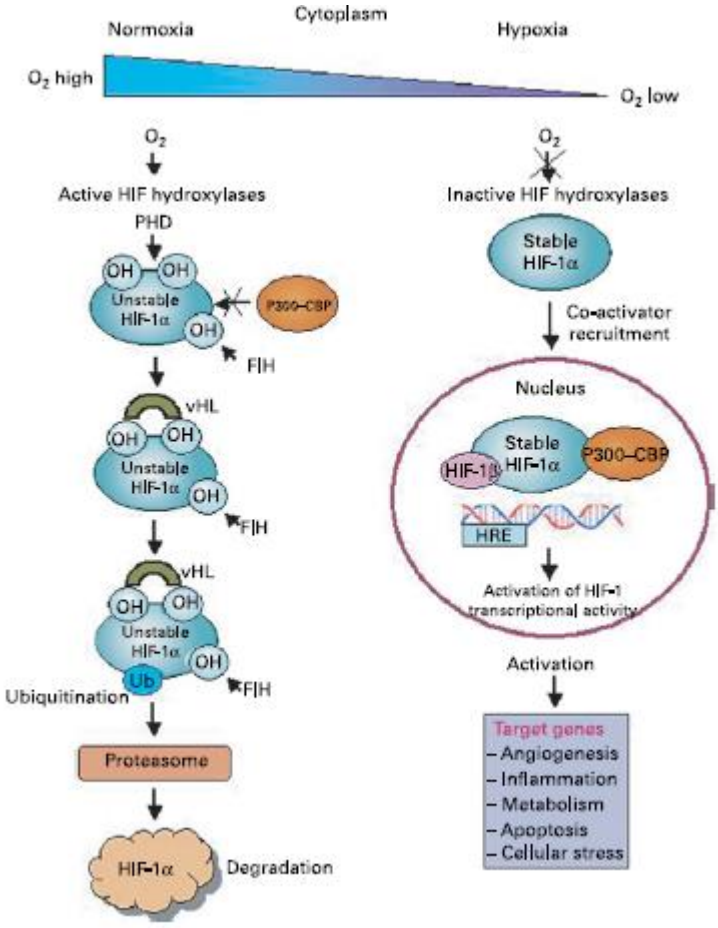


Figure 4- Schematic view of the molecular signalling response to hypoxia through the hypoxia-inducible factor (HIF) system.

FIH: factor inhibiting HIF-1; HRE: hypoxia-response element; p300–CBP: p300–cAMP response element binding protein; PHD: prolyl hydroxylase; Ub – ubiquitin; vHL: von Hippel–Lindau tumour suppressor protein. Figure adapted from Trayhurn *et al*. (38)

2.3. Alterations in Iron Metabolism

Iron is essential to cellular processes, such as energy production, biosynthesis and replication. Iron has a central role in erythropoiesis, being required to synthesis of heme, the prosthetic group of hemoglobin. (40)

Iron is provided by diet, but only about 10% of dietary iron is absorbed, being senescent erythrocytes the major source of iron for the synthesis of hemoproteins. Dietary iron only covers the daily iron loss (about 1-2mg/day). (40, 41)

Considering that erythrocytes have a lifespan of 120 days, about 20–25 mg of iron are required to the daily production of 20–25 mL of erythrocytes. Macrophages in the liver, spleen, and bone marrow phagocyte senescent or damaged erythrocytes; hemoglobin is degraded, heme is release, iron is extracted from heme and recycled to the extracellular fluid and plasma. (42, 43)

Iron from diet is absorbed by enterocytes, in proximal duodenum,in three forms: ferrous (Fe^{2+}), ferric (Fe^{3+}) and heme. 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; Fe^{2+} enters enterocytes by a facilitated transport mechanism, mediated by the divalent metal transporter 1 (DMT1). Heme iron enters directly into enterocytes as an intact iron-protoporphyrin complex, possibly by endocytosis or by binding to a heme receptor on the brush border of enterocytes, the recently described heme carrier protein 1 (HCP1). Inside the cell it is most likely to be cleaved by the intracellular microsomal enzyme, heme-oxygenase 1 (HO1), releasing Fe^{2+} iron and forming biliverdin.(40, 41)

Iron can be stored in enterocytes in the form of ferritin or it can be transferred across the basolateral membrane of the enterocyte to the bloodstream. Ferritin is a hollow spherical protein, whose cavity contains iron in ferric form complexed with hydroxide and phosphate anions. Its main functions are to store and sequester excess iron in a soluble, non-toxic form. Ferritin is regulated by cytokines like IL-6, IL-1 and IFN-γ.(44, 45)

Ferroportin (FPN1), a protein of the basolateral membrane of entherocytes transports Fe^{2+} to circulation, after iron oxidation by ferroxidases. (40) FPN1 is also expressed by the reticuloendothelial macrophages and lymphocytes. It is influenced by inflammation and is regulated by hepcidin, which binds to FPN1 inducing its internalization and lysosomal degradation (41, 45)

Transferrin (Tf) transports iron between the places where iron is absorbed or stored and the local of storage or usage. (40) It contains two globular domains, each one with a high affinity binding site to one molecule of Fe^{3+}. (40) The cellular uptake of transferrin-bound iron (TBI) is mainly mediated by the transferrin receptor (TfR) 1, located at the cell membrane. (40)

TBI is endocytosed via the cell membrane TfR1 and internalized into endosomal recycling vesicles, where is acidified and the Fe^{3+}iron is released. The iron-free (apo) transferrin-TfR1 complex returns to cell membrane and apotransferrin dissociates from TfR1 and diffuses away to be loaded with iron again. (41)

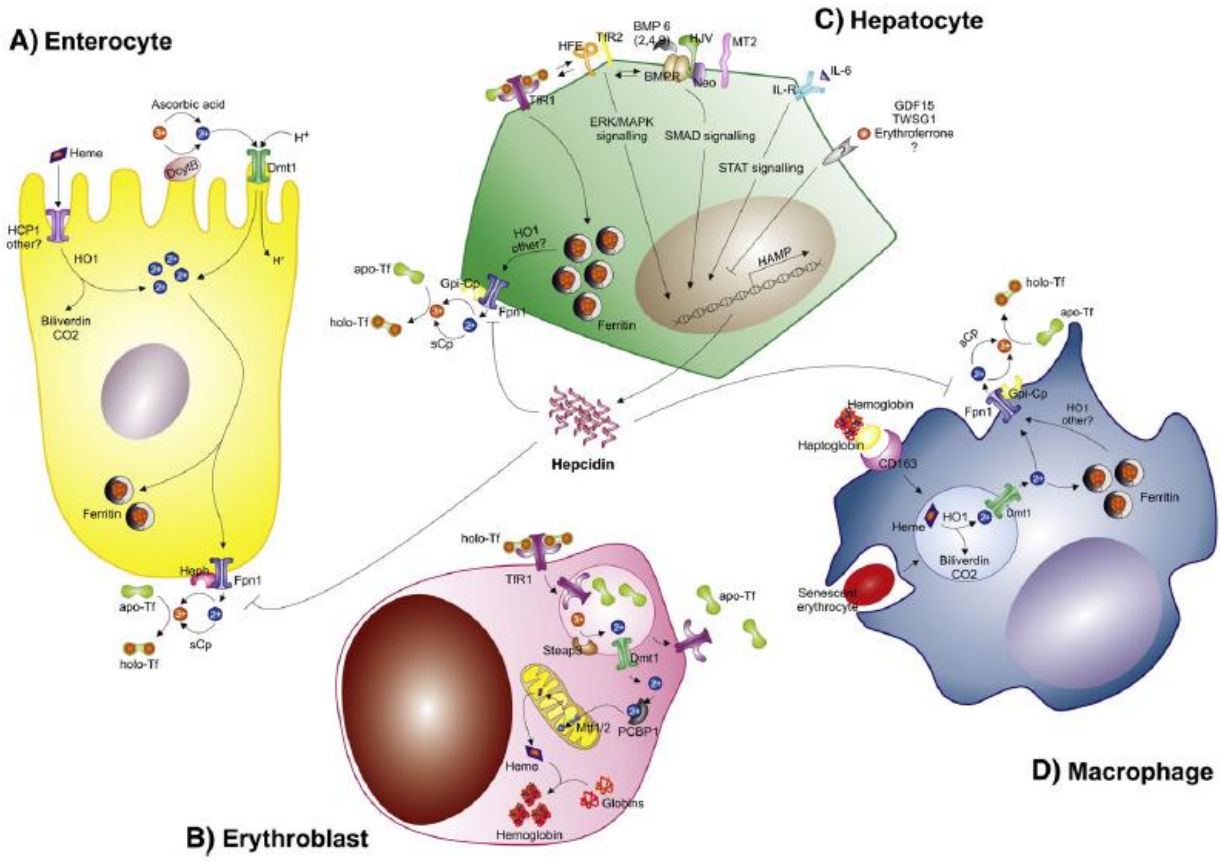


Figure 5 - Systemic iron metabolism regulation.
aCP: acyl carrier protein, BMP6: bone morphogenic protein 6; CO_2: carbon dioxide; Dmt1: divalent metal transfer; ERK/MAPK: extracellular signal–regulated kinases/mitogen-activated protein kinase; Fpn1: ferroportin 1; Gpi-Cp: ceruloplasmin anchored glycophosphatidylinositol; GDF15: growth differentiation factor; H^+: hydrogen ion; HAMP: hepcidin; HCP1: heme carrier protein; Heph: hephaestin; HFE: Human hemochromatosis protein; HJV: hemojuvelin; IL: interleukin; IL-R: interleukin receptor; PCBP1: Poly(RC) binding protein 1; STAT: signal transducer and activator of transcription; Tf: transferrin. Adapted from Silva *et al*. (40)

The major regulator of iron homeostasis is hepcidin a peptide hormone mainly synthetized by the liver, but synthetized also by other cell types, such as adipocytes. The expression of hepcidin is upregulated by *HAMP* gene, which is regulated by several factors, like systemic iron levels, hypoxia, anaemia, erythropoiesis, infection and inflammation. There are several hepatic proteins involved in the regulation of hepcidin synthesis, namely, hemojuvelin (Hjv), human hemochromatosis protein (HFE), TfR1 and TfR2. (40, 42, 43)

Hepcidin acts by triggering FPN1 internalization and, consequent degradation. Therefore, it regulates systemic iron status by reducing FPN1 levels, which leads to a decrease in the release of iron from enterocytes and macrophages.(40, 41)

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. In normal conditions, it is regulated through the bone morphogenetic protein-hemojuvelin (BMP-HJV) signaling pathway. (51, 52)

BMPs are a group of several proteins belonging to transforming growth factor β (TGF-β) superfamily, and they have pleiotropic roles in cellular differentiation, proliferation, and survival.(42)

Hemojuvelin (HJV) is a glycosylphosphatidylinositol-anchored membrane protein (mHJV) mainly expressed in skeletal muscle, heart, and liver. In conditions of iron deficiency or hypoxia, it is cleaved by furin at the C-terminus, generating soluble extracellular domain fragments (sHJV). The production of sHJV seems to downregulate hepcidin expression by competing with hepatocyte membrane-bound HJV for BMP binding. HJV seem to be cleaved also by matriptase-2 (MT2) and to suffer autocleavage . MT2 is expressed mainly in the liver and is a critical regulator of iron homeostasis. It cleaves membrane HJV, leading to sHJV release, thereby decreasing hepcidin expression. However, MT2 cleaves HJV producing one major soluble form of HJV that has decreased ability to bind to BMP6 and that can not suppress BMP6-induced hepcidin expression, suggesting that furin family proprotein convertases and MT2 cleave HJV in a site-specific manner. (40, 46, 47)

BMP6 binds to the activated BMP receptor complex, resulting in the phosphorylation of SMAD1/5/8. HJV is a BMP co-receptor and confers sensitivity. Phosphorylated SMAD1/5/8 binds with the common (Co)-SMAD, SMAD4, generating the transcriptional complex that binds to *HAMP* promoter and upregulates *HAMP gene* expression. (42, 46)

The function of HFE is not entirely known, but it is capable of forming high-affinity complexes with TfR1 in enterocytes. This binding allows the silencing of activity of HFE. Transferrin competes with HFE for binding to TfR1. An increase in transferrin saturation favours HFE dissociation, allowing its association with TfR2; this binding has lower affinity than association with TfR1 (40).Expression of TfR2, highly expressed in the liver, is decreased in iron deficiency and increased in iron overload. (40)

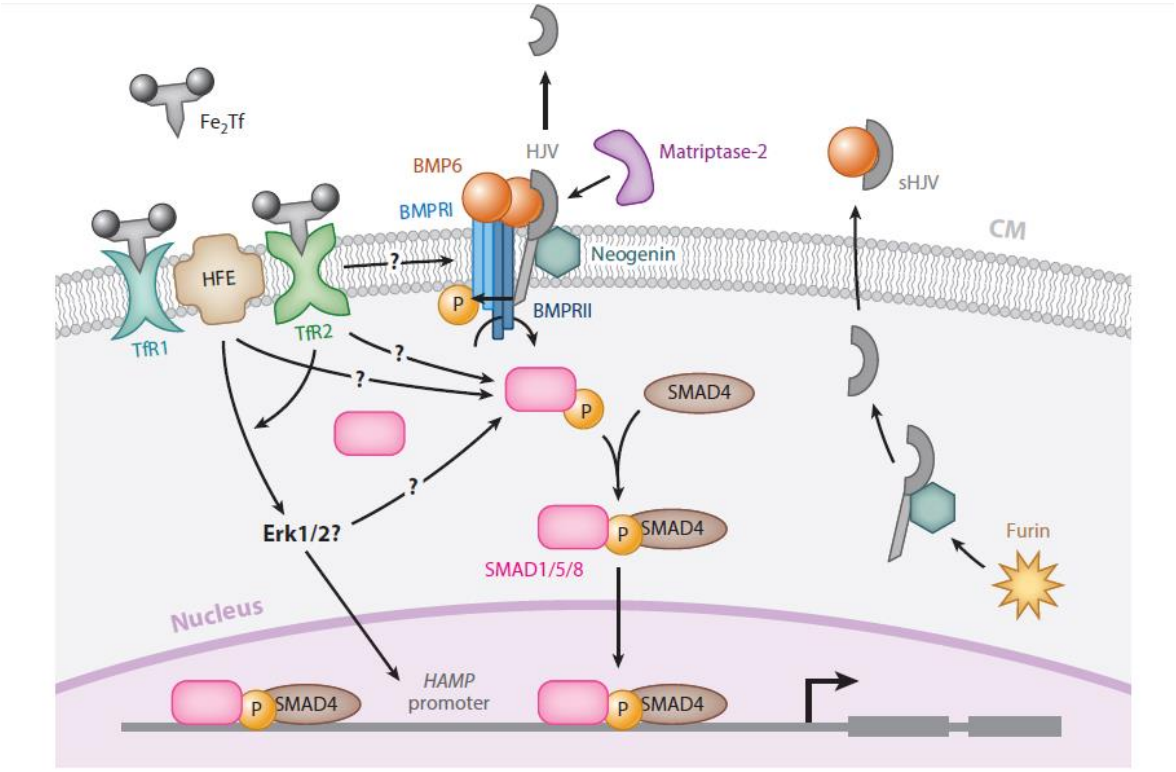


Figure 6- BMP/SMAD signalling in iron homeostasis.

BMP: bone morphogenic protein; BMPR-I and II: bone morphogenetic protein signalling receptors type I and type II; CM: cell membrane; ERK: extracellular signal-regulated kinase; HFE: human hemochromatosis protein; HJV: hemojuvelin; P: phospho-; sHJV: soluble hemojuvelin; Tf–: transferrin; TfR1 and 2: transferrin receptor 1 and 2. Adapted from Parrow *et al*. (46)

Chronic inflammation and hypoxia have a significant impact in iron status. (40, 44, 45, 48)

Hypoxia leads to an increase in erythropoiesis in order to overcome low body oxygen levels. HIF system is recognized as being the main modulator of both iron metabolism and erythropoiesis during hypoxia. The stabilized HIF regulates the expression of genes that encode the proteins Tf, TfR1, and erythropoietin (EPO).(40)

Hypoferremia is often observed in obesity. Several mechanisms have been proposed to explain the association between obesity and low iron status, namely, an inadequate dietary iron ingestion, an increase in iron requirements, as a consequence of a higher body mass, and an increase in blood volume. (44, 45)

However, the most accepted hypothesis is that iron deficiency is a consequence of the chronic low-grade inflammation state that characterizes obesity. It was reported that the chronical inflammatory state in obesity affects iron absorption. (44, 45)

Indeed, the reported association between hepcidin, chronic inflammation, and low iron status in obese populations, has been accepted as a potential mechanism by which iron status impairment occurs in obesity.(40, 44, 49)

In inflammatory pathway, IL-6 binds to its receptor, and initiates signalling through activated Janus kinase (JAK) 2, leading to phosphorylation of STAT3. Activated STAT3 then binds to a STAT3-responsive element (STAT3-RE) on the proximal hepcidin promoter. Hepcidin protein is secreted into the bloodstream resulting in ferroportin inhibition, leading to iron retention in reticuloendothelial macrophages and to a reduction of iron absorption in the intestinal epithelia. (42, 50)

Usually, in iron deficiency anaemia, hepcidin levels are downregulated, but in anaemia of inflammation these levels are upregulated by the inflammatory pathway, where proinflammatory cytokines, such as IL-6, stimulates *HAMP* expression through a STAT3 dependent mechanism. (51)

Adipose tissue is able to produce hepcidin, both at mRNA and protein levels. At mRNA level, adipose tissue *HAMP* expression is higher than hepatic expression, in obese patients and seems that this is caused by acute inflammatory changes. (51)
In spite of the knowledge that adipose tissue expression of HAMP is increased in obese patients, when compared with lean individuals, there are still controversies concerning the contribution of adipocytes to the circulating levels of hepcidin and to its relevance in iron metabolism. It remains unclear if it is upregulated by inflammation or iron status and if HJV levels play a role in this regulation (**Figure 7**). (51)

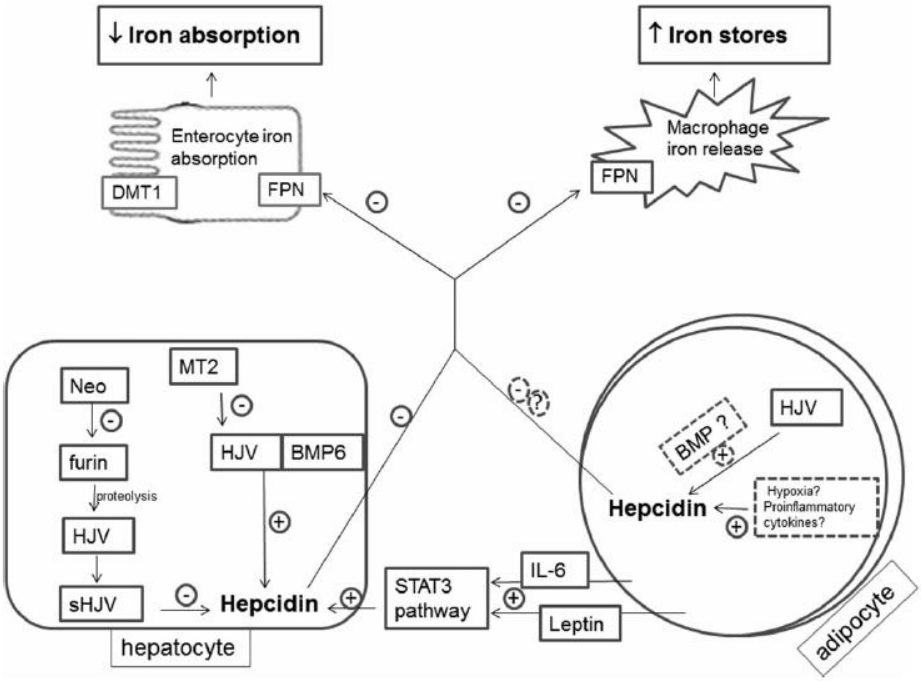


Figure 7– Schematic representation of the interaction between hepcidin and iron, in adipocyte and hepatocyte. BMP: bone morphogenic protein; DMT1: divalent metal transporter 1; FPN: ferroportin; HJV: hemojuvelin; IL: interleukin; MT2: matriptase-2; Neo: neogenin; sHJV: soluble hemojuvelin; STAT3: signal transducer and activator of transcription 3; -: suppress; +: induce; ?: doubt. Adapted from Coimbra *et al*. (51)

Chapter II – Aims

The aim of this study was to understand the role of subcutaneous and visceral adipose tissue in obesity related iron deficiency by comparing severe obese patients with normal weighted healthy individuals, at blood and adipose tissue levels.

Anthropometric measurements, haematological, biochemical and iron metabolism studies were performed for Obese and Control groups. To perform genetic studies in the adipose tissue, subcutaneous and visceral adipose tissue were carefully removed, when obese patients underwent bariatric surgery and controls underwent cholecystectomy.

We studied the gene expression of erythropoietic, iron regulatory proteins and inflammatory cytokines, such as, BMP6, EPOR, ferritin, hemojuvelin, hepcidin, IL-6, TNF-α and CRP for both groups.

Chapter III – Materials and Methods

1. Subjects

The protocol used was approved by the Committee on Ethics of *Hospital da Prelada*. Informed consent was obtained from all the patients enrolled in the study.
This study involved a group of severe or morbid obese patients (n=30) that were selected for laparoscopic adjustable gastric banding (LAGB). The selection criteria for LAGB were severe obesity and at least one comorbidity, or morbid obesity. A group of 17 patients submitted to cholecystectomy were enroled as a control group.
Subcutaneous and visceral adipose tissues biopsies were collected during surgeries.

2. Sample collection

Visceral and subcutaneous adipose tissues upon collection were immediately conserved in RNAlater® (Sigma-Aldrich, Inc., St.Louis, USA). Tissue samples were than stored at 4 °C for 24 hours; afterwards, were frozen at -20ºC.
Blood samples were collected, before surgeries, in order to obtain whole blood, plasma (ethylenediaminetetraacetic acid - EDTA), and serum. Aliquots of plasma and serum were stored at -80ºC until assayed.

3. Haematological determinations

The haematological determinations were performed on an automatized blood cell counter (ABX Micros 60-OT, Horiva ABX, Amadora, Portugal), and included red blood cell (RBC) count, haematocrit (Ht), haemoglobin concentration (Hb), mean cell haemoglobin (MCH); mean cell haemoglobin concentration (MCHC); mean cell volume (MCV); red cell distribution width (RDW), white blood cell (WBC) and platelet count. Cell morphology was evaluated in Wright stained blood films (Wright Stain, Sigma, St. Louis, Missouri, USA).
Reticulocyte count was measured by microscopic counting on blood smears after vital staining with New Methylene Blue (Reticulocyte Stain, Sigma, Missouri, USA).

4. Biochemical assays

The circulating plasma levels of erythropoietin (Human Erythropoietin Platinum ELISA, eBiocience, California, USA), sTfR (IVD Soluble Transferrin Receptor, R&D Systems Inc.,

Minneapolis, USA), hepcidin (Quantikine Human Hepcidin Immunoassay, R&D Systems Inc., Minneapolis, USA) and IL-6 (Human IL-6 HS Imunoassay, R&D Systems Inc., Minneapolis, USA), were determined by using an enzyme immunoassay.

Serum iron concentration was determined using a colorimetric method (Iron, Randox Laboratories Ltd., North Ireland, UK), whereas ferritin, transferrin and CRP were measured by immunoturbidimetry (Ferritin, Laboratories Ltd., North Ireland, UK; Transferrin, Laboratories Ltd., North Ireland, UK; CRP (latex) High-Sensitivity, Roche Diagnostics, Basel, Switzerland; respectively). Transferrin saturation (TS) was calculated using the formula: Transferrin Saturation (%)= $\frac{70.9 \text{ x serum iron levels (µg/dL}}{\text{transferrin (mg/dL)}}$. (52)

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5. Gene Expression Studies

5.1. RNA Isolation

All the equipment and materials were prepared to avoid contamination of samples with RNAses. To ensure that, diethylpyrocarbonate (DEPC) was used to treat all the metalic objects and glassware that would be in touch with the samples. The material was cleaned and immersed in DEPC for 2 hours and, finally, heated at 120 ºC for 1 hour.

RNA extraction from adipose tissues 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 method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction developed by Chomczynski and Sacchi. (53) In this method 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. Then, chloroform is added to the extract that, after centrifugation, forms three phases: an upper colourless aqueous (RNA) phase, a central white interphase (DNA) and the lower red organic phase (mostly proteins and lipids).
RNA is recovered from the upper phase by precipitation with isopropanol. After being recovered, RNA is washed with ethanol, resuspended in DEPC-treated water, and stored at -80 °C until required. Concentration and RNA purity were evaluated on NanoDrop® 1000 (Thermo Fisher Scientific, Massachusetts, USA) spectrophotometer.

5.2. 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 (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 method uses a RNA-dependent DNA polymerase with reduced RNAse H activity and increased thermal stability (an engineered Molony Murine Leukemia Virus Reverse Transcriptase).This protocol also includes an RNAse inhibitor to prevent RNA degradation by contamination. The kit requires a first short incubation of the template RNA with random hexamer primers (included in the kit) at 70 °C and a 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).

5.3. Gene Expression Analysis

Gene expression was analysed by real-time PCR performed in Step-One Real-Time PCR System (Applied Biosystems, California, USA) and data was analysed with the software provided.

For amplification, it was used the KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Massachusetts, USA). Primers were specifically designed for this study (**Table 4**).

PCR reactions were preformed using the following conditions: enzyme activation at 95 ºC for 30 seconds; denaturation at 95 ºC for 10 seconds; annealing for 20 seconds (temperature used for each gene is presented at **Table 4**). The melting curve was analysed to detect single-nucleotide polymorphisms and assess the specificity of amplification. Gene expression was normalized to alpha-tubulin gene, and relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method.

Table 4 - Primers and Annealing Temperatures used on Real-time PCR for gene expression analysis.

Primer	Forward (5'-3')	Reverse (3'-5')	Annealing Temperature
Alpha-Tubulin	CTGGAGCACTCTGATTGT	ATAAGGCGGTTAAGGTTAGT	56 ºC
HAMP	ATCAAAGTGTGGGATGTG	CAAGACCTATGTTCTGGG	60 ºC
EPOR	TATCTGACTCTGGCATCT	ATAAGGCTGTTCTCATAAGG	60 ºC
BMP6	TGCCAGTGCCTTATTACC	GTTACCAGACCTTATGCTACA	56 ºC
CRP	GTTTGGGTCCAGAGTGCTCA	GCTCTGCTGGGGCAATTCTA	60 ºC
TfRC	GGACGCGCTAGTGTTCTTCT	CATCTACTTGCCGAGCCAGG	59 ºC
HJV	ACTCAGTAGCCACCTCCCTC	TCACTGAGGTCAGGGAACCA	56 ºC
IL-6	TGCAATAACCACCCCTGACC	GTGCCCATGCTACATTTGCC	59 ºC
TNF-α	CCTGACATCTGGAATCTG	TCTAAGGAAGTCGGAA	57 ºC
Ferritin	TGCCGTTGTTCAGTTCTAATCAC	TCCAAGACAGCCACACCTT	60 ºC

BMP6: Bone morphogenic protein 6; CRP: C-reactive protein; EPOR: erythropoietin receptor; HAMP: hepcidin; HJV: hemojuvelin; IL-6: interleukin 6; TfRC: transferrin receptor 1; TNF-α: tumour necrosis factor-α

6. Statistical Analysis

All statistical analyses were performed using the Statistical Package for Social Science (SPSS, version 23 for Windows; IBM Corporation, New York, USA). A Shapiro-Wilk test was used to determine the distribution of the variables. Data is presented as mean ± standard deviation (SD) or as median levels (inter-quartile range) according to distribution of variables; data concerning gene expression is presented as mean ± standard error of the mean (SEM). To evaluate differences between groups, we used the unpaired Student's t-test for determinations presenting a Gaussian distribution, and the Mann–Whitney U test for those presenting a non-Gaussian distribution. The correlation analysis was performed by calculating the Pearson's or the Spearman's correlation coefficient, according to Gaussian or non-Gaussian distribution of the variables, respectively. A *P* value <0.05 was considered statistically significant.

Chapter IV – Results

In **Table 5**, the results for the demographic data and blood pressure are presented in both groups, Control (n=17; 7 females and 10 males) and Obese patients (n=25, 21 females and 4 males).
Weight, BMI, waist, hip and W:H ratio were significantly higher, while height was significantly lower in Obese group when compared with Control group.

Table 5 - Demographic data and blood pressure for Obese and Control Groups

	Control (n=17)	**Obese (n=25)**	***P***
Age (years)	55 ± 15	48 ± 9	0.053
Weight (kg)	72 ± 12	100 ± 12	<0.001
Height (m)	1.66 ± 0.07	1.60 ± 0.08	0.014
BMI (kg/m^2)	25.7 ± 3.2	39.1 ± 3.6	<0.001
Waist (cm)	93 ± 11 (n=14)	115 ± 10 (n=24)	<0.001
Hip (cm)	100 ± 11 (n=14)	124 ± 11 (n=24)	<0.001
W:H ratio	56 ± 6 (n=14)	72 ± 6 (n=24)	<0.001
SBP (mmHg)	129 ± 20 (n=13)	139 ± 16 (n=19)	0.133
DBP (mmHg)	85 ± 16 (n=19)	87 ± 10 (n=19)	0.601

Results are presented as mean ± standard deviation. BMI: Body Mass Index; DBP: Diastolic Blood Pressure; SBP: Systolic Blood Pressure; W:H ratio: Waist:Height ratio

In parallel with gene expression study, haematological, biochemical, iron metabolism and erythropoietin studies were performed, as a way to obtain more complete information about Obese and Control groups.

The results for the haematological studies in both groups are presented in **Table 6**. White blood cells (WBC), neutrophils and platelets counts were significantly increased in Obese group when compared with Control group.

The RBC count, haemoglobin concentration and haematocrit of Obese patients are similar to Control, however, there are significant morphological erythrocyte changes, namely, a decrease in MCV and MCH, and a trend towards a reduction in MCHC.

Ferritin is significantly and positively correlated with WBC (*P*=0.032, *r*=0.449) and lymphocytes (*P*=0.022, *r*=0.476).

Table 6 - Haematological study for Obese and Control groups

	Control (n=17)	**Obese (n=25)**	***P***
Haemoglobin (g/dL)	14.9 ± 1.3	14.2 ± 1.3	0.135
RBC (10^{12}/L)	4.93 ± 0.40	5.0 ± 0.39	0.616
Haematocrit (L/L)	42.9 [41.0-48.5]	42.5 [40.7-45.5]	0.745
MCV (fL)	90.5 ± 4.1	86.7 ± 5.7	0.048
MCH (pg)	30.3 [29.5-31.4]	28.7 [27.2-29.7]	0.007
MCHC (g/dL)	33.5 [32.8-34.1]	32.8 [32.0-33.4]	0.092
RDW (%)	13.1 [12.4-13.9]	13.3 [13.0-14.0]	0.296
Reticulocytes (x10^9/L)	50.70 [40.86-57.48]	47.73 [39.94-58.21]	0.943
RPI	1.02 [0.79-1.19]	0.90 [0.75-1.07]	0.899
WBC (x10^9/L)	6.41 ± 1.48	7.58 ± 1.05	0.010
Neutrophils(x10^9/L)	3.47 ± 0.90	4.60 ± 0.94	0.002
Eosinophils(x10^9/L)	0.15 [0.10-0.20]	0.20 [0.10-0.20]	0.745
Basophils(x10^9/L)	0.00 [0.00-0.10]	0.00 [0.00-0.10]	0.771
Monocytes(x10^9/L)	0.40 [0.33-0.60]	0.30[0.30-0.40]	0.132
Lymphocytes(x10^9/L)	2.28 ± 0.62	2.37 ± 0.40	0.621
Platelets(x10^9/L)	220 ± 46	288 ± 54	0.001

Results are presented as mean ± standard deviation and as median [interquartile ranges].
MCH: Mean cell haemoglobin; MCHC: Mean cell haemoglobin concentration; MCV: Mean cell volume; RBC: Red Blood Cells; RDW: Red Cell Distribution Width; RPI: reticulocyte production index; WBC: White Blood Cells

Table 7 shows the results for inflammatory biomarkers, iron metabolism and erythropoietin studies.
We found that IL-6, CRP, HAMP and sTfR were significantly increased in Obese group when compared with Control group.

We also found that hepcidin had a significant and positive correlation with ferritin (*P*=0.009, *r*=0.510); and a significant and negative correlation with erythropoietin (P=0.006, r=-0.532) and transferrin (*P*=0.014, r=-0.487); moreover, sTfR was significantly and negatively correlated with transferrin saturation (*P*=0.032, *r*=-0.431).

Table 7 – Biochemical, iron metabolism and erythropoietin studies in Obese and Control groups

	Control (n=17)	**Obese (n=25)**	***P***
IL-6 (pg/mL)	1.68 [1.09-2.04]	2.12 [1.68-3.79]	0.009
CRP (mg/dL)	0.14 [0.08-0.26]	0.56 [0.26-1.02]	0.001
Hepcidin (ng/mL)	23.5 [13.2-35.8]	56.7 [30.1-69.0]	0.003
Iron (mg/dL)	114.00 ± 33	102 ± 38	0.288
Ferritin (mg/dL)	136 [64-228] (n=16)	170 [92-269]	0.682
Transferrin (mg/dL)	266 ± 34	283 ± 55	0.260
Transferrin Saturation (%)	32.1 ± 7.7	27.0 ± 9.9	0.090
sTfR (nmol/L)	13.9 [11.8-20.2]	19.6 [14.8-26.4]	0.006
Erythropoietin (mlU/mL)	4.06 [2.58-5.76]	4.57 [2.94-6.28]	0.315

Results are presented as mean ± standard deviation and as median [interquartile ranges].
CRP: C-reactive Protein; IL-6: Interleukin 6; sTfR: Soluble Transferrin Receptor

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

Considering iron metabolism and erythropoietic markers (**Figure 8**), we found that in SAT only TfR was significantly overexpressed. The gene expression of the inflammatory markers (**Figure 9**) showed values similar to the Control group.

It was also found that in SAT, TfR expression was significantly and positively correlated with the expression of ferritin (*P*=0.002, r=0.631), BMP6 (*P*=0.009, *r*=0.570), HJV (*P*=0.021, *r*=0.490) and IL-6 (*P*=0.041, *r*=0.449).

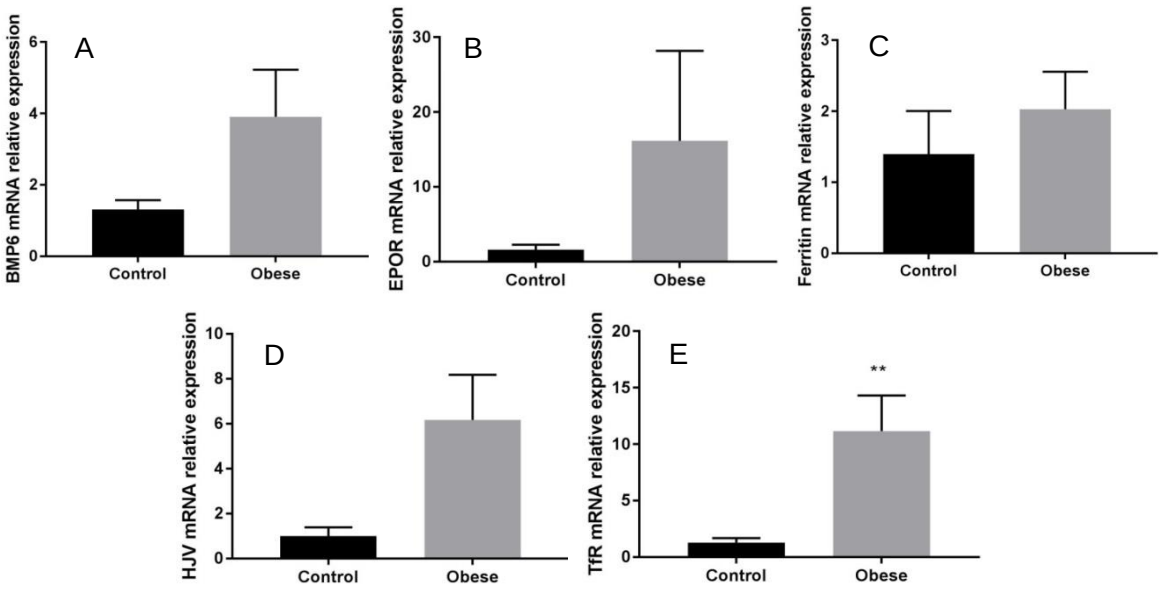


Figure 8 – Relative mRNA gene expression, in subcutaneous adipose tissue, for A) bone morphogenic protein 6 (BMP6), B) erythropoietin receptor (EPOR), C) ferritin, D) hemojuvelin (HJV) and E) transferrin receptor (TfR), in Control (n=8) and Obese (n=22) patients. Results are presented as mean ± standard error of the mean. $^{**}P< 0.001$ *vs* Control.

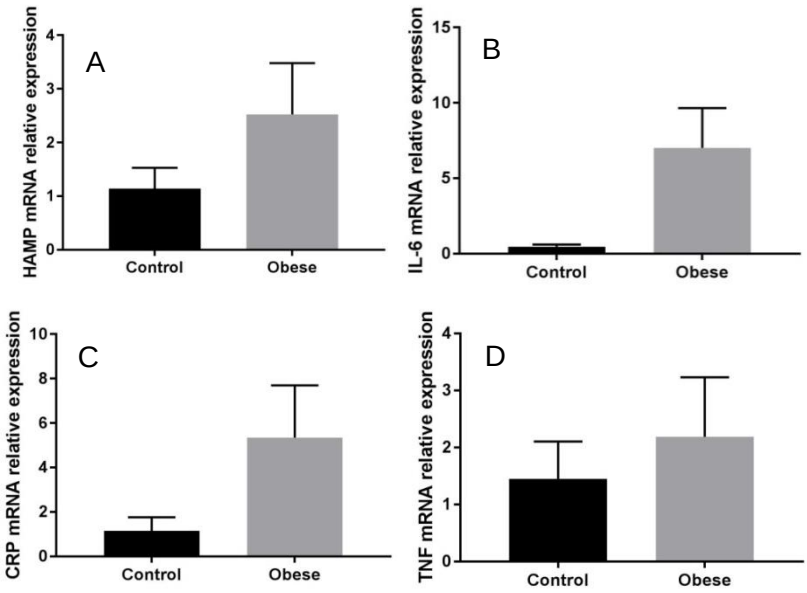


Figure 9 - Relative mRNA gene expression, in subcutaneous adipose tissue for A) hepcidin (HAMP), B) interleukin 6 (IL-6), C) C-reactive protein (CRP) and D) tumour necrosis factor α (TNFα) , in Control (n=8) and Obese (n=22) patients. Results are presented as mean ± standard error of the mean.

We also studied the gene expression of the same inflammatory, erythropoietic and iron metabolism markers in VAT, and found that the gene expression of hepcidin, ferritin and

CRP were significantly increased, when compared to VAT from the Control group (**Figure 10** and **Figure 11**). IL-6 gene expression showed a trend towards an overexpression (**Figure 11**) (*P*=0.077).

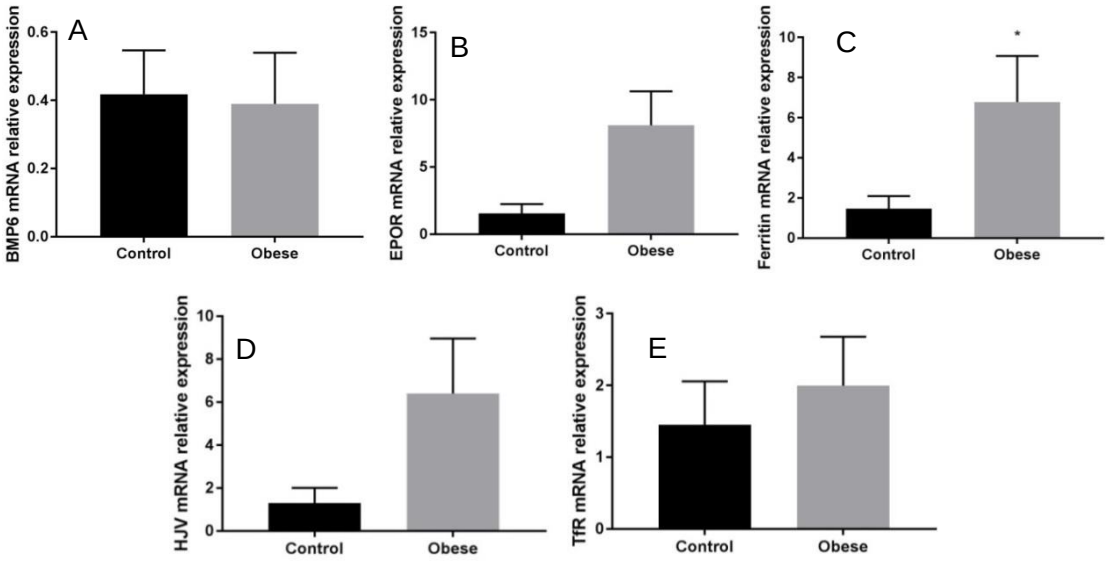


Figure 10 – Relative mRNA gene expression, in visceral adipose tissue, for A) bone morphogenic protein 6 (BMP6), B) erythropoietin receptor (EPOR), C) ferritin, D: hemojuvelin (HJV) and E) transferrin receptor (TfR). Results are presented as mean ± standard error of the mean.
**P*< 0.05 *vs* Control.

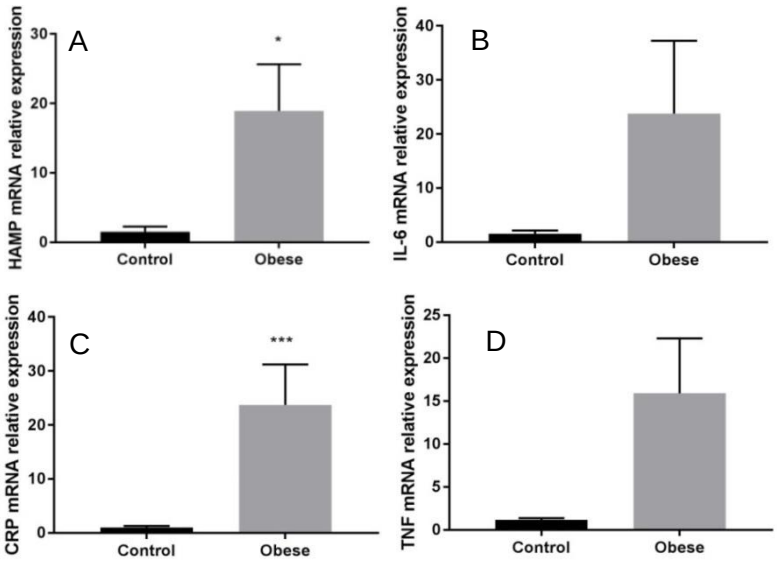


Figure 11 - Relative mRNA gene expression, in visceral adipose tissue for A) hepcidin (HAMP), B) interleukin 6 (IL-6), C) C-reactive protein (CRP) and D) tumor necrosis factor α (TNFα) in Control (n=8) and Obese (n=22) patients. Results are presented as mean ± standard error of the mean. **P*<0.05, ****P*<0.01 *vs* Control.

We also found that, in VAT, *IL-6* gene expression had significant and positive correlations with BMI (*P*=0.040, *r*=0.440), and with the gene expressions of CRP (*P*=0.001, r=0.642), ferritin (*P*=0.002, r=0.634), hemojuvelin (*P*=0.003, r=0.604), BMP6 (*P*=0.040, *r*=0.513) and hepcidin (*P*=0.048, *r*=0.447).

CRP gene expression also showed significant and positive correlations with the gene expression of BMP6 (*P*=0.000, *r*=0.791), hemojuvelin (*P*=0.000, *r*=0.834), hepcidin (*P*=0.003, r=0.623), ferritin (*P*=0.003, r=0.679) and EPOR (*P*=0.015, *r*=0.537).

Hepcidin gene expression was significantly and positively correlated with the gene expression of EPOR (*P*=0.002, r=0.682), BMP6 (*P*=0.007, r=0.579) and hemojuvelin (*P*=0.007, r=0.582), while ferritin expression was significantly and positively correlated with TNF-α (*P*=0.002, r=0.758), BMP6 (*P*=0.009, r=0.546) and hemojuvelin (*P*=0.010, r=0.536).

Chapter V – Discussion

No significant correlations were observed between gene expression of inflammatory, erythropoietic and iron metabolism markers, in VAT and in SAT, and the circulating levels of parameters studied.

In obesity, the unbalanced production of pro- and anti-inflammatory adipokines, the hypoxic milieu, the adipocyte hypertrophy and the infiltration of immune cells into the adipose tissue, contributes to the creation of a state of chronic low-grade inflammation. In agreement with published data (21, 24), we observed that obese patients, compared to normal weight controls, presented higher levels of inflammatory markers, such as IL-6, CRP and hepcidin, and higher leukocytes, namely neutrophils.

Obesity is accompanied by platelet hyperactivity (54), which favors the occurrence of thrombotic events; in our study, higher counts of platelets were also found.

Obesity is known to be often associated with iron deficiency (55-57). This disturbance in iron metabolism seems to be a consequence of the chronic low-grade inflammation state that characterizes obesity. Inflammation enhances hepcidin production, the major regulator of iron availability for erythropoiesis, and its synthesis occurs in response to pro-inflammatory cytokines, such as IL-6 (58). IL-6 induces hepatic hepcidin expression via JAK2/STAT3 signaling pathway. As referred, we found significantly higher hepcidin and IL-6 levels in obese patients. The hepatic hepcidin production is stimulated by pro-inflammatory cytokines, bone morphogenetic proteins and iron overload, being inhibited by iron deficiency, hypoxia, erythropoietin and erythropoietic products. (42, 43, 57) In accordance, we found that hepcidin correlated significantly and inversely with erythropoietin and transferrin.

Soluble TfR is a more reliable marker of iron status, in opposition to ferritin that is an acute-phase protein. Indeed, we observed that ferritin correlated significantly and positively with indicators of inflammation, namely with hepcidin and total leukocytes.(40) Soluble TfR reflects the functional iron compartment, increasing when the functional iron pool is decreased and with accelerated erythropoiesis.(40) Since no significant differences were found for sTfR concentration, reticulocyte and erythropoietin levels between obese patients and controls, the higher levels of sTfR observed in obese patients are likely to result from a disturbance in iron availability, due to the inhibition of iron absorption and

mobilization, by hepcidin, reducing the iron available for haemoglobin synthesis; indeed, sTfR was significantly and negatively correlated with transferrin saturation. The functional iron deficiency, probably, contributed to the morphological erythrocyte changes observed in obese patients - a significant decrease in MCV and MCH, and a trend towards a reduction in MCHC. This microcytic and hipocromic changes further strengthen the disturbance in iron availability.

It has been reported that the adipose tissue expresses hepcidin (27), and its regulator hemojuvelin.(47) As referred, hepatic production of hepcidin is up-regulated by IL-6, BMP and iron overload, through the BMP/SMAD pathway signaling. (51, 52) HJV bounded to the hepatocyte cell membrane participates as a BMP6 coreceptor in this pathway, regulating hepcidin expression, whereas sHJV antagonizes BMP6. Concerning the adipocyte production of hepcidin, the role of the BMP/SMAD pathway is not clear and it has been proposed that the inflammatory stimuli triggers this production.(51)

It was reported that differentiation of 3T3-L1 pre-adipocytes into adipocytes is associated with a consistent increase of ferritin mRNA expression (59) and that a higher gene expression of CRP occurs in adipose tissue of obese patients. (60) In the present study, we found that hepcidin, CRP and ferritin expressions were significantly higher, and IL-6 showed a trend towards an increase, in VAT of obese patients; moreover, IL-6 gene expression in VAT correlated positively and significantly with BMI, which is in accordance with an increasing production of this pro-inflammatory adipokine, with obesity. Moreover, IL-6 overexpression correlated positively and significantly with hepcidin, CRP and ferritin, which correlated significantly with TNF-α.
The increase in hepcidin and IL-6 expression and the correlation observed between them, point to a tight relationship between VAT hepcidin expression and inflammation. The correlation found for hepcidin, IL-6 and ferritin with BMP6 and hemojuvelin also suggest that hemojuvelin and the BMP/SMAD pathway may contribute to regulation of hepcidin visceral adipose tissue expression.
The increased gene expressions and the correlations between the inflammatory adipokines in VAT, show their important role in these adipose tissue, in obese patients.

The study of the gene expression of inflammatory, erythropoietic and iron metabolism markers, in the visceral and in the subcutaneous adipose tissue, showed that they present a different profile. Actually, VAT has been referred to be metabolically more active and more vascularized than SAT. (21)

In SAT of obese patients, we found only a higher expression of transferrin receptor, when compared to SAT of normal weight individuals, and the TfR expression correlated significantly with BMP6, hemojuvelin, ferritin and IL-6 expressions. This higher expression suggests that in obesity, iron transport into the subcutaneous adipocyte is enhanced. Hypoxia in the adipose tissue may have a relevant role in this mechanism, since TfR is regulated by stabilized HIF. It is known that HIF system is the main modulator of iron metabolism in hypoxic conditions, namely in obesity. (39, 40) Hepcidin expression was not significantly different in SAT of obese and normal weight patients. Apparently, SAT does not contribute in a significant manner for circulating levels of hepcidin, while VAT is more likely to contribute.

This study involved small groups of patients; thus, further studies, using a larger number of obese patients and controls, matched for gender and age, are needed in order to strength our results. Moreover, another gene should be used as housekeeping gene to reinforce data. It should be pointed out that adipose tissue collection is an invasive procedure, and that mRNA extraction from adipose tissue is not always successful, making the adipose tissue gene expression study a complex process.

In summary, concerning gene expression of inflammatory, erythropoietic and iron metabolism markers, in VAT and SAT showed a different profile, presenting VAT a more active metabolic role, with a high gene expression of pro-inflammatory adipokines that are able to induce disturbances in iron metabolism. Moreover, VAT presented an overexpression of IL-6 that is positively correlated with BMI, suggesting an increasing inflammation with adiposity.

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