NEW ADIPOKINES VASPIN AND OMENTIN, CIRCULATING LEVELS, GENE EXPRESSION IN ADIPOSE TISSUE AND RELATIONSHIP OF CIRCULATING LEVELS WITH NONALCOHOLIC FATTY LIVER DISEASE

David Gerardo Riesco Acevedo

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NEW ADIPOKINES VASPIN AND OMENTIN, CIRCULATING LEVELS GENE EXPRESSION IN ADIPOSE TISSUE AND RELATIONSHIP OF CIRCULATING LEVELS WITH NONALCOHOLIC FATTY LIVER DISEASE

Doctoral Thesis

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FEM CONSTAR que aquest treball titulat "**New adipokines vaspin and omentin, circulating levels, gene expression in adipose tissue and relationship of circulating levels with nonalcoholic fatty liver disease**", que presenta David Gerardo Riesco Acevedo per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Medicina i Cirurgia d'aquesta universitat i que compleix els requeriments per poder optar al títol de Doctor en Medicina.

Tarragona, 5 de Gener de 2016

El director de la tesi doctoral

Prof. Cristóbal Manuel Richart Jurado

La codirectora de la tesi doctoral

Dra. Maria Teresa Auguet Quintillà

DEDICADO:

A mis padres Victor y Raquel,

A mi esposa Karelys,

A mi hijo David,

Y a mi hermano Victor.

"Dondequiera que se ame el arte de la medicina

se ama también a la humanidad"

Platón

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I. LIST OF ABBREVIATIONS

ALT: alanine aminotransaminase

AST: aspartate aminotransaminase

AT: adipose tissue

BMI: body mass index

CK18: caspase-cleaved cytokeratin-18

CRP: c-reactive protein

CT: computed tomography

CVD: cardiovascular disease

DNL: *de novo* lipogenesis

FA: fatty acid

FABP4: fatty acid binding protein 4

FAS: fatty acid synthase

FATP: fatty acid transporter protein

FFA: free fatty acids

FxR: farnesoid X receptor

GGT: γ-glutamyl transferase

GLP-1: glucagon-like peptide-1

GNG: gluconeogenesis

GWAS: genome-wide association studies

HbA1c: glycosylated hemoglobin

HDL-C: high density lipoprotein cholesterol

HGP: hepatic glucose production

HOMA2-IR: homeostatic model assessment method insulin resistance

IL-1β: interleukin 1β

IL-6: interleukin 6

IL-7: interleukin 7

LDL-C: low density lipoprotein cholesterol

LPL: lipoprotein lipase

LPS: lipopolysacharide

LXRα: liver X receptor alpha

MetS: metabolic syndrome

MR: nuclear magnetic resonance

MUFA: monounsaturated fatty acids

NAFLD: non-alcoholic fatty liver disease

NAS: NAFLD activity score

NASH: non-alcoholic steatohepatitis

NEFA: nonsterified fatty acid

NFS: NAFLD fibrosis score

NL: normal liver

PPARα: peroxisome-proliferator-activated receptor α

PPARγ: peroxisome-proliferator-activated receptor γ

PPARδ: peroxisome-proliferator-activated receptor δ

PTX: pentoxyphylline

PUFAs: polyunsaturated fatty acids

QM: chylomicrons

RAS: renin–angiotensin system

ROS: reactive oxygen species

SAT: subcutaneous adipose tissue

SFA: saturated fatty acids

SNP: single nucleotide polymorphism

SREBP1c: sterol-regulatory-element-binding protein

SS: Simple steatosis

T2DM: type 2 diabetes mellitus

TCA: tricarboxylic acid cycle

TG: triglycerides

TGFβ1: transforming growth factor beta-1

TNFRI: tumour necrosis factor receptor I

TNFRII: tumour necrosis factor receptor II

TNFα: tumour necrosis factor alpha

TZD: thiazolidinedione

VAT: visceral adipose tissue.

VLDL: very low density lipoprotein

WAT: white adipose tissue

WC: waist circumference

WHR: waist-hip ratio

II. INTRODUCTION

1. Obesity as a 21st century epidemic and its global impact

Obesity is defined as a condition characterized by an excess of body fat mass and therefore of adipose tissue. Under normal conditions of living, obesity results from an imbalance between energy intake and the energy expenditure through physical activity [1].

The worldwide prevalence of obesity is increasing at an alarming rate in adults, adolescents and children, with adverse consequences for human health. The World Health Organization defines obesity as the epidemic of the 21st century. This "obesity epidemic" also parallels the rapid and substantive increase in our understanding of the molecular pathways and physiological systems underlying the regulation of energy balance.

Obesity is a chronic and multifactorial condition that results from the interaction between genotype and environment, with consequent increased systemic inflammation. To characterize obesity, body mass index (BMI) is used given its reproducibility, ease of use and ability to reflect adiposity in most of the population. BMI, which estimates excess weight relative to a certain height, is the ratio between the weight in kg and height in meters squared. Considering that obesity is established using BMI > 30 kg/m^2 as a cutoff point, the following classification is applied: 25.0–26.9 for grade I

overweight, 27.0–29.9 for grade II overweight (preobesity), 30.0–34.9 for grade I obesity, 35.0–39.9 for grade II obesity, 40.0–49.9 for grade III or morbid obesity (MO) and ≥ 50 for grade IV or extreme obesity [2].

The prevalence of obesity in the Spanish adult population aged between 25 and 64 years is estimated as 15.5%, with a higher prevalence in women (17.5%) than in men (13.2%) and a higher proportion of obese people in the Northwest region, Murcia, southern Spain and the Canary Islands. Grade II obesity affects 0.79% of men and 3.1% of women aged between 25 and 60 years, whereas 0.3% of males and 0.9% of women are morbidly obese. Data from the latest WHO survey show an absolute increase of 6% in obesity rates over the past 14 years [2]. For the population aged over 65 years, the estimated prevalence of obesity is 35%, with a prevalence of 30.9% among men and 39.8% among women [3].

According to current WHO data for 2014 the prevalence of overweight in the population over 18 years was 66.2 (59.3 to 72.2) % and the prevalence of obesity in the population over 18 years was 22, 8 (16.7 to 29.3) % [4] (**Figure 1)**.

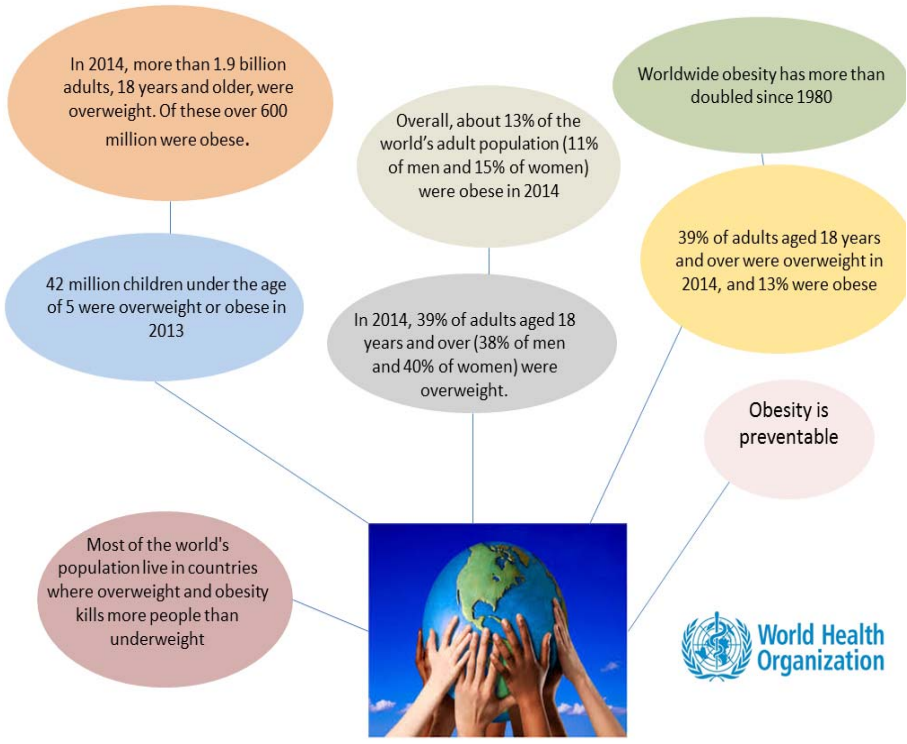

Figure 1. Epidemiology of Obesity. Data WHO report in February 2015.

Obesity is a known and independent risk factor for developing diabetes mellitus, insulin resistance (IR), dyslipidemia, high blood pressure, gallstones, sleep apnea syndrome, osteoarthritis, different neoplasms and thrombosis [5]. In fact, obese individuals are estimated to have increased mortality compared to normal-weight individuals. Despite considerable progress in diagnosis, prevention and treatment, cardiovascular disease remains the leading cause of death worldwide [6].

Metabolic syndrome (MetS), a determining factor for cardiovascular disease and type 2 diabetes (T2DM), is defined as a group of metabolic disorders including abdominal obesity [7], atherogenic dyslipidemia (hypertriglyceridemia and/or low high-density lipoprotein cholesterol (HDL-C)), high blood pressure and impaired glucose metabolism [8,9].

Increased visceral fat content is also associated with type 2 diabetes, insulin resistance and coronary heart disease [10]. Even within the normal BMI range, the accumulation of visceral fat remains an independent cardiovascular risk factor. This observation led researchers and physicians alike to consider that the clinical diagnosis of visceral obesity may be more important than the actual diagnosis of obesity by body mass index [7].

Central obesity and insulin resistance are considered the cornerstones in the pathogenesis of the metabolic syndrome. Insulin resistance is associated with other disorders that entail an additional increase in cardiovascular risk along with other abnormalities associated with lipid factors, changes associated with prothrombotic factors and proinflammatory factors [11].

In clinical practice, abdominal waist circumference and waist-hip ratio (WHR) are the anthropometric measurements commonly used to diagnose abdominal obesity. These measures are correlated with the total amount of visceral fat measured by abdominal CT scan [12].

For this reason, the Adult Treatment Panel-III (ATP-III) of the National Cholesterol Education Program (US) approved the use of abdominal waist circumference as an important component of the clinical diagnostic criteria of metabolic syndrome, using a waist circumference ≥ 102 cm in men and ≥ 88 cm in women as the cutoff points [7]. Obesity is associated with low-grade chronic inflammation, characterized by an increase in plasma levels of proinflammatory cytokines from adipose tissue (AT) and immune cells present in the tissue, and is causally involved in the development of insulin resistance [13].

Although numerous estimates have been made regarding the disease cost of obesity, its real impact on the health burden of a country was most vividly demonstrated by the UK government's Foresight Report, which showed that the medical costs of the chronic diseases induced or amplified by weight gain were so great that should obesity rates continue to increase, this problem would become a crippling burden on the health system and indeed would be unsustainable. Whereas many estimates of the economic impact of obesity in different societies exist, these often relate only to direct medical costs and do not include the far greater impact on the individuals' capacity to work and the burden placed on relatives later in life because of the additional physical and other handicaps involved. These indirect costs may amount to nearly 4% of a nation's gross domestic product [14].

2. Adipose tissue and its endocrine activity

Until late last century, adipose tissue was characterized by the storage and release of lipids. However, with the recognition of obesity as a major public health problem and progress in the study of the neuroendocrine system as a regulator of energy homeostasis, adipose tissue became a priority, making it possible to identify its extraordinarily dynamic behavior and highly active role as an endocrine organ [15,16,17].

Adipose tissue represents approximately 20% of body weight in men and 20–30% in women, making it one of the largest organs in the body.

In humans, fat formation occurs relatively early in the prenatal period between weeks 14 and 16. Throughout life, two particularly sensitive periods for changes in the cellularity of white adipose tissue (WAT) have been proposed, with a rapid expansion in adipose tissue mass occurring after birth and between 9–13 years [18].

Two types of adipose tissue are recognized based on their cellular structure, location, color, vascularization and function: brown adipose tissue and white adipose tissue.

Brown adipose tissue is present in a variety of mammals, being of special importance in those that hibernate. Its name originates from its brownish hue due to the high number of mitochondria and cytochromes.

In humans, brown adipose tissue is present only in newborns, and its function is to regulate thermogenesis. The thermogenic function of brown adipose tissue is due to its gene expression pattern that determines the presence of a protein called UCP-1 (uncoupling protein-1) in its mitochondria, which acts on the respiratory chain by uncoupling electron transport chain ATP synthesis in cellular oxidation [19].

White adipose tissue, to which we will be referencing from this point forward, actively participates in the regulation of various biological functions (Rasouli). WAT is distributed in the body in two main anatomical locations: the hypodermis regions for subcutaneous adipose tissue (SAT) and adipose tissue located around the organs for visceral adipose tissue (VAT) [20].

In females, subcutaneous fat is most abundant in the gluteo-femoral regions and the breast. In males, the main subcutaneous depots include the nape of the neck, the area over the deltoid and triceps muscles and the lumbosacral region, whereas excess fat is mainly stored in the visceral or intra-abdominal region, omentum, mesenteries and retroperitoneal area. This sexual dimorphism is responsible for the particular body shape of males and females, termed the android and gynecoid fat distribution. Furthermore, the fat distribution regional differences also result from variations in gene expression characteristics underpinning a diverse hormone receptor distribution, adipokine secretory profile and

expression pattern, as well as from specific local environmental features related to innervation and vascularization [21]. Numerous studies have revealed that android obesity correlates more often with elevated mortality and the risk of developing type 2 diabetes mellitus (T2DM), dyslipidemia, hypertension and atherosclerosis than gynecoid obesity [21].

Regarding lipid metabolism in adipose tissue, some of the hormones that modulate lipogenesis and lipolysis are catecholamines, insulin, glucagon, cortisol and ACTH. However, their actions are different depending on the type of adipose tissue involved [22].

Visceral adipose tissue has a higher triglyceride (TG) clearance rate than subcutaneous fat and is capable of greater lipolysis compared to subcutaneous adipose tissue [22]. Additionally, visceral adipose tissue has higher β-adrenergic-receptor expression, is more sensitive to steroids and has higher expression of inflammatory cytokines [23].

Adipocytes from visceral adipose tissue are larger, more resistant to insulin action and more metabolically active. Thus, visceral adiposity has greater influence on the appearance of comorbidities associated with obesity and it may be considered an indicator of adipose tissue metabolic dysfunction [24, 25].

Differences also exist between adipose tissues from the vascular standpoint. Unlike subcutaneous fat, visceral adipose tissue drains directly into the portal circulation [24].

Macrophage infiltration of adipose tissue is known to occur during the development of obesity, whereas the rate of monocyte infiltration is recognized to be higher in visceral than in subcutaneous adipose tissue [26, 27].

At the nervous system level, both visceral and subcutaneous adipose tissues are innervated by the autonomic nervous system and are under neuroendocrine control. Stimulation of the parasympathetic nervous system leads to an anabolic state with decreased lipolysis, whereas stimulation of the sympathetic nervous system produces a catabolic state with reduced adipogenesis and the stimulation of lipolysis [24].

With regard to its composition, adipose tissue is notably composed of adipocytes embedded in a web of loose connective tissue containing precursors of adipocytes, fibroblasts, immune cells (mainly macrophages and lymphocytes) and various other cell types [17].

Under maintained positive energy-balance circumstances, adipose mass expansion occurs initially through enlargement of the existing fat cells (hypertrophy). The persistence of these circumstances leads to severe obesity with an increase in the total fat cell number (hyperplasia). Prolongation of the positive energy balance over time leads to white adipose tissue

enlargement with hypertrophic growth predominating in adult-onset obesity [18].

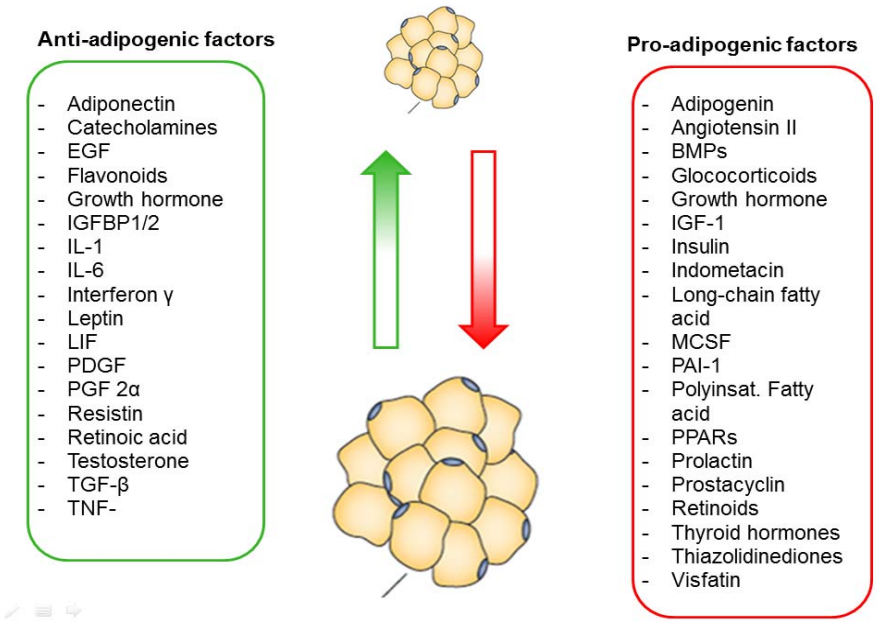

Figure 2. Factors affecting the regulation of adipogenesis BMPs, bone morphogenetic proteins; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1; IGFBP-1/2, insulin-like growth factor-binding protein 1 and 2; IL, interleukin; LIF, leukemia-inhibitory factor; MCSF, macrophage colony-stimulating factor; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; PGF, prostaglandin F; PPARs, peroxisome-activated receptors; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

The majority of the total lipid content stored in fat cells is represented by triacylglycerols (constituted mainly by oleic and palmitic acids) and to a smaller degree by diacylglycerols, phospholipids, unesterified fatty acids and cholesterol, which are continuously being mobilized and renewed. Mature white fat cells are mainly unilocular, containing only a single large

lipid droplet. In contrast, developing white adipocytes are transiently multilocular, exhibiting multiple smaller lipid droplets before these finally coalesce into a single large drop [28]. A narrow rim around the lipid droplet contains the cytoplasm with a thin basal lamina surrounding the cell. The cytoplasm contains few mitochondria with loosely arranged membranous cristae around the nucleus, exhibits a small Golgi zone and is filled with free ribosomes, containing only a limited number of short profiles of the granular endoplasmic reticulum (ER) with occasional lysosomes [28].

The adipocyte and its secretory products have been implicated in a wide variety of physiological processes. Adipose tissue produces and secretes hormone-like signaling molecules, cytokines and adipokines impacting multiple target organs [29]. Moreover, it expresses a wide range of receptors, which causes it to respond to numerous metabolic and endocrine stimuli involved in modulating blood pressure, glucose metabolism, inflammation and atherosclerosis [17, 29] **(Figure 2).**

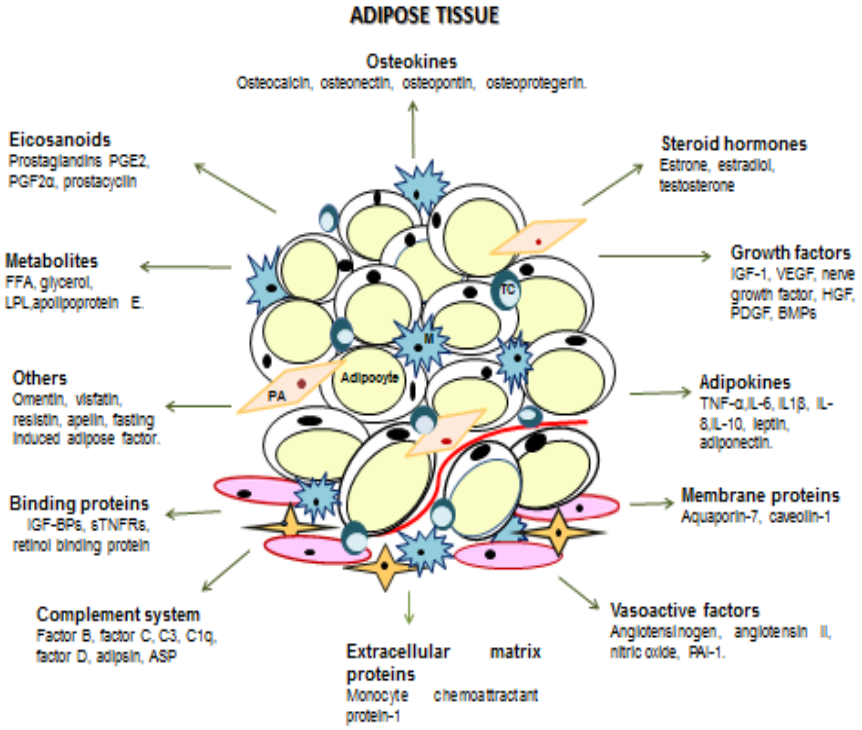

Figure 3. Main factors produced by adipose tissue, a multifunctional endocrine organ. ANP, atrial natriuretic peptide; ASP, acylation-stimulating protein; BMP, bone morphogenetic proteins; HGF, hepatocyte growth factor; IGF-1, insulin growth factor-1; IGF-BPs, insulin-like growth factor-binding proteins; IL, interleukin; LPL, lipoprotein lipase; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; PGE2, prostaglandin E2; PGF2α, prostaglandin F2α; sTNFRs, soluble receptors of tumor necrosis factor-alpha; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

3. Adipocytokines in visceral adipose tissue, subcutaneous adipose tissue and their involvement in inflammation

Adipose tissue is a highly active organ that is now recognized to be an active participant in energy homoeostasis and physiological functions such as immunity and inflammation [30].

Adipose tissue is known to express and secrete a variety of products known as “adipokines”, including leptin, adiponectin, resistin and visfatin, as well as cytokines and chemokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) **(Figure 3 and 4)**. The release of adipokines by either adipocytes or adipose tissue-infiltrated macrophages leads to a chronic subinflammatory state that could play a central role in the development of insulin resistance and type 2 diabetes and may increase the risk of obesity-associated cardiovascular disease [30].

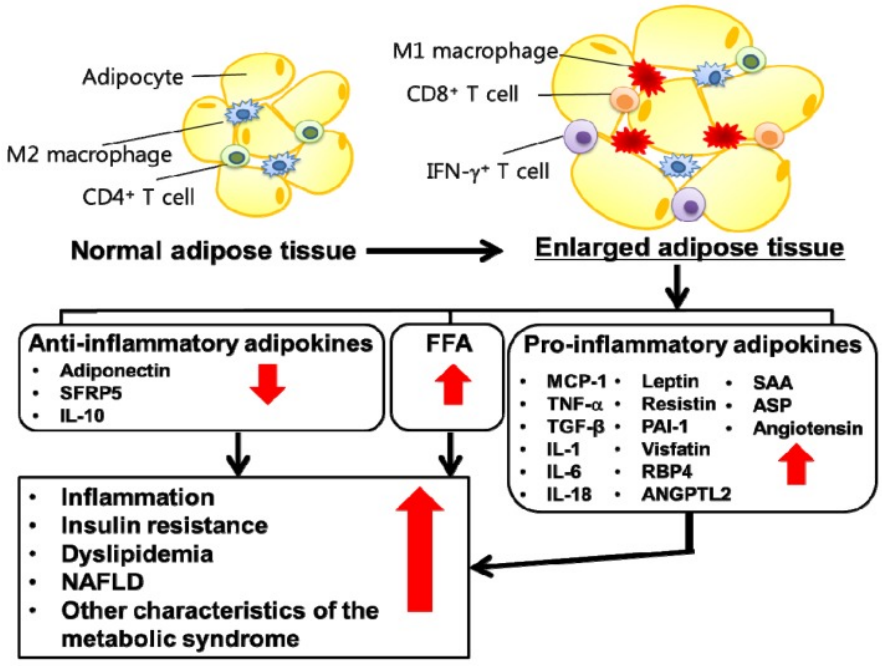

Figure 4. Secretion of inflammatory adipokines from adipose tissue in obese state. Secretion of inflammatory adipokines from adipose tissue in the obese state. In the obese state, the enlarged adipose tissue stores trigger the dysregulated secretion of adipokines and the increased release of free fatty acids. The free fatty acids and proinflammatory adipokines circulate to metabolic. tissues, including skeletal muscle and liver, and modify inflammatory responses as well as glucose and lipid metabolism, thereby contributing to metabolic syndrome. Additionally, obesity induces a phenotypic switch in adipose tissue from anti-inflammatory (M2) to proinflammatory (M1) macrophages. In contrast, the adipose production of insulin-sensitizing adipokines with anti-inflammatory properties, such as adiponectin, is decreased in the obese state. The red arrows indicate increased (pointing upward) or decreased (pointing downward) responses with obesity. ANGPTL, angiopoietin-like protein; ASP, acylation-stimulating protein; IL, interleukin; MCP-1, monocyte chemotactic protein; NAFLD, nonalcoholic fatty liver disease; PAI-1, plasminogen activator inhibitor-1; RBP-4, retinol-binding protein-4; SAA, serum amyloid A; SFRP5, secreted frizzled-related protein 5; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α. **Adapted from *Un Ju Jung et all. Int J Mol Sci. 2014 Apr; 15(4): 6184–6223.***

3.1 Proinflammatory molecules

3.1.1 Leptin

Leptin, a peptide hormone produced and secreted by adipose tissue in proportion to its mass, acts both centrally on the hypothalamus and peripherally to regulate several metabolic and inflammation-related functions. As the product of the obese gene (*ob*), leptin is a cytokine-like 16-kDa peptide whose effects are mediated through the long form of its receptor *Ob*-Rb [31, 32].

Leptin is produced proportionally to the body fat mass and controls appetite and energy expenditure through hypothalamic pathways [32, 33]. Most obese patients have high leptin levels, and the majority of obese subjects are leptin resistant, which establishes that obesity is the result of hormone resistance [34]. The mechanisms involved in leptin resistance are complex and likely involve both impaired leptin transport across the blood-brain barrier into the central nervous system (CNS) and induction of the suppressor of cytokine signaling-3 (SOCS3), resulting in decreased leptin signaling via the Janus kinase-signal transducer and activator of transcription (JAK-STAT) and phosphoinositol-3 (PI-3) kinase signaling pathways [35]. High-fat and more recently, high-fructose diets [36, 37], have been implicated in the induction of central leptin resistance via increased SOCS3 and/or

decreased signal transducer and activator of transcription (STAT) phosphorylation [38].

This hormone-like adipokine modulates body weight, food intake, fat stores, pancreatic islets cells, growth hormone levels, immunologic homeostasis, hematopoiesis, angiogenesis, wound healing, osteogenesis and gastrointestinal function [39, 40].

Leptin levels are proportional to insulin levels and inversely proportional to glucocorticoid concentrations [39, 41, 42]. Food deprivation decreases leptinemia, inhibits the immune response and causes lymphoid tissues to atrophy [43, 44].

Moreover, leptin induces the secretion of the proinflammatory cytokines interferon-gamma (IFN-γ) and interleukin-2 (IL-2) in stimulated T-cells from human peripheral or umbilical cord blood [31, 43]. Human monocytes are activated by leptin as shown by the expression of surface markers [45]. In addition to decreasing leptin levels, weight loss results in a lower leukocyte count and lower plasma levels of TNF-α, C-reactive protein (CRP) and Von Willebrand factor [31].

More recently, a role has been proposed for inflammation within the hypothalamus regarding the impairments of insulin and leptin signaling and perturbations of energy and glucose homeostasis [46]. Leptin has also been reported to bind to C-reactive protein and this binding may contribute to leptin resistance. Moreover, leptin may potentially promote a

proinflammatory state by increasing hepatic CRP expression [47].

3.1.2 Chemerin

Chemerin, a chemokine highly expressed in liver and white adipose tissue, was initially described as a protein with a complex immune system function [48, 49]. This protein has recently been identified as a new adipokine that regulates the development and metabolic function of adipocytes as well as glucose metabolism in the liver and skeletal muscle. Several research lines indicate that chemerin levels in plasma are elevated in obese patients and moreover and that these levels are correlated with various components of the metabolic syndrome. Thus, this double role of chemerin in inflammation and metabolism may provide a link between chronic inflammation and obesity, along with other obesity-associated diseases such as type 2 diabetes and cardiovascular disease [48].

Chemerin exerts its metabolic and immunomodulatory effects by binding to its receptor ChemR23 (CMKLR1). Two other G-protein-coupled receptors (GPRs) have been reported to bind chemerin with high affinity, namely, GPR1 [50] and C-C chemokine receptor-like 2 (CCLR2) [51]. GPR1 is most closely related to ChemR23; however, the expression pattern of GPR1 is different from ChemR23, with the main expression of GPR1 occurring in the liver, intestine, kidney and adipose tissue.

CCLR2 is expressed at high levels by lung endothelial cells and at lower levels by liver endothelium *in vivo* [52].

Furthermore, chemerin is crucial for normal adipocyte differentiation and modulates the expression of adipocyte genes involved in glucose and lipid homeostasis, such as glucose transporter-4, fatty acid synthase and adiponectin via its own receptor [53].

In obesity, chemerin expression and its circulating concentration are positively related to metabolic syndrome indices such as increased blood pressure and triglyceride levels. Weight loss induced by caloric restriction or bariatric surgery is associated with chemerin reduction [54, 55].
In a recent study, it was suggested that fasting levels of chemerin might be used as a biomarker to identify insulin resistance in healthy men without typical characteristics of metabolic disorders [56].

Studies demonstrate that chemerin regulates adipogenesis, causes insulin resistance, increases the risk for metabolic syndrome and modulates the immune system by enhancing the chemotaxis of dendritic cells and macrophages [57, 58].

Further studies are needed to determine the physiological role of chemerin in glucose metabolism and to identify chemerin's target tissues as well as relevant signal transduction pathways.

3.1.3 Resistin

Resistin is a small cysteine-rich protein secreted as a 94-amino-acid polypeptide that was first identified by Steppan during a study of the effects of peroxisome proliferator-activated receptor gamma (PPAR-γ) agonists on glucose homeostasis. Steppan named this protein 'resistin' for its property of 'resistance to insulin' in mice and this gene was later designated as *Retn* [59].

Expression of resistin mRNA is markedly increased by the proinflammatory cytokines interleukin-1 (IL-1), IL-6 and TNF-α, and by lipopolysaccharides (LPS) [60]. Moreover, resistin levels are mutually correlated with those of cell-adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) in patients with obstructive sleep apnea and in atherosclerotic patients are positively associated with other markers of inflammation, such as soluble tumor necrosis factor receptor (TNFR) type II and lipoprotein-associated phospholipase A2 [61, 62]. Similarly, stimulation of human macrophages with LPS led to increased resistin mRNA expression via a cascade involving the secretion of proinflammatory cytokines, whereas LPS administration to human volunteers is associated with dramatically increased circulating resistin levels, thus suggesting that this molecule may act as a critical mediator of the insulin resistance associated with sepsis and possibly other inflammatory conditions. In further support of its proinflammatory profile, resistin also up-regulates the

expression of vascular cell-adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 and CCL2 by human endothelial cells and induces these cells to release endothelin-1 [63].

Human resistin, however, is mainly secreted by peripheral blood mononuclear cells [64]. It competes with LPS for the binding to Toll-like receptor 4 and is thus involved in the inflammatory process. The expression of human resistin is predominantly localized in the macrophages and stromal cells in adipose tissue rather than the adipocytes [65, 66]. A high level of resistin gene expression was observed in human preadipocytes, which decreased during adipocyte differentiation. Notably, the relationship between resistin and insulin resistance in humans is complicated; it remains a subject of debate because some studies revealed a positive correlation between resistin and insulin resistance [67], whereas others failed to detect changes in resistin levels in obesity, insulin resistance or T2DM [68, 69].

As a secreted circulating protein, resistin may exert its functions in both endocrine and paracrine manners [66]. To date, resistin is widely accepted to play a pivotal role in several inflammatory conditions and in malignancies [70, 71].

3.1.4 Visfatin

Visfatin, discovered by Fukuhara *et al* and originally isolated as a presumptive cytokine named pre-B-cell colony-enhancing factor (PBEF) that enhances the maturation of B-cell precursors [72], is an adipokine mainly produced by visceral WAT. Visfatin was originally described as having a potential glucose-lowering effect because of its nicotinamide phosphoribosyltransferase (Nampt) activity [72].

Revollo *et al* demonstrated that the extracellular form of Nampt (eNampt/visfatin/PBEF), which is secreted through a non-classical secretory pathway, did not show insulin-mimetic effects *in vitro* or *in vivo* but rather exhibited robust nicotinamide adenine dinucleotide (NAD) biosynthetic activity. Haplodeficiency and chemical inhibition of Nampt resulted in significantly decreased NAD biosynthesis and glucose-stimulated insulin secretion in pancreatic islets *in vitro* and *in vivo*. Conversely, the administration of the Nampt reaction product nicotinamide mononucleotide (NMN) resulted in an amelioration of these defects. In summary, the current data suggest that adipose tissue as a natural source of eNampt/visfatin/PBEF may regulate β-cell function through the secretion of eNampt and extracellular biosynthesis of NMN [73].
Later studies showed that plasma visfatin was correlated with various metabolic states including obesity, increased visceral fat mass and diabetes in humans [74].

Visfatin also appears to mediate inflammatory responses in monocytes by the induction of the proinflammatory cytokines interleukin-IB (IL-1B), IL-6 and TNF-α. However, higher concentrations of visfatin increase the expression of anti-inflammatory cytokines, for example, interleukin-10 (IL-10). An important implication of this new adipokine in the inflammatory mechanisms of obesity beginning during childhood has been proposed [75].

A recent meta-analysis showed that plasma visfatin was increased in subjects with obesity, type 2 diabetes mellitus, metabolic syndrome and cardiovascular diseases [76]. Circulating visfatin was positively associated with insulin resistance, a finding that implied visfatin stimulation in hyperglycemia. Moreover, visfatin may have a direct role in vascular dysfunction and inflammation through iNOS up-regulation [77].

3.1.5 Retinol-binding protein-4 (RBP-4)

Retinol-binding protein-4 is a recently identified adipokine. It belongs to the lipocalin (LCN) family of proteins that transport small hydrophobic molecules. RBP-4 is a transport protein for retinol (vitamin A) in the circulation, which it transports from the liver to the peripheral tissues. Serum RBP-4 levels are increased and positively correlated with BMI in obese non-diabetic and diabetic subjects [78].

Although RBP-4 is primarily secreted by the liver, visceral WAT is also an important source of this adipokine [79]. In experimental models, Yang *et al* showed that serum RBP-4 contributed to insulin resistance via the induced expression of gluconeogenic enzymes in the liver and impaired insulin signaling in muscle [80]. The same authors reported that RBP-4 was increased in human obesity and type 2 diabetes. In the latter, a positive relationship was found with RBP-4 and insulin-resistance severity in obese, glucose-intolerant, type-2 diabetics and nonobese subjects with a strong family history of T2DM [81].

To date, the majority of clinical studies in children and adolescents support the hypothesis that RBP-4 has a relevant role in obesity and the development of insulin resistance and T2DM [82]. It appears to be involved in the early phases of the development of insulin resistance and other components of the metabolic syndrome, and as such, RBP-4 could be used as an additional marker for the early detection of subjects predisposed to developing T2DM, enabling early and vigorous intervention [82]. Therefore, longitudinal studies investigating the prognostic value of RBP-4 are particularly needed in children and adolescents predisposed to developing these conditions.

3.1.6 Interleukin-6

Interleukin-6 (IL-6) is a proinflammatory factor produced by monocytes, fibroblasts and the stromal vascular fraction of

visceral WAT [83]. Visceral more than subcutaneous adipose tissue has a greater ability to secrete IL-6. However, adipose tissue is composed of numerous cells other than adipocytes, and most of the IL-6 is derived from the stromal vascular fraction, composed of endothelial cells and monocytes/macrophages [84]. In the absence of inflammation, adipose tissue appears to contribute to 15–30% of circulating IL-6 [85].

IL-6 is a multifunctional cytokine that targets several tissues and cell types. One of its major actions is control of the hepatic production of inflammatory proteins such as C-reactive protein. A positive relationship exists between IL-6 levels in adipose tissue and circulating CRP levels, which is an important cardiovascular risk factor [86]. Visceral adipose tissue produces three times more IL-6 than subcutaneous adipose tissue. This may explain, in part, the deleterious role of central obesity in cardiovascular diseases. Indeed, IL-6 visceral adipose tissue production could have a direct effect on hepatic metabolism as its venous drainage goes directly to the liver through the portal vein. Thus, IL-6 produced by intra-abdominal adipose tissue could directly contribute to visceral obesity-related hypertriglyceridemia by stimulating the hepatic secretion of triglycerides and very low density lipoprotein (VLDL) [87].
Chronic elevation of IL-6 plasma levels and the increased cardiovascular risk related to an inflammatory state could be causes of insulin resistance. The proposed mechanism of this

biological effect is the reduction of glucose transporter-4 and insulin receptor substrate-1 expression in response to IL-6 exposure [88]. IL-6 also plays an anti-inflammatory indirect and dual role by reducing TNF-α and interferon-gamma stimulating interleukin-1 receptor antagonist (IL-1RA) [32].

It is also possible that the chronic elevation of IL-6 circulating levels, more so than acute IL-6 secretion, has a weak or no effect on muscle *in vivo*, whereas it could contribute to whole-body insulin resistance, particularly in liver and adipose tissue [89].

3.1.7 Tumor necrosis factor-alpha

Tumor necrosis factor-alpha (TNF-α) was originally described to induce the necrosis of tumors after acute bacterial infection. However, this cytokine is also involved in inflammation, autoimmune diseases, tumorigenesis, metastasis, viral replication, septic shock and fever. A large variety of agents may induce its production, such as endotoxin, viruses, cytokines (e.g., IL-1) and drugs. TNF-α is suppressed by interferon-α (IFN-α), IFN-b, IL-6 and by the immunosuppressive cytokines interleukin-4 (IL-4) and IL-10. Many drugs may inhibit the production of TNF-α, such as dexamethasone and cyclosporin A. TNF-α is produced by many cell types, ranging from the cells of the immune system (monocytes, T lymphocytes, B lymphocytes,

polymorphonuclear cells) to tumor cells, fibroblasts and smooth muscle cells [90].

TNF-α is a proinflammatory cytokine produced by numerous cells, but mainly macrophages and lymphocytes. In adipose tissue, TNF-α is primarily produced by resident macrophages. Adipocytes also produce TNF-α in rodents, whereas in humans, such production occurs at low levels. In rodents, TNF-α is involved in the pathophysiology of insulin resistance. One of the possible mechanisms by which this cytokine interferes with insulin sensitivity is through the abnormal phosphorylation of insulin receptor substrate (IRS)-1. However, as previously mentioned, TNF-α is poorly expressed in human adipose tissue, with no differences in fat depots. Moreover, its expression is slightly modified in human obesity, and the absence of its production in subcutaneous adipose tissue in obese and lean men has been demonstrated by direct arteriovenous balance measures [91]. This finding indicates that adipose tissue is poorly or indirectly involved in the increased circulating concentrations of TNF-α observed in obesity. Nevertheless, it warrants consideration that TNF-α in experiments may accelerate atherosclerosis, mainly through the induction of adhesion molecule expression, VCAM-1, ICAM-1, MCP-1 and E-selectin in endothelial and vascular smooth muscle cells, resulting in altered endothelium-dependent vasodilatation, and the promotion of endothelial cell apoptosis [92].

3.1.8 Lipocalin-2

Lipocalin-2 (LCN-2), also known as neutrophil gelatinase-associated lipocalin (NGAL), siderocalin, or 24p3, is a 25-kDa glycoprotein that binds and transports small hydrophobic molecules, such as retinoic acid, hormones and fatty acids. It is produced by immune cells, mainly neutrophils and adipocytes [93].

In obese human subjects and animals, the circulating concentration of lipocalin-2 is augmented and positively correlated with body-fat mass, arterial blood pressure (ABP), the insulin resistance index, and abnormal lipid profiles [94].
In mice, deficiency of this adipokine protects against the development of endothelial and cardiometabolic dysfunctions associated with genetic or dietary obesity. Moreover, when fed a high-fat diet (HFD), the administration of recombinant lipocalin-2 protein promotes endothelial dysfunction and induces adipose tissue inflammation [95, 96].
An augmented lipocalin-2 level is likely to play a causative role in obesity-related cardiovascular complications, including hypertension and heart disease [94].
LCN2 was initially shown to possess antibacterial properties, suppressing bacterial growth by binding and sequestering bacterial siderophores [97, 98].

It is not known whether lipocalin-2-evoked endothelial dysfunction is related to non-esterified free fatty acids (NEFA)-mediated lipotoxicity under obese conditions. NEFAs up-

regulate and activate lipocalin-2 by promoting deamidation. The deamidated lipocalin-2 accumulates in arteries, inducing endothelial dysfunction and causing elevated arterial blood pressure [99].

3.2 Anti-inflammatory molecules

3.2.1 Adiponectin

Adiponectin was discovered almost simultaneously by four groups and designated as adipocyte complement-related protein of 30 kDa (Acrp30). It is produced exclusively by the adipocyte fraction of adipose tissue [85]. Whereas it is adipocyte protein that links visceral adiposity with insulin resistance and atherosclerosis, adiponectin is unique among adipocyte-derived hormones in that its circulating concentrations are lower in obese that in normal-weight humans and animals [100]. Adiponectin circulates as multimers, trimers, hexamers and higher molecular weight multimeric oligomers [100, 101]; it has both an adaptor protein, APPL1, as well as two receptors, Adipo-R1 and Adipo-R2 that are the main adiponectin receptors with respect to glucose and lipid metabolism [102].
Adiponectin mRNA expression varies according to the tissue, being lower in visceral than in subcutaneous tissue [103].

The high-molecular-weight forms of the hormone have been implicated as being the most biologically active in increasing hepatic insulin sensitivity [100].

Circulating adiponectin levels are negatively correlated with fasting insulin concentrations and positively correlated with insulin sensitivity [100, 104]. Low circulating adiponectin concentrations are predictive of the development of insulin resistance, T2DM and cardiovascular disease in humans [105, 106]. In addition to its effects on insulin sensitivity, adiponectin exhibits a vascular-protective effect early in the atherogenesis process by interfering with the regulation of adhesion molecule expression on vascular endothelial cells [107] and the transformation of macrophages into foam cells [108], also by modulating smooth muscle cell proliferation [109]. Adiponectin induces endothelial VCAM-1, ICAM-1, and pentraxin-3 expression [32]. The hormone is an antioxidant and increases endothelial nitrous oxide production, acting to protect the vasculature by reducing platelet aggregation and vasodilatation [108, 110].

Adiponectin also has anti-inflammatory effects that inhibit the activation of macrophages and protects against the development of atherosclerosis in vascular endothelium [111]. It appears that many adiponectin anti-inflammatory properties derive from the anti-TNF-α effects that could partially explain its protective role in atherosclerosis. In contrast, adiponectin expression in human adipocytes is reduced by TNF-α and IL-6 [112].

Relevant to its insulin-sensitizing actions, adiponectin decreases glucose production by isolated hepatocytes by

decreasing the mRNA expression of two essential gluconeogenesis enzymes, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, and activates AMP kinase, resulting in increased fat oxidation in liver and skeletal muscle leading to decreased ectopic fat deposition in these insulin target tissues [113].

In humans, plasma adiponectin concentrations are influenced by age and gender, and the inverse relationship between adiponectin and body adiposity is more closely related to visceral rather than subcutaneous fat distribution [114].

Although adiponectin does not cross the blood brain barrier, it may have effects in the CNS via increasing cytokine expression in brain endothelial cells [115].

3.2.2 Apelin

Apelin is a peptide produced and secreted by adipocytes, the stromal vascular fraction and cardiovascular tissues. The gene encoding the apelin receptor (APJ) shares the greatest sequence identity with the angiotensin receptor. In humans, apelin appears to function as a paracrine hormone and its levels are significantly increased by insulin in obese patients [116]. In addition to its role as an adipokine, apelin is expressed in the central nervous system (with particularly high expression in the hypothalamus), heart, skeletal muscle and stomach [117].

Apelin and APJ expression are found in many tissues where they play roles in satiety, immune function and fluid balance [118]. A positive correlation exists between plasma apelin levels and BMI. Furthermore, apelin administration has been found to decrease body adiposity and the serum levels of insulin and triglycerides in obese mice fed a high-fat diet. These findings suggest the presence of resistance to apelin, in a manner similar to insulin and leptin [119, 120, 121].

Apelin increases the serum adiponectin level and decreases that of leptin. It regulates insulin resistance by influencing serum adiponectin levels, energy expenditure and the expression of uncoupling proteins in brown adipose tissue in mice [122].

Apelin expression also closely correlates with TNF-α in AT of lean and obese individuals, and *in vitro* studies of cultured explants of human AT show an up-regulation of apelin in response to TNF-α [123].

In particular, the ability of apelin to decrease lipolysis and induce vasodilatation with the subsequent reduction in blood pressure may open new therapeutic avenues [124].

3.2.3 Nesfatin

Nesfatin-1 (NEFA/nucleobindin-2-encoded satiety and fat-influencing protein-1) is an 82-amino-acid peptide encoded in the N-terminal region of its precursor, nucleobindin-2 (NUCB-

2) [111, 125]. NUCB-2 was predicted to be processed by prohormone convertases (PC) to form nesfatin-1 [125]. Administration of the full-length nesfatin-1 or its 30-amino-acid midsegment (M30), considered its bioactive core, reduces food intake and fat mass in rodents [126, 115]. Naresh Ramesh *et al* and others have identified nesfatin-1-like immunoreactivity in the pancreatic islet beta cells of rats, mice [126] and humans [114] and found an insulinotropic effect for this peptide *in vitro* and *in vivo* [114, 126]. Nesfatin-1 increases glucose-stimulated insulin secretion from beta cells by direct action involving Ca2+ influx through L-type calcium channels [127].
Nesfatin-1 is emerging as an evolutionarily conserved, NUCB-2-derived multifunctional peptide affecting major organ systems in vertebrates [128].

When originally discovered, nucleobindin-2 was named because of its very high sequence similarity with another secreted protein, nucleobindin-1 (NUCB-1). Both NUCB-1 and NUCB-2 are homologous multi-domain Ca2+ and DNA binding proteins encoded by two unlinked genes. Human NUCB-1 and NUCB-2 exhibit 62% amino acid sequence identity and are remarkably conserved within the nesfatin-1 region [129, 128]. NUCB-1 has been found within the nucleus, endoplasmic reticulum and cytoplasm of cells in the stomach, intestine, adrenal glands, pituitary, ovary and testis [130].
The endogenous expression profile of NUCB-1 was characterized recently by immunofluorescence staining,

showing NUCB-1 localization in the endocrine pancreas along with insulin, glucagon, somatostatin, ghrelin and pancreatic polypeptide immunopositive cells [130].

Overall, nesfatin-1 is now emerging as a multifunctional peptide with reproductive, cardiac and endocrine functions [131,132,128].

Recently, nesfatin-1 was found to exert cardiovascular actions in the brain by increasing sympathetic nerve activity, thereby increasing mean arterial pressure [133]. Furthermore, subjects with acute myocardial infarction had relatively lower serum nesfatin-1 concentrations than healthy volunteers [134]. This finding indicates that nesfatin-1 may play a role in atherosclerosis. Thus, nesfatin-1 is hypothesized to contribute to PAD development in T2DM [135].

While the above studies determined nesfatin-1 in tissues, the identity of NUCB-2/nesfatin-1-expressing cells within the intestine is not known. Considering that nesfatin-1 is insulinotropic and considering incretins, which are predominant insulinotropins that are primarily secreted from the intestine, further research is warranted to determine whether nesfatin-1 is present in enteroendocrine cells [136].

3.2.4 Interleukin-7

Interleukin-7 (IL-7) is a constitutively secreted cytokine primarily produced in bone marrow and peripheral lymphoid organs; it has recently been identified as a new adipokine

whose expression and secretion are increased in the obese human's adipose tissue [31, 137].

Whether this endogenous production of IL-7 has any physiological function in AT and metabolism remains unknown. IL-7 has been shown to induce the production of proinflammatory IL-1, IL-6, IL-8 and TNF-α by monocytes [31, 138].

3.2.5 Interleukin-10

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that attenuates the inflammatory processes induced by TNF-α, IL-6 and IL-1 while up-regulating the release of IL-1RA [139]. IL-10 is negatively correlated with BMI, percent fat mass and fasting glucose levels [140].

In another study, circulating levels of IL-10 were increased in obese people and were positively associated with weight, BMI, waist, waist-hip ratio, fat mass, systolic pressure and, interestingly, the titers of adenoviruses and enteroviruses [141]. Low levels of IL-10 are associated with both the metabolic syndrome and type 2 diabetes. Although IL-10 is associated with insulin sensitivity, the mechanism of action is unknown. Weight loss is correlated with increased IL-10 plasma levels and reduced TNF-α and IL-6 plasma levels in obese subjects [141].

3.3 Acute-phase proteins

3.3.1 C-reactive protein

C-reactive protein (CRP) was discovered as a substance in the sera of patients with pneumococcal pneumonia that reacted with polysaccharide C of *Streptococcus pneumonia* [142]. Circulating CRP is synthesized and secreted predominantly by hepatocytes in response to proinflammatory cytokines such as TNF-α, IL-1 and IL-6 [143, 144].

CRP is a well-known marker of whole-body systemic low-grade inflammation, and data suggest that CRP is implicated in the development of cardiovascular disease [145]. Increased levels of CRP are reported to be associated with metabolic syndrome, obesity, atherosclerosis, unstable angina, insulin resistance and diabetes [146].

Higher levels of CRP are positively associated with measures of adiposity [147], whereas better fitness is associated with lower CRP in children and adults [148].

3.4 Omentin, a novel adipokine

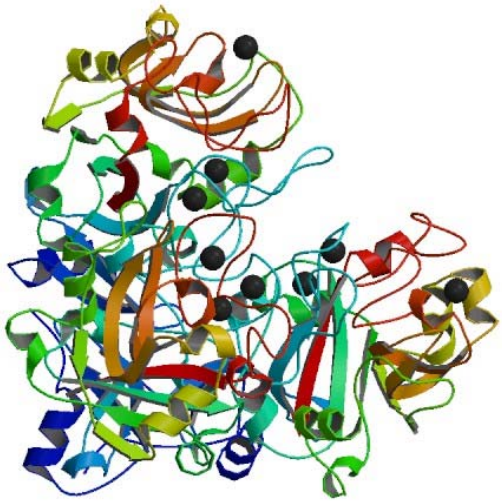

Figura 5. Omentin Molecule. *Adapted from pdb.org*

Omentin/intelectin was initially described in intestinal Paneth cells; omentin/intelectin (Figure 5) associates with galactofuranose within the carbohydrate moieties of bacterial cell walls and has been implicated in the gut defensive mechanisms against pathogenic bacteria, for example, *Escherichia coli* [149]. Additionally, a homolog of omentin/intelectin designated as omentin-2 has been reported, sharing 83% amino acid identity with omentin/intelectin.

Omentin-1 is the major circulating form of omentin and has been predominantly studied by researchers. Its gene is located in the 1q22-q23 chromosomal region, which is considered to be linked to T2DM in various populations [150].

Omentin expression varies throughout the body (heart, lungs, ovary, placenta, endothelial cells, human epicardial fat, thymus, small intestine, colon and reticulocytes [151, 152]; however, its main tissue of production is now considered to be visceral adipose tissue [153].

Recently, omentin has been reported as a novel adipokine preferentially produced and secreted by visceral AT (predominantly expressed in AT stromal vascular cells) compared with subcutaneous AT; *in vitro* experiments revealed that omentin enhances insulin-stimulated glucose uptake in human adipocytes and triggers Akt signaling. Akt is a serine/threonine protein kinase that plays an important role as a second messenger in multiple cellular functions, for example, glucose metabolism, cell proliferation and apoptosis [153].

3.4.1 Omentin and inflammation

C-reactive protein and TNF-α-induced nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) activation in human endothelial cells are significantly decreased by omentin; moreover, the changes in CRP levels are predictive of changes in circulating omentin levels after metformin treatment in overweight, insulin-resistant women with polycystic ovary syndrome (PCOS), another chronic inflammatory disorder [154]. Thus, omentin plausibly may have an anti-inflammatory role in proinflammatory states. Additionally, because omentin is mainly expressed in the stromal vascular cells of visceral adipose tissue, this potential anti-inflammatory role could be

important in modulating the proinflammatory elements in visceral AT. Future studies are needed to address this concept [154].

Omentin levels are decreased in the omental tissue in Crohn's disease, which is an inflammatory disease resulting from a chronic transmural intestinal inflammatory state [151]. Omentin is known to inhibit VCAM-1 expression induced by TNF-α, possibly demonstrating an anti-inflammatory role [155].

Among other inflammatory conditions that have been studied is rheumatoid arthritis, in which low omentin levels were present in synovial fluid compared to healthy controls, indicating omentin-1 as a possible biomarker for the osteoarthritic degenerative process [156]. The gene expression of omentin is enhanced in the airway epithelial cells of patients with asthma [157]. Lower levels of omentin in smokers appear to be related to a susceptibility to infections. Its levels in psoriasis are decreased, although increased levels of omentin are present in psoriatic arthritis [158].

3.4.2 Omentin in obesity, diabetes and insulin resistance

Omentin appears to enhance insulin-stimulated glucose uptake in cultured human adipocytes and may act as a modulator of vascular function [159]. Additionally, circulating omentin levels and omental AT omentin mRNA expression were found to be

significantly lower in impaired glucose-tolerant (IGT) and T2DM subjects compared with matched controls [160].

Circulating omentin and omentin gene expression in visceral AT were reported to be decreased in obese subjects. Moreover, circulating omentin levels were negatively correlated with markers of obesity, that is, body mass index, waist circumference and circulating leptin; thus, obesity and possibly leptin may regulate omentin levels [150].

Circulating omentin levels have been reported to be significantly increased after weight loss induced by a hypocaloric diet; this finding was associated with a parallel improvement in insulin sensitivity [161]. Furthermore, in human omental AT explants, insulin and glucose have been shown to decrease omentin mRNA expression, protein levels and secretion into conditioned media. Additionally, hyperinsulinemic induction via a prolonged insulin-glucose infusion in healthy subjects culminated in reduced circulating omentin levels [151].

Interestingly, circulating omentin levels were elevated in patients with nonalcoholic fatty liver disease (NAFLD) and this feature was an independent predictor of hepatocyte ballooning. This observation is paradoxical considering that obesity is positively associated with NAFLD [162].

3.4.3 Omentin and cardiovascular disease

In an interesting study, the authors found reduced levels of omentin-1 in plasma and subcutaneous adipose tissue in

subjects with nascent metabolic syndrome versus controls, independent of obesity. The term nascent MetS was used by the authors to describe subjects with MetS without the confounding presence of diabetes and/or cardiovascular diseases. The presence of omentin deficiency in the syndrome further broadens the role of omentin as an anti-inflammatory adipokine [163]. Circulating omentin concentration could be a useful marker of endothelial function as it was associated significantly with endothelial-dependent vasodilation [164].

Shibata and coauthors have examined the presence and levels of omentin in patients with coronary artery disease (CAD) versus control subjects after adjustment for conventional CAD risk factors and suggested that omentin levels may serve as a novel biomarker for CAD, independently of the cardiovascular medication used [165]. Omentin is expressed not only in visceral adipose tissue but also in epicardial adipose tissue, which is a potential explanation for its role in coronary artery disease [166]. Thus, omentin-1 functions as an adipokine that attenuates vascular inflammation. Taken together, omentin-1 may modulate obesity-related metabolic and cardiovascular disorders via an anti-inflammatory mechanism [166].

3.5 Vaspin, a novel adipokine

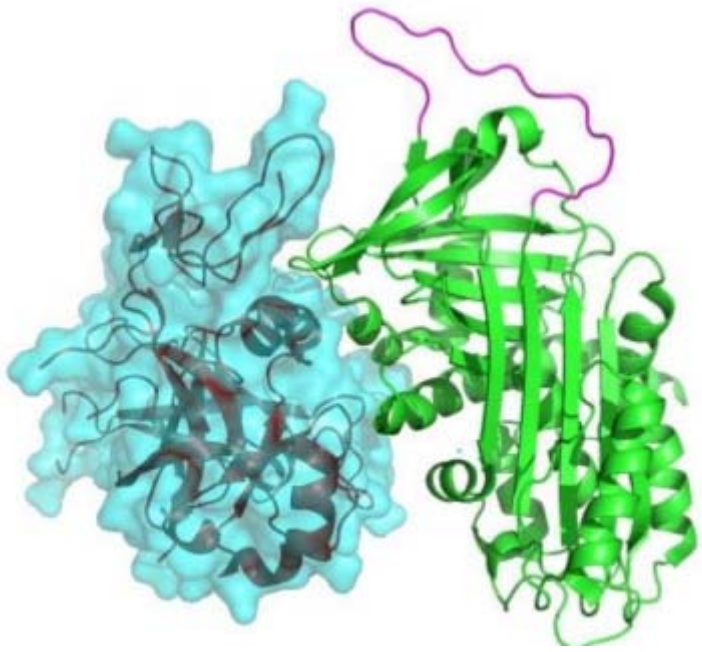

Figura 6. Vaspin Molecule. *Adapted from uscnk.com*

One of the most recently discovered adipokines is vaspin (Figure 6), a VAT-derived serine protease inhibitor with insulin-sensitizing effects, belonging to the serpin superfamily, clade A (Serpina12) [167]. Vaspin is a novel 392–395-amino-acid adipokine that was identified in the visceral white adipose tissue of obese, diabetic Otsuka Long-Evans Tokushima fatty (OLETF) rats, an animal model characterized by central obesity and T2DM. In this experimental model, visceral vaspin expression and its plasma level were decreased with worsening of diabetes and body weight loss. Vaspin has also been demonstrated to improve glucose tolerance and insulin

sensitivity in diet-induced obese rodents and may also normalize altered gene expression related to insulin resistance [167].

Recombinant vaspin administration in DIO (diet-induced obesity) mice significantly improved their glucose tolerance and insulin sensitivity. This beneficial effect results in normalizing plasma glucose levels and modifying the expression of genes involved in the pathogenesis of insulin resistance, such as resistin, leptin, TNF-α, glucose transporter-4 and adiponectin. Based on these data, it has been assumed that vaspin serves as an insulin sensitizer with anti-inflammatory effects and might act as a compensatory mechanism with target white adipose tissue, which is activated in response to the decreased insulin sensitivity [167, 168].

Nakatsuka *et al* have indicated that changes in the vaspin gene are responsible for its compensatory effects on the metabolic abnormalities with regard to obesity. They demonstrated that vaspin-transgenic mice were protected against diet-induced obesity, glucose tolerance impairment and fatty liver, whereas vaspin-deficient mice developed glucose intolerance due to up-regulation of the endoplasmic reticulum stress markers [169].

Vaspin has been presented as a circulating serpin, which serves as a ligand for the cell-surface receptor complex GRP78/MTJ-1 in the liver after ER stress-induced translocation to the plasma membrane. Vaspin exerts its anti-inflammatory action through binding to GPR78, a glucose-regulated protein,

and the subsequent signals beneficially affect ER stress-induced metabolic disorders [169]. In another study, Nakatsuka *et al* demonstrated that vaspin served as a ligand for a cell-surface GRP78/voltage-dependent anion channel complex in endothelial cells as well and thus exerts antiapoptotic, proliferative and protective effects on vascular walls in rat models with streptozotocin-induced diabetes mellitus [170]. These reactions display the molecular basis of the direct correlation between this adipokine and the ER stress responses of endothelial cells and in the presence of obesity [169, 170]. Furthermore, vaspin protects endothelial cells via an inhibitory effect on NFkB [171].

In human adipose tissue, vaspin mRNA expression was undetectable in lean individuals (BMI < 25 kg/m^2), whereas it was fat depot-specific in obese subjects [172]. Visceral vaspin mRNA expression positively correlated with BMI and percentage body fat and negatively correlated with plasma glucose concentration by a two-hour oral glucose tolerance test (OGTT). Subcutaneous vaspin mRNA expression negatively correlated with waist-to-hip ratio, fasting plasma insulin concentration and the glucose infusion rate during the steady state of euglycemic-hyperinsulinemic clamping. It has been suggested that the induction of vaspin mRNA expression in human adipose tissue might represent a compensatory mechanism associated with obesity, insulin resistance and T2DM [172]. Blüher summarized the findings that vaspin is predominantly localized in mature adipocytes, isolated from

different fat depots, and is also expressed in skin, hypothalamus, pancreatic islets and gastric cells, whereas a stromal or vascular endothelial cell expression has not been established [173].

3.5.1 Vaspin and metabolic syndrome

Several studies have shown a positive correlation between vaspin gene expression and the components of the metabolic syndrome. Kloting *et al* have investigated vaspin mRNA expression as an indicator for obesity and its association with anthropometric and metabolic parameters in visceral and subcutaneous adipose tissue samples. Although vaspin has not been detected in white adipose tissue in individuals with normal weight, it has been established in both VAT and SAT in obese subjects with T2DM [174, 175]. A significant correlation has been demonstrated between vaspin levels and obesity class, total body fat percentage, insulin resistance and glucose intolerance. Additionally, subcutaneous mRNA expression of vaspin significantly correlates with waist-hip ratio, immunoreactive insulin and the fasting glucose infusion rate in the steady-state condition. Contrary to expectations, vaspin mRNA expression has been found only in 23% of VAT and 15% of SAT samples, and no significant correlation has been demonstrated between visceral vaspin gene expression and VAT and SAT areas [174]. These findings are supported by another study that has reported vaspin mRNA expression predominantly in non-adipose cells [176].

3.5.2 Vaspin and cardiovascular disease

VAT-derived factors, including adipocytokines such as vaspin, are believed to have local and endocrine roles in the development of initial and advanced atherosclerosis in obese subjects by affecting the endothelium, vascular smooth muscle cells and macrophages, thus disrupting vascular homeostasis [177,178].

Kobat *et al* have demonstrated lower vaspin levels in subjects with CAD compared to controls, and this tendency has been confirmed in a control group with higher systolic blood pressure in comparison to controls with normal blood pressure. Hence, vaspin might be used as a predictor of CAD [179].

In females, Choi *et al* have indicated a significant correlation between plasma vaspin concentrations and the presence and severity of coronary stenosis, calculated by the Agatston score [180]. The results of Karbek *et al* and Esaki *et al* have confirmed the positive association between vaspin and coronary atherosclerosis [181].

Cura *et al* have confirmed the absence of an association between vaspin levels and the severity of stenosis but have found elevated vaspin levels in subjects with acute ischemic stroke [182].

Controversial results have been reported in two other studies, demonstrating that vaspin levels are significantly lower in subjects with CAD in comparison with healthy controls and correlate with CAD severity [179, 183].

4. Nonalcoholic fatty liver disease

The healthy liver contains little fat, although up to 5-10% fat by histology is defined as normal. Once liver fat exceeds 5-10% and when this finding is not due to excess alcohol consumption or other known causes of liver disease, the condition is termed nonalcoholic fatty liver disease. NAFLD covers a spectrum of liver disease ranging from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH), and cirrhosis due to NAFLD [184].

NASH includes the presence of simple steatosis, lobular inflammation and hepatocellular injury [185, 186] (ballooning) with or without fibrosis. Whereas simple steatosis is generally a benign, non-progressive clinical entity, NASH may progress to cirrhosis [186]. NAFLD is becoming an increasingly important health issue because it is the most common cause of chronic liver disease in the Western world, and its incidence is increasing rapidly [187].

The insulin resistance associated with obesity and inactivity is characterized by multiple metabolic abnormalities, which are better predictors of cardiovascular disease and T2DM than obesity alone [188]. A cluster of some of these features have been called metabolic syndrome. The liver fat accumulation due to non-alcoholic causes has an important role in the pathogenesis of insulin resistance and components of the metabolic syndrome [189].

NAFLD has been found to be associated with components of the metabolic syndrome (MetS) such as obesity, dyslipidemia, insulin resistance (IR) and T2DM [190, 191] and thought to represent the hepatic manifestation of MetS. Then, many people with NAFLD are not obese, and many people with NAFLD do not have diabetes. The presence and severity of NAFLD is an independent risk factor of cardiovascular and chronic kidney disease; suggesting that NAFLD is a multisystem disease (**Table 1**). It is noteworthy that although the primary liver pathology in NAFLD affects hepatic structure and function, causing morbidity and mortality from cirrhosis, liver failure and HCC, the majority of deaths among NAFLD patients are attributable to cardiovascular disease (CVD) [192, 193].

Table 1. Histological scoring system.

Conditions with established associations	Conditions with emerging associations
Obesity	Polycystic ovary syndrome
Insulin resistance	Hypothyroidism
Type 2 Diabetes Mellitus	Obstructive sleep apnea
Dyslipidemia	Hypopituitarism
Metabolic syndrome*	Hypogonadism
	Pancreato-duodenal resection

*Metabolic syndrome, as defined by the Adult Treatment Panel (ATP) III criteria, is defined by the presence of three or more of the following: 1) waist circumference greater than 102 cm in men or greater than 88 cm in women; 2) triglyceride level higher than 150 mg/dL (1.7 mmol/L) or drug treatment for elevated triglycerides; 3) high-density lipoprotein (HDL) cholesterol level lower than 40 mg/dL (1.03 mmol/L) in men and less than 50 mg/dL (1.29

mmol/L) in women or on drug treatment for low HDL; 4) systolic blood pressure ≥ 130 mmHg or diastolic pressure ≥ 85 mmHg or treatment for hypertension; and 5) fasting plasma glucose level ≥ 110 mg/dL or drug treatment for elevated blood glucose ***Adapted from Grundy et al. Diagnosis and management of the metabolic syndrome: An American Heart Association/National Heart, Lung, and Blood Institute scientific statement. Circulation. 2005 25;112(17):2735-52.***

4.1 Epidemiology of NAFLD

Epidemiology and natural history of NAFLD remain incompletely understood, with the incidence of fatty liver disease having been examined in only a few studies [194]. The estimated prevalence of NAFLD varies widely, likely secondary to differences in the populations studied and the methods used to detect NAFLD. The prevalence of suspected NAFLD based on elevated aminotransferases without imaging is between 7% and 11% of the general population; however, this is likely an underestimation because aminotransferases may be normal in individuals with NAFLD [194]. The prevalence based on abdominal ultrasound is between 20 and 30% of the population in the West, although the detection of steatosis by this technique requires fatty infiltration of at least one-third of the liver parenchyma [195]. By liver biopsy, the prevalence of significant hepatic steatosis in potential living donors for liver transplantation was 20% [196].

The prevalence of NAFLD worldwide varies between different countries and regions, which may be divided into three different groups of high, low and unknown prevalence [197]. The high-prevalence group is mostly represented by "Western"

countries wherein the population has an “urban” lifestyle. The prevalence rates are persistently and rapidly increasing in these countries due to the increase in obesity, IR, lipid impairments and T2DM related to suboptimal dietary habits and sedentary lifestyles. The prevalence of NAFLD in these populations ranges from 20% to 30%, and the prevalence of NASH ranges from 3% to 16% [198].

Ethnicity has a significant impact on the prevalence of NAFLD. In the Dallas Heart Study, the prevalence of hepatic steatosis was 45% in Hispanics, 33% in non-Hispanic Caucasians and 24% in African Americans [199]. This difference in prevalence was only partially explained by differences in obesity and insulin resistance, particularly in African Americans, in whom the prevalence of NAFLD was lower than in Caucasians with similar risk factors. This condition is highly prevalent in Asian populations as well. For example, in a Korean study of potential liver donors who underwent liver biopsy, the presence of NAFLD was 51%, with 10% having > 30% steatosis and 2.2% having NASH [200].

NAFLD tends to be more frequent in men than in women (42% versus 24%, respectively), although the frequency may be higher among postmenopausal women [201]. The prevalence of NAFLD increases with age, from less than 20% in people under the age of 20 years to more than 40% in individuals over 60 years. However, NAFLD has also been described in the

pediatric population with a prevalence of 2.6%, which may rise to within the range of 10–80% in obese children [202].

A number of studies have examined the prevalence of NAFLD in patients attending obesity clinics or undergoing bariatric surgery and reported prevalence of NAFLD in these groups ranges from 57% to 91%, whereas the prevalence of NASH ranged from 26% to 37% and that of unsuspected cirrhosis from 1.6 to 1.7% [200, 203, 204].

As a rule, the prevalence of NAFLD is higher in males and increases with increasing age, and it is influenced by the diagnostic method and the characteristics of the population, especially lifestyle habits. The prevalence of NAFLD is 80–90% in obese adults, 30–50% in patients with diabetes and up to 90% in patients with hyperlipidemia [204].

4.2 Natural history of NAFLD

The evolution of NAFLD requires years of follow-up and multiple biopsies [205]. The majority of patients with simple steatosis will not develop NASH, approximately 5% of patients with NAFLD develop cirrhosis. Although the simple steatosis may not be totally benign, a percentage of patients will progress to NASH [206]. However, patients with NASH are certainly at high risk for histologic progression and the development of cirrhosis. The age and degree of inflammation found in the initial liver biopsy are risk factors for progression to advanced fibrosis [207]. Compared to simple steatosis, NASH has higher liver-related mortality with an odds ratio (OR) of 5.71 for NASH and an OR of 10.06 for NASH with advanced fibrosis [208]. Ekstedt *et al*, studied the natural history of NAFLD doing repeat biopsies, they reported that 47% of patients with SS progressed to NASH [209].

The underlying mechanisms by which NASH or T2DM increase the risk of developing HCC are not fully understood; however, mechanisms involved in inflammation, metabolic stress and IR that are shared between NASH and T2DM may also be involved in HCC development [210].

4.3 NAFLD diagnosis and staging

Some patients undergoing thoracic and abdominal imaging for reasons other than liver symptoms, signs or biochemistry may

demonstrate unsuspected hepatic steatosis. While this phenomenon is not uncommon in clinical practice, studies have not systematically examined the characteristics or natural history of NAFLD in this patient population. The American Association for the Study of Liver Diseases (AASLD), the American College of Gastroenterology and the American Gastroenterological Association (AGA) published new guidelines for the diagnosis and management of NAFLD. They stipulated that the diagnosis of NAFLD requires **1)** evidence of hepatic steatosis either by imaging or by histology, **2)** the absence of significant alcohol consumption and **3)** the absence of secondary causes of hepatic steatosis. Common alternative causes of hepatic steatosis are significant alcohol consumption, hepatitis C, medications, parenteral nutrition, Wilson's disease and severe malnutrition [211].

NAFLD should be suspected in individuals who are obese or diabetic or who have metabolic syndrome. However, NAFLD is frequently underdiagnosed because it is mostly asymptomatic. Several non-invasive methods are used to diagnose NAFLD. In clinical practice, plasma liver aminotransferase levels and ultrasound are the most common diagnostic techniques [204]. Elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in the absence of other liver diseases may support NAFLD; moreover, an AST/ALT ratio less than 1 is also seen in NAFLD and supports NASH. Both aminotransferase levels and ultrasonography are widely

available, easy to perform and low in cost; however, they have low sensitivity for fatty liver. Other imaging techniques, such as computed tomography (CT) and nuclear magnetic resonance (MR) may also detect liver steatosis. CT provides a semiquantitative method and may be used to diagnose moderate to severe hepatic steatosis, although it has relatively low sensitivity and is not usually used for this purpose. MR remains the gold-standard technique: it is highly sensitive and specific for steatosis (with 5.5% intrahepatic TG content considered diagnostic for NAFLD), involves no radiation and allows quantification of liver steatosis; however, MR has limited availability and requires expensive hardware and software, rendering each test costly [204]. Alternatively, the fatty liver index (FLI) is an algorithm based on four markers: body mass index, waist circumference, triglycerides and γ-glutamyltransferase (GGT); the FLI has been confirmed as accurately identifying NAFLD [212].

Liver biopsy is currently the gold standard for characterizing liver histology (the degree of hepatocyte injury and the levels of fibrosis and inflammation) in NAFLD patients. However, this procedure is invasive and expensive and involves some morbidity and, very rarely, a mortality risk. Nevertheless, liver biopsy should be considered in those patients with fatty liver who, after imaging techniques, laboratory abnormalities and/or non-invasive methods, are considered to be at increased risk of having NASH and advanced fibrosis [213].

Because liver biopsy still remains the gold standard, a significant interest exists in developing non-invasive biomarkers for identifying steatohepatitis in NAFLD. In this regard, circulating levels of caspase-cleaved cytokeratin-18 (CK-18) fragments have been investigated as a promising biomarker for the presence of steatohepatitis in patients with NAFLD [214]. The number of hepatic progenitor cells and ductular reaction, assessed by CK-7 immunostaining, was used as an indicator of different fibrosis patterns in 38 pediatric NASH patients [215]. Although the incidence of CK-7-positive centrilobular hepatocytes using immunostaining was 64.3% in 14 needle biopsy liver specimens belonging to NASH patients, CK-7 immunostaining was not associated with the stage of fibrosis or the grade of steatosis. Significantly higher caspase 3 and 8 activity was observed in patients with NASH than in simple steatosis in a small sample of 50 NAFLD patients. Mean caspase 3 and 8 activity scores were comparable between patients with normal and patients with elevated ALT levels [216]. Moreover, recent breakthroughs have enabled non-invasive techniques to be used to diagnose the level of fibrosis/inflammation (**Figure 7**).

FibroScan, also known as transient elastography, is another non-invasive method for assessing liver fibrosis. This method measures liver stiffness, and although it was originally designed for the hepatitis C population, it is being used in the NAFLD population to determine the stage of fibrosis. However,

it has been shown to provide inaccurate liver stiffness measurement in overweight and obese patients [212].

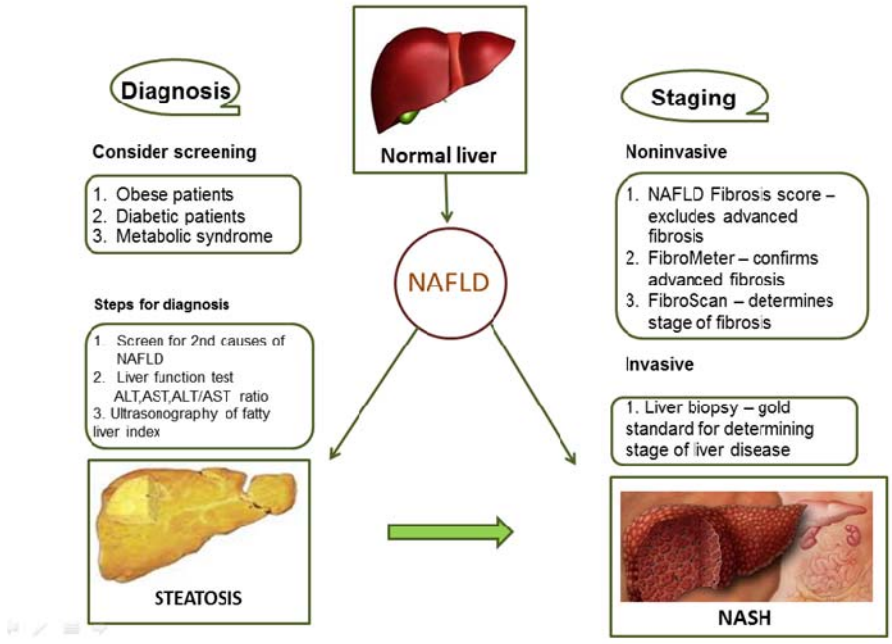

Figure 7. Diagnosis and staging of non-alcoholic fatty liver disease. **Adapted from Schwenger K et al. Clinical approaches to NAFLD. *World J Gastroenterol 2014; 20(7):1712-23.***

4.4 Histopathology of NAFLD

Matteoni *et al* proposed important pathological classifications of NAFLD/NASH [217], the author distinguished between NASH and non-NASH. They defined histological NAFLD types 1 and 2 as “non-NASH”, whereas types 3 and 4 were classified as NASH. Brunt *et al* [218] proposed a semiquantitative grading and staging system for NASH. The authors graded steatosis, inflammation and ballooning degeneration, and the staging

was based on the degree of fibrosis. This classification is only applicable to NASH and not to the entire spectrum of NAFLD. The pathology committee of the NASH CRN group therefore developed and validated a histological scoring system for use in NAFLD, based on Brunt's classification. The NAFLD activity score (NAS) system is applicable to both adults and pediatric patients. The NAS represents the sum of the scores for steatosis (0–3), lobular inflammation (0–3) and ballooning degeneration (0–2). A NAS of 5 or more correlates with the diagnosis of NASH and scores less than 3 correlate with "not NASH", whereas scores 3 or 4 are uncertain for the diagnosis of NASH. Fibrosis is scored separately to yield a fibrosis stage of between 0 and 4 [206].

The histologic spectrum of NAFLD is characterized by steatosis, lobular inflammation, ballooning of hepatocytes, fibrosis and other features that may or may not be present, such as Mallory-Denk bodies and portal inflammation.

4.5 Pathogenesis of NAFLD

The molecular mechanism underlying NAFLD progression has been interpreted in terms of a "double-hit" process, which was proposed in 1998 by Day *et al* [219]. The "first hit" includes the accumulation of TG and free fatty acids (FFAs) in hepatocytes. Insulin resistance and hyperinsulinemia, it is associated with weight gain or obesity, this component of the "first hit" results in hepatic steatosis. These changes have been postulated as

resulting in increased sensitivity to the “second hit”, which involves proinflammatory cytokines, mitochondrial dysfunction, oxidative stress and subsequent lipid peroxidation, leading to hepatocyte damage, inflammation and the development of steatohepatitis, suggesting that in the “first hit”, TG accumulation predisposes to further liver damage in the pathogenesis of NASH. However, this theory has recently been replaced by a more complex model in which 1) hepatic TG accumulation appears to be a benign sign of hepatic steatosis, whereas FFAs and TG-derived metabolites may be the true lipotoxic agents that contribute to the development of NASH and 2) TG breakdown via metabolic lipases contributes to the pathogenesis and progression of NAFLD [220].

The classic “two-hit” hypothesis has been modified into the “multiple parallel hits” hypothesis to explain the development of NAFLD and the progression from simple steatosis to NASH [221]. In the “multi-hit” hypothesis, imbalanced lipid metabolism and insulin resistance are considered as the “first hit”, leading to the development of hepatic steatosis. After steatosis develops, the liver becomes more vulnerable to many hits that may act in parallel, including gut-derived bacterial toxins, adipokine/cytokine imbalance, mitochondrial dysfunction, oxidative damage, and dysregulated hepatocyte apoptosis, release of profibrogenic factors and proinflammatory mediators from impaired organelles, and hepatic stellate cell and Kupffer cell activation. These factors may collectively stimulate

inflammation, apoptosis, fibrosis and finally tumor development, Iron accumulation and genetic factors are also involved in the development of NASH.[222, 223] (**Figure 8**).

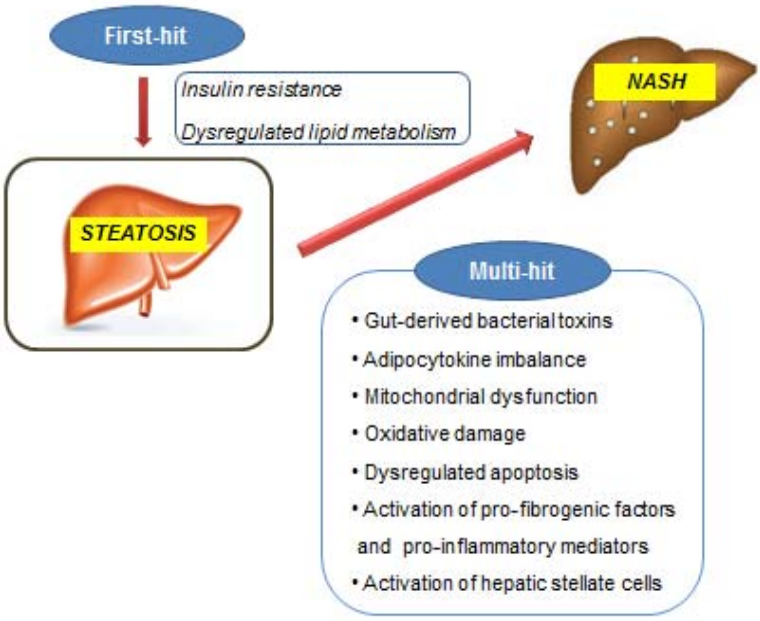

Figure 8. The multi-hit hypothesis of NAFLD pathogenesis. The "first hit", insulin resistance and lipid metabolism dysregulation, leads to the development of simple steatosis and susceptibility of the hepatocytes to "multi-hits". ***Adapted from Int. J. Mol. Sci. 2014;15,6184-6223***.

4.6 Pathophysiological Mechanisms Leading to NAFLD

4.6.1 Development of hepatic steatosis

The unequivocal histologic hallmark of NAFLD in both adults and children is steatosis, defined as the histologic manifestation of intracytoplasmic hepatic lipid accumulation in the form of triglycerides. The generally accepted dogma in NAFLD pathogenesis is that TG accumulation occurs when hyperinsulinemia and **insulin resistance**, commonly associated with obesity and T2DM, are present [223]. In NAFLD patients, the presence of IR is common at the liver, adipose tissue and muscle level [224].

However, several mechanisms may lead to a fatty liver: 1) increased FFA supply due to increased lipolysis from both visceral/subcutaneous adipose tissue and/or increased intake of dietary fat, 2) decreased free fatty acid oxidation, 3) increased *de novo* hepatic lipogenesis (DNL) and 4) decreased hepatic very low-density lipoprotein-triglyceride secretion [225]. Free fatty acid delivery to the liver accounts for almost two-thirds of its lipid accumulation [226].

Elevated peripheral fatty acids and DNL therefore predominantly contribute to the accumulation of hepatic fat in

NAFLD [227]. Triglyceride synthesis appears to be an adaptive, beneficial response in situations where hepatocytes are exposed to potentially toxic triglyceride metabolites. Thus, evidence is increasing that the accumulation of fat in the liver in many instances cannot be regarded as pathology or disease but rather as a physiologic response to increased caloric consumption [228, 229, 230].

Inflammation results in a hepatocyte stress response, which may lead to lipid accumulation and therefore could precede steatosis in NASH. Patients with NASH may present without any or much steatosis, suggesting that inflammation could occur first [231](**Figure 9**).

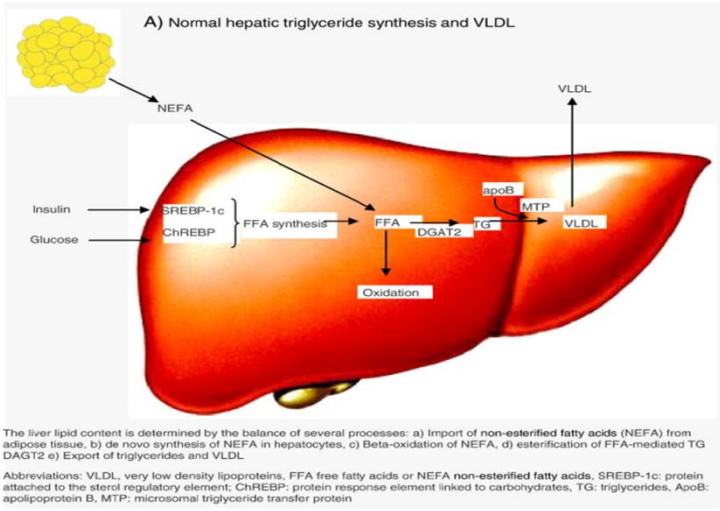

Figure 9. Normal hepatic triglyceride synthesis and VLDL**.** ***Adapted from Brea A et all. Non-alcoholic fatty liver disease and cardiovascular risk. International Journal of Cardiology, 20 August 2013, Pages 1109–1117***

Under IR conditions, the ability of insulin to stimulate liver glycogen synthesis and suppress hepatic glucose production (HGP) is diminished, resulting in increased plasma glucose concentrations [232]. The adipose tissue becomes resistant to the antilipolytic effect of the insulin. Insulin fails to inhibit the hormone-sensitive lipase, the enzyme that regulates the release of FFA from adipose tissue, resulting in increased delivery of FFA to the liver [233, 234]. Skeletal muscle IR typically accompanies insulin resistance at other sites. The independent effect of muscle insulin resistance on the exacerbation of NAFLD has been demonstrated in rodents [235, 236] and translated to humans, in which selective muscle insulin resistance in healthy young lean individuals has been shown to predispose them to increased hepatic *de novo* lipogenesis, hepatic TG accumulation and atherogenic dyslipidemia after consuming high-carbohydrate meals.

4.6.2 Progression of steatosis to NASH

In the process of developing NASH, multiple pathways or "multiple hits" are required to develop inflammation, cellular injury and fibrosis [223]. 'Hits' that may contribute include direct hepatic lipotoxicity, oxidative stress, mitochondrial dysfunction, hepatocyte apoptosis, hepatic stellate cell and Kupffer cell activation, activation of profibrogenic factors and proinflammatory mediators, hormones derived from adipose

tissue (adipokines), endotoxins of intestinal origin, iron accumulation and genetic factors. All these mechanisms are not mutually exclusive, although they are more likely to act in a coordinated and cooperative manner [224].

The current theory of lipotoxicity focuses on an increase in the flux of FFAs within hepatocytes, which is a direct consequence of an increased dietary intake of saturated fatty acids (SFAs), *de novo* lipogenesis and adipose tissue lipolysis in the setting of insulin resistance and impairment of compensatory oxidative processes. As a result, toxic lipid FFA-derived metabolites are generated, such as ceramides, diacylglycerols, lysophosphatidylcholine and oxidized cholesterol metabolites, which act as reactive oxygen species (ROS), causing lipotoxic hepatocellular injury manifested as endoplasmic reticulum stress, inflammation, apoptosis and necrosis [237] (**Figure 10**).

Oxidative stress is a condition due to an altered balance between the production of ROS and the antioxidant defensive capacity. These cytotoxic ROS and lipid peroxidation products may diffuse into the extracellular space, affecting Kupffer cells and hepatic stellate cells (HSC) [238,239].

This cellular oxidative stress from hepatocytes and the direct uptake of FFA or free cholesterol in Kupffer cells induces the activation of nuclear transcription factors such as NF-kβ, which regulates the synthesis of several proinflammatory cytokines,

such as 1) tumor necrosis factor-alpha, which activates the caspase pathway and leads to hepatocyte apoptosis, 2) transforming growth factor beta-1 (TGFβ-1), which activates collagen synthesis by HSC, 3) the Fas ligand that causes 'fratricide deaths' between adjacent hepatocytes and 4) interleukin-8 (IL-8), a powerful neutrophil chemotactic [240].

Endotoxin (lipopolysaccharide), a key constituent of many bacteria present in our microbiota, also considered a metabolic organ, plays a central role in innate immune responses and has been considered the so-called ''second hit'' in previous NASH models [219]. Patients with NAFLD demonstrate increased gut permeability, which importantly has been associated with the severity of liver steatosis but not with the degree of NASH-associated inflammation [241].

Aron-Wisnewsky *et al* summarized the influence of gut microbiota in stimulating fat deposition and promoting NASH through five mechanisms: 1) it promotes obesity by improving the energy yield from food, 2) it regulates gut permeability, low-grade inflammation and immune balance, 3) it modulates dietary choline metabolism, 4) it regulates bile acid metabolism and 5) it increases endogenous ethanol production by bacteria [242]. All these factors are molecular mechanisms by which microbiota may induce NAFLD and its progression. When tight junctions are impaired, intestinal permeability increases, leading to the delivery of gut-derived bacterial products such as endotoxin (LPS) to the liver via the portal vein. Ruiz *et al*

demonstrated that plasma endotoxin levels were elevated in fatty liver patients, which were further increased in individuals with NASH, and were associated with a rise in TNF-α gene expression in the hepatic tissue [243].

Some studies have shown that increased hepatic iron concentration in patients with NAFLD is associated with IR and may therefore indirectly contribute to disease progression [244, 245]. Ferritin levels reflect total body iron stores, and as such, raised levels indicate iron overload. Studies suggest that ferritin may act as a cytokine to induce the release of further tissue cytokines and activate Kupffer and stellate cells, thereby exacerbating fibrosis [246, 247].

Genetic factors might be attractive candidates to explain why a certain percentage of patients with fatty liver develop inflammation. NAFLD is a heritable disorder, suggesting genetic components exist that predispose to these traits. Polymorphisms in patatin-like phospholipase-3 (PNPLA-3), encoding a protein of unknown function with homology to lipid acyl hydrolases, have been strongly associated with increased hepatic fat content in NAFLD [248].

Considering that NAFLD is part of a syndrome that strongly overlaps with obesity and insulin resistance, it therefore appears likely for common genetic aspects for all those diseases to exist [249].

4.6.3 Role of adipokines in the pathogenesis of NAFLD

Adipose tissue has been recognized in the last decade as a highly active endocrine and immune organ with the capacity to produce various mediators including adipocytokines and cytokines both in health and disease. The balance/imbalance of an adipose tissue ‘‘mediator cocktail’’ may profoundly affect not only the status of the adipose tissue but also particularly that of important target organs such as the liver [250]. Adipokines have been reported as promoting insulin resistance [251,252], leading to enhanced delivery of FFA to the liver and then to hepatic steatosis. Moreover, these molecules may cause direct damage to the liver or act indirectly by increasing oxidative stress, liver fibrosis and tumor development by activating the oncogenic factor Stat3 [221].

Clinical studies suggest that the expression of adipokines may vary in patients with NAFLD/NASH compared to healthy controls. In particular, serum levels of proinflammatory adipokines such as leptin, resistin, visfatin, TNF-α and IL-6 are significantly higher in NAFLD/NASH patients, whereas levels of insulin-sensitizing adipokines with anti-inflammatory properties, such as adiponectin, are significantly reduced [253] (**Figure 10**). Although adipose tissue secretes the majority of adipokines, they are also produced by other organs. In this regard, in human liver biopsies, hepatic adiponectin receptor

mRNA increased in biopsy-proven NASH [254]. In contrast, Kaser S *et al* found low mRNA expression of adiponectin and adiponectin receptor 2 (AdipoR2) in the liver of patients with NASH compared to those with SS, and AdipoR2 expression was inversely related to alanine aminotransferase and the fibrosis stage [255]. More recently, Moschen *et al* demonstrated in a prospective study that rapid weight loss after bariatric surgery results in a significant improvement of both histological and biochemical liver parameters, accompanied by an increase in adiponectin serum levels, as well as hepatic mRNA adiponectin expression [256]. Hepatic TNF-α and TNF-α receptor-1 (TNFR-1) expression was increased in patients with NASH compared with an obese group of similar age without NASH. In these NASH patients, more advanced fibrosis was also accompanied by increased hepatic expression of TNF-α [257].

Regarding IL-6, Wieckowska *et al* demonstrated a marked increase in hepatic IL-6 expression in NASH patients compared to those with SS or a normal liver, and its expression positively correlated with the degree of inflammation, stage of fibrosis and IR [258]. In another study, weight loss resulted in a dramatic decrease in hepatic IL-6 expression [259].

Recently, Chuan Shen *et al* have demonstrated that hepatic resistin overexpression in NASH patients is associated with the severity of liver inflammation and fibrosis [260]. Finally, our research group has recently found serum and hepatic visfatin

to be higher in morbidly obese women with NAFLD than in those with normal liver histology [249].

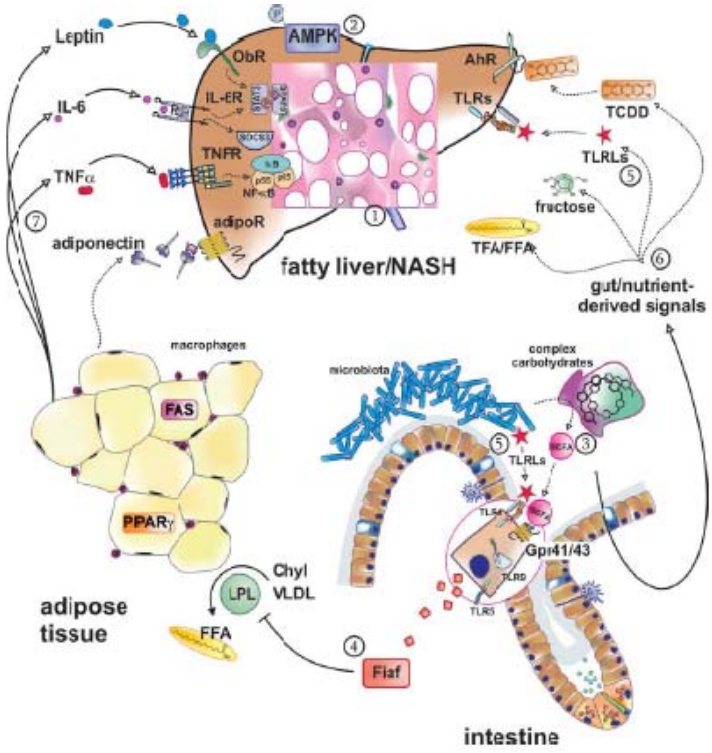

Figure 10. The multiple parallel hits model. Lipotoxicity: (1) A liver loaded with lipids consisting primarily of trigylcerides might reflect a benign process because trigylcerides might exert mostly protective effects. Furthermore, hyperleptinemia leads to oxidation of hepatic lipids, thereby also protecting this organ from lipotoxicity. When the capacity of peripheral and central organs of detoxifying ‘‘aggressive lipids’’ fails, lipotoxic attack of the liver might begin. Inflammation may precede steatosis in NASH. Gut-derived signals: Many signals beyond endotoxin might affect hepatic steatosis and inflammation. Several pathways have been identified how the gut microbiota might influence host energy metabolism: (2) Absence of the microbiota in germ-free mice correlates with increased activity of phosphorylated AMPK in the liver and the muscle (not shown). (3) Some of the breakdown products of polysaccharides are metabolized to SCFAs. SCFAs such as propionate and acetate are ligands for the G protein– coupled receptors Gpr41 and Gpr43. Shortage of SCFAs might allow the evolution of systemic inflammatory events. Such mechanisms elegantly combine diet, microbiota, and the epithelial cell as ‘‘nutrient sensor.’’ (4) The microbiota decreases epithelial expression of fasting-induced adipocyte factor (Fiaf), which functions as a circulating lipoprotein lipase (LPL) inhibitor and therefore is an important regulator of

peripheral fat storage.(5) Several TLRs, such as TLR5 or TLR9, are not only able to affect microbiota but also to regulate metabolism, systemic inflammation, and insulin resistance, thus highlighting the role of the innate immune system in metabolic inflammation as observed in NASH. (6) Various nutrients such as trans fatty acids (TFAs), fructose or aryl hydrocarbon receptor (AhR) ligands such as 2,3,7,8-tetrachlorodibenzodioxin (TCDD) may directly lead to steatosis/liver inflammation. Adipose tissue–derived signals: Signals derived from the adipose tissue beyond toxic lipids might play a central role in NAFLD/NASH. (7) Here, adipocytokines such as adiponectin and leptin, certain proinflammatory cytokines such as TNFa or IL-6, and others (the death receptor Fas, PPARc) are of key relevance. The cytokine/adipocytokine milieu might be critical because ob/ob-adiponectin tg mice, although becoming severely obese, are not insulin-resistant. This suggests that in the hierarchy of processes soluble mediators play the central role. Adipose-derived mediators might indeed affect target organs such as the liver, because JNK1 adipose-deficient mice are protected from diet-induced obesity, and experiments have demonstrated that this effect is mediated mainly by IL-6 (a cytokine), which is of key importancein human obesity**. *Adapted from Herbert Tilg and Alexander R. Moschen. Evolution of Inflammation in Nonalcoholic Fatty Liver Disease: The Multiple Parallel Hits Hypothesis. HEPATOLOGY, Vol. 52, No. 5, 2010*.**

III. HYPOTHESIS AND OBJECTIVES

Considering that:

1. In humans, the ability of different tissues, such as adipose tissue, to secrete omentin, vaspin and other adipokines has been demonstrated.

2. The available data associate circulating levels of omentin and vaspin, and circulating levels of other proinflammatory and anti-inflammatory adipocytokines, with the presence and degree of obesity. However, references regarding the relationship between the circulating levels of omentin/vaspin and gene expression in adipose tissue are scarce and sometimes contradictory.

3. Recently available data associate circulating levels of omentin/vaspin with different metabolic syndrome parameters and the presence of its hepatic manifestation: nonalcoholic fatty liver disease. The associations between the circulating levels of omentin/vaspin and the presence and histological type of nonalcoholic liver disease, that is, simple steatosis (SS) or nonalcoholic steatohepatitis (NASH), are poorly studied.

4. Liver biopsy is the current gold standard to diagnose, monitor progress and determine prognosis of nonalcoholic fatty liver disease. At present, there is no clinical or biochemical marker, or any validated mathematical model, which by combining clinical and biochemical data, would quantify the probability of having histologically advanced nonalcoholic fatty liver disease, or its progression from simple steatosis to NASH.

The objectives of this study are as follows:

1. To analyze the circulating levels of omentin and vaspin and their gene expression in visceral and subcutaneous adipose tissue in a group of morbidly obese women versus a control group of normal-weight women and to study their relationship with the different clinical-biochemical variables that constitute metabolic syndrome.

2. To analyze the relationship between the circulating levels of omentin/vaspin and gene expression in subcutaneous and visceral adipose tissue with the presence of type 2 diabetes mellitus and insulin resistance in a group of morbidly obese women.

3. To analyze the relationship between circulating levels of omentin/vaspin and the presence of nonalcoholic fatty liver disease in a group of morbidly obese women.

4. To evaluate the clinical use of the determination of circulating levels of omentin and vaspin as biomarkers for the presence and stage of nonalcoholic fatty liver disease.

IV. MATERIALS AND METHODS

1. Patients and methods

The study was approved by the institutional review board. All participants gave written informed consent for participation in the medical research. We analyzed the circulating levels of vaspin and omentin in 71 Spanish women of European descent: 31 were lean (BMI < 25 kg/m^2) and 40 were morbidly obese (BMI > 40 kg/m^2). We also analyzed vaspin and omentin gene expression in paired samples of subcutaneous and visceral adipose tissues from 46 patients: 6 were lean (BMI < 25 kg/m^2), and 40 were morbidly obese (BMI > 40 kg/m^2). We additionally analyzed 40 liver samples from MO women. NAFLD was diagnosed by the following criteria: 1) liver pathology, 2) an intake of less than 10 g of ethanol/day and 3) appropriate exclusion of other liver diseases.

Adipose tissue samples were obtained from morbidly obese women and from control women who underwent bariatric surgery by laparoscopic gastric bypass and elective surgery, respectively. Subcutaneous adipose tissue biopsies were obtained from the right hypochondrion region and visceral adipose tissue biopsies were obtained from the greater epiploon region. The liver biopsies were also obtained during the planned bariatric surgery. All liver biopsies were performed under clinical indications.

For each type of surgery, the samples were obtained by the same specialist. Morbidly obese women and controls had similar ages. The weight of all subjects was stable for at least three months before surgery. The exclusion criteria were as follows: 1) concurrent use of medications known to produce hepatic steatosis, 2) patients using lipid-lowering medications including PPAR-α or -γ agonists, 3) diabetic women who were receiving insulin or on medication likely to influence endogenous insulin levels, 4) menopausal and post-menopausal women and subjects receiving contraceptive treatment and 5) patients who had an acute illness, current evidence of acute or chronic inflammatory or infectious diseases or end-stage malignant diseases.

The liver samples were scored by two experienced hepatopathologists using the methods described previously [261, 262].

According to their liver pathology, the patients were subclassified into the following groups: 1) MO with normal liver (NL) histology (n = 4), 2) MO with simple steatosis (micro/macrovesicular steatosis without inflammation or fibrosis (n = 18) and 3) MO with nonalcoholic steatohepatitis (NASH) (Brunt grade 1–3, n = 18).

2. Laboratory methodology

2.1 Anthropometric measurements

BMI was calculated as weight divided by height squared (kg/m^2) according to the criteria of the World Health Organization [2]. Waist circumference was measured at the height of the iliac crest.

Morbidly obese women were further subclassified according to the presence or absence of the metabolic syndrome. The MetS and metabolic risks are defined according to the US National Cholesterol Education Program Adult Treatment Panel III guidelines and modified as recommended in the latest American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement by adopting a lower cutoff for fasting glucose (5.6 mmol/L). MetS was defined as having three of the following metabolic risk factors: 1) central obesity (waist circumference 88 cm in women), 2) hypertriglyceridemia (fasting triglycerides 1.69 mmol/L (150 mg/dL)), 3) low HDL cholesterol (fasting HDL < 1.29 mmol/L (50 mg/dl) in women), 4) glucose intolerance (fasting glucose 5.6 mmol/L (100 mg/dL)) and 5) hypertension (blood pressure 130/85 mmHg obtained as the mean of two seated readings obtained after resting for at least 10 minutes or on regular antihypertensive medications) [262].

2.2 Analytical methods

2.2.1 Biochemical and immunoanalysis

Basal, fasting blood samples were drawn after an overnight fast to determine glucose, insulin and standard laboratory parameters. Plasma and serum samples were stored at -80°C until analytical measurements were performed except for glucose, which was determined immediately after the blood was drawn.

Fasting plasma glucose and lipid profile (triglycerides, total cholesterol and high-density lipoprotein cholesterol) were measured using the usual enzymatic methods in an ADVIA Centaur autoanalyzer. Low-density lipoprotein cholesterol was calculated as the difference between the total cholesterol, HDL and triglyceride content/5 when TG was < 400 mg/dL. The plasma insulin concentration was measured by commercial chemiluminescence assay for ADVIA Centaur (Siemens Medical Solutions S. L., Barcelona, Spain) according to the manufacturer's instructions. The homeostasis model assessment of insulin resistance (HOMA2-IR) was completed using the HOMA Calculator version 2.2.2 (http://www.dtu.ox.ac.uk, accessed May 2010). Glycosylated hemoglobin (HbA1c) was measured by a chromatographic method (Glico Hb Quick Column Procedure, Helena Laboratories, Beaumont, TX, USA). Circulating levels of TNFR-1, TNFR-2 (Biosource Europe S.A., Nivelles, Belgium), IL-6 (Quantikine, R&D Systems, Minneapolis, MN, USA), adiponectin, HMW adiponectin (Linco Research, Inc., St.

Charles, MO, USA), resistin (Biovendor, Modrice, Czech Republic), leptin (Biovendor, Modrice, Czech Republic), RBP-4, lipocalin-2 (Biovendor, Modrice, Czech Republic), serum vaspin (Adipogen, Seoul, South Korea) and plasma omentin (Apotech, Axxora, Nottingham, UK) were measured in duplicate using enzyme-linked immunosorbent assays (ELISA) following the manufacturer's instructions. The TNFR-1 assay sensitivity was 50 pg/mL, and the interassay and intra-assay coefficients of variation were less than 5.7 and 1.7%, respectively. The TNFR-2 assay sensitivity was 0.1 ng/mL, and the interassay and intra-assay coefficients of variation were less than 3.2 and 3.3%, respectively. The IL-6 assay sensitivity was 0.039 pg/mL, and the interassay and intra-assay coefficients of variation were less than 9.6 and 6.9%, respectively. The adiponectin assay sensitivity was 0.78 ng/mL, and the interassay and intra-assay coefficients of variation were less than 8.4 and 7.4%, respectively. The HMW adiponectin assay sensitivity was 0.5 ng/mL, and the interassay and intra-assay coefficients of variation were less than 3.8 and 2.6%, respectively. The resistin assay sensitivity was 33 pg/mL, and the interassay and intra-assay coefficients of variation were less than 6.9 and 3.4%, respectively. The leptin assay sensitivity was 0.2 ng/mL, and the interassay and intra-assay coefficients of variation were less than 7.6 and 4.4%, respectively. The RBP-4 assay sensitivity was 0.02 g/L, and the interassay and intra-assay coefficients of variation were less than 1.1 and 2.2%, respectively. The LCN-2 assay

sensitivity was 0.01 ng/mL, and the interassay and intra-assay coefficients of variation were less than 5.6 and 4.4%, respectively. The vaspin assay sensitivity was 12 pg/mL, and the interassay and intra-assay coefficients of variation were less than 6 and 4%, respectively. The omentin assay sensitivity was 0.4 ng/mL, and the interassay and intra-assay coefficients of variation were less than 6 and 2.7%, respectively.

2.2.2 Analysis of Human Vaspin and Omentin Gene Expression

Total RNA was isolated from adipose tissues using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol and digested with DNase I (RNase-Free DNase set, Qiagen). RNA quality was evaluated by measuring the 260/280-nm absorbance ratio (≥ 1.8) and by electrophoresis. First-strand cDNA was synthesized using an equal amount of total RNA with the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Real-time quantitative PCR was performed in a final volume of 20 µL, which contained 10 ng of reverse-transcribed cDNA, 10 µL of 2X Taq Man Fast Universal PCR Master Mix (Applied Biosystems) and 1 µL Taq Man Assay predesigned by Applied Biosystems® for the detection of vaspin, omentin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as a housekeeping gene. All reactions were performed in triplicate and were carried out in 96-well plates by using the 7900HT Fast Real-Time PCR system (Applied Biosystems).

2.3 Statistical analysis

All the values reported are expressed as the means ± SEM (standard error of the mean) or as means ± SD (standard deviation) and were analyzed using the statistical package SPSS/PC+ for Windows (v.20.0, Chicago, IL, USA). One-way ANOVA with post-hoc Tukey test was used to compare continuous variables between groups. The strength of association between variables was calculated using Pearson's method and Spearman's ρ-correlation test. Multiple linear regression analysis with backward variable selection was performed to identify independent predictors of HOMA2-IR. The validity of the regression model and its assumptions was assessed with the plot of residuals vs predicted values. The data were normally distributed. Logistic regression analysis was performed to identify independent predictors of the metabolic syndrome. Vaspin and omentin circulating levels were age- and BMI-adjusted in some analyses. P values < 0.05 were considered to be statistically significant.

ROC curves were used to evaluate the biomarker performance of circulating omentin levels in the diagnosis of NAFLD and NASH compared to liver biopsy, which is the current reference method. The three values of optimum cutting were selected based on obtaining a first cutoff of high sensitivity (90%), a second cutoff that prioritized specificity (90%) and a third that included the best combination of sensitivity and specificity according to the Youden index.

V. RESULTS

1. Serum vaspin and omentin levels in normal-weight and morbidly obese women

Table 2 shows the baseline characteristics of the group of control women with normal weight (BMI < 25 kg/m^2) and the group of morbidly obese women (BMI > 40 kg/m^2). The two groups exhibited similar ages.

Patients from the MO group showed a significant increase in glucose, insulin, HOMA2-IR and glycosylated hemoglobin A1c compared to the control group. Blood pressure was increased in the MO group.

The lipid profile significantly differed in the two groups with high triglyceride levels in the MO group and significantly decreased HDL-C levels in MO women compared to the control group.
Applying the metabolic syndrome ATP III criteria [262], 86% of MO women met the criteria, compared with 2.5% of women in the control group. In the statistical analysis, a significant increase was evident in the circulating levels of AST, ALT and GGT in the subgroup of MO women.

Table 2. Baseline characteristics of the study cohort

	CONTROL (N = 31)	MORBID OBESITY (N = 40)	P VALUE
Age (years)	45.53 (1.55)	46.37 (3.34)	ns
Weight (kg)	56.90 (6.96)	122.28 (17.07)	**<0.001**
BMI (kg/m²)	23.21 (0.46)	48.22 (1.07)	**<0.001**
WC(cm)	80.40 (2.16)	134.00 (3.10)	**<0.001**
SBP(mmhg)	120.91 (4.22)	134.99 (4.07)	**0.020**
DBP(mmhg)	70.04 (1.96)	76.70 (2.80)	**<0.001**
Glucose(mg/dl)	94.06 (2.53)	117.70 (4.25)	**0.001**
Insulin(mu/l)	8.25 (1.32)	22.69 (3.94)	**0.001**
Hba1c (%)	4.55 (0.07)	5.63 (0.29)	**<0.001**
HOMA2-IR	1.11 (0.18)	2.60 (0.24)	**0.001**
HDL-C (mg/dl)	59.83 (2.87)	40.97 (1.33)	**<0.001**
Triglycerides (mg/dl)	95.13 (9.06)	185.52 (11.88)	**<0.001**
AST (u/l)	21.36 (7.56)	46.08 (32.97)	**<0.001**
ALT (u/l)	20.08 (13.79)	44.48 (25.85)	**<0.001**
GGT(u/l)	20.59 (29.68)	32.63 (33.82)	ns
FA (u/l)	61.58 (20.50)	71.47 (21.01)	**0.022**

Mean (SEM) * Significant differences vs the control group ($p < 0.05$)

The circulating levels of adipocytokines studied, comparing both groups, are shown in **Figure 11**.

Regarding the analysis of serum vaspin levels depending on the degrees of obesity, the mean serum vaspin±SD was 0.87±0.96 µg/L in MO women and 1.66±2.09 µg/L in the control group; statistical analysis showed no significant differences of the MO group compared to the control group.

The analysis of circulating omentin levels in the MO group showed significant differences from the control group: in the MO group, the mean±SD was 1.97±2.15 ng/ml; in the control group, 5.27±5.33 ng/ml.

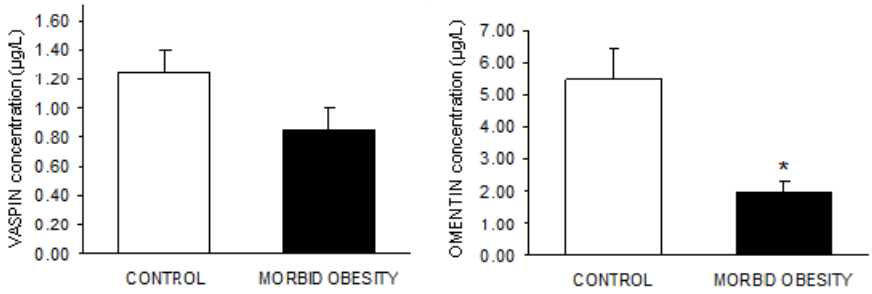

Figure 11 Vaspin and omentin concentrations in control women and morbidly obese women. * Indicates statistically significant differences between groups ($p < 0.05$).

2. Correlation of circulating levels of vaspin and omentin with metabolic variables and circulating levels of other adipocytokines.

No correlation was observed between serum vaspin levels and the metabolic variables studied (**Table 3**). The relationship between serum vaspin levels and the presence of MetS diagnostic criteria was also studied; however, we were unable to show correlation between the two.

Table 3. Correlation of vaspin and omentin levels with metabolic variables

Circulating levels	**VASPIN**		**OMENTIN**	
Variables	**r**	**p-value**	**r**	**p-value**
BMI (Kg/m²)	-0.181	0.134	-0.212	0.096
WC (cm)	-0.448	**0.007**	-0.332	0.078
Glucose (mg/dL)	-0.002	0.989	-0.276	**0.029**
Insulin (mU/L)	-0.015	0.903	-0.252	0.054
HOMA2-IR	0.004	0.976	-0.274	**0.037**
HbA1c (%)	-0.181	0.218	0.011	0.943
HDL-C (mg/dL)	0.166	0.183	-0.178	0.182
Triglycerides (mg/dL)	-0.218	0.072	-0.101	0.433
SBP (mmHg)	-0.002	0.986	-0.179	0.201
DBP (mmHg)	-0.019	0.887	-0.072	0.608

BMI: body mass index, WC: waist circumference, HOMA2-IR, homeostasis model assessment of insulin resistance; HDL-C: high-density lipoprotein cholesterol, SBP: systolic blood pressure, DBP: diastolic blood pressure. Bolded p-values indicate significant correlations ($p < 0.05$).

However, serum vaspin levels correlated inversely with serum lipocalin-2 and IL-6 leptin levels (**Table 4**).

Table 4. Correlation of vaspin and omentin levels with circulating adipo/cytokine levels

Circulating levels	VASPIN		OMENTIN	
Variables	r	p-value	r	p-value
HMW Adiponectin (µg)	0.178	0.173	0.078	0.557
Adiponectin (µg/L)	0.145	0.250	0.013	0.431
Resistin (µg/L)	-0.178	0.157	-0.150	0.236
LCN-2 (µg/L)	-0.336	**0.016**	0.088	0.559
IL-6 (ng/L)	-0.290	**0.029**	0.033	0.812
RBP-4 (mg/dL)	0.015	0.915	0.013	0.931
TNFR-1 (µg/L)	-0.241	0.057	0.004	0.976
TNFR-2 (µg/L)	-0.051	0.689	0.036	0.787
Leptin (µg/L)	-0.290	**0.041**	0.195	0.199

HMW adiponectin: high-molecular-weight adiponectin, LCN-2: lipocalin-2, IL-6: interleukin-6, RBP-4: retinol-binding protein-4, TNFR-1: tumor necrosis factor receptor-1, TNFR-2: tumor necrosis factor receptor-2. Bolded p-values indicate significant correlations ($p < 0.05$).

The omentin plasma levels were inversely correlated with glucose and HOMA2-IR levels (**Table 3**). When the relationship between the circulating levels of omentin and the presence of metabolic syndrome diagnostic criteria was investigated, a negative correlation with the metabolic syndrome was demonstrated. After adjusting for age and BMI, the sample subjects were subclassified into three tertiles according to their omentin levels: tertile 1 (> 4.33 ng/ml), tertile 2 (2.30 to 4.33 ng/ml) and tertile 3 (< 2.30 ng/ml). In logistic regression analysis, low circulating omentin levels in tertiles 2 and 3 were associated with the presence of metabolic syndrome diagnostic criteria, whereas tertile 1 patients with higher omentin levels were not related to the development of MetS (**Table 5**).

Table 5. Logistic regression analysis for the presence of the metabolic syndrome according to the omentin tertile circulating levels

	OR	95% CI	p-value
OMENTIN tertile 2 vs 1	25.00	4.41 – 141.68	<0.001
OMENTIN tertile 3 vs 1	90.00	11.46 – 706.71	<0.001

Model 1. Omentin tertiles (ng/ml) adjusted for BMI and age: 1 (> 4.33); 2 (4.33–2.30); 3 (< 2.30). Tertile 1 as reference with OR = 1.

In considering whether a correlation existed between omentin plasma levels and circulating levels of other adipocytokines, no correlation could be established (**Table 6**).

Table 6 Correlations between the number of diagnostic criteria of metabolic syndrome met by the patients

Variables	**Number of criteria of Metabolic syndrome**	
	r	p-value
Vaspin (µg/L)	-0.209	0.83
Omentin (µg/L)	-0.264	0.045
BMI (Kg/m^2)	0.737	**<0.001**
WC (cm)	0.783	**<0.001**
HOMA2-IR	0.589	**<0.001**
Glucose (mg/dl)	0.710	**<0.001**
SBP (mmHg)	0.636	**<0.001**
DBP (mmHg)	0.250	**0.006**
HDL-C (mg/L)	-0.750	**<0.001**
Triglycerides (mg/dl)	0.704	**<0.001**

Vaspin and omentin circulating levels and different parameters. BMI: body mass index, WC: waist circumference, HOMA2-IR: homeostasis model assessment of insulin resistance, SBP: systolic blood pressure, DBP: diastolic blood pressure. Bolded p-values indicate significant Spearman's correlations ($p < 0.05$).

3. Vaspin gene expression in subcutaneous and visceral adipose tissue in normal-weight and morbidly obese women.

In a second part of the study, the gene expression of adipokines was analyzed in subcutaneous and visceral adipose tissues, comparing the morbidly obese group (n = 40) with the control group of normal-weight individuals (n = 6). The results are shown in **Figure 12**. A significant increase in vaspin gene expression was shown in both subcutaneous and visceral adipose tissues in the MO group compared to the control group. No differences were found between vaspin gene expression in visceral and subcutaneous adipose tissues in individuals of the MO group ($p = 0.192$) compared with the control group ($p = 0.934$).

Vaspin gene expression in both types of subcutaneous and visceral adipose tissues is not correlated with obesity, glucose metabolism, insulin resistance parameters, blood pressure and other adipokines or the inflammatory parameters studied (data not shown)**.**

4. Omentin gene expression in subcutaneous and visceral adipose tissues in normal-weight and morbidly obese women.

Gene expression (mRNA) in subcutaneous adipose tissue was similar in the morbidly obese group and the control group. However, the present study demonstrated that omentin gene

expression in visceral adipose tissue was significantly lower in morbidly obese individuals compared to normal-weight controls. When comparing both adipose tissues, omentin gene expression was observed to be higher in visceral adipose tissue relative to its gene expression in subcutaneous tissue in both the MO (p = 0.009) and control (p = 0.034) groups.

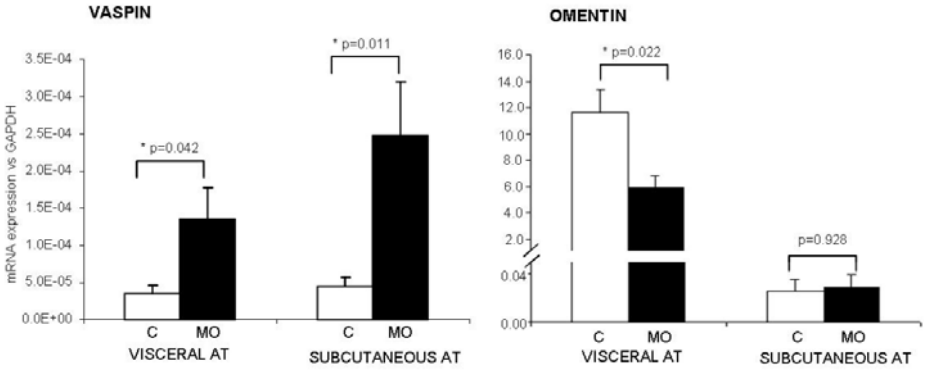

Figure 12. mRNA Vaspin and omentin expression in visceral and subcutaneous adipose tissue.

Omentin mRNA gene expression in both visceral and subcutaneous adipose tissues was not correlated with obesity, glucose metabolism or insulin resistance parameters. No relationship could be established for either of the two types of adipose tissue between omentin gene expression and lipid metabolism, blood pressure and other adipokines or the cytokines studied (data not shown).

5. Serum omentin and vaspin in morbid obesity in relation to the presence and histologic type of nonalcoholic fatty liver disease.

The circulating levels of vaspin and omentin and their association with NAFLD and the histologic type, that is, hepatic simple steatosis (SS) and nonalcoholic steatohepatitis, were studied in the group of morbidly obese women. Liver tissue samples of MO patients were classified according to histological criteria into two subgroups: normal liver (NL) (4 cases) and NAFLD (36 cases). At the baseline analysis of both groups, a significant increase in AST, ALT and GGT levels was observed in the group with liver disease (NAFLD) with respect to the subgroup of patients without impaired liver histology (NL). No differences were observed between the two groups with respect to the other parameters analyzed: age, weight, BMI, SBP, DBP, fasting glucose, fasting insulin, HbA1c, HOMA2-IR, HDL-C and TG (**Table 7**).

Table 7. Baseline characteristics of the morbidly obese women based in liver histology

MORBID OBESITY					
	NORMAL LIVER (n=4)		NAFLD (n=36)		p-value
AGE (years)	44.05	(10.71)	47.55	(11.11)	n.s
WEIGHT (Kg)	122.62	(15.43)	122.79	(18.14)	n.s
BMI (Kg/m^2)	48.46	(7.33)	48.02	(5.66)	n.s
PC (cm)	133.85	(14.90)	132.48	(11.54)	n.s
TAs (mmHg)	134.00	(15.58)	133.66	(26.34)	n.s
TAd (mmHg)	76.38	(15.69)	78.99	(17.73)	n.s
GLUCOSA(mg/dL)	103.11	(27.96)	119.06	(38.61)	n.s
INSULINEMIA(Mu/L)	17.48	(11.19)	21.24	(22.73)	n.s
HbA1C (%)	5.30	(0.80)	6.14	(1.82)	n.s
HOMA2-IR	2.13	(1.31)	2.74	(2.75)	n.s
HDL-C (mg/dL)	40.02	(9.68)	41.21	(8.10)	n.s
TRIGLIC (mg/dL)	158.32	(44.76)	165.70	(73.56)	n.s
AST (U/L)	31.91	(11.01)	48.58	(27.03)	*** 0.004**
ALT (U/L)	32.47	(11.79)	48.11	(27.31)	*** 0.005**
GGT (U/L)	19.94	(10.40)	38.01	(24.90)	*** 0.025**
FA (U/L)	65.35	(18.92)	75.29	(22.62)	n.s

Mean standard deviation. Comparison between the subgroup with normal liver and NASH subgroup. * Significant differences p <0.05.

The comparison study and analysis of the circulating levels of adipocytokines, between the two subgroups (NL and NAFLD) showed that the circulating omentin levels tended to be higher

in the subgroup of MO women whose liver histology was indicative of NAFLD compared with MO women having normal liver histology. However, these differences were not significant considering the n of the normal liver group (NL MO: 0.38±0.43, NAFLD MO: 1.81±2.04). Because this trend was observed, serum omentin levels were studied in the different liver histology subgroups of NL, SS and NASH. The omentin plasma levels were statistically higher in the NASH group compared to the SS group (SS MO: 1.07±1.52, NASH MO: 2.30±2.03).

When analyzing serum vaspin levels, no significant differences were identified by liver histology type (NL MO: 0.76 ± 1.11, SS MO: 0.85 ± 1.18, NASH MO: 1.26±0.95).

Regarding the rest of the adipokines studied, no significant differences were observed (data not shown).

6. Assessment of the omentin effect on the presence of NASH

According to the previous results, this study evaluated the diagnostic efficacy of omentin plasma levels as a biomarker of NAFLD in the group of MO patients with liver histology indicative of NAFLD; additionally, it evaluated the behavior of omentin plasma levels in the NASH subgroup, determining a cutoff point and area under the curve to establish the diagnosis of both entities (**Figure 13**).

Regarding NAFLD, the area under de curve of omentin circulating levels was 0.64. Therefore omentin is not a valid biomarker of NAFLD.

In the NASH group, an optimal omentin cutoff level of 1.83 and an area under the curve of 0.71 were obtained, with a sensitivity of 58.8 % and specificity 76.2%, reaching the level of statistical significance. The negative predictive value (NPV) for the NASH group was 91.9 % (**Figure 13**).

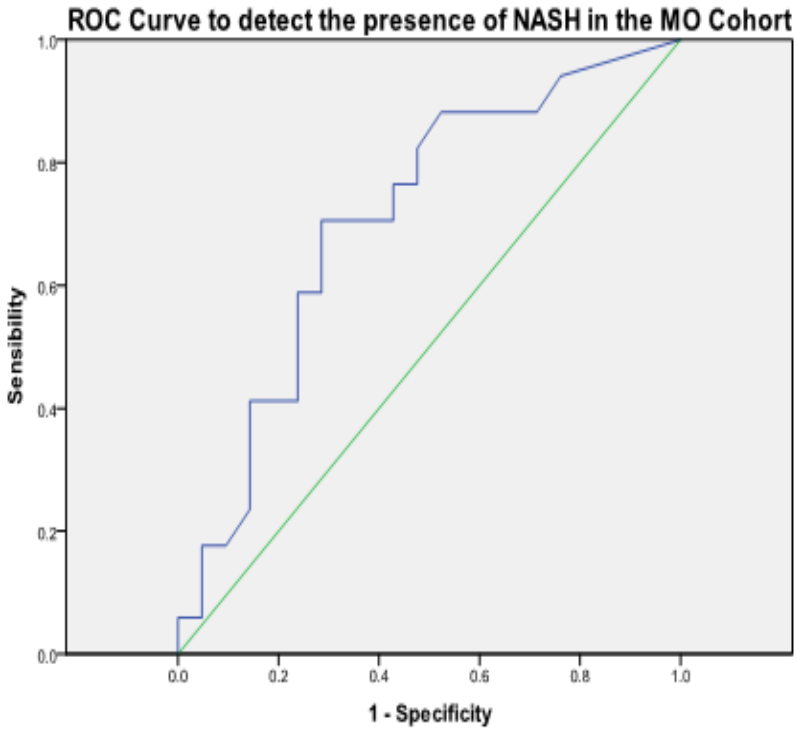

Figure 13. ROC curve to detect the presence of NASH in MO cohort.

VI. GENERAL DISCUSSION

Although it has been reported that abnormalities in the circulating levels of vaspin and omentin and the gene expression of both factors are related to BMI and markers of insulin sensitivity in metabolic syndrome patients, the findings of various authors to date are confusing. The first objective in this study was to measure vaspin and omentin circulating levels and mRNA expression in subcutaneous and visceral adipose tissues in morbidly obese women and to compare them to age-matched controls. We also assessed the relationship between these two adipokines and biochemical markers of metabolic syndrome and other adipocytokines.
Because few studies have been conducted to determine the relationship between vaspin and omentin in the presence of NAFLD, the second most important objective of the present study was to relate the circulating levels of vaspin and omentin to the presence of NAFLD.

The main results of this study are that omentin circulating levels are diminished in morbid obesity, mRNA vaspin expression is higher in the VAT and SAT of morbidly obese patients and mRNA omentin expression is lower in the VAT of morbidly obese women and, finally, omentin circulating levels are increased in patients with NASH.

Regarding the relationship of vaspin to obesity and metabolic syndrome, we demonstrated that serum vaspin levels were not increased in morbidly obese women and that they did not

correlate with BMI and markers of glucose or lipid metabolism. In studies involving humans, results are controversial. A meta-analysis encompassing six studies including 1826 obese individuals and 11 studies including 1570 subjects with T2DM provides evidence of higher vaspin levels in obesity and T2DM and emphasizes the pivotal role of vaspin in the progression of metabolic and glucose abnormalities, thus its promising potential as a cardiovascular risk marker [263]. Saboori *et al* demonstrated that the serum vaspin level was significantly higher in obese women [264]. Youn *et al* reported that elevated vaspin serum concentrations correlated with obesity and impaired insulin sensitivity, although not in patients with type 2 diabetes [269]. In obese children, Lee *et al* observed a negative correlation between vaspin concentration and HOMA-IR [265]. A significant difference in vaspin levels has been observed in subjects with newly diagnosed T2DM and MetS compared to a group without MetS [266].

However, in agreement with our results, von Loeffelholz *et al* have shown that no association exists between serum vaspin and HOMA-IR in nondiabetic humans [267]. Seeger *et al* also reported that circulating vaspin was not independently associated with markers of glucose metabolism [268], whereas Briana *et al* demonstrated that vaspin concentrations did not correlate with insulin levels in maternal, fetal and neonatal samples [269], as occurred in our population.

The exact reasons for the variability in serum vaspin concentrations remain debatable. A difference in the

measurement ranges between ELISA and RIA human vaspin systems have been observed [269,271]. Moreover, Breitfeld *et al* conducted a genome-wide association study and identified several single nucleotide polymorphisms (SNPs) in the vaspin locus of chromosome 14 associated with serum vaspin levels and inferred that genetic variations are the most likely reason for serum vaspin variations [270].

Regarding the relationship between vaspin levels and other adipocytokines in the circulation, in our study, serum vaspin levels correlated inversely with levels of LCN-2 and IL-6. LCN-2 has been reported to be an adipokine that appears to be an independent risk factor for hyperglycemia and insulin resistance in humans; moreover, it has also been related to inflammation [272]. IL-6 is a known proinflammatory cytokine that is also increased in obesity [273]. Taken together, these findings might suggest that vaspin has an anti-inflammatory profile.

Alternatively, vaspin mRNA expression was significantly higher in our morbidly obese cohort in the SAT and VAT. Kloting *et al* have reported that vaspin expression was not detectable in lean subjects but that it was present in both the SAT and VAT of obese patients. Its levels were significantly correlated with parameters of obesity, insulin resistance and impaired glucose tolerance. However, we have been unable to support their findings. In untreated OLETF rats, vaspin expression and its serum levels decreased as diabetes worsened and body

weight fell. The expression and serum levels were normalized by treatment with insulin or pioglitazone, suggesting that vaspin exerts a defensive action against insulin resistance. In contrast, the administration of recombinant human vaspin improved insulin sensitivity and glucose tolerance, and reversed the expression of those genes that may promote insulin resistance such as leptin, resistin and TNF-α in diet-induced obese mice [274].

In summary, serum vaspin levels are inversely related in our study to IL-6, whereas SAT and VAT vaspin expression is significantly higher in morbidly obese women. Additionally, as noted above, the literature confirms that vaspin has an insulin-sensitizing effect. Thus, in conjunction with our results, this finding suggests that vaspin has a compensatory role in the inflammatory complications of obesity.

Regarding omentin, another important finding of our study is that plasma omentin levels are significantly lower in the morbidly obese and that these levels inversely correlate with glucidic metabolism parameters, in accordance with other authors [150, 153, 275, 276]. The exact factors contributing to markedly reduced circulating omentin levels in obesity and diabetes remain to be determined. Considering the results of previous studies [150,180], the increased insulin levels typically found in patients with obesity and T2DM might be an important contributor preceding decreased omentin levels. Another possible factor contributing to decreased omentin levels could

be excessive adiposity and obesity-associated metabolic complications. This possibility is supported by the finding of increased circulating levels of omentin in patients with anorexia nervosa with severely reduced body fat content [277.] and the finding that serum omentin levels were significantly increased after bariatric surgery, whereas its mRNA expression in SAT was significantly reduced after the surgery, described by Urbanova *et al* and Lapointe *et al* [276,278].

In the same context, we found a negative correlation with systolic blood pressure. Urbanova *et al* have also reported that serum omentin concentrations negatively correlated with parameters of metabolic syndrome [276]. We also demonstrate that patients with omentin levels in the lowest tertile were 90 times more likely to have the MetS than those in the highest tertile, after adjustment for age and BMI. Moreover, women with omentin levels in the second tertile were 25 times more likely to have MetS.

Omentin expression in visceral adipose tissue was significantly lower in the morbidly obese women in our study, in agreement with the results of Souza *et al* [150]. Moreover, Cai *et al* demonstrated that omentin mRNA expression decreased in overweight/obese individuals and decreased further when overweight/obesity was combined with type 2 diabetes. Thus, omentin expression is negatively correlated wiGENCth fasting insulin, HOMA-IR and BMI [160].

In the study of Urbanova *et al*, omentin mRNA expression in subcutaneous adipose tissue did not correlate with any of the anthropometric and biochemical parameters studied [276]. However, these authors did not study visceral adipose tissue.

We performed this analysis in light of the few studies to analyze circulating levels of vaspin and omentin in patients with NAFLD and the suggestion that circulating levels of these molecules might serve as useful biomarkers in the histologic diagnostic of NAFLD [289, 290]. Regarding vaspin levels, we did not observe differences between the groups. Vaspin was associated with liver histology in two studies with biopsy-proven NAFLD patients. Kukla *et al* found a positive relationship between circulating levels of vaspin and hepatocyte ballooning in an obese NAFLD cohort [291]. Aktas *et al* indicated that vaspin positively correlated with liver fibrosis, independent of confounding factors such as sex, age and metabolic and histological parameters [292]. The latter study group found significantly elevated circulating vaspin levels in NAFLD patients compared with healthy controls, whereas Kukla *et al* only found significantly higher adipokine levels in NAFLD patients with hepatocyte ballooning. Circulating vaspin levels were not found to be associated with the histologic findings in a recent study with non-diabetic, non-obese NASH patients by Genc *et al* [282]. Although they also reported significantly higher levels in NASH patients versus normal liver controls, the significance was unapparent after adjustment for

the metabolic risk factors. Therefore, the role of vaspin in NAFLD at this time is unclear [279].

The results obtained suggest that circulating omentin levels tended to be higher in MO women with NAFLD compared to MO women with normal liver histology. Subsequently, we aimed to analyze whether differences existed between circulating omentin levels of patients with SS and NASH. Surprisingly, we found that circulating omentin levels were increased in patients with NASH. In this regard, Yilmaz *et al* found similar but not identical results. They suggested that serum omentin levels are raised in patients with NAFLD regardless of potential confounders and represent an independent predictor of hepatocyte ballooning [281]. As previously mentioned, serum omentin is generally considered to be negatively associated with insulin resistance and obesity [280,150]. Although these observations might suggest that omentin levels should be lower in NAFLD, not higher, the present knowledge of the mechanisms by which omentin might be regulated in nonalcoholic fatty liver disease does not allow us to derive conclusions regarding this topic. A salient finding of the present analysis was the increase in serum omentin levels in our NASH patients. No information is available concerning this finding, and additional studies are required to identify reasons for this paradoxical increase. In particular, future studies must delineate whether levels of omentin are higher in

the NASH population because of a compensatory counter-regulatory mechanism.

The performance of omentin for the diagnosis of NASH showed an excellent area under the receiver operating characteristics (ROC) curve. The main adipokines and cytokines involved in the pathogenesis of NAFLD include adiponectin, leptin, resistin, visfatin, TNF-α and interleukin-6 [293]. Adiponectin modifies insulin receptor function and influences hepatocellular free fatty acid metabolism [283]. Adiponectin levels were low in NAFLD patients [284]. Hypoadiponectinemia in NAFLD is part of the metabolic disturbances associated with MetS, with a significant inverse relationship found between serum adiponectin levels and hepatic fat content [285]. Another prominent adipocytokine associated with NAFLD is leptin. Increases in leptin levels and decreases in adiponectin levels occurred similarly in patients with simple steatosis or NASH during obesity reversal after bariatric surgery, suggesting that these changes are due to morbid obesity and occur independently of liver disease [286]. In a separate study, leptin and adiponectin levels remained independent predictors for NASH in obese patients [287]. Serum levels of retinol-binding protein-4, another adipokine associated with insulin resistance, were also higher in NAFLD patients compared with controls [288].

In sum, omentin could represent a good biomarker of NASH, probably in conjunction with other adipokines. Further studies should be performed to confirm our results.

The major limitation of the present study is the relatively small number of subjects in the sample. Although our specific cohort of morbidly obese women showed a clear relationship between the MetS and omentin levels without the interference of confounding factors, these results cannot be extrapolated to other obesity groups or men. Second, due to the difficulty of obtaining tissue samples, the expression results must be confirmed in larger study populations. Another limitation of the study is that it is cross-sectional. We could not prove a causal link between the levels of omentin and the development of MetS or the levels of vaspin and anti-inflammatory action. Similarly, our cohort of severely obese women made it possible to establish clear relationships between NASH and circulating omentin levels without the interference of such confounding factors as gender or age. Further prospective studies are required to explain these phenomena.

VII. CONCLUSIONS

The conclusions of this study are as follows:

1. No differences were found between circulating levels of vaspin in morbidly obese women compared to the normal-weight control group. Serum vaspin levels were not related to the metabolic variables studied or to metabolic syndrome parameters. **A significant inverse relationship was found with lipocalin-2, leptin and IL-6 levels.**

2. **The circulating levels of omentin were decreased significantly in the group of morbidly obese women** compared to the normal-weight control group**.** These levels showed an inverse correlation with glucose and HOMA2-IR levels; similarly, **low circulating levels of omentin were significantly associated with the presence of metabolic syndrome diagnostic criteria.** A significant relationship with other adipokines could not be established.

3. **Vaspin gene expression in both subcutaneous and visceral adipose tissues was significantly increased in the MO group compared to the control group.** However, no differences could be found between vaspin

gene expression in visceral and subcutaneous adipose tissues.

4. Vaspin gene expression in both types of visceral and subcutaneous adipose tissues was not correlated with obesity, glucose metabolism or the metabolic syndrome parameters studied.

5. Omentin gene expression in subcutaneous adipose tissue was similar in the morbidly obese group and the control group. However, **omentin gene expression in visceral adipose tissue was shown to be significantly lower in morbidly obese individuals** when compared to normal-weight controls**. Omentin gene expression was observed to be higher in visceral adipose tissue with respect to gene expression in subcutaneous tissue in both groups.**

6. Omentin gene expression in both visceral and subcutaneous adipose tissues did not correlate with obesity, glucose metabolism or insulin resistance parameters. No relationship could be established between omentin gene expression and lipid metabolism, blood pressure or other adipokines.

7. **Circulating levels of omentin tended to be higher in the subgroup of MO women who demonstrated liver histology indicative of NAFLD** compared to MO women having normal liver histology**.**

8. **Omentin levels were significantly increased in the group of MO individuals with nonalcoholic steatohepatitis** compared to those MO individuals with liver steatosis**.**

9. **The performance of omentin for the diagnosis of NASH showed an excellent area under the receiver operating characteristics curve**, suggesting its potential utility as a biomarker.

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IX. ANNEX

UNIVERSITAT ROVIRA I VIRGILI
NEW ADIPOKINES VASPIN AND OMENTIN, CIRCULATING LEVELS, GENE EXPRESSION IN ADIPOSE TISSUE AND RELATIONSHIP OF CIRCULATING LEV
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New adipokines vaspin and omentin. Circulating levels and gene expression in adipose tissue from morbidly obese women

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Abstract

Background: Vaspin and omentin are recently described molecules that belong to the adipokine family and seem to be related to metabolic risk factors. The objectives of this study were twofold: to evaluate vaspin and omentin circulating levels and mRNA expression in subcutaneous and visceral adipose tissues in non-diabetic morbidly obese women; and to assess the relationship of vaspin and omentin with anthropometric and metabolic parameters, and other adipo/cytokines.

Design: We analysed vaspin and omentin circulating levels in 71 women of European descent (40 morbidly obese [BMI ≥ 40 kg/m^2] and 31 lean [BMI ≤ 25]). We assessed vaspin and omentin gene expression in paired samples of visceral and subcutaneous abdominal adipose tissue from 46 women: 40 morbidly obese and 6 lean. We determined serum vaspin and plasma omentin levels with an Enzyme-Linked Immunosorbent Assay and adipose tissue mRNA expression by real time RT-PCR.

Results: Serum vaspin levels in the morbidly obese were not significantly different from those in controls. They correlated inversely with levels of lipocalin 2 and interleukin 6. Vaspin mRNA expression was significantly higher in the morbidly obese, in both subcutaneous and visceral adipose tissue.
Plasma omentin levels were significantly lower in the morbidly obese and they correlated inversely with glucidic metabolism parameters. Omentin circulating levels, then, correlated inversely with the metabolic syndrome (MS). Omentin expression in visceral adipose tissue was significantly lower in morbidly obese women than in controls.

Conclusions: The present study indicates that vaspin may have a compensatory role in the underlying inflammation of obesity. Decreased omentin circulating levels have a close association with MS in morbidly obese women.

Keywords: circulating levels morbid obesity, mRNA tissue expression, omentin, vaspin

Background

The incidence of obesity is rising rapidly in industrialized and developing countries. Increased abdominal visceral fat is associated with insulin resistance, type 2 diabetes, and coronary heart disease [1]. Reduction of visceral fat mass by omentectomy has significant positive and long-term effects on glucose metabolism, insulin sensitivity, and metabolic profiles in obese subjects [2]. In the last decade, numerous studies have revealed that adipose tissue secretes a variety of bioactive substances that could explain the epidemiologic relationship between visceral fat mass and increased metabolic risk. These substances, termed adipokines, include leptin [3], adiponectin [4], resistin [5], lipocalin 2(LCN2) [6], fatty acid binding proteins (AFABP) [7], plasminogen activator inhibitor-1 [8], interleukin 6 (IL 6) [9], and various growth factors. They are considered to play an important role in interactions between a

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variety of systems, including adrenal, immune, and central and peripheral nervous systems [10]. In obese subjects, expression, synthesis and release of pro-inflammatory cytokines (tumor necrosis factor-alpha [TNF-α], IL 6) and adipokines (leptin, resistin) is enhanced but anti-inflammatory adipokines such as adiponectin are decreased [11].

Recently, vaspin (visceral adipose tissue-derived serine protease inhibitor) was identified as a member of the serine protease inhibitor family. Vaspin cDNA was isolated from visceral white adipose tissues (WAT) of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, an animal model of abdominal obesity with type 2 diabetes [12]. Vaspin is highly expressed in rat adipocytes from visceral WAT at the age when obesity and insulin plasma concentrations reach a peak [13]. Vaspin improves insulin sensitivity in mice [13]. However, in humans the effect of vaspin on insulin sensitivity is uncertain and the correlation between vaspin and body mass index (BMI) is also unclear [14,15].

Omentin 1 was identified as a novel adipokine predominantly secreted by visceral stromal vascular cells but not adipocytes [16,17]. Furthermore, in vitro experiments revealed that treatment with recombinant omentin-1 enhances insulin-mediated glucose uptake in human subcutaneous and omental adipocytes, while increasing Akt/PKB phosphorylation [17]. In cultured adipocytes, omentin 1 production is decreased by D-glucose and insulin [18,19].

Nevertheless, in studies involving humans, the plasma concentration of omentin-1-the major circulating isoform in human plasma-is decreased in patients with type 1 diabetes mellitus [18,19] and not affected by glucose ingestion [20]. In addition, omentin plasma levels and omentin gene expression in visceral adipose tissue are decreased in obesity [19].

Although some studies have focused on the abnormal levels of vaspin and omentin in metabolic syndrome patients and mRNA expression, particularly with reference to BMI and markers of insulin sensitivity, the regulation of these molecules and their relationship with other adipokines in morbidly obese patients has not been specifically studied.

Our objective in this study was to measure the circulating levels of vaspin and omentin and mRNA expression in subcutaneous and visceral adipose tissue in morbidly obese women and make a comparison with age-matched control women (we studied only women to avoid sex differences). We also assessed the relationship between these two adipokines and biochemical markers of metabolic syndrome, levels of other adipokines, and pro-inflammatory cytokines.

Methods

Subjects

The study was approved by the institutional review board. All participants gave written informed consent for participation in medical research. We analyzed the circulating levels of vaspin and omentin in 71 Spanish women of European descent: 31 lean (BMI < 25 Kg/m2) and 40 morbidly obese (BMI > 40). We also analyzed vaspin and omentin gene expression in paired samples of subcutaneous and visceral adipose tissue from 46 patients: 6 lean (BMI < 25 Kg/m2) and 40 morbidly obese (BM I > 40). Adipose tissue samples were obtained from morbidly obese women and from control women who underwent bariatric surgery by laparoscopic gastric by-pass and elective surgery, respectively. Subcutaneous adipose tissue biopsies were taken from the right hypocondrion region and visceral adipose tissue biopsies were taken from the greater epiploon region. For each type of surgery, samples were obtained by the same specialist. Morbidly obese women and controls were age matched. The weight of all subjects was stable for at least three months before surgery. Those patients who had an acute illness, acute or chronic inflammatory or infective diseases, or an end-stage malignant disease, were excluded from. Liver and renal diseases were specifically excluded by biochemical work-up. Control or morbidly obese patients diagnosed as type 2 diabetes mellitus or receiving hypolipemiant treatment were also excluded from the study.

Anthropometric Measurements

BMI was calculated as weight divided by height squared (kg/m^2) (according to the criteria of the World Health Organization [21]. Waist circumference (WC) was measured at the height of the iliac crest.

Diagnosis of Metabolic Syndrome

Morbidly obese women were further subclassified according to the presence or absence of the metabolic syndrome (MS). The MS and metabolic risks are defined according to the US National Cholesterol Education Program Adult Treatment Panel III guidelines and modified as recommended in the latest American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement [22] by adopting a lower cutoff for fasting glucose (5.6 mmol/L). The MS was defined as having 3 of the following metabolic risk factors: (1) central obesity (waist circumference 88 cm in women), (2) hypertriglyceridemia (fasting triglycerides 1.69 mmol/L (150 mg/dL)), (3) low HDL cholesterol (fasting HDL <1.29 mmol/L (50 mg/dl) in women), (4) glucose intolerance (fasting glucose 5.6 mmol/L (100 mg/dL)), and (5) hypertension (sitting blood pressure 130/85 mm Hg

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obtained as a mean of two readings taken after resting for at least 10 minutes or on regular antihypertensive medications).

Analytical Methods

Basal, fasting blood samples were taken after an overnight fast to determine glucose, insulin, and standard laboratory parameters. Plasma and serum samples were stored at -80°C until analytical measurements were performed, except for glucose, which was determined immediately after blood was drawn.

Fasting plasma glucose and lipid profile (triglycerides, total cholesterol, and high-density lipoprotein cholesterol) were measured using the usual enzymatic methods in an ADVIA Centaur auto analyzer. Low-density lipoprotein cholesterol was calculated as the difference between total cholesterol, HDL, and triglyceride content/5 if it was <400 mg/dL. Plasma insulin concentration was measured by commercial chemiluminescence assay for ADVIA Centaur (Siemens Medical Solutions S. L., Barcelona, Spain) according to the manufacturer's instructions. The homeostasis model assessment of insulin resistance (HOMA2-IR) was completed using the HOMA Calculator version 2.2.2 (http://www.dtu.ox.ac.uk accessed May 2010). Glycosylated hemoglobin (HbA1c) was measured by a chromatographic method (Glico Hb Quick Column Procedure, Helena Laboratories, Beaumont, TX).

Circulating levels of TNF-RI, TNF-RII (Biosource Europe S.A., Nivelles, Belgium), IL 6 (Quantikine, R&D Systems, Minneapolis, USA), adiponectin, HMW adiponectin (Linco Research, Inc., St. Charles, USA), resistin (Biovendor, Modrice, Czech Republic), leptin (Biovendor, Modrice, Czech Republic), RBP4, lipocalin 2 (Biovendor, Modrice, Czech Republic), serum vaspin (Adipogen, Seoul, South Korea) and plasma omentin (Apotech, Axxora, Nottingham, UK) were measured in duplicate using enzyme-linked immunosorbent assays (ELISA) following the manufacturer's instructions. TNF-RI assay sensitivity was 50 pg/mL and the inter-assay and intra-assay coefficients of variation were less than 5.7 and 1.7%, respectively. TNF-RII assay sensitivity was 0.1 ng/mL and inter-assay and intra-assay coefficients of variation were less than 3.2 and 3.3%, respectively. IL 6 assay sensitivity was 0.039 pg/mL and inter-assay and intra-assay coefficients of variation were less than 9.6 and 6.9%, respectively. Adiponectin assay sensitivity was 0.78 ng/mL and inter-assay and intra-assay coefficients of variation were less than 8.4 and 7.4%, respectively. HMW adiponectin assay sensitivity was 0.5 ng/mL and inter-assay and intra-assay coefficients of variation were less than 3.8 and 2.6%, respectively. Resistin assay sensitivity was 33 pg/mL and inter-assay and intra-assay coefficients of variation were less than 6.9 and 3.4%, respectively. Leptin assay sensitivity was 0.2 ng/mL and inter-assay and intra-assay coefficients of variation were less than 7.6 and 4.4%, respectively. RBP4 assay sensitivity was 0.02 g/L and inter-assay and intra-assay coefficients of variation were less than 1.1 and 2.2%, respectively. LCN 2 assay sensitivity was 0.01 ng/mL and inter-assay and intra-assay coefficients of variation were less than 5.6 and 4.4%, respectively. Vaspin assay sensitivity was 12 pg/mL and inter-assay and intra-assay coefficients of variation were <6% and <4%. Omentin assay sensitivity was 0.4 ng/mL and inter-assay and intra-assay coefficients of variation were less than 6 and 2.7%, respectively.

Analysis of Human Vaspin and Omentin Gene Expression

Total RNA was isolated from adipose tissues with the RNeasy mini kit (Qiagen) according to the manufacturer's protocol and digested with DNase I (RNase-Free DNase set, Qiagen). RNA quality was evaluated by measuring the 260/280 nm absorbance ratio (≥1.8) and by electrophoresis. First-strand cDNA was synthesized using an equal amount of total RNA with the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Real-time quantitative PCR was performed in a final volume of 20 μL, which contained 10 ng of reverse-transcribed cDNA, 10 μL of 2X Taq Man Fast Universal PCR Master Mix (Applied Biosystems) and 1 μL Taq Man Assay predesigned by Applied Biosystems® for the detection of vaspin, omentin, and GAPDH, used as a housekeeping gene. All reactions were performed in triplicate and were carried out in 96-well plates by using the 7900HT Fast Real-Time PCR systems (Applied Biosystems).

Statistical Analysis

All the values reported are expressed as mean ± SEM (standard error of the mean) and were analyzed using the statistical package SPSS/PC+ for Windows (v.15.0 Chicago, Illinois, USA). Differences between groups were calculated using either Student's t test or the One-way ANOVA analysis. The strength of association between variables was calculated using Pearson's method for parametric variables and the Spearman Rho correlation test for non-parametric contrasts. Multiple linear regression analysis with backward variable selection was performed to identify independent predictors of HOMA2-IR. The validity of the regression model and its assumptions was assessed with the plot of residuals vs. predicted values. Data were normally distributed. Logistic regression analysis was performed to identify independent predictors of the metabolic syndrome. Vaspin and omentin circulating levels were age and BMI adjusted in some analyses. P values < 0.05 were considered to be statistically significant.

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Results

Population studied

The baseline patient characteristics given in Table 1 show the mean and SEM of the variables of interest. Patients were separated into control subjects (BMI < 25 kg/m^2), and morbid subjects (BMI > 40 kg/m^2). The two groups were well matched for age.

Biochemical analyses indicated that obese women had significantly higher levels of glucose, insulin, HOMA2-IR and HbA1c than the control group. Blood pressure was also increased in the morbidly obese women. The lipidemic profile differed significantly between groups. Obese patients showed higher triglyceride levels and lower HDL cholesterol.

Serum vaspin

Mean ± SD serum vaspin was 0.87 ± 0.96 μg/liter in the morbidly obese women and not significantly different from the values of the control group (1.66 ± 2.09 μg/liter) (Figure 1).

Serum vaspin concentrations did not correlate with metabolic variables (Table 2) and inversely correlated with levels of LCN2, leptin and IL 6 (Table 3).

We investigated the relationship between vaspin circulating levels and the presence of the MS but we found no correlation (Table 4). As expected, the MS correlated with the BMI, WC, HOMA2-IR, fasting glucose, blood pressure, HDL cholesterol and triglycerides.

Subcutaneous and visceral vaspin mRNA expression

Vaspin mRNA expression was significantly higher in morbidly obese women than in controls, in both SAT and VAT (Figure 2). We found no differences between visceral and subcutaneous vaspin expression in the adipose tissues of the obese group (p = 0.192) and the control group (p = 0.934) (Figure 2).

SAT and VAT vaspin mRNA expression did not correlate with obesity, glucose metabolism, insulin resistance parameters, blood pressure, other adipokines studied or inflammatory parameters (data not shown).

Table 1 Baseline characteristics, anthropometric measurements, and metabolic analysis of the population studied

	CONTROL (n = 31)	MORBID OBESE (n = 40)	*p*-value
BMI (Kg/m^2)	23.21 ± 0.46	48.22 ± 1.07	**<0.001**
WC (cm)	80.40 ± 2.16	134.00 ± 3.10	**<0.001**
AGE (years)	43.53 ± 3.08	46.37 ± 1.54	0.414
GLUCOSE (mg/dL)	94.06 ± 2.53	117.70 ± 4.25	**0.001**
INSULIN (mU/L)	8.25 ± 1.32	22.69 ± 3.94	**0.001**
HOMA2-IR	1.11 ± 0.18	2.60 ± 0.24	**<0.001**
HbA1c (%)	4.55 ± 0.07	5.63 ± 0.29	**0.001**
HDL-C (mg/dL)	59.83 ± 2.87	40.97 ± 1.33	**<0.001**
TRIGLYCERIDES (mg/dL)	95.13 ± 9.06	183.52 ± 11.88	**<0.001**
SBP (mm Hg)	120.91 ± 4.22	134.99 ± 4.07	**0.020**
DBP (mm Hg)	70.04 ± 1.96	76.70 ± 2.80	0.056

BMI: body mass index, WC: waist circumference, HOMA2-IR: homeostatic model assessment method insulin resistance, HDL-C: high density lipoprotein cholesterol, SBP: systolic blood pressure, DBP: diastolic blood pressure. Data are the mean ± SEM. * indicates significant differences vs. control group (p < 0.05).

Plasma omentin-1

Mean ± SD plasma omentin was 1.97 ± 2.15 ng/mL in the morbidly obese group and significantly different from that of the control group (5.27 ± 5.33 ng/mL) (Figure 1).

Plasma omentin levels inversely correlated with fasting glucose and HOMA2-IR (Table 2) and did not correlate with the adipokines or cytokines studied (Table 3). When we investigated the relationship between omentin circulating levels and the presence of the MS, we found that omentin correlated negatively with the MS (Table 4). After adjustment for age and BMI, we subclassified the subjects in three tertiles in accordance with their omentin levels: tertile 1 (>4.33 ng/ml); tertile 2 (4.33-2.30 ng/ml); tertile 3 (<2.30 ng/ml). In a logistic regression analysis, the lowest omentin levels (tertile 2 and 3) were associated with the presence of MS while the highest tertile (tertile 1) was not (Table 5).

Visceral and subcutaneous omentin expression

Omentin mRNA expression in SAT was similar in morbidly obese women and controls. However, omentin expression in VAT was significantly lower in morbidly obese women than in controls (Figure 2). When comparing both adipose tissues, we found that omentin expression was higher in VAT than in SAT in both the obese group (p = 0.009) and the control group (p = 0.034) (Figure 2).

SAT and VAT omentin mRNA expression did not correlate with obesity, glucose metabolism or insulin resistance parameters. Neither was there any correlation with lipid metabolism, blood pressure or the other adipokines and cytokines studied (data not shown).

Discussion

It has been reported that abnormalities in the circulating levels of vaspin and omentin and the gene expression of both factors are related to BMI and markers of insulin sensitivity in metabolic syndrome patients, although to date, the findings of various authors are confusing. Our objective in this study was to measure vaspin and omentin circulating levels and mRNA expression in subcutaneous and visceral adipose tissue in morbidly obese women and to compare them to age-matched controls. We also assessed the relationship between these two adipokines and biochemical markers of metabolic syndrome and other adipo/cytokines.

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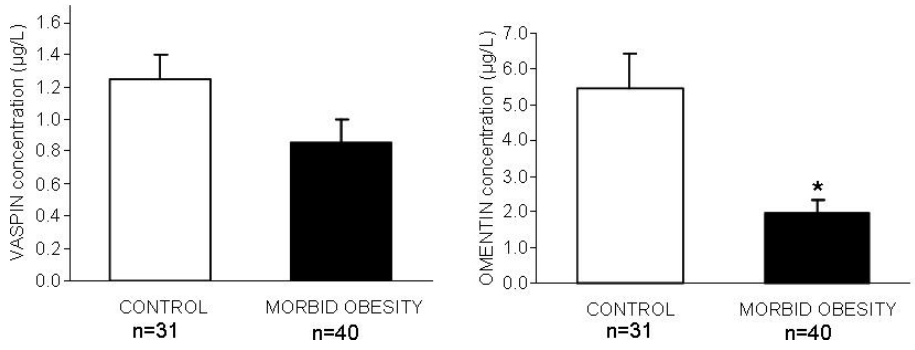

Figure 1 Vaspin and omentin concentrations in control women and morbidly obese women. * Indicates statistically significant differences between groups ($p < 0.05$).

In the current study, we demonstrate that serum vaspin levels are not increased in morbidly obese women and that vaspin levels do not correlate with BMI, markers of glucose or lipid metabolism.

In studies involving humans, and as has been mentioned in the introduction, how vaspin serum levels correlate with BMI, markers of insulin sensitivity and glucose metabolism is unclear. Youn et al. have reported that elevated vaspin serum concentrations correlated with obesity and impaired insulin sensitivity, although not in patients with type 2 diabetes [14]. In obese children, Lee et al. have observed a negative correlation between vaspin concentration and HOMA-IR [23]. However, in agreement with our results, von Loeffelholz et al. have shown that there is no association between serum vaspin and HOMA-IR in nondiabetic humans [24]. Seeger et al. have also reported that circulating vaspin is not independently associated with markers of glucose metabolism [15], and Briana et al. have shown that vaspin concentrations do not correlate with insulin levels in maternal, fetal and neonatal samples [25], as occurs in our population.

To our knowledge, this is the first time that the relation between vaspin levels and other adipo/cytokines in the circulation has been studied.

In our study, serum vaspin levels correlate inversely with levels of LCN2 and IL 6. It has been reported that LCN2 is an adipokine that seems to be an independent risk factor for hyperglycemia and insulin resistance in humans. It has also been related to inflammation [26]. IL 6 is a known proinflammatory cytokine that also increased in obesity [9]. Taken together, these findings might suggest that vaspin has an anti-inflammatory profile.

On the other hand, vaspin mRNA expression is significantly higher in our morbidly obese cohort in SAT and VAT. Kloting et al. have reported that vaspin expression was not detectable in lean subjects but that it was present in both the SAT and VAT of obese patients. Its levels were significantly correlated with parameters of obesity, insulin resistance and impaired glucose

Table 2 Correlation of vaspin and omentin levels with metabolic variables

Circulating levels	VASPIN		OMENTIN	
Variables	**r**	***p*-value**	**r**	***p*-value**
BMI (kg/m^2)	-0.181	0.134	-0.212	0.096
WC (cm)	-0.448	**0.007**	-0.332	0.078
Glucose (mg/dL)	-0.002	0.989	-0.276	**0.029**
Insulin (mU/L)	-0.015	0.903	-0.252	0.054
HOMA2-IR	0.004	0.976	-0.274	**0.037**
HbA1c (%)	-0.181	0.218	0.011	0.943
HDL-C (mg/dL)	0.166	0.183	-0.178	0.182
Triglycerides (mg/dL)	-0.218	0.072	-0.101	0.433
SBP (mm Hg)	-0.002	0.986	-0.179	0.201
DBP (mm Hg)	-0.019	0.887	-0.072	0.608

BMI: body mass index, WC: waist circumference, HOMA2-IR, homeostatic model assessment method insulin resistance; HDL-C: high density lipoprotein cholesterol, SBP: systolic blood pressure, DBP: diastolic blood pressure. Bolded p-values indicate statistically significant correlations ($p < 0.05$).

Table 3 Correlation of vaspin and omentin levels with circulating adipo/cytokine levels

Circulating levels	VASPIN		OMENTIN	
Variables	**r**	***p*-value**	**r**	***p*-value**
HMW adiponectin (µg/L)	0.178	0.173	0.078	0.557
Adiponectin (µg/L)	0.145	0.250	0.103	0.431
Resistin (µg/L)	-0.178	0.157	-0.150	0.236
LCN2 (µg/L)	-0.336	**0.016**	0.088	0.559
IL6 (ng/L)	-0.290	**0.029**	0.033	0.812
RBP4 (mg/dL)	0.015	0.915	0.013	0.931
TNFRI (µg/L)	-0.241	0.057	0.004	0.976
TNFRII (µg/L)	-0.051	0.689	0.036	0.787
Leptin (µg/L)	-0.290	**0.041**	0.195	0.199

HMW adiponectin: high molecular weight adiponectin, LCN2: lipocalin 2, IL6: interleukin 6, RBP4: retinol binding protein 4, TNFRI: tumor necrosis factor receptor I, TNFRII: tumor necrosis factor receptor II. Bolded p-values indicate statistically significant correlations ($p < 0.05$).

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Table 4 Correlations between the number of diagnostic criteria of metabolic syndrome met by the patients

	Number of criteria of Metabolic syndrome	
Variables	**r**	***p*-value**
VASPIN (μg/L)	-0.209	0.083
OMENTIN (μg/L)	-0.264	**0.045**
BMI (kg/m^2)	0.737	**<0.0001**
WC (cm)	0.783	**<0.0001**
HOMA2-IR	0.589	**<0.0001**
Glucose (mg/dL)	0.710	**<0.0001**
SBP (mm Hg)	0.636	**<0.0001**
DBP (mm Hg)	0.250	**0.006**
HDL-C (mg/dL)	-0.750	**<0.0001**
Triglycerides (mg/dL)	0.704	**<0.0001**

Vaspin and omentin circulating levels and different parameters.
BMI: body mass index, WC: waist circumference, HOMA2-IR: homeostatic model assessment method insulin resistance, SBP: systolic blood pressure, DBP: diastolic blood pressure.
Bolded p-values indicate statistically significant Spearman's correlations ($p < 0.05$).

tolerance [27]. However, we have not been able to support their findings.

In untreated OLETF rats, vaspin expression and its serum levels decreased as diabetes worsened and body weight fell. The expression and serum levels were normalized by treatment with insulin or pioglitazone, suggesting that vaspin exerts a defensive action against insulin resistance. On the other hand, the administration of recombinant human vaspin improved insulin sensitivity and glucose tolerance, and reverses the expression of those genes that can promote insulin resistance such as leptin, resistin and TNF-α, in diet-induced obese mice [13].

To sum up, in our study serum vaspin levels are inversely related to IL 6. SAT and VAT vaspin expression is significantly higher in morbidly obese women. In addition, as mentioned above, the literature confirms that vaspin has an insulin-sensitizing effect. In conjunction with our results, then, this suggests that vaspin has a compensatory role in the inflammatory complications of obesity.

The second important finding of our study is that plasma omentin levels are significantly lower in the morbidly obese and that these levels inversely correlate with glucidic metabolism parameters, in accordance with the findings of de Souza et al. [19] and Yang et al. [17]. In the same context, we found a negative correlation with systolic blood pressure.

We also demonstrate that patients with omentin levels in the lowest tertile were 90 times more likely to have the MS than those in the highest tertile, after adjustment for age and BMI. Moreover, women with omentin levels in the second tertile were 25 times more likely to have the MS.

Omentin expression in visceral adipose tissue is significantly lower in the morbidly obese women in our study in agreement with the results of Souza et al [19]. Also, Cai et al. demonstrate that omentin mRNA expression decreases in overweight/obese individuals and decreases further when overweight/obesity is combined with type 2 diabetes. Thus, omentin expression is negatively correlated with fasting insulin, HOMA-IR and BMI [28].

The major limitation of the present study is the relatively small number of subjects in the sample. Although our specific cohort of non-diabetic morbidly obese women showed a clear relationship between the MS and omentin levels without the interference of confounding factors, these results are not extrapolable to other obesity groups or men. Secondly, due to the difficulty of obtaining tissue samples, the expression results

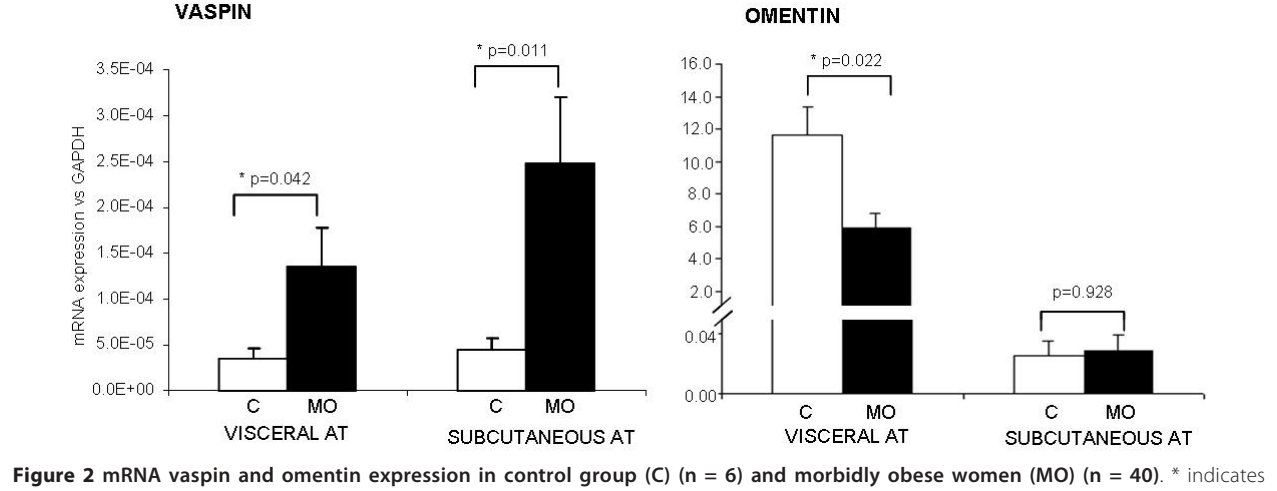

Figure 2 mRNA vaspin and omentin expression in control group (C) (n = 6) and morbidly obese women (MO) (n = 40). * indicates statistically significant differences between control and morbidly obese subjects ($p < 0.05$).

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Table 5 Logistic regression analysis for the presence of the metabolic syndrome according to the omentin tertile circulating levels

	OR	95% CI	p-value
OMENTIN tertile 2 vs 1	25.00	4.41-141.68	**<0.001**
OMENTIN tertile 3 vs 1	90.00	11.46-706.71	**<0.001**

Model 1. Omentin tertiles (ng/ml) adjusted for BMI and age: 1 (>4.33); 2 (4.33-2.30); 3 (<2.30). Tertile 1 as reference with OR = 1.

need to be confirmed in larger study populations. Another limitation of the study is that it is cross-sectional. We could not prove a causal link between the levels of omentin and the development of MS or the levels of vaspin and anti-inflammatory action. Further prospective studies are required to explain these phenomena.

Conclusion

To sum up, our data suggest that vaspin is likely to have anti-inflammatory/protective action in morbid obesity. Also, decreased omentin levels have a close association with MS in women with morbid obesity. Future studies should also include parameters for weight loss and exercise to observe their effect on vaspin and omentin levels.

Abbreviations

BMI: body mass index; DBP: diastolic blood pressure, HOMA2-IR: homeostatic model assessment method insulin resistance; HMW: adiponectin, high molecular weight adiponectin; IL6: interleukin 6; LCN2: lipocalin 2; MS: metabolic syndrome; RBP4: retinol binding protein 4; SAT: subcutaneous adipose tissue; SBP: systolic blood pressure; TNF-α: tumor necrosis factor-alpha, TNF-RI: tumor necrosis factor receptor I; TNF-RII: tumor necrosis factor receptor II; VAT: visceral adipose tissue; WC: waist circumference.

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Authors' contributions

YQ, BM, AC, MB and CA carried out the molecular genetic studies and the immunoassays. TA participated in the design of study, performed the statistical analyses and was involved in drafting the manuscript. XT carried out the molecular genetic studies and performed the statistical analysis. MO performed the statistical analysis. DR, JAP, MH and FS made substantial contributions to the conception and design, acquisition of data, and analysis and interpretation of data. They were also involved in drafting the manuscript. DdC and CR revised the draft and gave final approval for publication. All authors read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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