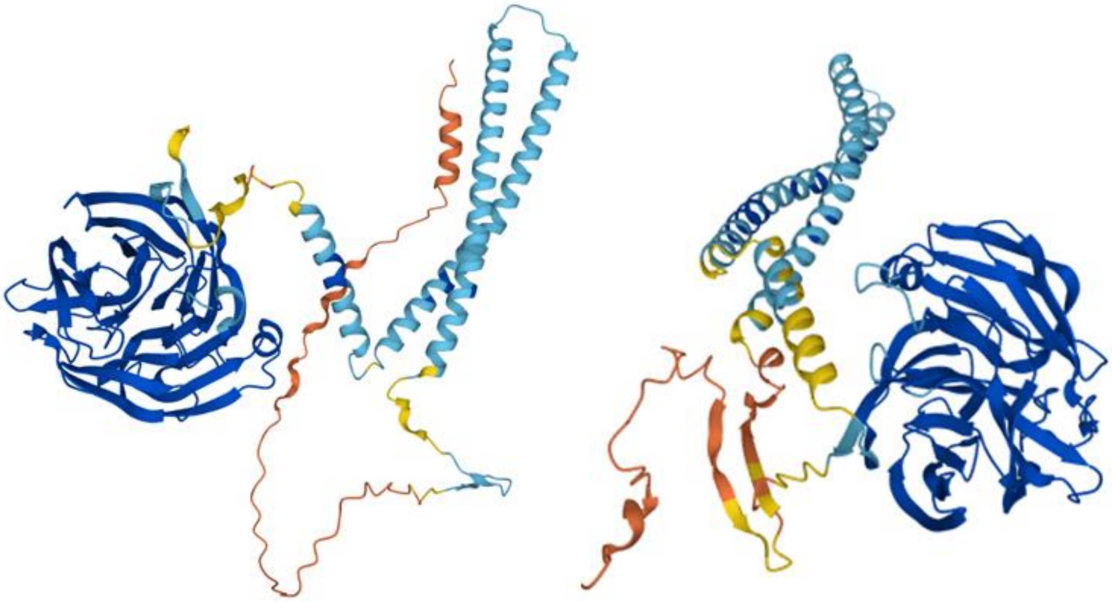
Olfactomedin proteins and their potential involvement in nonalcoholic fatty liver disease

FINAL DEGREE PROJECT



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Biochemistry and Molecular Biology

Universitat Rovira i Virgili, Tarragona 2022

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This work was carried out from the results obtained in the External Practices and have been made in the *Grup d'Estudi en Malalties Metabòliques Associades a la Inuslino-Resistència* (GEMMAIR) under the tutoring of Maria Teresa Auguet Quintillà and Laia Bertran Ramos.

1. ABSTRACT

To provide new insights into the roles of Olfactomedin (OLFM) 2 and OLFM4 related to nonalcoholic fatty liver disease NAFLD pathogenesis, in this study, we used RT-qPCR to analyse the mRNA expression of OLFM2 in liver biopsies and OLFM4 in jejunum samples, in a cohort of women with morbid obesity classified according to their hepatic histology. Our results showed that OLFM2 increased as NAFLD progresses and OLFM4 present positive correlations with interleukins and jejunal TLR9. In conclusion, OLFM2 in liver seems to play a relevant role in NAFLD progression, while OLFM4 in jejunum could be involved in gut dysbiosis related-inflammation events.

Keywords: Olfactomedin; nonalcoholic fatty liver disease; gut-liver axis; inflammation.

2. ABBREVIATIONS

A.U, arbitrary units

ALP, Alkaline phosphatase

ALT, Alanine aminotransferase

AMA, antimitochondrial autoantibody

ANA, antinuclear autoantibody

AP-1, activating protein 1

APRI, AST-to-platelet ratio index

AST, Aspartate aminotransferase

BMI, Body mass index

c-AMP, cyclic adenosine monophosphate

CVD, cardiovascular diseases

DBP, Diastolic blood pressure

DCA, Deoxycholic acid levels

ER, Endoplasmic reticulum

ERK1/2 MAPK, mitogen-activated protein kinases pathway

FAS, fatty acid synthase

FDA, Food and Drugs agency

FIB-4, fibrosis-4 index

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase

GGT, Gamma-glutamyltransferase

GM-CSF, granulocyte-monocyte colony-stimulating factor

GRIM-19, gene associated with retinoic interferon-induced mortality 19

H.pylory, helicobacter pylori

HbA1c, Glycosylated haemoglobin

HCC, Hepatocellular carcinoma

HDL-C, High density lipoprotein cholesterol

HOMA1-IR, homeostatic model assessment method-insulin resistance

HSC, Hepatic stellate cells

IgA, immunoglobulin A

IISPV, Pere Virgili Health Research Institute

IL, Interleukin

IR, Insulin resistance

JNK1, C-Jun N-terminal kinase

LDL-C, Low density lipoprotein cholesterol
LGR5, Leucine-rich repeat-containing G-protein coupled receptor 5
MO, Morbid obesity
MS, metabolic syndrome
NAFLD, Nonalcoholic fatty liver disease
NASH, Nonalcoholic steatohepatitis
NFS, fibrosis score
NF-κβ, nuclear factor Kappa B
NL, Normal liver
NW, Normal weight
OLFM 2, Olfactomedin 2
OLFM 4, Olfactomedin 4
PI3K, phosphoinositol 3-kinases
PPAR, Peroxisome proliferator-activated receptor
ROS, reactive oxygen species
ROS, reactive oxygen species
RUNX1, Runt-related transcription factor 1
SBP, Systolic blood pressure
SFRP5, Secreted frizzled-related protein 5
SMA, anti-smooth muscles autoantibody
SS, Simple steatosis
STAT3, The signal transducer and activator of transcription 3
T2DM, Type 2 diabetes mellitus
T3, triiodothyronine
T4, thyroxin
TLR, Toll-like receptor
TMA, trimethylamine
TMAO, Trimethylamine N-oxide
TNF-α, Tumor necrosis factor α
TSH, thyroid-stimulating hormone
WNT, Wingless-MMTV Integration Site
WNT5A, WNT family member 5a

3. INTRODUCTION

3.1 Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is a general term used for a variety of liver conditions, characterised by the presence of fat inside the hepatocytes in patients who do not consume alcohol in significant quantities (< 20 g/day in men and < 10 g/day in women) and in absence of secondary causes of hepatic fat accumulation, such as the use of steatogenic medication or other causes of concomitant liver disease [1,2].

NAFLD is considered a metabolic disorder whose main risk factors are obesity, dyslipidaemia, type 2 diabetes mellitus (T2DM), metabolic syndrome (MS) and insulin resistance (IR). Other important factors that are associated with NAFLD are smoking, alcohol consumption, high-fat or high-carbohydrates diet, etc (Table 1) [3,4].

Table 1: Primary and secondary factors that induce NAFLD.

NAFLD primary factors	NAFLD Secondary factors
Obesity	Drugs
Type 2 diabetes mellitus	Alteration of the anatomy of the small intestine
Dyslipidaemia	Other metabolic diseases
Childhood obesity	Infections (hepatitis virus…)
Insulin resistance syndrome	Excess of carbohydrates in diet

NAFLD, Nonalcoholic fatty liver disease

3.1.1 NAFLD prevalence

NAFLD is currently the most prevalent chronic liver disease worldwide, and it has become a growing health problem. NAFLD is a widely recognized medical condition that represents an important increasing cost for the National Health System every year [5].

Its incidence is at the present increasing along with that of T2DM and obesity. The estimated global prevalence of NAFLD is 25% in adults, but in overweight people, it grows up to about 58%, or increase up to 98% in non-diabetic morbidly obese (MO) people [2]. Previous studies suggested that NAFLD prevalence in women increases, reaching the highest values between 60 and 69 years old and decreasing after 70 years old. This fact has been attributed to natural changes in female physiology, such as the characteristic adipose distribution, sexual hormones, IR and obesity among others[6]. In addition, in the United States, NAFLD is the most common form of chronic liver disease, affecting about a quarter of the population [3,4,6]. In this sense, the prevalence of the disease varies between different continents (Figure 1A); specifically, the prevalence on main European countries is quite homogeneous (Figure 1B).

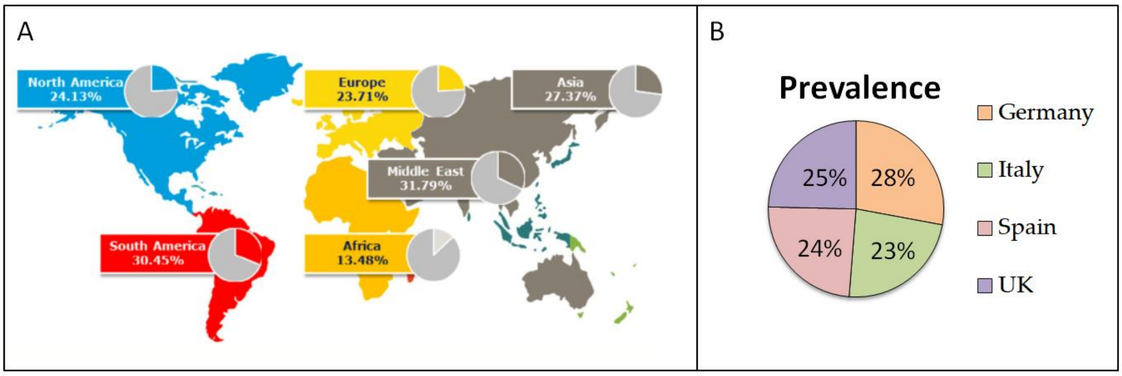

Figure 1: *A) Map of NAFLD distribution around the world. B) Graphical representation of NAFLD prevalence in European countries* [7]

3.1.2 NAFLD pathophysiology

As we have already mentioned, the main characteristic of NAFLD is the excessive accumulation of fat (triglycerides) in more than 5% of hepatocytes, and this fact represents the key point of the steatosis process [1,3]. In this sense, the spectrum of the disease ranges from the presence of hepatic simple steatosis (SS) without inflammation, to the development of nonalcoholic steatohepatitis (NASH), which is characterized by lobular inflammation and hepatocyte ballooning presenting or not fibrosis [8–10]. NASH is the severe form of NAFLD, since it can progress to liver failure, cirrhosis, and even to hepatocellular carcinoma (HCC)[11,12]. The development of liver fibrosis is most strongly associated with morbidity and mortality, also increasing the cardiovascular risk [13]. The progression process of NAFLD was represented in Figure 2 [14].

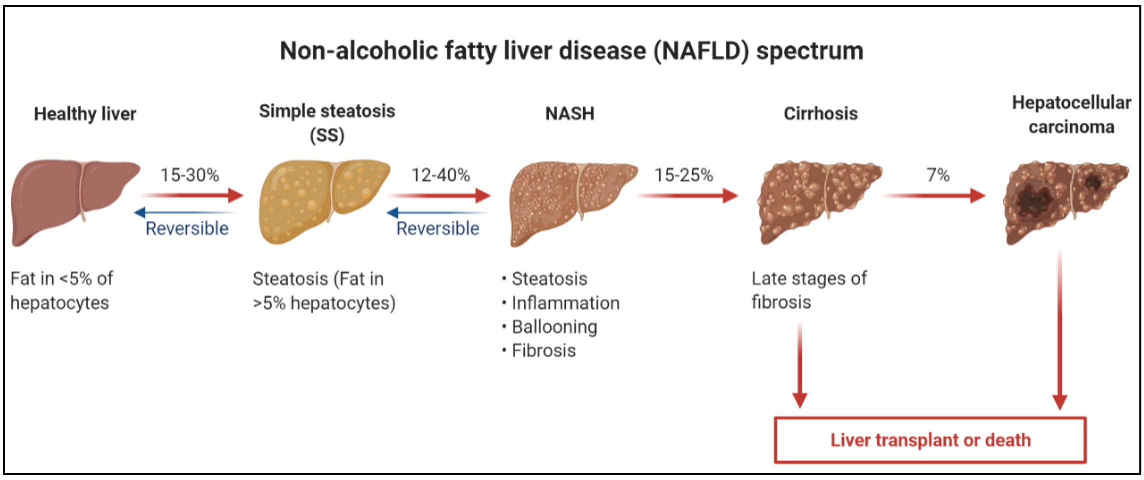

Figure 2*:* Spectrum of NAFLD. Source: Self-made.

The presence of oxidative stress induced by steatosis process, has been identified as the starting point of the progression from SS to NASH [15]. Years ago, the pathophysiology of NAFLD was proposed to be produced by a double-hit process, with an initial state that corresponds to steatosis and later, it gives rise to steatohepatitis, where liver damage is more severe and inflammation appeared [16]. Currently, the multiple-hit hypothesis has been widely recognized (figure 3). It describes that the imbalance in lipid metabolism, sedentary lifestyle, obesity, hepatic lipid accumulation, gut microbiota dysbiosis and IR triggers the appearance and progression of NAFLD. At this point, the liver is more vulnerable to receive multiple damage that can act in parallel, such as bacterial toxins release derived from the gut dysbiosis, oxidative stress, death of hepatocytes, deliver of pro-inflammatory cytokines, etc. Over time and collectively, these factors can trigger fibrosis and inflammation leading to NASH and even HCC [16,17]. In addition, it is known that the development of NASH is related to systemic inflammation and other pathological processes such as endoplasmic reticulum stress, alterations in innate immunity, toll-like receptor (TLR) signalling, intestinal disorders, and mitochondrial dysfunction [11].

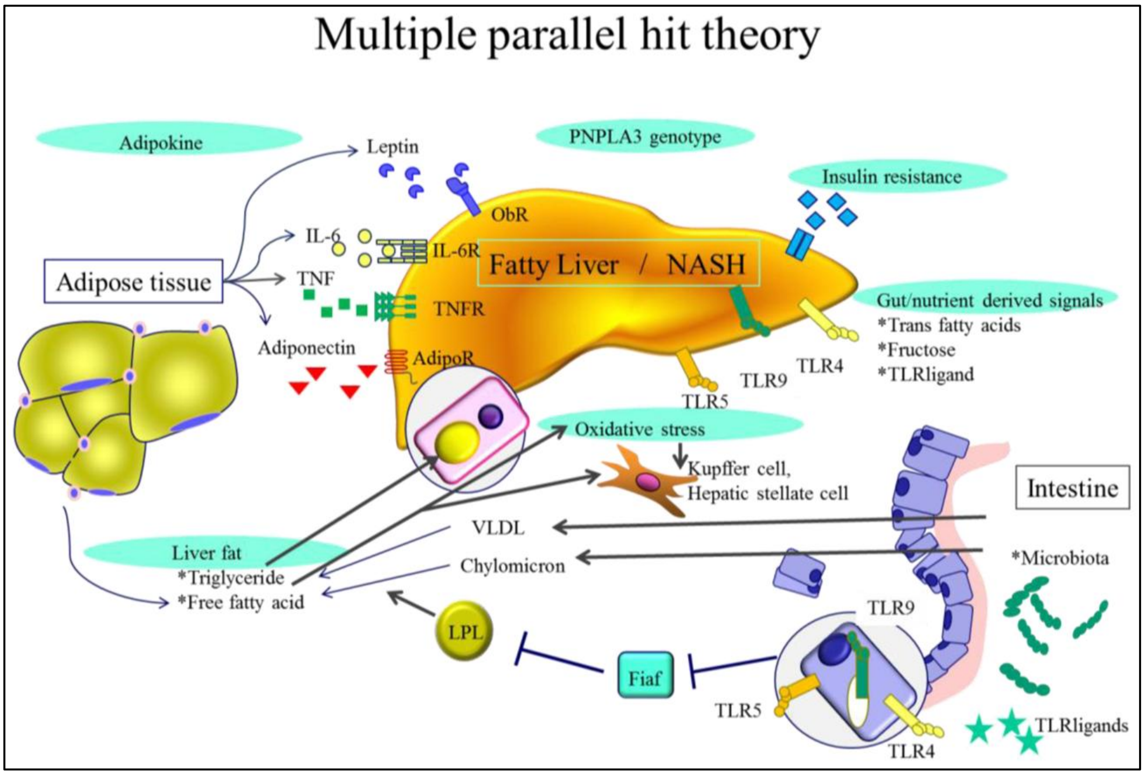

Figure 3: *The multiple-hit hypothesis in NAFLD. IL, interleukin; TNF, tumor necrosis factor; ObR, ob/leptin receptor; TNFR, tumor necrosis factor receptor; AdipoR, adiponectin receptor; VLDL, very low density lipoprotein; LPL, lipoprotein lipase; PNPLA3, patatin-like phospholipase 3; NASH, nonalcoholic steatohepatitis; TLR, Toll-like receptors; Fiaf, fasting-induced adipose factor* [18].

The reason why most NAFLD patients presented a benign disorder, while others have an advanced and severe disease is unknown. However, risk factors such as obesity, diet, age over 45 years old, liver enzyme abnormalities (aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio greater than 1.0), high albumin levels, presence of fibrosis in liver biopsy, etc. could help in predicting which individuals are at risk to develop a severe progression of the disease in the future[11,19].

3.1.3 Diagnosis

The main problem in NAFLD diagnosis is that most patients are asymptomatic, so many diagnoses are made incidentally by detecting abnormalities in routine biochemical tests or liver steatosis on images taken for other reasons [20]. In this sense, the diagnosis of NAFLD should be based on four main aspects:

- Detection of hepatic steatosis (in >5% of hepatocytes)
- Discard other related etiologies
- Rule out alcohol consumption in the patient
- Discard other causes of chronic liver disease such as hepatitis B or C

Moreover, NALFD should be suspected in obese, diabetic or people presenting MS, with abnormal liver functions; although other factors need to be evaluated (Table 2).

Table 2: Main factors considered for NAFLD diagnosis in clinical practice.

Factors to be evaluated during NAFLD diagnosis
Personal and familiar history of T2DM, hypertension and CVD
Hepatitis B/C infection
Alcohol consumption: < 20 g/d (women), < 30 g/d (man)
High levels of hepatic enzymes (AST, ALT)
Waist circumference, BMI, change in body weight
History of steatosis-associated to drug use
Serum total and HDL-c, triacylglycerol, uric acid
Undertaken due to clinical suspicion
Hemochromatosis testing: Ferritin and transferrin saturation
Thyroid disease: TSH level (T3/T4)
Celiac disease: IgA and tissue transglutaminase
Polycystic ovarian syndrome
Autoimmune disease: ANA, AMA, SMA
Wilson’s disease: Ceruloplasmin
Alpha-1 antitrypsin deficiency: Alpha-1-antitrypsin level

Fast blood glucose, hemoglobin A1c
Ultrasound

NAFLD, nonalcoholic fatty liver disease; T2DM, type 2 diabetes mellitus; CVD, cardiovascular diseases; AST, aspartate-aminotransferase; ALT, alanine-aminotransferase; BMI, body mass index; HDL-c, high density lipoprotein-cholesterol; TSH, thyroid-stimulating hormone; T3, triiodothyronine; T4, thyroxin; IgA, immunoglobulin A; ANA, antinuclear autoantibody; AMA, antimitochondrial autoantibody; SMA, anti-smooth muscles autoantibody [5,21].

In this regard, the diagnosis is based on non-invasive techniques such as serological methods, evaluation of transaminases and imaging tests (computerized tomography, ultrasound and resonance), which detect if there is fat in the liver, its quantity or signs of cirrhosis. Liver elastography attempts to determine the presence or absence of fibrosis [22]. However, they cannot identify inflammation, ballooning, acidophilic bodies, and other features present in NASH, so they are not able to diagnose NASH without fibrosis [23]. If there is suspicion of it, invasive techniques are needed, such as percutaneous liver biopsy, that is the most commonly used in order to perform histopathological techniques to determine NASH parameters in the liver sample. Unfortunately, the biopsy is an invasive method, it carries some risks and can cause bleeding and pain. Nevertheless, it is the technique that gives the most accurate and stratified diagnosis since it provides information about inflammation, ballooning, fibrosis and necrosis presence and degree, and for this reason, it is the *gold standard* diagnostic method[2].

Therefore, it is necessary to understand more about the pathophysiology of NAFLD in order to discover new biomarkers that allow an early, non-invasive and accurate diagnosis method[23].

Until now, non-invasive biomarkers of fibrosis in bloodstream have been identified, such as AST-to-platelet ratio index (APRI), fibrosis-4 index (FIB-4) or NAFLD fibrosis score (NFS). They are very useful since the biopsy only reports the histopathological profile of a specific area of the liver (which has been extracted), and these biomarkers give an idea of the global hepatic fibrosis stage [24]. The NFS uses clinical and biochemical values to predict the severity of liver affectation, and it is the most validated non-invasive technique to assess NAFLD. In addition, another useful way of detecting fibrosis is the Fibroscan, which can be performed before the biopsy. It evaluates the rigidity of the tissue and it allows the detection of fibrosis and cirrhosis [25]. Unfortunately, there are no non-invasive biomarkers of NASH stage without fibrosis, and for this reason it is really necessary to characterize the precise point where the disease changes from benign to severe [22] to find new biomarkers in this regard. On the other hand, interleukins and other cytokines, gut microbiota-derived metabolites and other involved molecules can be proposed as potential non-invasive biomarkers to evaluate the progression of NAFLD [26] and this is the reason why there are several studies in this field [27–31], nevertheless, further research is needed.

3.1.4 Treatment

After NAFLD diagnosis, the patient needs to follow some therapeutic strategies in order to reverse and control the disease and prevent its progression to NASH. Currently, the therapy for NAFLD patients is focused on 3 important points [32]:

- Weight loss (changes in lifestyle, drugs for the treatment of obesity and, if necessary, bariatric surgery)
- Pharmacological approaches
- Screening and prevention of hepatic complications such as NASH, cirrhosis or hepatocellular carcinoma.

Although there is not a specific intervention to treat NAFLD, changing the lifestyle such as including a balanced diet and practicing physical exercise is the strategy that gives the best results [5]. This strategy can be supplemented with drugs if the patient worsens, as shown Table 3.

Table 3: Drugs used for the treatment of NAFLD [2]

Representative drug	Main mechanism of action	Main/serious side effects	Evidence of benefit in NAFLD/NASH
Metformin	Improves insulin sensitivity	Gastrointestinal upset	Recommended in patients with T2DM and NAFLD
Pioglitazone	Modulates tissue insulin sensitivity through PPAR signalling	Worsening heart failure	Recommended in patients with NASH and T2DM
Exenatide/liraglutide	Suppresses appetite, helps weight loss and enhances endogenous insulin production	Gastrointestinal upset	Recommended in obese/overweight T2DM and NAFLD
Sitagliptin/linagliptin	Enhances endogenous insulin production	Gastrointestinal upset	Suggested in obese/overweight T2DM with NAFLD
Vitamin E	Reduces oxidative stress	Haemorrhagic stroke	Recommended in patients with NASH and without diabetes
Pentoxyphylline	Raises c-AMP and reduces TNF-α	Upper gastrointestinal upset	Suggested in NASH
Atorvastatin	Lowers plasma lipids	Muscle pains and myopathy	Suggested in patients with dyslipidaemia & NAFLD

NAFLD, Nonalcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis; T2DM, Type 2 diabetes mellitus; PPAR, Peroxisome proliferator-activated receptor; c-AMP, Cyclic adenosine monophosphate; TNF-α, Tumor necrosis factor α.

If the body mass index (BMI) is greater than 35 kg/m^2 (severe obesity), metabolic bariatric surgery has suggested to be considered, because the beneficial results have been reported after this intervention in NAFLD patients with MO [32]. However, there is currently no specific and completely effective treatment approved by the Food and Drugs agency (FDA) efficient enough to treat NAFLD, and for this reason, research into new therapies is booming, and there are ongoing clinical trials [33,34].

In addition, given the huge influence of the gut-liver axis in NAFLD progression, the modulation of gut microbiota with prebiotics, probiotics and antibiotics has been suggested to be a good strategy to prevent or treat the disease in a complementary manner [35]. Despite all these facts, if liver damage is advanced (cirrhosis, or appearance of carcinoma), a liver transplant is proposed [4,11].

3.1.5 Intestinal dysbiosis in NAFLD

NAFLD is a metabolic and multisystemic disease very related to the intestinal dysbiosis [36]. Intestinal dysbiosis is a situation where there is an imbalance in the gut microbiota between beneficial microorganisms and pathogens. When the outcome of this event is an excess of pathogenic flora, this could have harmful effects in human health [37,38].

Intestinal dysbiosis triggers an increase in the permeability of the intestinal barrier. This fact makes microbiota-derived mediators such as lipopolysaccharides, short-chain fatty acids, choline metabolites, bile acids and ethanol endogenous to be released into the bloodstream. Since there is a cross-talk between the liver and the intestine (gut-liver axis) through the portal vein circulation (approximately the 75% of the blood that reaches the liver comes from the portal bloodstream), when the permeability of the intestinal barrier is corrupted, the liver is exposed to derived-components of the gut microbiota. These are recognized by TLRs that promote the release of cytokines and chemokines, which help to induce inflammation and liver injury, triggering NAFLD progression (Figure 4)[35,39].

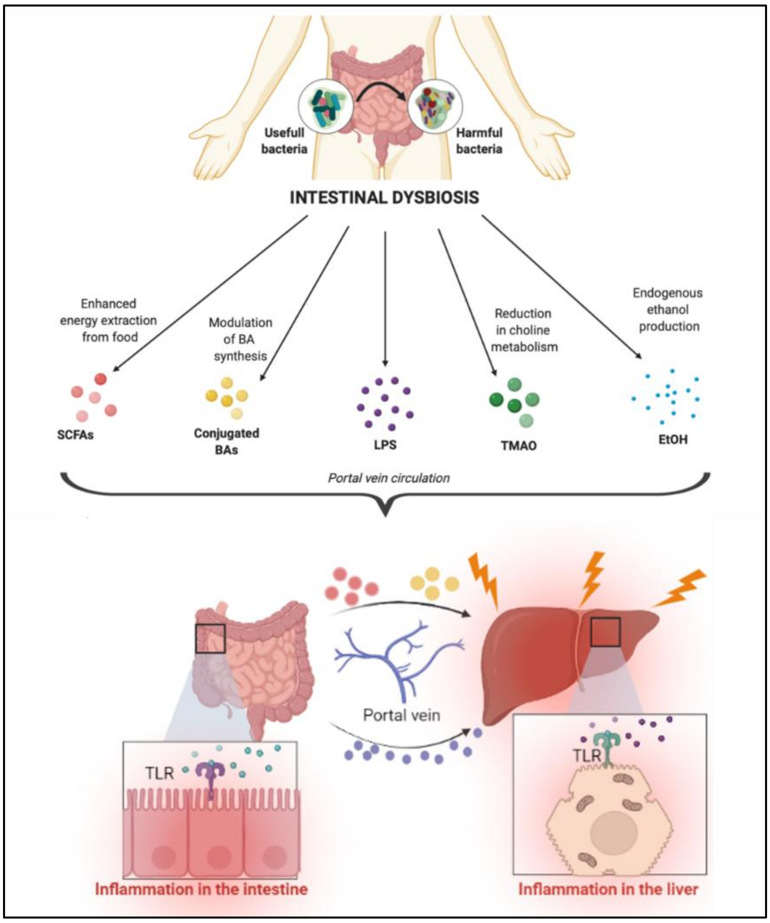

Figure 4: *Graphical summary of the intestinal dysbiosis impact into liver health. SCFAs, Short Chain Fatty Acids; BA, bile acids; LPS, lipopolysaccharide; TMAO, trimethylamine oxide; EtOH, ethanol; TLR, Toll-like receptors. Adapted by*[11].

In addition to increased intestinal permeability, gut dysbiosis can also induce endogenous alcohol production, intestinal endotoxemia, upregulation of *de novo* hepatic lipogenesis, reduced choline metabolism, triacylglycerol synthesis and IR aggravation [11].

In summary and taking all the above-mentioned information into account, the mechanisms of NAFLD pathogenesis are complex and still not completely clear, so it requires further research. For example, the discovery of new proteins involved in NAFLD pathogenesis mechanisms of action could help to define in more detail the disease and, what is more, perhaps to find new therapeutic targets.

3.2 Olfactomedin proteins

Olfactomedins (OLFMs) are a family of glycoproteins that were discovered in the 1991s [40]. They were found to be secreted in the nasal lumen of the lower mucosal layer, and for this reason they received the name of "olfactomedins". At first, it was suggested that OLFMs could play a role in the differentiation signalling in chemosensory neurons, although the hypothesis has not been proved [40,41].

OLFM homologues were widely distributed throughout the brain and the adrenal medulla. Proteins sharing a 250 amino acid domain homologous to olfactomedin were found to be generally expressed in different species, from nematodes to humans and thought to mediate protein-protein interactions in embryonic development. However, they have only been found in multicellular organisms, indicating that they must have a role in cell-cell signalling and cell interactions [42].

In addition to participate in haematopoiesis and early development of the nervous system, OLFMs have been associated with diseases such as gastrointestinal cancer and glaucoma, since the interruption of their expression leads to developmental disturbances and lethality [43].

Thanks to phylogenetic studies made by Zeng and collaborators [44], OLFMs are classified into seven subfamilies (I-VII) showed in Figure 5. All of them are secreted glycoproteins, with the exception of the group II and VI that are transmembrane proteins [43].

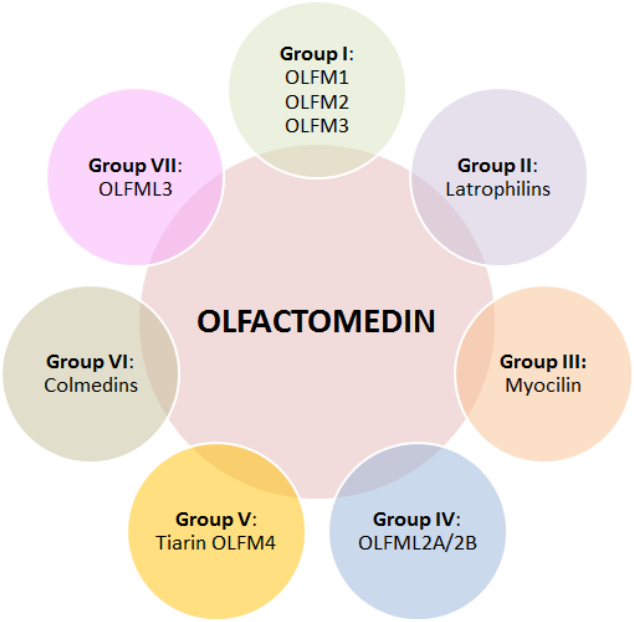

Figure 5: *Graphical representation of the OLFM subfamilies. OLFM, olfactomedin* [42].

Regarding transmembrane OLFMs, in group VI we find the gliomedins, which contain the OLFM domain in the extracellular region. This domain can suffer homo-oligomerization, be integrated into the matrix and perform its function, which is to intervene in the formation of Ranvier's nodes. On the other hand, group II includes latrophilins, which are independent receptors for calcium coupled to G protein with an extracellular olfactomedin domain at the N-terminal. They have an effect similar than gliomedins, in addition to intervening in the cell adhesion.

Secreted OLFMs seems to have an associated role with early differentiation of the nervous system. Also, membrane-associated extracellular OLFMs are involved in the maintenance of neuronal stability. Moreover, proteins with OLFM domains have been suggested to facilitate protein-protein interactions, intercellular interactions, and cell adhesion [43]. Despite these evidences, it is already necessary to understand more about its expression and the molecular pathways they trigger.

3.2.1 Olfactomedin 2

The OLFM2 (also known as OlfC or olfactomedin-related endoplasmic reticulum-localized-2, noelin-2) protein is encoded by the *olfm2* gene, located on chromosome 19p13.2. Its coding sequence is made up of 6 exons that transcribe the OLFM2 mRNA from three different promoters. This molecule is a secreted glycoprotein that is conserved in different species (human-mouse 96%) and can form dimers and heterodimers [42]. OLFM2 is part of the family I together with OLFM1 and 3, with which it has similarities. This glycoprotein is mainly expressed in neurons and has a high expression in adipose tissue and skin [45].

OLFM2 has demonstrated to be related with retina and eye development [46], given that severe mutations in closely related olfactomedin domain-containing *olfm2*, induce ocular malignancies such as glaucoma [47–49]. Moreover, it seems that circulating levels of OLFM2 in plasma can be used as potential biomarker of arteriosclerotic disorders such as restenosis [50]. In addition, OLFM2 has been suggested as a predicting biomarker of survival in patients with HCC presenting vascular invasion [51].

On the other hand, González-García *et al.* study examined the role that OLFM2 could have in energy balance in OLFM2-KO mice. This animal model (some under a standard diet and others on a high-fat diet) presented lower adiposity, higher energy expenditure and a lower respiratory quotient, indicating that energy is derived from fat metabolism. In addition, the global absence of OLFM2 in these animals produced enhanced brown adipose tissue thermogenesis (browning) and protection against IR. On the other hand, an overexpression of this gene produced the opposite outcome such as weight gain and decreased browning [45]. To conclude, these authors suggested that central OLFM2 had a key role in the regulation of energy metabolism, leading to a catabolic phenotype and a possible resistance to obesity regardless of diet (figure 6) [45].

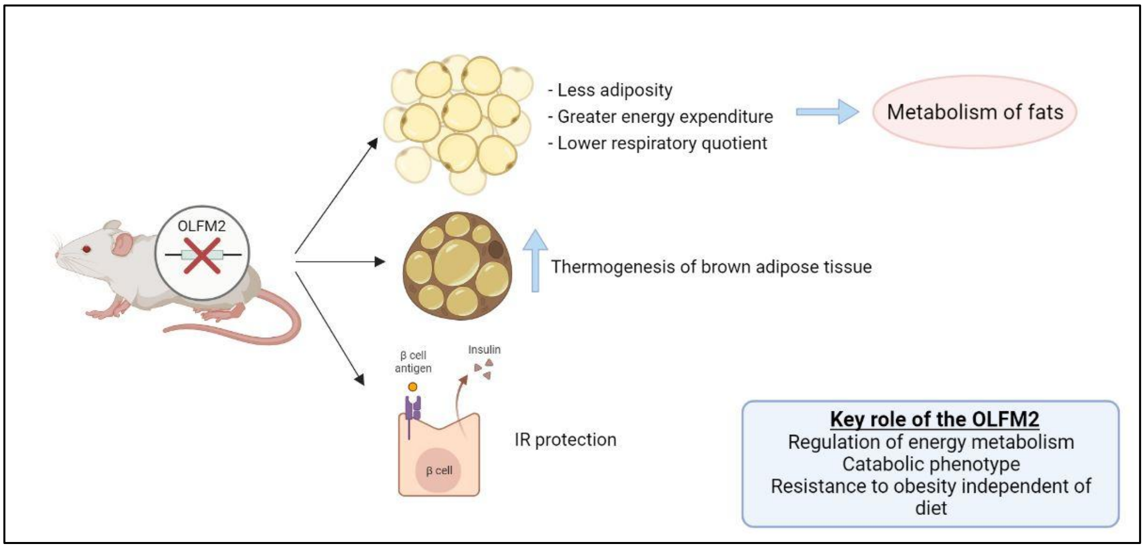

Figure 6: *Role of OLFM2 regarding energy balance in animal models. Self-made.*

3.2.2 Olfactomedin 4

OLFM4, also known as GW112 or hGC-1, is a glycoprotein with an OLFM domain in the C-terminal region, that belongs to the group V. The gene that encodes is on chromosome 13q14.3, and it is expressed in the cytoplasm, nucleus, membrane, mitochondria and neutrophils. OLFM4 is found in the colon, prostate and small intestine, and also is present in other organs but to a lesser extent. It seems that an aberrant expression of this protein is associated with pathological events (inflammatory bowel disease, gastric mucosa infected with *Helicobacter pylori* (H. pylori) and other gastrointestinal malignancies) [52].

OLFM4 expression is related to many proteins including, cadherins, NOd1, cathepsins, the gene-associated with retinoic interferon-induced mortality 19 (GRIM-19), etc. Therefore, this glycoprotein regulates important cellular functions (anti-inflammatory, apoptosis, cell adhesion, proliferation, etc.), although further studies are still required to complete its knowledge [53].

In this sense, a lot of studies have been performed in relation to OLFM4 in different tissues and organs. For example, this protein acts as a target gene for important signalling pathways involved in the gut and gastrointestinal cancers [54,55]. Liu *et al.* reported that OLFM4 expression was increased in neutrophils and macrophages during H. pylory infection, having a regulatory role in the host's immune response. In addition, it was shown that OLFM4 is a target gene of the NF-κB pathway, and that it could exert a negative feedback on the activation of this pathway, leading an anti-inflammatory role by downregulating the host's immune system against bacterial infection. The mice used in the study, being deficient in OLFM4-KO, showed a reduction in infection rate, and the immune response to the bacteria was improved (Figure 7A)[56].

At the same time, the anti-inflammatory role of OLFM4 can also be observed in Crohn's disease and ulcerative colitis, where its mRNA levels are enhanced in the intestinal epithelium, which is the one that suffers from inflammation in this pathology. Therefore, OLFM4 has an important role in the defending the stomach and colon (Figure 7B).

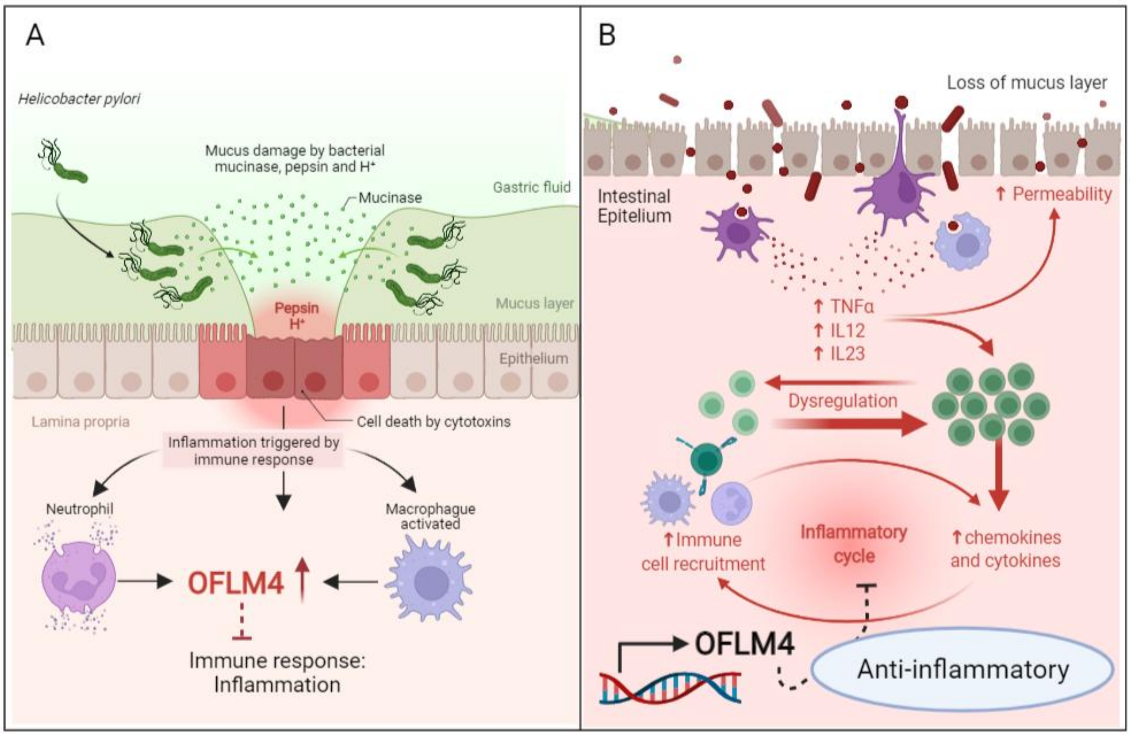

Figure 7: *Role of OLFM4 in defending the gastric mucosa infected by H. pylori (A) and the colon (B). H^+, hydrogenion; OLFM 4, olfactomedin 4; TNFα, Tumor necrosis factor α; IL 12, Interleukin 12; IL 23, Interleukin 23.*

In addition to these findings, a study in human myeloblasts also reported that OLFM4 is regulated by the transcription factor Nf-κB, which control several mechanisms implicated in tumour cells such inflammation, proliferation, angiogenesis, metastasis, etc [57]. These authors demonstrated that sargramostim, a synthetic version of GM-CSF which stimulates the production of white blood, induced OLFM4 expression. This induction was made through PI3K-dependent ROS generation, which activates ERK1/2 MAPK, a pathway that upregulated OLFM4 expression mediated by Nf-κB and transcription factor AP-1. This transcription factors binds to the 5'upstream promoter region of the *olfm4* gene (Figure 8). These facts suggest that OLFM4 is involved in the regulation of cell apoptosis and proliferation of cancer cells [53,56].

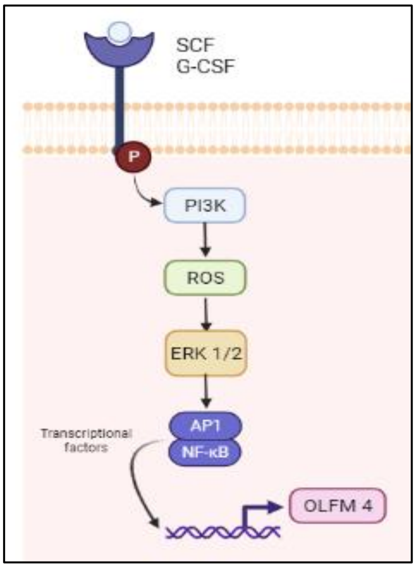

Figure 8: *Representation that OLFM4 expression regulated by the transcription factor NF-κB, was induced by sargramostim. SCF, stem cell factor; G-CSF, sargramostim granulocyte-macrophage colony-stimulating factor; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; ERK 1/2, mitogen-activated protein kinases; AP1, activator protein 1; Nf-κB, nuclear factor kappa light chain enhancer of activated B cells; OLFM4, olfactomedin 4.*

Apart from being related to the Nf-κB pathway, the *olfm4* gene is a target of the Notch signalling pathway. Notch gene has been related to intestinal cell proliferation and differentiation processes [58], and it has been seen to be involved in hepatic repair and liver fibrosis [59] and also in HCC [60]. In the same line, it has been seen that an important pro-inflammatory cytokine that promotes inflammatory bowel disease is tumour necrosis factor alpha (TNF-α), which together with components of the Notch pathway act synergistically to positively regulate the expression of OLMF4, proposing a role in the regulation of intestinal cell differentiation and proliferation [52].

On the other hand, Ashizawa *et al*. carried out a study in patients with HCC and observed that OLFM4 was related to this cancer. It has been shown that the expression of this glycoprotein was increased in several types of neoplasms, promoting tumour progression [61]. In addition, the LGR5 receptor that is expressed in HCC, induces the expression of OLFM4 through the Wnt signalling pathway, which inhibits GRIM19, a tumour suppressor gene, producing the activation of the oncogenic transcriptional factor "signal transducer and activator of transcription 3" (STAT3). In this sense, GRIM19 induces tumour cell destruction, while STAT3 increases tumour invasion, and inhibits apoptosis (Figure 9) [61]. Therefore, the inhibition of OLFM4-STAT3 signalling together with the activation of GRIM19 could be a therapeutic target to prevent the progression of HCC.

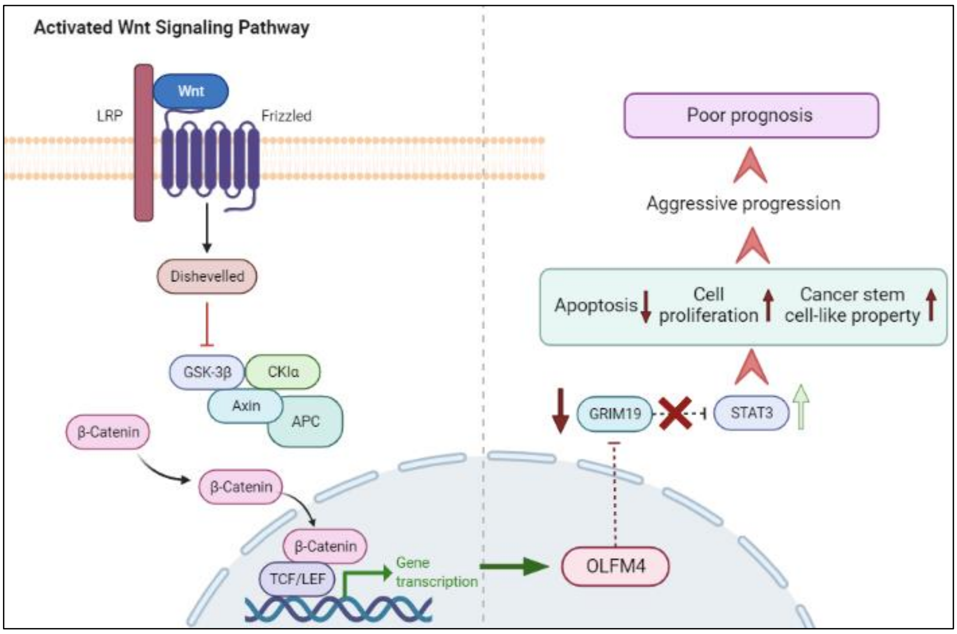

Figure 9: *Relationship of OLFM4 with hepatocellular carcinoma through WNT pathway. LRP, Lipoprotein Receptor-related Protein; Wnt, Wingless-MMTV Integration Site; GSK-3β, Glycogen synthase kinase 3 beta; CKI α, Cyclin-dependent kinase inhibitors alpha; APC, adenomatous polyposis coli; TCF/LEF, T-cell growth factor/lymphoid enhancing factor; OLFM4, olfactomedin 4; GRIM19, gene associated with IFN-retinoid-induced mortality 19; STAT3, signal transducer and activator of transcription 3.*

In summary, numerous signalling pathways such as Wnt [3,62], nuclear factor kappa B (NF-κB) [53] and Notch [58,63], regulate the expression of OLMF4 and are strongly related to pathological processes in diseases such as NAFLD [64–66]. Moreover, OLFM4 is involved in important cellular processes such as cell adhesion, apoptosis, proliferation, inflammation, etc. in many tissues [52,67,68], so it could be a potential biomarker for different pathologies, especially related to intestine and liver.

3.2.3 Expression of OLFM2 and OLFM4 in human tissues

The classification of human proteins based on their expression in different organs and tissues is essential to better understand diseases and the human biological system. In this regard, Fagerberg *et al.* analysed the gene expression of different proteins, including OLFM2 and OLFM4, using quantitative transcriptomics (RNA-Seq) and the data obtained were represented in a recent version of the Human Protein Atlas (www.proteinatlas.org) [69]. In this way, protein expression patterns linked with the results of numerous individual antibody-based immunostains were seen [70]. Thanks to these experiments, it was shown that the expression of OLFM2 mRNA mainly occurred in neurons, but in addition, this expression was seen in liver and adipose tissue (Figure 10A). Figure 10B shows a section of liver tissue of a women stained with eosin and haematoxylin and with an immunostaining against OLFM2, that can be seen as brown spots within the hepatocytes [71].

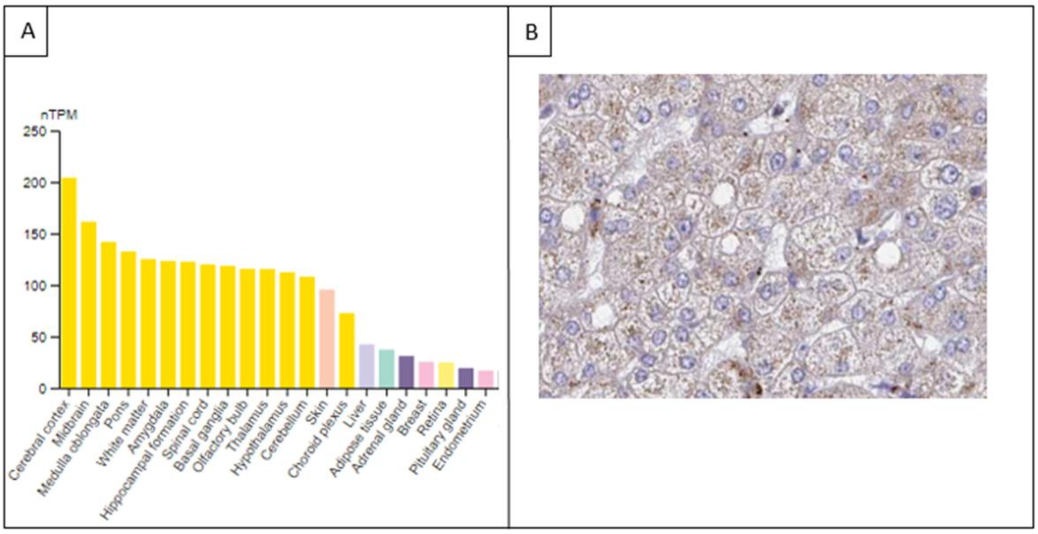

Figure 10: *(A) Distribution of the OLFM2 mRNA expression in different tissues (B) Immunohistochemistry analysis in a section of liver tissue stained with eosin and haematoxylin and with an immunostaining against OLFM2 protein (brown spots)* [71].

On the other hand, OLFM4 mRNA expression is mainly expressed in the small intestine and duodenum (Figure 11A). OLFM4 was mainly expressed in the microvilli of the small intestine (Figure 11B), generally in the cytoplasm and cell-membrane [72].

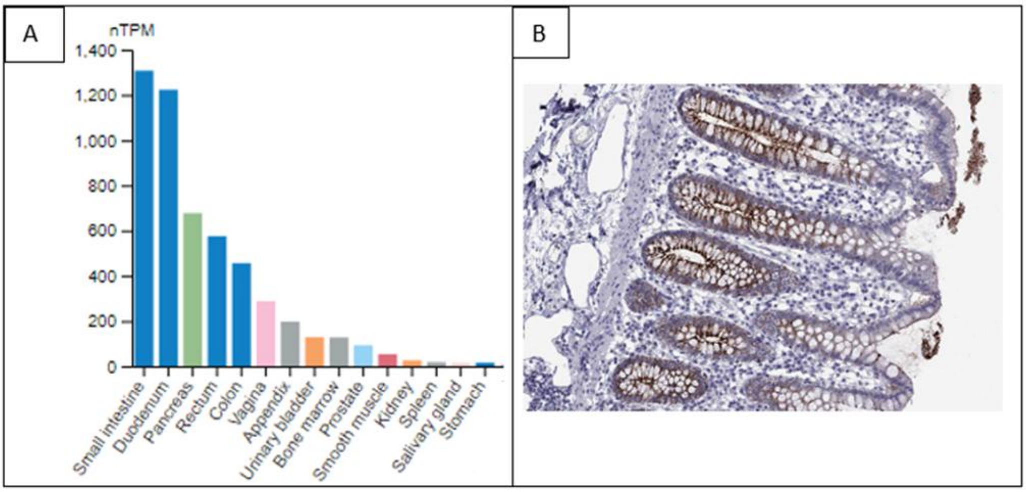

Figure 11: *(A) Distribution of the OLFM4 mRNA expression in different tissues (B) Immunohistochemistry analysis in a section of liver tissue stained with eosin and haematoxylin and with an immunostaining against OLFM4 protein (brown spots)* [72].

The expression values of OLFM2 and OLFM 4 reported in the different tissues and the category of tissue specificity can be seen in Table 4[69,73].

Table 4: Expression values of OLFM2 and OLFM 4 in some human tissues.

Ensembl gene ID	Liver	Small Intestine	Duodenum	Colon	Category
ENSG00000105088 (OLFM2)	35,60	2,30	2,45	2,95	Mixed high
ENSG00000102837 (OLFM 4)	0,24	972,61	921,30	368,59	Mixed high

OLFM2, olfactomedin 2; OLFM4, olfactomedin 4

In this sense, since the high expression of OLFM2 in liver and the huge expression of OLFM4 in intestine, and given the previous evidences of these proteins in several metabolic and inflammatory diseases, we wanted to study the potential function of OLFM2 and OLFM4 in NAFLD pathogenesis.

4. HYPOTHESIS AND OBJECTIVES

NAFLD is a multisystemic metabolic condition related to obesity, since it is one of the main comorbidities. Moreover, it has been also related to intestinal dysbiosis due to the huge influence of the gut-liver axis cross-talk into NAFLD progression, which triggers the delivery of toxins into the liver due to the disruption of the intestinal barrier. OLFM proteins have been mostly involved in embryonic development and cancer processes, but they still have unknown functions; for example, OLFM2 is a glycoprotein related to obesity, insulin resistance and energy metabolism regulation, and OLFM4 is linked to immunity and gastric and colorectal inflammation. The Human Protein Atlas has reported OLFM2 expression in liver and OLFM4 expression in small intestine samples.

Hypothesis:

In this scenario, we hypothesize that OLFM2 in liver and OLFM4 in gut play relevant roles NAFLD pathogenesis.

Objectives:

1. To provide new insights about OLFM2 and OLFM4 roles, in gut-liver axis communication-related to NAFLD.
2. To evaluate the role of OLFM2 in liver biopsies of MO-NAFLD patients to analyse its involvement in NAFLD pathogenesis.
3. To evaluate the role of OLFM4 in jejunum samples of MO-NAFLD patients to study its implication in NAFLD mediated by intestinal dysbiosis.

5. MATERIAL AND METHODS

5.1 Subjects

The institutional review board (Institut Investigació Sanitària Pere Virgili (IISPV) CEIm; 23c/2015; 11 May 2015) approved this research. Informed written consent was obtained from all participants. The cohort was formed by 69 Caucasian women with MO (BMI > 40 kg/m2). Liver and jejunum biopsies were collected during a planned laparoscopic bariatric surgery and liver samples were indicated for clinical diagnosis. The exclusion criteria were as follows: (1) an intake of ethanol higher than 10 g/day or other toxins; (2) patients who had infectious disease or neoplastic disease or an acute or chronic hepatic disease; (3) menopausal women or women using contraceptives; (4) women with diabetes receiving insulin or another medication that can modulate endogenous insulin levels and (5) patients treated with fibrates.

5.2 Sample size

The work is mainly focus on define the specific role of OLFM2 in hepatic tissue and OLFM4 in jejunum samples in MO patients with or without NAFLD. To achieve our objective, sample size has been calculated using GRANMO calculator (https://www.imim.es/ofertadeserveis/software-public/granmo/) accepting an alpha risk of 0.05 and a beta risk of less than 0.2 in a bilateral contrast, 25 subjects in the first group (NL: control group) and 50 in the second (NAFLD: SS and NASH) are needed to detect as statistically significant the difference between two proportions, which for group 1 is expected to be of 0.33 and group 2 of 0.67. The ARCSINUS approach has been used.

5.3 Liver pathology

Liver samples were classified with the method described elsewhere [17,74], using haematoxylin and eosin and Masson’s trichrome stains, and then, they were scored by an experienced hepatopathologist. According to their hepatic histopathology, women with MO were classified into normal liver (NL) (n = 27) and NAFLD (n = 42). Patients with NAFLD were subclassified SS (micro/macrovesicular steatosis without inflammation or fibrosis, n = 26), and NASH (Brunt grades 1–2, n = 16). None of the patients with NASH in our cohort presented fibrosis. In order to give visual information, Figure 12 shows the histological features, grading, and staging of NL and NAFLD (SS and NASH) with our own images.

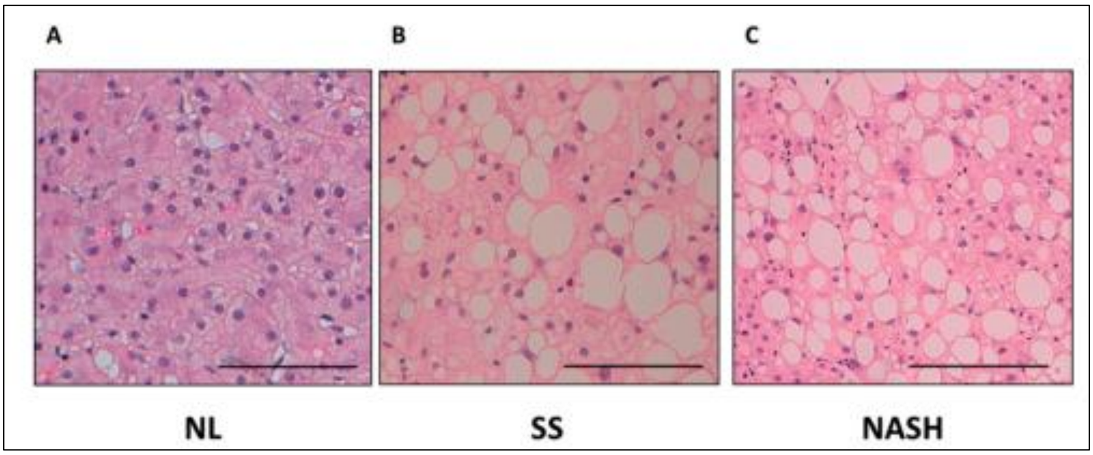

Figure 12: *Image showing histological features of the liver, stained with eosin-haematoxylin, from a woman with MO, (A) and NL, women with SS (B), and women with NASH (C). X400, scale bar:100µm* [75].

5.4 Biochemical Analyses

Physical, anthropometric, and biochemical evaluation were performed from all the studied cohort. Blood samples were extracted through a BD Vacutainer® system by specialized nurses, after overnight fasting and before bariatric surgery. Venous blood samples were obtained in tubes with or without ethylenediaminetetraacetic acid, which were separated in plasma and serum aliquots by centrifugation (3500 rpm, 4 ◦C, 15 min). Conventional automated analyser was used to analyse biochemical parameters. IR was estimated using homeostatic model assessment for IR (HOMA1-IR). Cytokines, such as interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-17, TNF-α and adiponectin were determined using multiplex sandwich immunoassays and the MILLIPLEX MAP Human Adipokine Magnetic Bead Panel 1 (HADK1MAG-61K, Millipore, Billerica, MA, USA), the MILLIPLEX MAP Human High-Sensitivity T Cell Panel (HSTCMAG28SK, Millipore, Billerica, MA, USA), and the Bio-Plex 200 instrument, according to the manufacturer's instructions. Absolute quantification of circulating bile acids, choline, trimethylamine (TMA), trimethylamine N-oxide (TMAO), betaine and short-chain fatty acids were analysed by liquid chromatography coupled to triple-quadrupole-mass spectrometry (LC-QqQ). All these analyses were assessed at the Center for Omic Sciences (Rovira i Virgili University-Eurecat).

5.5 Gene expression in liver and jejunum

Hepatic and jejunal samples were collected during bariatric surgery and conserved in tubes with RNAlater (Qiagen, Hilden, Germany) at 4 ◦C. Then samples were processed and stored at −80 ◦C. RNeasy mini kit (Qiagen, Barcelona, Spain) was used to extract total RNA from liver and jejunum. Reverse transcription to cDNA was performed with the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Madrid, Spain). Real-time quantitative PCR was carried out with the TaqMan Assay predesigned by Applied Biosystems for the detection of OLMF2 (Hs01017934_m1) in liver and OLFM4 (Hs00197437_m1) in jejunum, also we evaluated some hepatic lipid metabolism-related genes such as sterol-regulatory-element-binding protein 1c (SREBP1c) (Hs01088691_m1), liver X receptor alpha (LXRα) (Hs00173195_m1), fatty acid synthase (FAS) (Hs00188012_m1); other related hepatic genes such as runt-related transcription factor 1 (RUNX1) (Hs01021970_m1) and c-Jun N-terminal kinase 1 (JNK1) (Hs01548508_m1); and TLRs in jejunum (TLR2 (Hs02621280_s1), TLR4 (Hs00152939_m1), TLR5 (Hs05021301_s1), TLR9 (Hs00370913_s1)). The expression of each gene was calculated standardized to the expression of 18S RNA (Fn04646250_s1) for hepatic genes, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs02786624_g1) for jejunal genes, after they was normalized using the control group (NL) as a reference. All reactions were duplicated in 96-well plates using the QuantStudio™ 7 Pro Real-Time PCR System (Applied Biosystem, Foster City, CA, USA).

5.6 Statistical analysis

The data was analysed using the SPSS/PC+ for Windows statistical package (version 27.0; SPSS, Chicago, IL, USA). The distribution of variables was obtained using the Kolmogorov–Smirnov test. All results were expressed as the median and the interquartile range (25^{th} - 75^{th}). The different comparative analyses were assessed using Mann–Whitney U test to compare groups. The strength of the association between variables was calculated using Spearman's method. P-values < 0.05 were statistically significant. Graphics were elaborated using GraphPad Prism software (version 7.0; GraphPad, San Diego, CA, USA).

6. RESULTS

6.1 Baseline characteristics of patients

The clinical and biochemical measurements of the cohort formed by women with MO (BMI > 40 kg/m^2) classified according to the hepatic histology as NL (n=27), SS (n=26) and NASH (n=16), are shown in Table 5. The participants did not present significant differences between groups in terms of weight, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), HOMA1-IR, insulin, glycosylated haemoglobin (HbA1c), cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), AST, ALT and gamma-glutamyltransferase (GGT). However, in this analysis, we found higher levels of glucose and alkaline phosphatase (ALP) in SS group than in NL, elevated levels of triglycerides in NASH cohort compared to NL women and increased levels of ALP in SS subjects in comparison to NASH patients.

Table 5. Anthropometric and biochemical variables of women in the studied cohort.

Variables	**NL (*n*=27)**	**SS (*n*=26)**	**NASH (*n*=16)**
Weight (kg)	117.00(107.00-131.00)	114.00(108.98-128.60)	110.50(104.33-120.75)
BMI (kg/m^2)	43.50(40.89-46.88)	44.35(40.87-46.80)	44.19(40.69-45.80)
SBP (mmHg)	120.00(100.00-132.50)	117.50(108.50-127.00)	115.00(102.00-127.00)
DBP (mmHg)	63.00(57.50-73.00)	62.00(59.50-73.75)	64.00(55.00-70.00)
HOMA1-IR	2.05(1.03-3.45)	2.52(1.38-3.68)	1.63(1.26-4.23)
Glucose (mg/dL)	85.00(76.00-93.00)	93.00(87.25-107.00)*	91.50(82.25-101.75)
Insulin (mUI/L)	9.57(5.55-16.82)	10.17(7.23-13.93)	7.19(5.14-26.02)
HbA1c (%)	5.50(5.30-5.70)	5.55(5.30-5.95)	5.55(5.15-6.13)
TG (mg/dL)	106.50(94.00-136.00)	117.50(82.25-172.50)	153.00(116.50-256.50)*
Cholesterol (mg/dL)	170.00(148.25-209.50)	171.15(136.25-194.25)	183.90(152.75-229.50)
HDL-C (mg/dL)	40.60(32.05-48.50)	43.50(33.75-47.00)	37.80(33.50-48.50)
LDL-C (mg/dL)	107.90(86.00-134.20)	104.10(77.20-126.25)	94(79.30-128.03)

AST (UI/L)	20.00(15.50-36.50)	23.00(17.00-35.00)	27.00(17.25-43.50)
ALT (UI/L)	22.50(16.00-37.50)	31.00(22.00-32.25)	32.00(16.25-41.00)
GGT (UI/L)	18.00(15.25-26.25)	21.00(16.00-32.25)	25.50(18.00-28.75)
ALP (Ul/L)	58.50(49.25-71.25)	74.00(64.00-86.25)*	63.00(55.00-74.50)$

*NL, normal liver; SS, simple steatosis; NASH, nonalcoholic steatohepatitis; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA1-IR, homeostatic model assessment method-insulin resistance; HbA1c, glycosylated haemoglobin; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; ALP, alkaline phosphatase. Data are expressed as the median (interquartile range). *Significant differences vs NL group ($p < 0.05$). $Significant differences vs SS group ($p < 0.05$).*

6.2 Evaluation of Relative mRNA Abundance of OLFM2 and OLFM4 According to Hepatic Histology

To achieve the aim of this study, that was to explore the role of OLFMs in NAFLD progression, we evaluated the OLFM2 mRNA hepatic expression in liver samples in a cohort of women with MO. Moreover, given that it has been reported the importance of the gut-liver axis in NAFLD progression, we also analyzed the OLFM4 mRNA jejunal.

First, when we analysed OLFM2 and OLFM4 relative mRNA expressions between NL and NAFLD in hepatic and jejunal samples, respectively. We only reported significant differences between groups in the case of OLFM2 ($p = 0.005$). Then, we subclassified the patients according to the hepatic histopathological grades into NL, SS and NASH. In this sense we found a higher expression of hepatic OLFM2 in SS and NASH women compare to NL cohort (Figure 13A). Later, when the subjects were divided depending on NASH presence an increase of OLFM2 relative expression in NASH group was observed when compared to non-NASH, as shown Figure 13B. However, regarding OLFM4 we did not report significant differences between groups (Figure 13C and D).

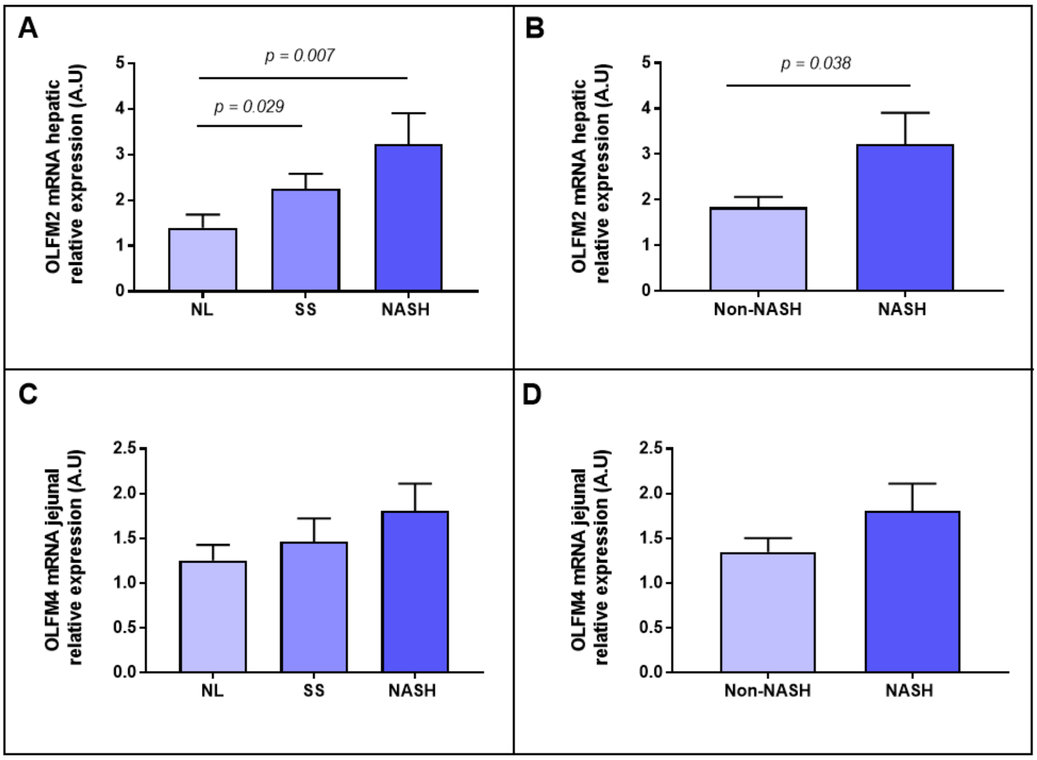

Figure 13. *Differential relative mRNA abundance of OLFM2 in hepatic tissue between women with MO (A) classified as NL, SS and NASH and (B) classified according to the presence or absence of NASH. Differential relative mRNA abundance of OLFM4 in jejunal tissue between women with MO (C) classified as NL, SS and NASH and (D) classified according to the presence or absence of NASH. OLFM, olfactomedin; NL, normal liver; SS, simple steatosis; NASH, nonalcoholic steatohepatitis; A.U arbitrary units. Differences between groups were calculated using Mann-Whitney test and $p < 0.05$ was considered statistically significant.*

6.3 Evaluation of Relative mRNA Abundance of OLFM2 and OLFM4 According to Steatosis Severity

To deepen the knowledge of the link between of OLFMs and NAFLD, first we wanted to focus on the hepatic lipid content degree, so we classified the cohort into different grades of steatosis. In this sense, we reported an enhanced expression of hepatic OLFM2 mRNA in moderate and severe stages of steatosis in comparison to those subjects without it (Figure 14A); while we did not show significant differences when OLFM4 was analysed, as graphically represented in Figure 14B.

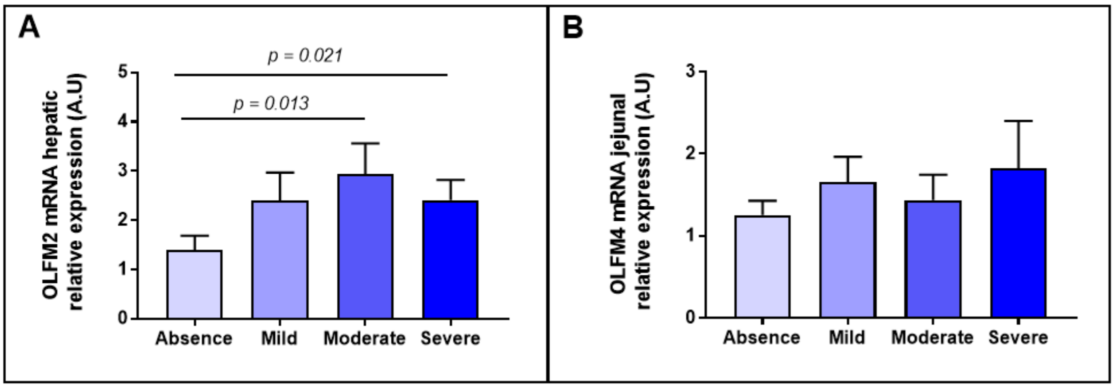

Figure 14: *Differential relative mRNA abundance of (A) OLFM2 in hepatic tissue and (B) OLFM4 in jejunal samples between women with MO classified according to differences grades of steatosis into: absence, mild, moderate and severe. OLFM, olfactomedin. A.U arbitrary units. Differences between groups were calculated using Mann-Whitney test. p < 0.05 was considered statistically significant.*

6.4 Evaluation of Relative mRNA Abundance of OLFM2 and OLFM4 According to NASH-Related Parameters

Later, we wanted to focus the analysis on the main parameters of the advanced stage of NAFLD, such as inflammation and hepatocellular ballooning, so we have evaluated their link with OLFM2 and OLFM4. Concerning portal inflammation, we did not observe differential expressions of OLFM2 in liver and OLFM4 in jejunum between groups (Figures 15A and B). Regarding lobular inflammation, we only found an increase of OLFM2 hepatic expression in the cohort presenting this type of inflammation (Figure 15C), but we did not find differences in the regard of OLFM4 jejunal expression (Figure 15D). Moreover, non-significant differences in OLFM2, neither OLFM4, were reported when the cohort was classified by the presence of hepatocyte ballooning (Figure 15E and F).

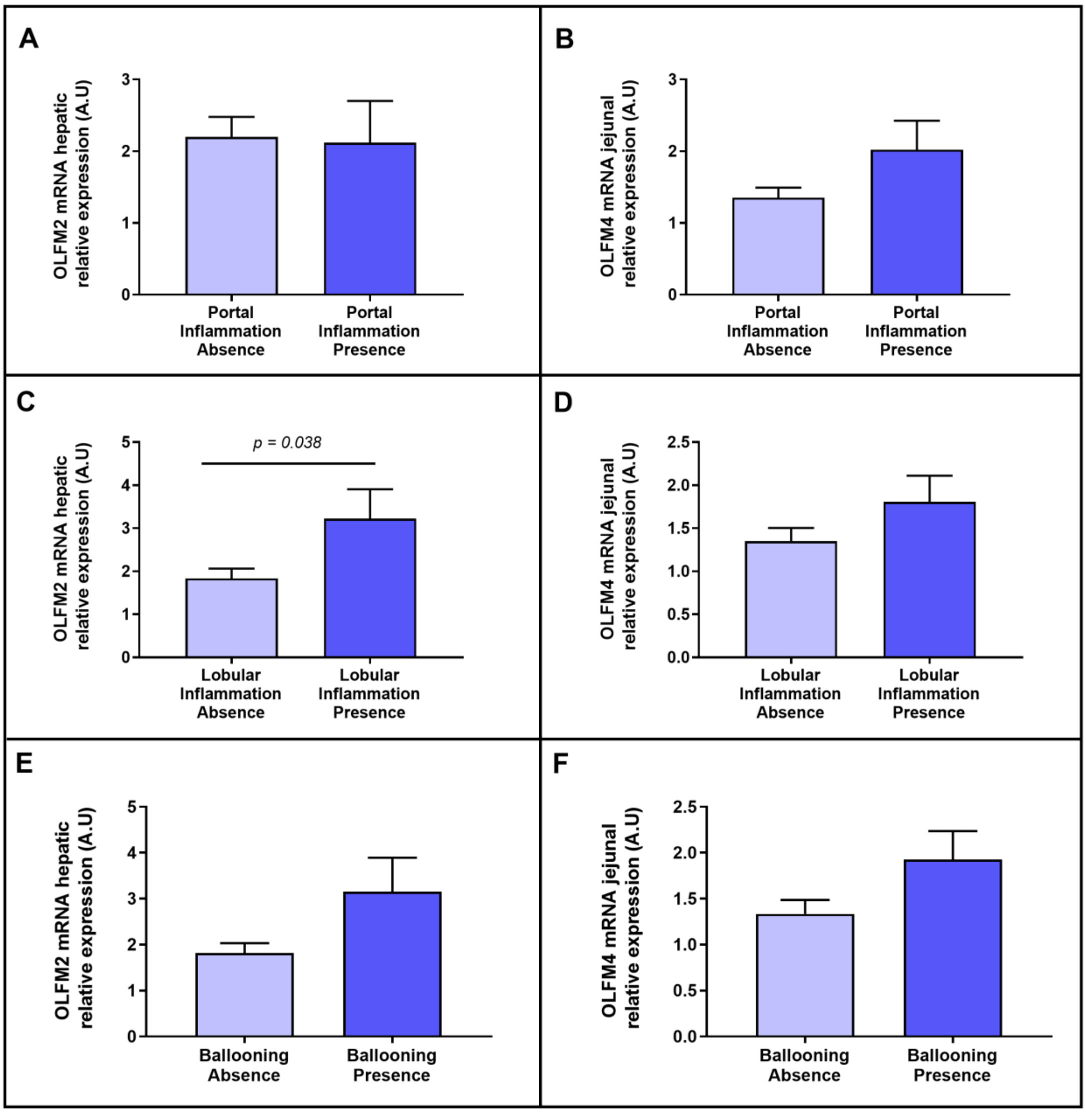

Figure 15: *Differential relative mRNA abundance of hepatic OLFM2 between, (A) absence or presence of portal inflammation, (C) lobular inflammation absence or presence and (E) ballooning absence or presence. Differential relative mRNA abundance of jejunal OLFM4 between, (B) absence or presence of portal inflammation, (D) lobular inflammation absence or presence and (F) ballooning absence or presence. OLFM, olfactomedin. A.U arbitrary units. Differences between groups were calculated using Mann-Whitney test. p <0.05 was considered statistically significant.*

6.5 Correlations Between Relative mRNA Abundance of Hepatic OLFM2 and Jejunal OLFM4, with Clinical and Biochemical-Related Parameters

To broad the study of OLFM2, we analysed their correlations with different parameters related to metabolism and inflammation in a cohort of women with MO. First, we observed positive correlations between hepatic OLFM2 mRNA expression and glucose, cholesterol, TMAO, DCA levels and FAS, RUNX1 and JNK1 mRNA expressions in liver, as shown figure 16A-G, respectively. Also, we found negative association between hepatic OLFM2 mRNA expression and weight, jejunal TLR4 and jejunal TLR5, as was graphically represented in figure 16H-J.

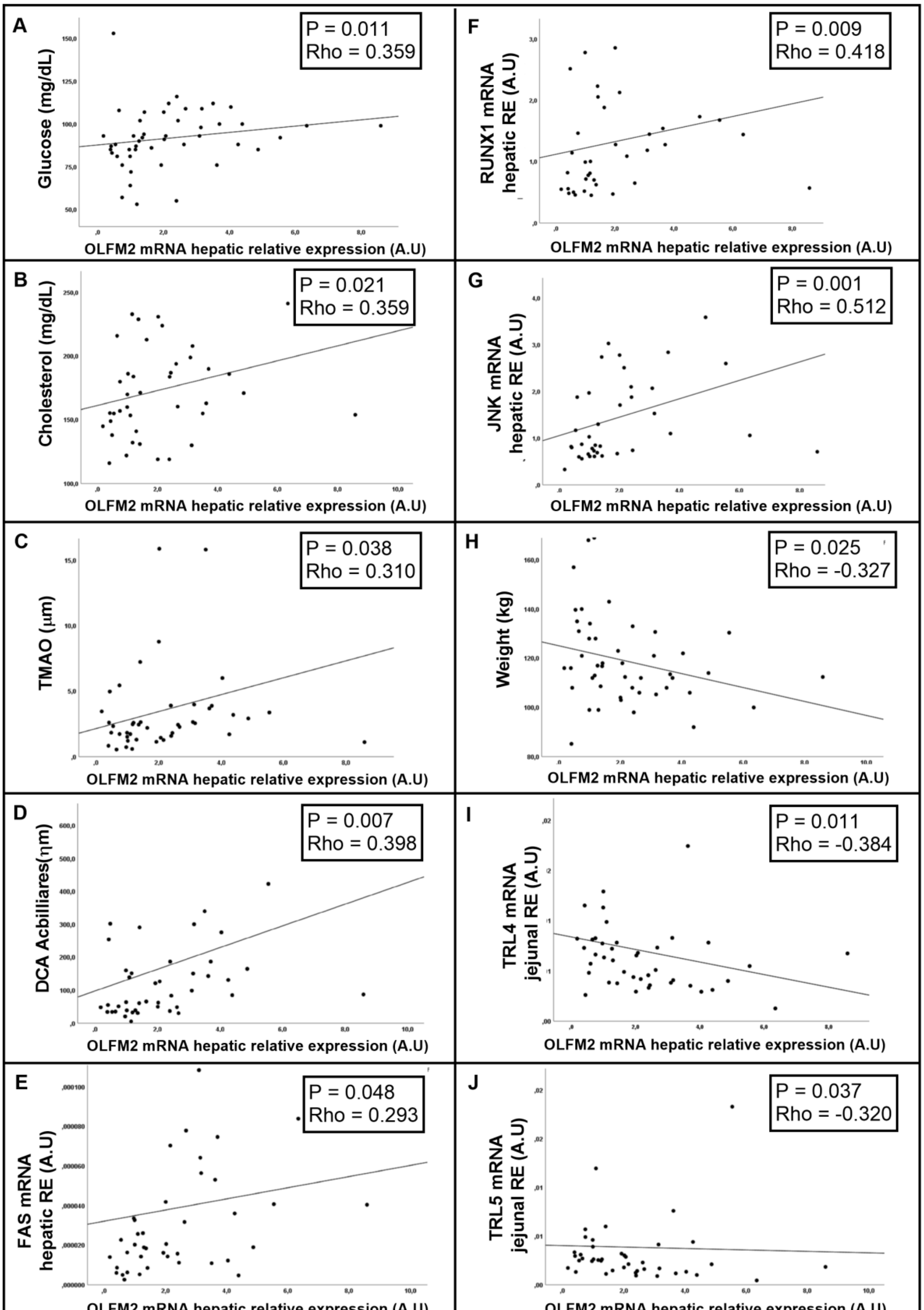

Figure 16: *Significative correlations between OLFM2 hepatic mRNA expression and (A) glucose, (B) cholesterol (C) TMAO, (D) DCA levels and (E) FAS, (F) RUNX1, (G) JNK1 in liver, (H) weight, (I) jejunal TLR4 and (J) TLR5 using Spearman's method. OLFM, olfactomedin; RUNX1, runt-related transcription factor 1; JNK1, Jun N-terminal kinase ; TMAO, Trimethylamine N-oxide dihydrate ; DCA, deoxycholic acid; TLR, toll like receptor FAS, fatty acid synthase; RE, relative expression; A.U, arbitrary units. Differences between groups were calculated using Mann-Whitney test. p < 0.05 was considered statistically significant.*

On the other hand, if we focus on the OLFM4 in jejunum, we observed positive correlations between this OLFM and IL-8, IL-10, IL-17 levels and jejunal TLR9, as shown Figure 17A, B, C and D, respectively. In addition, we found a negative association between jejunal OLFM4 mRNA expression with glucose (p = 0.047, Rho = -0.274).

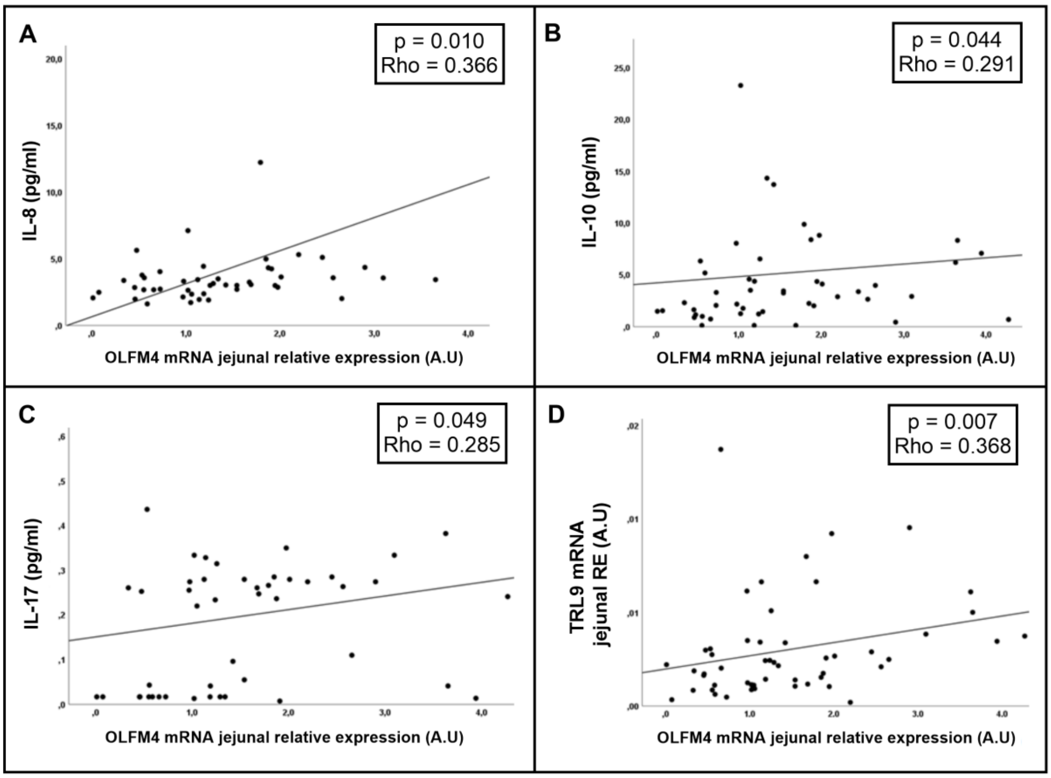

Figure 17: *Significantly positive correlations between OLFM4 jejunal mRNA expression (A) IL-8, (B) IL-10, (C) IL-17 levels and (D) TLR9 in jejunum using Spearman's method. OLFM, olfactomedin; TLR, toll like receptor; IL, interleukin; RE, relative expression; A.U, arbitrary units. Differences between groups were calculated using Mann-Whitney test. p < 0.05 was considered statistically significant.*

7. DISCUSSION

This study is important as it is the first to report an interesting association between OLFMs and NAFLD in hepatic and jejunal samples of women with MO. It is already known that more in depth studies of the molecular mechanisms involved in NAFLD are necessary to find new therapeutic targets. In this sense, given that OLFMs are strongly linked to cellular processes [42,43] and have been related to some disorders such obesity, HCC and intestinal diseases among others [45,52,56,67,68], we wanted to evaluate the possible roles of OLFM2 and OLFM4 in NAFLD pathogenesis. In this regard, our results showed that OLFM2 hepatic mRNA expression enhances as NAFLD progresses; we found increased hepatic OLFM2 mRNA abundance in moderate and severe degrees of steatosis, and in patients presenting lobular inflammation. Unfortunately, we did not report significant differences between groups regarding OLFM4 jejunal expression. However, we found interesting correlations between hepatic OLFM2 and jejunal OLFM4 with some metabolites, biochemical parameters and hepatic and jejunal related genes.

Regarding our first finding, when patients were classified according to their hepatic histopathology, OLFM2 mRNA expression in the liver increased when hepatic health worsened. Moreover, a significant increase in OLFM2 expression in the presence of NASH was observed when the cohort was classified into non-NASH and NASH groups. These facts seem to suggest that OLFM2 is involved in NAFLD progression, especially in NASH. Although OLFM2 mRNA abundance has been found in liver tissue [69], its expression has not been previously evaluated, and this protein has not been related to NAFLD. Therefore, OLFM2 in liver tissue seems to play a role in NAFLD progression in subjects with obesity; however, further studies are needed in this field.

Then, when we evaluated the relationship of hepatic OLFM2 and jejunal OLFM4 based on the steatosis stage of the disease, we found a significant increase in hepatic OLFM2 in patients with moderate and severe steatosis grades. Later, when we analysed the role of these OLFMs in accordance with steatohepatitis parameters, such as lobular and portal inflammation and hepatocyte ballooning, we found an increase in hepatic OLFM2 expression in patients with lobular inflammation. These findings indicate that OLFM2 increases as disease progresses and it was highly increased in NASH, which is usually characterized by hepatic inflammation and elevated lipid content [14,76]. As we have previously mentioned, this is a novel finding; thus no previous information exist in this regard, not even reports on the expression of this protein in the liver and more studies are necessary to corroborate this evidence.

Unfortunately, although OLFM4 seems to play relevant roles as anti-inflammatory molecule in *H. pylori* infection [56] and gastric disorders [67,68], which could suggest that OLFM4 is involved in NAFLD pathogenesis through microbiota changes and the gut-liver axis; in our study the mRNA expression of this protein in jejunal samples was not reported significantly different between hepatic histopathological groups. In the literature, there are no other studies in this regard. Therefore, if we want to delve into this relationship, additional studies should be carried out to confirm our findings.

To deepen the knowledge of OLFMs is regards to NAFLD, we analysed several correlations. To mention, positive associations between hepatic OLFM2 mRNA were found with hepatic FAS and cholesterol and glucose levels, which are parameters closely related to NAFLD. When there is an excess of carbohydrates in the diet, glucose can be stored as lipids in the liver, and FAS, a lipogenic enzyme, synthesizes triglycerides from free fatty acids; therefore, its action enhances hepatic steatosis, the principal characteristic of NAFLD [77,78]. In addition, another own study reportedincreased FAS expression and high glucose levels in people with NAFLD [79], so our current results are consistent with previously mentioned publications showing dysregulation of hepatic lipogenesis in NAFLD[80]. Regarding cholesterol, there is some evidence that excessive cholesterol intake is involved in the development of NAFLD [81]. All this information suggests that these positive correlations of OLFM2 in the liver with glucose and cholesterol levels and hepatic FAS, corroborate our previous hypothesis that hepatic expression of OLFM2 increases as NAFLD progresses; at the same time, the metabolic imbalance also worsens [82].

Additionally, we found a negative significant correlation between hepatic OLFM2 and weight. This association is difficult to explain, given that González-García *et al.* observed that a global lack of OLFM2 induces weight-loss [45] and in the current study we have observed a negative association between these parameters. This contradiction can be explained by the fact that our groups of subjects are comparable in terms of weight, but we have a great variability inside the cohort (patients with a BMI > 40kg/m^2). Also, in these types of extreme obesity, perhaps some correlations may have been skewed by excessive BMI.

On the other hand, we also observed a positive correlation of hepatic OLFM2 with DCA and TMAO levels. TMAO is a gut microbiota-dependent metabolite, and some publications [80,83,84] reported increased TMAO levels in patients with NAFLD. The same authors suggested that TMAO could contribute to the development and severity of this disease due to its role in glucose regulation, cholesterol homeostasis and lipid-absorption [83,85]. An increase in DCA levels, which are involved in the metabolism of ingested lipids, was also reported in NAFLD patients [86]. Additionally, Grzych *et al.* showed high levels of DCA in yhe presence of NASH [87]. Therefore, this positive association of hepatic OLFM2 with DCA and TMAO levels, remains consistent with our previous results, reinforcing the hypothesis that OLFM2 may play an important role in the progression of NAFLD.

Later, we wanted to analyse whether OLFM2 in the liver is associated with jejunal TLRs, since these are involved in gut-liver axis crosstalk that can influence NAFLD appearance and progression [88]. In this regard, although we previously observed that hepatic OLFM2 mRNA expression is enhanced when the disease becomes severe, we observed a negative correlation between jejunal TLR4 and 5 and hepatic OLFM2 mRNA expression. This is a contradictory result given that TLRs are known to be involved in NAFLD progression [88], but this fact may be becouse women in our cohort followed a very low-calorie diet for 3 weeks before bariatric surgery [89] and in this sense, Macedo Rogero *et al*. demonstrated that omega 3 polyunsaturated fatty acids, usually included in the very low-calorie diet [90], attenuate the activation of the TLR4 signalling pathway, exerting an anti-inflammatory effect [91] and inhibiting lipid accumulation [92].

Concerning TLR5, this receptor has been shown to not necessarily be altered by diet [93]. Otherwise, TLR5 seems to play a key role in liver protection against intestinal dysbiosis-induced NAFLD in mice [94], preventing gut inflammation related disorders [95]. Moreover, an increase in TLR5 seems to contribute to liver regeneration events [96]. Hence, in this case, the negative correlation of TLR5 with OLFM2 mRNA expression in the liver makes sense given that OLFM2 increases as NAFLD worsens, while TLR5 in the gut could be decreased due to the high inflammation pattern induced by intestinal dysbiosis and liver damage. Similarly, mouse models without TLR5 expression were used to present obesity, hepatic steatosis and IR [97], the main characteristics of our study cohort.

Finally, regarding OLFM2, we have also found a positive correlation with the mRNA expression of RUNX1 and JNK1 in the liver. These associations make sense since RUNX1 and JUNK1 have been reported to be overexpresses in liver of NAFLD patients [3,75,98], the first with a possible protective role in the first stages of the disease, and the second related to hepatocytes death in livers with steatosis [75,99].

Focusing on OLFM4, as stated above, its relative mRNA expression in the jejunum did not present significant differences between NAFLD groups, but we did observe positive associations with IL-8, IL-10 and IL-17 levels and jejunal TLR9 expression. In this sense, circulating IL-8 and IL-17 are defined as proinflammatory cytokines that present enhanced circulating levels in NAFLD [100–102]. Therefore, in this work OLFM4 seems to be related to a proinflammatory state, despite previously being linked to an anti-inflammatory role in other tissues [56,103]. Hence, we cannot definitively conclude that OLFM4 in the jejunum is involved in gut-dysbiosis-related inflammation because our patients presented a low-grade chronic inflammation due to obesity [104], but it would be interesting to evaluate OLFM4 jejunal abundance in lean subjects.

IL-10 is a well-defined anti-inflammatory cytokine [105] with protective effects in liver injury [106]; but high levels of circulating IL-10 have sometimes been reported in subjects with obesity [107]. Given that IL-10 attenuates the secretion of proinflammatory cytokines, a continuous increase in IL-10 levels could be explained by competition of IL-10 with proinflammatory cytokines in an attempt to balance the inflammatory state of these patients [107]. Moreover, IL-10 exhibits feedback regulation to inhibit proinflammatory cytokine production [108]. These findings could explain the positive correlation between OLFM4 mRNA in the jejunum, which was previously correlated with proinflammatory cytokines, and IL-10 circulating levels, since this anti-inflammatory cytokine seems to counteract the low-grade chronic inflammatory pattern of our MO patients.

TLR9 is a receptor that is highly linked to metabolism and inflammatory events [109], and it has been suggested to play a relevant role in NAFLD pathogenesis [88]. Additionally, it was clinically demonstrated that TLR9 is a key driver of NASH [109,110]. Hence, the positive association of TLR9 with OLFM4 jejunal expression also makes sense, as it is consistent with its correlation with proinflammatory cytokines. This finding reinforces the possible inflammatory role of OLFM4 in the jejunum, but more studies are needed.

Finally, we found a negative correlation between jejunal OLFM4 mRNA expression and glucose levels. It was reported that OLFM4 is an inhibitor of GRIM-19 [61], which joins in the first step of the electron transport chain, necessary for the metabolism of glucose homeostasis and the consequent release of insulin [111]. On the other hand, Wenli Liu *et al*. demonstrated in animal models that pancreatic OLFM4 inhibited mitochondrial activity under high glucose conditions; in other words, by inhibiting OLFM4 in the pancreas, glucose and insulin concentrations decreased when fasting [111]. In the regard of this study, there is a negative correlation between OLFM4 in jejunum and glucose levels, which contradicts Wenli Liu *et al.* findings, but this situation may be done since our cohort followed a very low-calorie diet and T2DM treatment, so they do not present high concentrations of glucose. In addition, in this previous study the OLFM4 analysis was performed in pancreatic tissues of mice, while ours was assessed in jejunum samples of women presenting MO.

In summary, we have carried out a novel study showing that the hepatic expression of OLFM2 seems to increase as NAFLD progresses, and this protein seems to be associated with NASH and with a severe steatosis pattern in NAFLD patients. Additionally, OLFM4 was suggested to be more related to inflammation that occurs due to intestinal dysbiosis, usually linked to NAFLD. In this sense, we are pioneers in demonstrating a relationship between OLFM2 and NAFLD and between OLFM4 and inflammation related to gut dysbiosis. However, there are some limitations of this study. In the literature, there is little or no experience in this field. Also, this is a cross-sectional study, so a causal relationship cannot be confirmed. Last, a cohort of patients made up of only MO women was used, so these results cannot be extrapolated to other sexes or other groups of people with obesity, overweight or normal weight. Hence, future studies are needed to understand the specific role of these OLFMs in the pathogenesis of NAFLD and their relationship with the gut-liver axis and inflammation.

8. CONCLUSIONS

As sum up, the novel finding of this study is that hepatic OLFM2 seems to play a relevant role in NAFLD progression in women with MO, since its expression enhanced in NASH and is correlates with severe steatosis and an inflammatory state. Moreover, we suggest that OLFM4 in the jejunum could be involved in gut dysbiosis related-inflammatory events. There are necessary more studies in this field to corroborate these hypotheses.

9. ACKNOWLEDGEMENTS

I feel the need to thank the teachers who have been part of my education, both professionally and personally, to get me to the point where I am now. Even more important to my family, friends and partner who have always supported me without conditions, making me feel accompanied and safe in every decision, dedicating their time, advice and effort.

I have to thank my academic tutor Anna Hernández Aguilera and Carmen Aguilar Crespillo, Dr. Teresa Auguet Quintillà and especially Laia Bertran Ramos, who are part of the GEMMAIR research group. Without them this work would not have been possible, they have welcomed me as one of the group, they have advised me, they have selflessly guided me throughout the project, they have dedicated all the time necessary to prepare the final degree project and that is why I want to express my sincere thanks. I finish this stage, more prepared, with more knowledge about my future, more confident and eager to continue training in the field of research.

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