Universitat de Girona

NONALCOHOLIC FATTY LIVER DISEASE AS A PREDICTOR OF ATHEROSCLEROSIS IN OBESE SUBJECTS

A CROSS-SECTIONAL STUDY

AUTOR: Estefanía Sánchez de Gea

TUTOR: Jose Manuel Fernández Real

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1 ABBREVIATIONS

ALT: Alanine aminotransferase

AST: aspartate aminotransferase

AUC: Area under curve

BMI: body mass index

CEIC: Clinical research Ethics Committee

CHD: cardiovascular heart disease

c-IMT: carotid Intima-Media Thickness

CK-18: Cytokeratin 18

CKD: Chronic kidney disease

CV: coefficient of variation

CVD: Cardiovascular disease

DBP: diastolic blood pressure

FFA: free fatty acids

FRS: Framingham risk score

HCC: hepatocellular carcinoma

HCV: hepatitis C virus

HOMA-IR: insulin resistance index

HTN: hypertension

LPS: lipopolysaccharides

MetS: metabolic syndrome

NAFL: Non-alcoholic fatty liver

NAFLD: Non-alcoholic fatty liver disease

NASH: Nonalcoholic steatohepatitis

PNPLA3: Patatin-like phospholipase domain-containing 3

POS: polycystic ovary syndrome

PUFAs: n-6 polyunsaturated fatty acids

SBP: systolic blood pressure

SD: standard deviation

T2DM: type 2 diabetes mellitus

TLR: toll-like receptor

US: ultrasound

WMA: World Medical Association

2 ABSTRACT

BACKGROUND: Non-alcoholic fatty liver disease (NAFLD) is considered to be the next global epidemic. The prevalence of NAFLD is increasing worldwide paralleling the epidemic of obesity and rise of diabetes mellitus.

NAFLD is the leading cause of chronic liver disease, with a prevalence as high as 30% in the general population but cardiovascular diseases are the first cause of death among these subjects. This suggests that the threshold to develop policies of monitoring for cardiovascular disease should be implemented in patients with NAFLD.

It has been demonstrated that NAFLD is highly associated with atherosclerosis. It appears that NAFLD may induce insulin resistance, oxidative stress, inflammation, dyslipidemia and fluctuation of adipokines associated with atherosclerosis.

OBJECTIVE: This study aim to evaluate the possible influence of NAFLD on markers of subclinical atherosclerosis (c-IMT and/or plaques).

METHODS: We studied 400 consecutive obese subjects (282 women; mean age, 45,6± 9,33; BMI 43,87 ± 6,22 and 118 men; mean age 43,89 ± 9,28; BMI 43,87 ± 6,22). Hepatic steatosis and atherosclerosis [carotid intima-media thickness (cIMT) >0.8 mm and/or presence of plaques] were evaluated ultrasonographically.

RESULTS: NAFLD and age were the best predictors of atherosclerosis using c-IMT, in both obese men and women. NAFLD in obese women could predict not only c-IMT but also the presence of atheroma plaques.

CONCLUSIONS: Non-alcoholic fatty liver disease and age could be predictors for carotid atherosclerosis in obese subjects.

KEY WORDS: Nonalcoholic fatty liver disease, Nonalcoholic steatohepatitis, Atherosclerosis, obesity, risk factor, age, c-IMT, Framingham 10-years risk score.

3 INTRODUCTION

3.1. DEFINITION OF NAFLD

Nonalcoholic fatty liver disease is a term used to describe excessive liver fat accumulation (≥5% of hepatocytes by histology or ≥5.6% by nuclear magnetic resonance techniques) causing cellular dysfunction (lipotoxicity) when there are not any secondary causes of hepatic steatosis such as alcohol consumption (>21 drinks/wee or >30 g/day in men and >14 drinks/week or >20 g/day in women), viral hepatitis, medications (amiodarone, methotrexate, valproate), autoimmune hepatitis, hemochromatosis, Wilson's disease, iron overload or toxins(1,2).

Non-alcoholic fatty liver disease represents a hepatic manifestation of the metabolic syndrome which is characterized by the presence of 3 or more of the following: 1) abdominal obesity (waist circumference > 102 cm in men, >88 cm in women), 2) hypertriglyceridemia (>150 mg/dl), 3) low high-density lipoprotein (HDL) levels (<40 mg/dL in men, <50 mg/dL in women), 4) hypertension (>130/80 mm/Hg), and 5) high fasting glucose levels (>110 mg/dL) (3). Moreover, obesity, which is an important risk factor for the development of NAFLD, may result in insulin resistance and metabolic syndrome (4). Liver injury usually occurs in the presence of these features, mediated by inflammatory cytokines, mitochondrial dysfunction secondary to nutrient excess, oxidative stress and extrahepatic factors such as impaired adipose tissue signaling, the effect of gut microbiota and gene polymorphisms such as PNPLA3 and TLF6 which are currently being explored (5).

3.2. SPECTRUM OF FATTY LIVER DISEASE

Current knowledge suggests that there is a natural history from normal liver to nonalcoholic fatty liver (NAFL) or isolated steatosis (hepatic macrovesicular fat accumulation in at least 5% of hepatocytes without necroinflamatory or fibrotic changes on liver biopsy), nonalcoholic steatohepatitis (NASH; diagnosed by three necessary features: macrovesicular steatosis, hepatocytes ballooning degeneration and lobular inflammation) and finally to end-stage cirrhosis and/or hepatocellular carcinoma (HCC)(3,6,7). (See **FIGURE 1)**

NASH is usually associated with some degree of fibrosis, but any stage of fibrosis may also be present in NAFL without any features of NASH. Most studies have shown that fibrosis influences liver-related and overall mortality independently, regardless of the presence or severity of other histological features(8).

The grade of steatohepatitis indicates the necroinflamatory activity and the degree of cell injury that is present. The stage of the disease reflects the degree of fibrosis or how far an individual has progressed toward cirrhosis. In order to classify and capture the entire spectrum of disease, several systems for grading and staging NAFLD have been proposed, such as the SAF score, which separately assesses the grade of steatosis (S, from S0 to S3), the grade of activity (A from A0 to A4 by adding grades of ballooning and lobular inflammation, both from 0 to 2) and the stage of fibrosis (F from F0 to F4). Another scoring system is the NAFLD Activity Score proposed by the NIDDK NASH CRN and is based on the notion that in liver disease, necroinflamatory lesions and the stage of fibrosis should be evaluated separately because the former are potentially more reversible than the latter (5,8,9). (See **TABLE 1**)

The progression from NASH to cirrhosis and HCC has been well established. Nevertheless the progression and relationship between NAFL and NASH is unclear and is currently being studied (8,10).

TABLE 1. Non-alcoholic steatosis or non-alcoholic fatty liver disease activity score is determined by evaluating the steatotic and inflammatory activity (4).

NAS	Steatosis	Ballooning	Inflamation, lobular
0	<5% (0)	None (0)	None (0)
3	5%-33% (1)	Rare or few (1)	1-2 foci per 20 x field (1)
6	34%-66% (2)	Many (2)	2-4 foci/20 x field (2)
8	>66% (3)	Many (2)	>4 foci/20 x field (3)

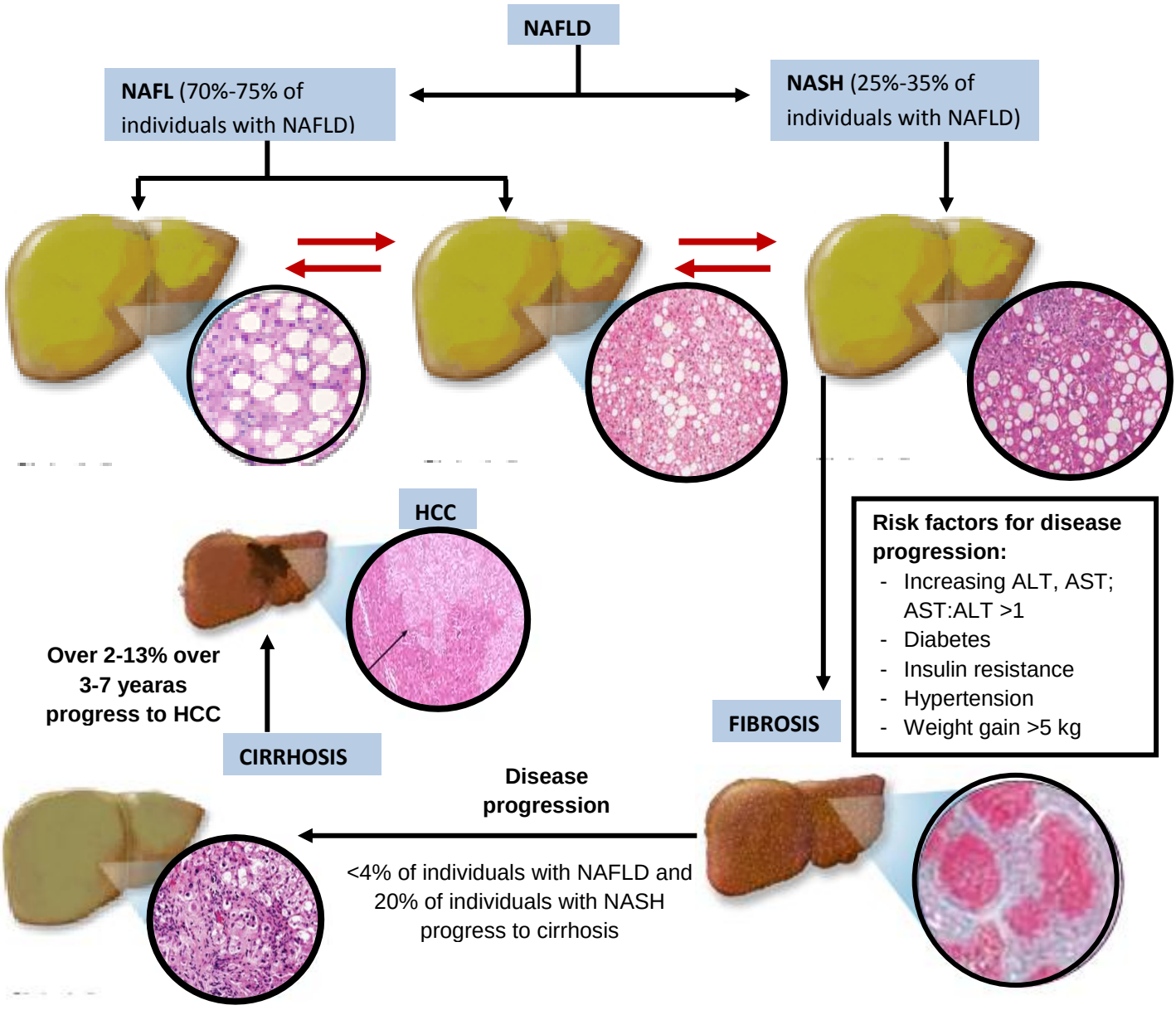


FIGURE 1. Histological subtypes of NAFLD and their implication on Disease Progression. Data adapted from (11–15)**.**

3.3. EPIDEMIOLOGY

NAFLD has become a major cause of liver disease worldwide in the last 20 years paralleling the epidemic of obesity and rise of diabetes mellitus prevalence, two diseases strongly related with NAFLD.(16)

A recent meta-analysis study showed that 25% of the adult population worldwide has NAFLD. The prevalence of NAFLD varies according to region, ranging from 14% in Africa to 23.71% in Europe, 24.13% in North America, 27% in Asia (despite the lower BMI throughout this region), 31% in South America and 32% in the Middle East (17,18).

Furthermore, there are differences in the prevalence between different races: 45% in Hispanics, 33% in Caucasian Americans, 24% in African Americans (19) and 25% in Asians (20). (See **TABLE 2).**

There is also an increase in the prevalence of NAFLD with age: in pediatric population the prevalence is 2.6% which may rise to the range of 10-80% in obese children (21). In adults under the age of 30 the prevalence of NAFLD is around 22% and more than 40% in over 70s (17). (See **TABLE 2).** Nevertheless it remains unclear if the higher prevalence and severity of the disease with age is simply a cumulative end result of incremental components of metabolic syndrome and longer duration of NAFL/NASH in these populations (10,22).

Region	Prevalence (%)	95% CI (%)	Mean age	Prevalence (%)	95% CI (%)
Africa	**13.48**	(5.69-28.69)	**Children**	**2.6**	-
Asia	**27.37**	(23.29-31.88)	**30-39**	**22.43**	(15.38-31.52)
Europe	**23.71**	(16.12-33.45)	**40-49**	**26.53**	(22.37-31.16)
Middle East	**31.79**	(13.48-58.23)	**50-59**	**27.4**	(19.56-36.93)
North America	**24.13**	(19.73-29.15)	**60-69**	**28.9**	(19.25-40.94)
South America	**30.45**	(22.74-39.44)	**70-79**	**33.99**	(32.08-35.95)
Overall	**25.24**	(22.1-28.65)	**Overall**	**24.29**	(20.96-27.96)

TABLE 2. NAFLD Prevalence stratified by Region and Mean Age. Data adapted from (17,21)

Some studies reported that women had higher risk of developing NAFLD, but these studies were not population-based and were subject to potential ascertainment bias (10). In addition, other studies showed a higher prevalence of fatty liver in men (23). Together, these findings highlight uncertainties regarding the influence of gender on NAFLD, and more studies are needed to better clarify this. Sex hormones may be one of the possible causes of these differences (3). In fact, a recent study showed that prevalence of NAFLD was similar in pre- and intrapuberal boys and higher in the postpubertal groups, whereas in girls NAFLD was most common in the intrapuberal group and lower in the postpubertal group (24). In addition, postmenopausal women are at higher risk of NAFLD (3,10,25).

Regarding NAFLD incidence, there are a small number of studies. One meta-analysis study obtained results only for Asia [52.34 per 1,000 (95% CI: 28.31-96.77) person-year] and Israel [28.01 per 1,000 (95% CI: 19.34-40.57) person-year] (17).

The same meta-analysis studied the prevalence of NASH among NAFLD patients with indication for biopsy: 63.45% for Asia, 69.25% for Europe, and 60.64% for North America; and among NAFLD patients without indication for biopsy: 6.67% for Asia and 29.85% for North America (17).

There is an association between NAFLD and the presence of the components of metabolic syndrome (MetS), i.e. abdominal obesity, dyslipemia, hypertension and type 2 diabetes mellitus (T2DM).(18). **TABLE 3** shows the prevalence of some of the different metabolic comorbidities associated with NAFLD and NASH.

Non-alcoholic fatty liver disease is strongly linked to obesity (BMI> 30 kg/m^2), with a reported prevalence as high as 80% in obese patients and only 16% in individuals with normal BMI and without metabolic risk factors (26,27). Visceral fat accumulation has been linked to NAFLD susceptibility in nonobese subjects as opposed to subcutaneous fat and BMI (28).

Risk factors	Region	NAFLD	NASH
Obesity	Overall Asia Europe North America Oceania South America	**51%** **64%** **37%** **57%** **-** **-**	**82%** **-** **95%**
Diabetes	Overall	**22%**	**43%**
Hyperlipidemia/Dyslipemia	Overall	**69%**	**72%**
Hypertrigliceridemia	Overall	**41%**	**83%**
Hypertension	Overall	**39%**	**68%**
MetS	Overall	**42%**	**71%**

TABLE 3. NAFLD and NASH risk factors Prevalence (%) stratified by Region. Data adapted from (17)

3.4. NATURAL HISTORY AND PROGNOSIS

The principal causes of mortality in NAFLD population are firstly cardiovascular disease (25-43%) and then non-liver malignancy (19-28%), liver-related disease (9-15%) and infection (5-11%)(14).

Patients who have steatosis alone are more likely to suffer the consequences of cardiovascular or nonhepatic cancer-related illnesses but are not at increased risk of liver-related mortality. Nevertheless non-alcoholic steatohepatitis presents a much greater risk of developing end-stage liver disease or liver related mortality, particularly when fibrosis is present (8,13). (See **FIGURE 2).** In addition, NASH patients are at higher risk of cardiovascular disease than those with steatosis alone, emphasizing the role of chronic inflammation in the pathogenesis of atherosclerosis in these patients (29).

It is thought that fibrosis progression in patients with NAFL is uncommon, whereas NASH progresses more frequently (approximately 9% of patients with NASH present advancement of their fibrosis) (10,17) (See **FIGURE 2).**

Progression to cirrhosis or hepatocellular carcinoma (HCC) only occurs in 2.5% of patients with NASH, and it is usually slower than other chronic liver diseases. The progression of NAFLD or NASH to cirrhosis has been estimated

to be 57 and 28 years, respectively, compared to 20-30 years for hepatitis C virus (HCV) infection (30).

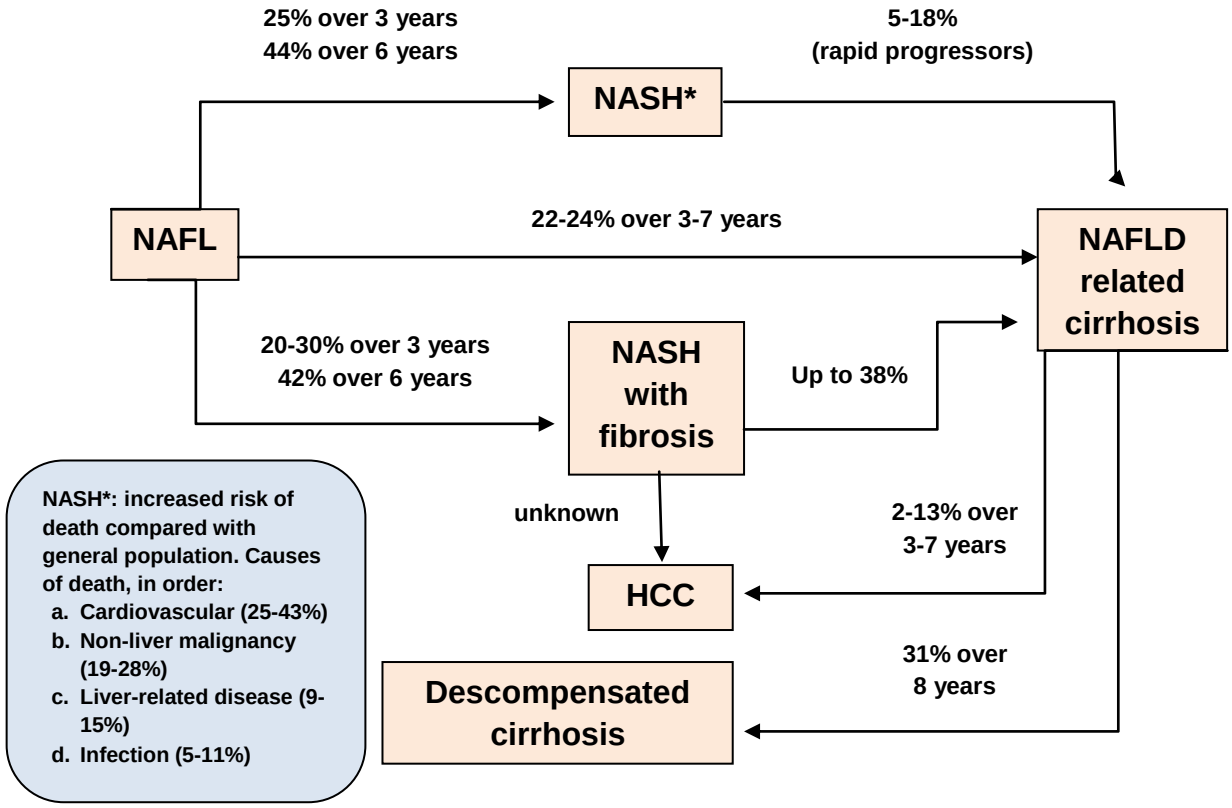


FIGURE 2. Natural history of non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH) with or without fibrosis, cirrhosis, and hepatocellular carcinoma. Data adapted form (3,13,14,31)

3.5. PATHOGENESIS

The pathogenesis of fatty liver has similar mechanisms to those that have alcoholic fatty liver, and presents two stages (the “two-hit” process): The first hit is the accumulation of triglycerides in hepatocytes, which increases the vulnerability of the liver to various possible “second hits” that in turn lead to inflammation, hepatocytes damage, fibrosis, and cellular death characteristics of NASH. This second hit is the result of numerous conditions acting in parallel, such as oxidative stress, abnormal lipid metabolism, genetic predisposition, lipotoxicity, mitochondrial dysfunction, altered production of cytokines and adipokines, endoplasmatic reticulum stress, and gut dysbiosis. The inability to quell these injurious processes contribute to liver damage, progressive fibrosis that can lead to cirrhosis, and the development of hepatocellular cancer in some patients (11,32–34). As the occurrence and progression of this disease is not

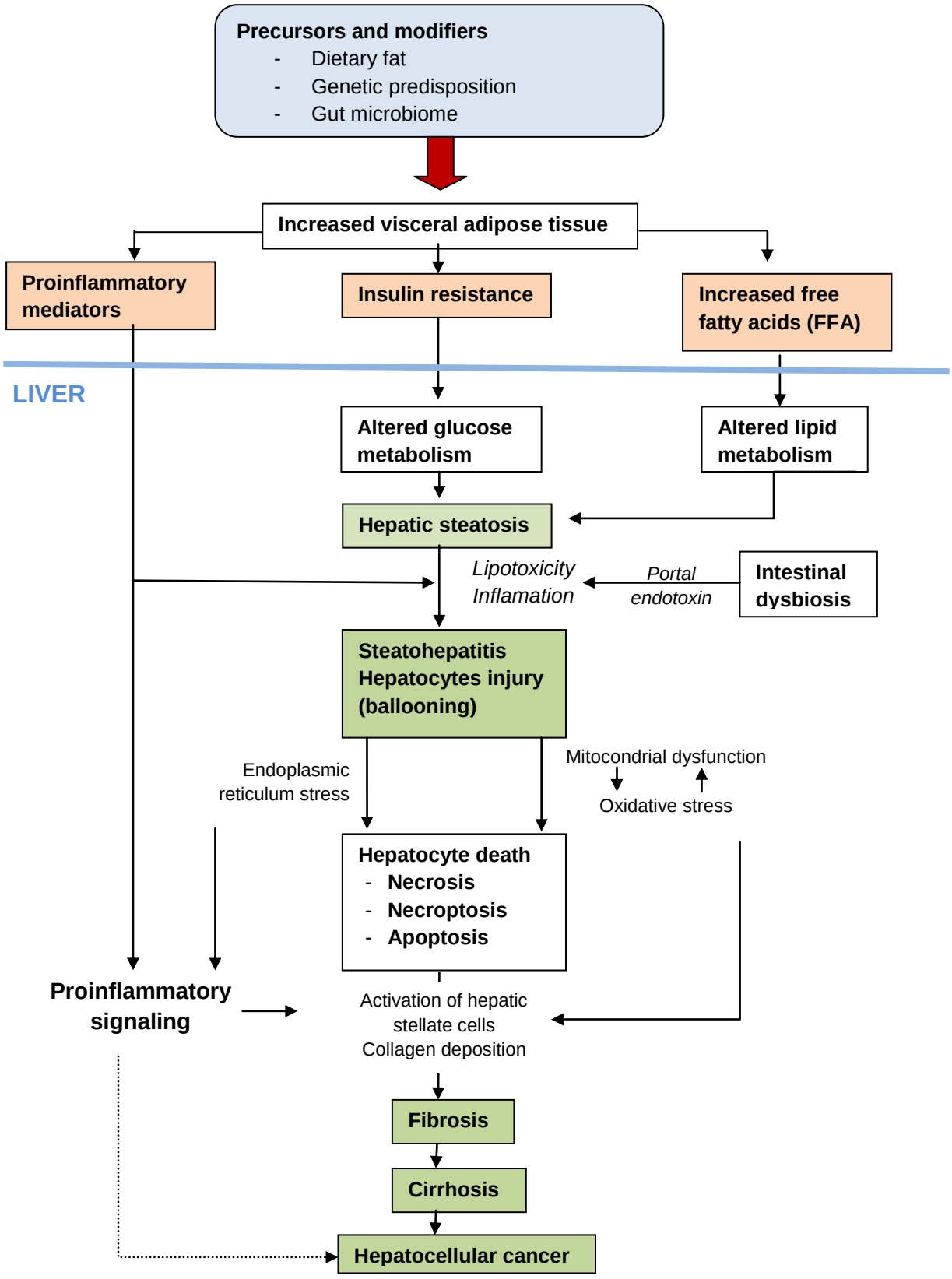
attributed to a single pathogenic mechanism, current paradigms suggest a "multiple parallel hits" hypothesis for the pathogenesis of NAFLD (18) (see **FIGURE 3).**

Recent studies have shown that hyperinsulinemia and increased insulin resistance play an important role in the pathogenesis of NASH, even in patients with normal glucose tolerance. High concentrations of insulin would block mitochondrial oxidation of fatty acids, leading to the deposition of triglycerides and fatty acids in the liver favoring oxidative stress and liver damage (33).

Another pathway that likely contributes to liver inflammation in NAFLD patients is gut microbiota and their metabolic products (such as ethanol and other volatile organic compounds). The liver acts as a barrier between the gut and the systemic circulation by removing toxins, but it could be damaged by different mechanisms. One of the possible mechanisms is through the type of diet, such as those rich in fructose and/or n-6 polyunsaturated fatty acids (PUFAs) that could directly contribute to hepatic lipid accumulation and inflammation and indirectly altering the composition of gut microbiota –a process known as dysbiosis. This may alter the intestinal mucosal barrier by increasing the permeability of tight junctions, leading to the translocation of bacteria or bacterial products, such as lipopolysaccharides (LPS), which bind to toll-like receptors (TLR) 4 and activate the production of proinflammatory cytokines, subsequently resulting in inflammation. Another probable mechanism is when the specialized macrophages in the hepatic sinusoids (Kupffer cells) are damaged, or when the gut´s mucosal barrier is impaired by inflammation or portal hypertension (HTN), a metabolic endotoxinemia may develop (18,34).

The mechanisms of fatty liver pathogenesis are still not well known and continue to be the objective of a continuous and intense investigation. The current risk factors that are being studied and associated with fatty liver will be explained below (18).

FIGURE 3. Mechanisms involved in the Pathophysiology of Nonalcoholic Fatty liver (11)**.**



3.6. RISK FACTORS

There are a lot of studies that have evaluated associations between NAFLD/NASH and several host and environmental factors. In the Epidemiology section, the prevalence and characteristics of some of the risk factors associated with NAFLD/NASH [ethnicity, age, gender and components of metabolic syndrome (abdominal obesity, dyslipemia, hypertension, hypertriglyceridemia and T2DM)] have been commented.

Other clinical associations that are being studying are diet, lifestyle, smoking, alanine aminotransferase, genetic factors (PNPL3 gen), chronic kidney disease, polycystic ovary syndrome, obstructive sleep apnea, colonic adenomas, hyperuricemia, vitamin D deficiency, hyperferritinemia, hypothyroidism and pancreatic steatosis (3). One of the most important associations is with cardiovascular pathologies.

It has been demonstrated that NAFLD leads to an increased risk of cardiovascular events and mortality. The mechanism linking NAFLD and atherosclerosis is poorly understood and may be related to insulin resistance, inflammation, oxidative stress, lipid disorders and fatty hormone levels (35) There is convincing evidence that worsening grades of NAFLD contribute to progressive cardiometabolic risk, such that NASH represents a marker as well as a mediator of increased cardiovascular risk more than simple steatosis(36). Carotid intima media thickness (c-IMT) is a reliable index of subclinical atherosclerosis and a mirror of atherosclerosis progression in NAFLD patients (35). Observational studies suggest that NAFLD is associated with increased c-IMT and carotid plaques in both children and adults (37). Therefore, NAFLD is closely associated with atherosclerosis and it seems to an early risk factor for atherosclerosis.

3.5. CLINICAL FEATURES

Most patients with NAFLD, including NASH, are asymptomatic, although fatigue, malaise and a sensation of fullness or discomfort on the right side of the right upper quadrant of the abdomen occurs in approximately one third of patients with NAFLD (3,6,9,38).

The physical findings include: hepatomegaly, which can be difficult to detect during physical examinations of obese patients; acanthosis nigricans, identified as increased pigmentation around the neck and on the elbows, knuckles, or other joints, which is associated with insulin resistance. A minority of people present signs of chronic liver disease such as spider telangiectasias, muscle wasting or palmar erythema. Icterus, ascites and variceal hemorrhage point to the presence of cirrhosis (6,9).

3.8. DIAGNOSIS

The gold standard method to diagnose NAFLD is liver biopsy. Nevertheless noninvasive surrogate markers are poised to replace liver biopsy for the diagnosis of advanced stage fibrosis and cirrhosis(39).

The diagnosis of patients with NAFLD is often made accidentally due to many patients are asymptomatic. Altered levels of liver enzymes or fatty liver characteristics are usually found in imaging studies when a patient undergoes testing for an unrelated symptom or condition. It is important to know that people with NAFLD currently remains undiagnosed in the great majority of afflicted individuals (9).

Elevations in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels can be associated with an increased risk of disease progression. Nevertheless, some patients with advance disease often have normal liver enzyme levels (11).

Some biomarkers like cytokeratin 18 (CK-18) are able to predict NASH among those with NAFLD with an area under the receiver operating characteristic curve of 0.82 (95% CI 0,78-0,88), based on a meta-analysis of 13 studies(40).

Ultrasonography is the most appropriate imaging test due to its cost and easy applicability. It is very sensitive detecting steatosis, but is less sensitive detecting fibrosis. Steatosis can also be observed by magnetic resonance imaging (33).

3.10. TREATMENT

Management of weight and overall fitness is the aim of treatment for all patients. It has been seen that both diet type and exercise can improve NAFLD. Mediterranean diet, rich in fiber and polyunsaturated fatty acids have been shown to reduce hepatic steatosis, while the high cholesterol diet has been associated with higher NAFLD since it promotes de novo lipogenesis. Coffee intake has also shown a reduction of NAFLD(39)

Bariatric surgery in properly selected patients can be very effective, not only in resolving or greatly improving NAFLD, but also in improving survival in patients with obesity-related comorbidities, particularly those related to cardiovascular disease and cancer (11,39)

Vitamin E, Insulin sensitizers, pentoxifylline, and omega-3 polyunsaturated fatty acids have demonstrated improve NAFLD. Other drugs in early development are: obeticholic acid and GFT505 (11,33,39).

4 JUSTIFICATION

Non alcoholic fatty liver disease (NAFLD) is becoming a major public health problem due to its increase in its incidence and prevalence worldwide, along the epidemic of obesity and diabetes. It affects 25% of the adult population and increases year after year.

NAFLD is one of the main causes of chronic pathology of the liver and could become one of the main causes of liver transplantation in the future.

Although there has been a relationship between NAFLD and fibrosis, cirrhosis and hepatocellular carcinoma, one of the main causes of death among these patients are cardiovascular diseases.

NAFLD is characterized by insulin resistance and is strongly associated with type 2 diabetes and obesity. It is a marker of pathological ectopic fat accumulation combined with a low-grade chronic inflammatory state. This disease results in several deleterious pathophysiological processes, including abnormal glucose, fatty acid and lipoprotein metabolism, increased oxidative stress, deranged adipokine profile, hypercoaguablitiy, endothelial dysfunction, and accelerated progression of atherosclerosis.

The pathophysiology of atherosclerosis in NAFLD is not well established yet. There are many studies, especially In Western countries, showing the association of NAFLD with atherosclerosis and other cardiovascular disease. Nevertheless, these associations are not yet fully clarified and more studies of prevalence in other countries are needed, as well as causality studies.

5 HYPOTHESIS

The presence of moderate or severe steatosis in obese people with NAFLD could predict a higher value of carotid intima-media thickness (c-IMT) with greater cardiovascular risk.

6 OBJECTIVE

The main aim of this study is to investigate the prevalence of atherosclerosis in obese patients with non-alcoholic fatty liver disease (NAFLD) and whether such an association is independent of classical risk factors (type 2 diabetes mellitus, tobacco, metabolic syndrome, insulin resistance and hypertension).

7 PATIENTS AND METHODS

7.1. STUDY DESIGN

This study has been designed as a cross-sectional study in order to observe the association between NAFLD in obese people and atherosclerosis. We studied 400 consecutive obese subjects (282 women; mean age 45.6 ± 9.33 years; body mass index 44.3 ± 6.08 and 118 men; mean age 43.89 ± 9.28; body mass index 43.87 ± 6.22) from the Hospital Josep Trueta. They were recruited, from January 2010 to December 2014, in the *Institut D'investigació Biomédica de Girona* (IDIBGI) in Catalonia (Spain) for the ongoing multicenter FLORINASH Project. Their investigation was designed to evaluate the role of intestinal microflora in adults with NAFLD.

7.2. PARTICIPANTS

7.2.1. INCLUSION CRITERIA

- Age 25-65 years
- Body mass index (BMI)>30 kg/m2
- Ability to understand study procedures

7.2.2. EXCLUSION CRITERIA

- History of cardiovascular disease (coronary heart disease, stroke, heart failure, peripheral vascular disease or congenital heart disease).
- Infection in the previous month.
- Serious chronic illness.
- Ethanol intake >20 g/day in women or >30g/day in men.
- Use of medication that might interfere with insulin action.

7.3. SAMPLING

7.3.1. SAMPLE STRATIFICATION

Sample recruitment took place in the period from January 2010 to December 2014 (4 years and 11 months) and non-probabilistic consecutive sampling method was used. This sampling consisted of selecting patients who met the inclusion and exclusion criteria from the Endocrinology outpatient clinic of the Hospital Josep Trueta. Candidates received an explanation of the purpose and nature of all procedures (**ANNEX 1**) and were invited to participate voluntarily by signing the informed consent (**ANNEX 2**).

7.3.2. SAMPLE SIZE

To calculate the sample size, the free online application GRANMO was used, taking into account that the prevalence of atherosclerosis in obese people admitted to the Endocrinology department of Hospital Josep Trueta is around 25%.

Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test, 242 subjects were deemed to be necessary in the group with moderate or severe steatosis and 242 in the group with slight or without steatosis (1160 subjects in total). These sample numbers recognize as statistically significant an expected proportion difference of 0.12 in the group with moderate or severe steatosis and 0.22 in the group with slight or without steatosis. A drop-out rate of 10%, corresponding to incomplete data collection sheets has been anticipated in the calculation of sample numbers.

7.4. DATA COLLECTION, VARIABLES AND METHODS

Data was reported to the study database according to the Case Report Form (CRF) (**ANNEX 3**), in which all variables are included.

Data was collected by the research team by means of anthropometric measurements, vascular and abdominal ultrasonography. Tests conducted 8 hours later after measured blood for plasma lipids, glucose and insulin. Glucose and lipid levels were determined by standard laboratory methods. Serum insulin was measured in duplicate by a monoclonal immunoradiometric assay (Medgenix Diagnostics, Fleunes, Belgium). The intra-assay CV was 5.2% at a concentration of 10 mU/l and 3.4% at 130 mU/l. The interassay CVs were 6.9% and 4.5% at 14 and 89 mU/l, respectively.

7.4.1. Independent variable

- **Hepatic steatosis**: was graded as absent, mild, moderate or severe according to conventional criteria and evaluated by ultrasound (41,42). A Siemens Acuson S2000 (Mochida Siemens Medical System, Tokyo, Japan) ultrasound system with a 3.5 MHz convex transducer to scan the liver was used. Two radiologists, blinded to clinical and laboratory data, evaluated images independently.

7.4.2. Dependent variables

- **Atherosclerosis**: was considered present when c-IMT >0.8 mm and/or plaques were present:
  - c-IMT: Values >0.80 mm were considered increased. These measurements were manually measured in the far wall of each common carotid artery in a proximal segment and in a plaque-free segment 10 mm from the bifurcation. The mean c-IMT value for each subject was calculated from these four measurements
  - Plaque: defined as a focal thickening ≥ 1.2 mm in any of 12 carotid segments (near and far walls of the right and left common carotid arteries, bifurcation and internal carotid artery)
  - All carotid arteries were scanned by a Siemens Acuson S2000 (Mochida Siemens Medical System, Japan) ultrasound system with a 7.5 MHz linear array transducer. Images were independently evaluated by two radiologists blinded to clinical and laboratory data and were evaluated according to the Mannhem Consensus (43).

- **Framingham Risk Scores (FRS) Calculation**: were calculated using a standard score sheet that is gender specific and includes the following variables: age, blood pressure, total cholesterol, HDL cholesterol, smoking history and history of diabetes.
- The FRS sheet was used to estimate a **10-year probability of developing CHD** in our patients with NAFLD.

7.4.3. Confounding variables

In order to establish an independent association between steatohepatitis and the presence of atherosclerosis, we must take into account other possible variables that could be involved in the development of cardiovascular diseases:

- **Gender (Male/Female):** patients were stratified by sex in order to examine differences between these two groups in the association between NAFLD and carotid atherosclerosis.
- **Age (years)**
- **Body mass index (kg/m2)**
- **Waist circumference (cm)**
- **Total cholesterol (mg/dl)**
- **HDL cholesterol (mg/dl):**
- **Fasting trygliceride (mg/dl)**
- **Fasting glucose (mg/dl):**
- **Glucose AUC (mg/dl/min)**
- **Fasting insulin (mU/l)**
- **Insulin AUC (mU/l/min)**
- **HOMA-IR (mean ± SD):** insulin resistance was determined by the homeostasis model assessment of insulin resistance.
- **Serum aspartate aminotransferase (U/l), alanine aminotransferase (U/l) and gamma-glutamyltransferase (U/l):** were determined using enzimatic methods.
- **Ultrasensitive CRP (mg/dl)**
- **Systolic blood pressure (mmHg)**
- **Diastolic blood pressure (mmHg)**
- **Current smoking:** defined as any self-reported smoking (non smoker, former smoker >1 year, current smoker at least 1 cigarette/day last 6 months.

8 STATISTICAL ANALYSIS

Statistical analyses were performed with IBM SPSS statistics version 23.

1. Descriptive analysis

The results are expressed as percentages (%) or frequencies for categorical variables, as mean ± standard deviation (SD) for normal distribution quantitative variables and as median and interquartile range for quantitative variables without normal distribution.

The main variables (hepatic steatosis, cIMT, Framingham Risk Score, presence of plaques of atherosclerosis) have been represented graphically.
The qualitative and quantitative discrete variables have been represented in a bar diagram and circular charts, while the continuous quantitative variables have been represented with histogram.

It has been differentiated between men and women in the analysis of these variables.

2. Bivariate Analysis

It was used student's t-test to determine differences in quantitative variables, and the chi-square test to determine differences in qualitative variables related to the presence of atherosclerosis. A bivariate analysis was also made on the confounding variables to observe their possible contribution to the presence of atherosclerosis. To examine possible sex differences in the associations between NAFLD and carotid atherosclerosis, we also analyzed men and women separately.

HOMA-IR and fasting insulin/mU/l/min were expressed as median and interquartile range. A p value of <0.05 was considered statistically significant.

The differences showed in atherosclerosis measurements were represented in pie charts for qualitative variables and in error bars for quantitative variables.

3. Multivariate Analysis

Multiple linear regression analyses was used to explore the independent associations between NAFLD and all variables that showed significant differences in the bivariate analysis performed previously. Stratification by sex was performed. First, c-IMT was the dependent variable, whereas fatty liver and the rest of confounding variables were the independent variables. Then was used the atherosclerotic plaque and/or c-IMT> 0.8 as dependent variable. A p value of <0.05 was considered statistically significant.

8 RESULTS

1. Descriptive analysis

The prevalence of NAFLD in the 400 obese subjects (282 women; mean age 45.6 ± 9.33 years; body mass index 44.3 ± 6.08 and 118 men; mean age 43.89 ± 9.28; body mass index 43.87 ± 6.22) was 59,6%. There was some differences between sexes: in obese men, there were 22,9% of patients without steatosis, 25,4% with slight steatosis, 32,2% with moderate steatosis and 19,5% with severe steatosis. In obese women, there were 47,9% of patients without steatosis, 25,9% with slight steatosis, 18,8% with moderate steatosis and 7,4% with severe steatosis. (See **FIGURE 4**).

Carotid atherosclerosis (cIMT >0.8 or carotid plaque) was detected in 129 subjects (32,2%) (39,8% of men and in 29,1% of women). (See **FIGURE 5**).

The mean value of c-IMT was 0,60 ± 0,14 mm (0,66 ± 0,14 mm in men and 0,58 ± 0,14 mm in women) (See **FIGURE 6**).

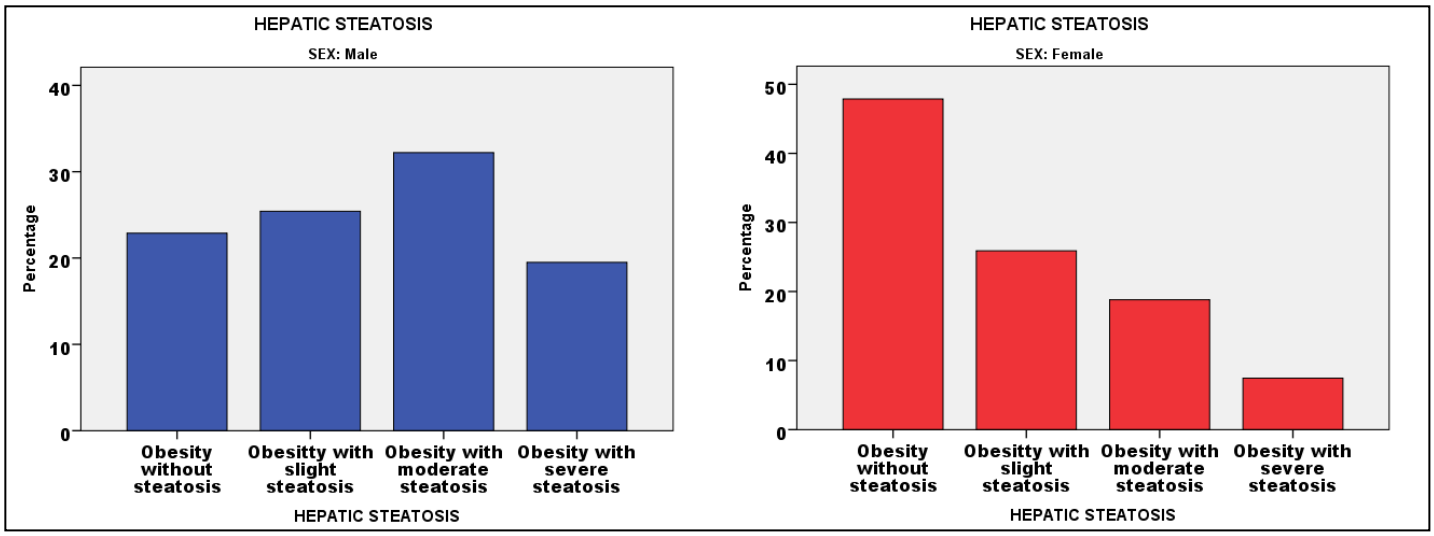


FIGURE 4. Prevalence of steatohepatitis between males and females.

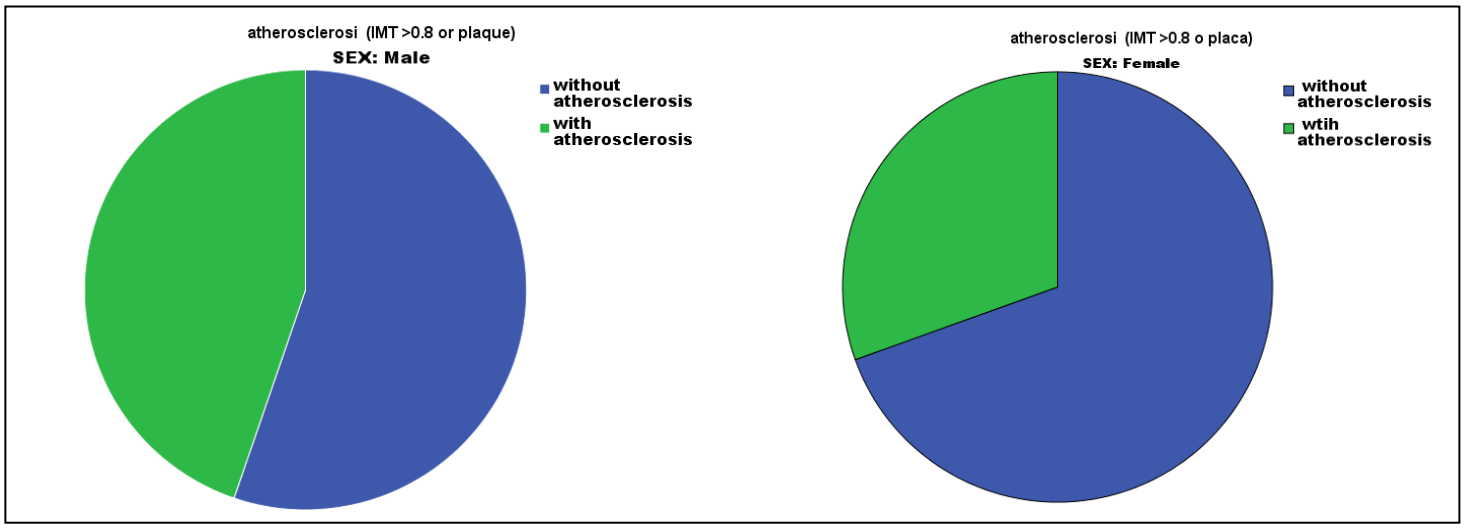


FIGURE 5. Prevalence of atherosclerosis between males and females.

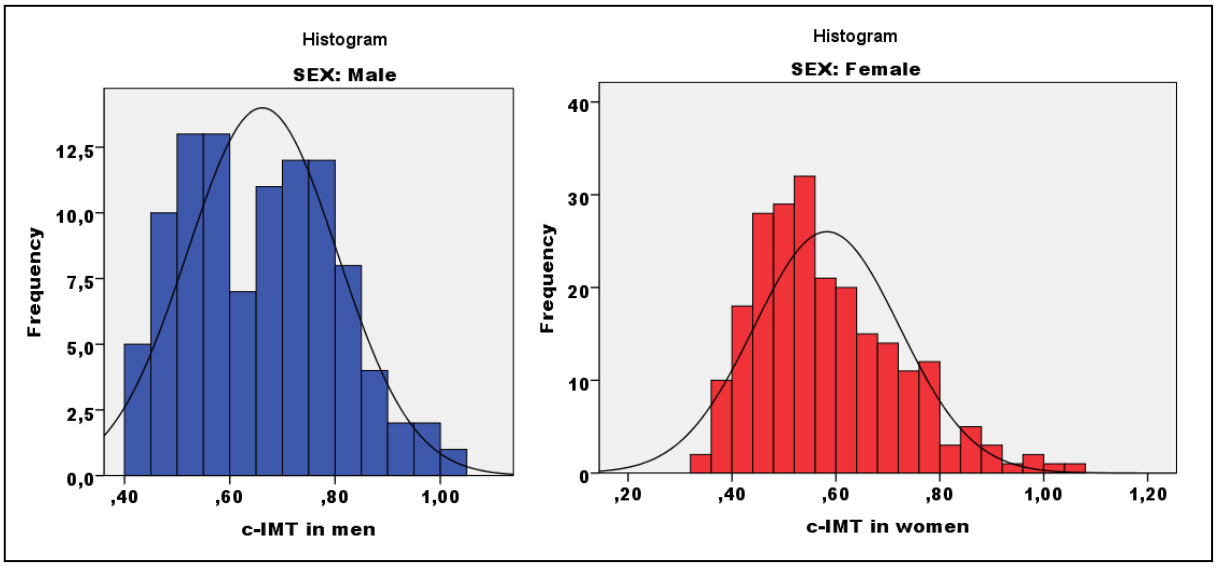


FIGURE 6. Histogram of c-IMT between males and females.

2. Bivariate Analysis

The sample was stratified by sex. C-IMT, Framingham 10-year risk score and atherosclerosis (c-IMT>0,8 and/or plaques) were significantly different according to the different stages of NAFLD in both men and women (See **TABLE 4 and FIGURE 5).**

Quantitative variables were expressed as mean ± standard deviation. In men, waist circumference, HDL cholesterol, aspartate aminotransferase and alanine aminotransferase were significantly different between groups while in women all confounding variables were significantly different between groups (See **TABLE 5).**

TABLE 4. Bivariate associations between NAFLD and atherosclerosis variables stratified by sex.

	MEN			WOMEN		
	HEPATIC STEATOSIS			HEPATIC STEATOSIS		
	Without steatosis or slight steatosis (n=57)	Moderate or severe steatosis (n=61)	p-value	Without steatosis or slight steatosis (n=208)	Moderate or severe steatosis (n=74)	p-value
Final mean value overall carotid segments (mm)	0,59 ± 0,11	0,71 ± 0,14	**<0,001**	0,53 ± 0,108	0,72 ± 0,14	**<0,001**
c-IMT >0.8 and/or atherosclerosis plaque (no/yes)*	37/14	21/33	**0,002**	167/33	20/49	**<0,001**
Framingham point score	9,93 ± 5,33	9,85 ± 5,55	0,938	10,65 ± 6,48	14,21 ± 5,74	**<0,001**
Calculated 10-year risk coronary heart disease risk (%)	10,65 ± 7,80	10,91 ± 8,65	0,870	2,78 ± 3,88	5,35 ± 6,64	**0,003**
Increased cIMT (>0.8 mm) (no/yes)*	46/9	34/27	**0,010**	191/16	38/35	**<0,001**

***These variables are represented in frequencies.

TABLE 5. Bivariate associations between NAFLD and confounding variables with gender stratification.

	MEN			WOMEN		
	HEPATIC STEATOSIS			HEPATIC STEATOSIS		
	Without steatosis or slight steatosis (n=57)	Moderate or severe steatosis (n=61)	p-value	Without steatosis or slight steatosis (n=208)	Moderate or severe steatosis (n=74)	p-value
Age (years)	43,9 ± 9,6	43,8 ± 8,9	0,933	44,0 ± 9,5	49,9 ± 7,1	**<0,001**
Body mass index (kg/m2)	43,3 ± 6,1	44,4 ± 6,32	0,346	43,8 ± 6,2	45,6 ± 5,4	**0,033**
Waist circumference (cm)	128,1 ± 13,7	135,1 ± 11,1	**0,004**	119,5 ± 14,2	125,2 ± 11,1	**0,001**
Systolic blood pressure (mmHg)	141,6 ± 15,8	143,2 ± 19,9	0,629	136,3 ± 18,6	146,1 ± 21,0	**<0,001**
Diastolic blood pressure (mmHg)	81,1 ± 9,44	84,1 ± 18,35	0,263	74,96 ± 11,3	79,5 ± 10,54	**0,003**
Total cholesterol (mg/dl)	192,4 ± 36,3	191,8 ± 32,9	0,929	192,6 ± 33,9	202,6 ± 37,2	**0,033**
HDL cholesterol (mg/dl)	43,2 ± 7,48	39,9+-7,06	**0,017**	50,1 ± 11,45	46,9 ± 12,54	**0,048**
Fasting tryglycerides (mg/dl)	128,5 ± 59,1	150,2 ± 65,8	0,065	115,0 ± 58,3	141,3 ± 61,0	**0,001**
Fasting glucose (mg/dl)	97,5 ± 28,66	96,6 ± 16,74	0,845	93,36 ± 16,3	107,6 ± 25,2	**<0,001**
Glucose AUC (mg/dl/min)	17360,6 ± 3920,1	18497,7 ± 3528,2	0,124	16661,7 ± 3678,6	19997,3 ± 4297,7	**<0,001**
Fasting insulin (mU/l/min)*	16,2 (10,6-20,4)	16,10 (9,2-21,7)	0,408	10,7 (6,95-16,05)	10,8(6,35-17,08)	**0,001**
Insulin AUC (mU/l/min)	11277,1 ± 6298,7	13335,9 ± 8755,3	0,198	8022,0 ± 4907,07	9572,45 ± 5511,8	**0,051**
HOMA-IR (mean +-SD)*	3,98 (2,51-5,21)	3,6 (2,25-5,28)	0,830	2,53 (1,60-3,84)	2,57 (1,47-4,25)	**0,004**
Aspartate aminotranferase (U/l)	24,17 ± 9,25	28,45 ± 12,31	**0,044**	20,21 ± 7,91	25,05 ± 12,32	**0,004**
Alanine aminotransferase (U/l)	30,71 ± 19,03	44,52 ± 24,32	**0,001**	21,84 ± 13,45	30.99 ± 16,16	**<0,001**
Gamma-glutamyltranferase (U/l)	34,13 ± 18,90	36,00 ± 20,050	0,605	26,42 ± 25,70	39,07 ± 34,61	**0,005**
Ultrasensitive CRP (mg/dl)	0,65 ± 0,50	0,85 ± 0,70	0,086	0,93 ± 0,78	1,18 ± 0,91	**0,041**
Current smoking (no/yes)	36/21	35/26	0,387	166/42	59/15	0,097

* These variables were measured with median and interquartile range

3. Multivariate Analysis

Multiple linear regression analyses were performed to analyze the possible independent associations between NAFLD and the variables that showed significant differences in bivariate analysis. Stratification by sex was performed.

First of all, with c-IMT value as the dependent variable, and fatty liver and the rest of confounding variables as the independent variables. Hepatic steatosis, age and cholesterol levels (See **TABLE 6**) independently predicted cIMT values in men, whereas hepatic steatosis, age, systolic blood pressure and diastolic blood pressure independently predicted cIMT values in women (See **TABLE 7**).

Finally, in another model of multiple regression analysis with c-IMT >0,8 and/or atherosclerosis plaques as dependent variable. Age and fasting glucose but not hepatic steatosis independently predicted atherosclerosis in men (See **TABLE 8**) while in women, age and hepatic steatosis independently predicted atherosclerosis (See **TABLE 9**).

TABLE 6. Lineal regression analyses in men. C-IMT was the dependent variable.

Predictors	Non-standardized coefficients		Standardized coefficients	t	Sig.
	B	Standard error	Beta		
(Constant)	-,109	,221		-,491	,625
Hepatic steatosis	,048	,015	,353	3,227	**,002**
Patient age	,007	,002	,477	4,388	**,000**
BMI	-,001	,004	-,056	-,376	,708
Waist circumference	,001	,002	,058	,419	,676
SBP	,001	,001	,073	,518	,606
DBP	,002	,002	,167	1,179	,243
Cholesterol	,001	,000	,295	2,753	**,008**
HDL Cholesterol	-,002	,002	-,091	-,855	,396
Triglycerides	,000	,000	-,105	-,866	,390
Glucose	,000	,002	-,033	-,273	,786
Area under curve SOG glucose	2,316E-6	,000	,053	,409	,684

area under curve SOG insulin	-2,513E-6	,000	-,135	-1,205	,233
HOMA-IR	,003	,003	,110	,990	,326
Aspartate aminotransferase	-,001	,003	-,082	-,368	,714
Alanine aminotransferase	,000	,001	,070	,313	,756
Gamma-glutamyltranferase	,000	,001	-,067	-,577	,566
Ultrasensitive CRP	,020	,028	,074	,724	,472

TABLE 7.Lineal regression analyses in women. C-IMT was the dependent variable.

	Non-standardized coefficients		**Standardized coefficients**		
Predictotrs	B	Standard error	Beta	**t**	**Sig.**
(Constante)	,230	,109		2,104	,037
Hepatic steatosis	,068	,010	,467	6,952	**,000**
Patient age	,005	,001	,345	5,606	**,000**
BMI	-,002	,002	-,064	-,834	,406
Waist circumference	,000	,001	,048	,638	,525
SBP	,001	,001	,211	2,811	**,006**
DBP	-,002	,001	-,181	-2,543	**,012**
Cholesterol	,000	,000	,045	,713	,477
HDL Cholesterol	-,001	,001	-,102	-1,552	,123
Triglycerides	-5,263E-5	,000	-,023	-,335	,738
Glucose	,000	,001	-,011	-,128	,899
area under curve SOG glucose	3,028E-6	,000	,092	1,189	,236
area under curve SOG insulin	-9,985E-7	,000	-,037	-,566	,572
HOMA-IR	-,001	,003	-,033	-,427	,670
Aspartate aminotransferase	,001	,001	,048	,517	,606
Alanine aminotransferase	7,620E-5	,001	,008	,084	,933
Gamma-glutamyltranferase	7,215E-5	,000	,016	,262	,794
Ultrasensitive CRP	,012	,010	,073	1,288	,200

TABLE 8.Lineal regression analyses in men. Carotid atherosclerosis (cIMT>0.8 and/or plaques) was the dependent variable.

Predictors	**Non-standardized coefficients**	**Standardized coefficients**	**t**	**Sig.**

	B	Standard error	Beta		
(Constant)	-1,491	,853		-1,748	,086
Hepatic steatosis	,079	,058	,169	1,369	,176
Patient age	,031	,006	,598	5,048	**,000**
BMI	-,002	,014	-,027	-,172	,864
Waist circumference	,001	,006	,018	,114	,909
SBP	,003	,004	,105	,673	,504
DBP	,005	,006	,132	,844	,402
Cholesterol	,003	,002	,213	1,767	,083
HDL Cholesterol	-,001	,008	-,022	-,189	,851
Triglycerides	,001	,001	,123	,928	,358
Glucose	-,014	,006	-,304	-2,324	**,024**
area under curve SOG glucose	1,587E-5	,000	,104	,760	,450
area under curve SOG insulin	-1,258E-5	,000	-,194	-1,563	,124
HOMA-IR	,020	,010	,224	1,872	,067
Aspartate aminotransferase	-,005	,010	-,109	-,474	,637
Alanine aminotransferase	,005	,005	,241	1,005	,319
Gamma-glutamyltranferase	-,001	,003	-,054	-,425	,672
Ultrasensitive CRP	,038	,100	,042	,375	,709

TABLE 9.Lineal regression analyses in women. Carotid atherosclerosis (cIMT>0.8 and/or plaques) was the dependent variable.

	Non-standardized coefficients		Standardized coefficients		
Predictors	B	Standard error	Beta	**t**	**Sig.**
(Constant)	-,405	,390		-1,037	,301
Hepatic steatosis	,225	,034	,484	6,595	**,000**
Patient age	,017	,003	,337	5,011	**,000**
BMI	-,016	,007	-,204	-2,394	**,018**
Waist circumference	,004	,003	,132	1,559	,121
SBP	,002	,002	,073	,845	,399
DBP	-8,188E-5	,003	-,002	-,027	,979
Cholesterol	-,001	,001	-,043	-,618	,537
HDL Cholesterol	-,003	,003	-,080	-1,112	,268
Triglycerides	,000	,001	,047	,629	,530
Glucose	,001	,003	,021	,217	,828

area under curve SOG glucose	-6,941E-6	,000	-,064	-,747	,456
area under curve SOG insulin	-2,811E-6	,000	-,032	-,446	,657
HOMA-IR	,012	,013	,080	,931	,353
Aspartate aminotransferase	,000	,005	,004	,043	,966
Alanine aminotransferase	,001	,003	,045	,423	,673
Gamma-glutamyltranferase	-,001	,001	-,097	-1,455	,147
Ultrasensitive CRP	,030	,035	,055	,878	,381

10 DISCUSSION AND CONCLUSION

In this study, was examined NAFLD in obese subjects as a predictor of atherosclerosis among other anthropometric and metabolic parameters of cardiovascular risk, reflected in c-IMT and plaques in ultrasounds of obese adults. Framingham 10-years risk score was also evaluated.

NAFLD represents an emerging public health problem worldwide, affecting around 25% of the adult population in the world. The prevalence of NAFLD varies widely according to the population studied and definition used. In general, the prevalence of NAFLD reaches 50-80% in obese subjects, 40-70% in patients with metabolic syndrome and 20-40% in type 2 diabetes mellitus(17,29). This evidence was consistent with the results of the present study, performed in a general obese subjects who underwent health examination and found that 59,6% of participants had NAFLD disease.

Although NAFLD can progress to liver cirrhosis and hepatic carcinoma, the majority of deaths in patients with NAFLD are related to CVD (13). Abnormal findings of noninvasive markers for subclinical atherosclerosis in NAFLD may reveal an underlying link between NAFLD and CVD. The exact role of NAFLD in the pathogenesis of atherosclerosis is unclear, but some studies reported that abnormal glucose, fatty acid and lipoprotein metabolism, increased oxidative stress, endothelial dysfunction, subclinical inflammation and other metabolic abnormalities are probably involved(36). The data regarding

relationships among markers for subclinical atherosclerosis are limited and inconsistent. Furthermore, the causal association between NAFLD and CVD is rather weak (44). Moreover, it is unclear which atherosclerotic marker is more clinically meaningful in patients with NAFLD.

There are some studies showing the higher c-IMT and carotid plaques prevalence between NAFLD population (45,46) . In obesity there are other factors that could affect c-IMT, including type 2 diabetes, insulin resistance and secreted proinflammatory factors like free fatty acids, adiponectin, tumour necrosis factor-α or other adipocytokines (47). In addition, other studies have shown that in healthy patients, the association between atherosclerosis and steatosis is not very significant, after adjusting for waist circumference and BMI, which could mean that atherosclerosis, is not independent risk marker for atherosclerosis(48). This could be due to excess adiposity.

Detecting early vascular damage is important in patients with NAFLD and our finding that steatosis has a strong association with atherosclerosis, indirectly supports previous studies suggesting that hepatic fat accumulation itself could be atherogenic (36) .

According to our results, NAFLD and age were the best predictors of atherosclerosis using c-IMT, in both obese men and women. NAFLD in obese women could predict not only c-IMT but also the presence of atheroma plaques. These findings add to those reported by Kunutsor *et al*. (49), who demonstrate that there are age interactions in the association of NAFLD with the risk of CVD in a general Caucasian population.

This study almost shows differences between sexes. On the one hand, not only NAFLD and age, but also cholesterol levels were the best predictors of atherosclerosis measured with c-IMT in men, whereas NAFLD, age, systolic blood pressure and diastolic blood pressure independently predicted c-IMT in women. These results supports previous studies showing the association of non-alcoholic fatty liver with hypertension and dyslipidemia, two components of the metabolic syndrome (50,51).

In conclusion, this study shows that NAFLD and age, both in men and women, are independent predictors of atherosclerosis. This leads to the

importance of ultrasound evaluation in patients with this disease, especially middle-age patients.

12 LIMITATIONS AND BIASES

Before interpreting the results of this study, some limitations have to be commented.

This study could be influenced by a significant selection bias due to subjects were recruited from a study (FLORINASH) with a different primary outcome objective (evaluate the role of intestinal microflora in adults with NAFLD).

As this study has a cross-sectional design it cannot establish causal inferences. Only a longitudinal approach could determine the real effects of steatosis on carotid atherosclerosis.

Another limitation is data collection. It was used ultrasound to diagnose NAFLD. This technique is dependent operator and normally detects NASH only when more than 30% of the hepatocytes are affected by fat. Ultrasound was also used to measure atherosclerosis (c-IMT and plaque). In order to minimize the inter-observer variability, all US images were recorded and examined by the same professional.

Taking into account the sample size measured before, a larger sample might enable other robust indicators of atherosclerosis in obese adults to be identified. Furthermore the number of men being studied appears to be disproportionately small to arrive at definite conclusion.

It was used a case report form in each visit (**ANNEX 1**) in order to minimize the interviewer bias.

In this study was consider c-IMT as a surrogate of atherosclerosis. It has been associated with prevalent and incident cardiovascular disease but is clearly not the same.

Finally, the age range of the study subjects was relatively high. Therefore, the influence of disease related variables like obesity would be very different depending on the duration of disease exposure.

13 ETHICAL CONSIDERATIONS

This study respects all ethical considerations and human rights reflected in the Declaration of Helsinki for medical research involving human subjects, developed by the World Medical Association (WMA).

All personal and clinical information will remain confidential and only used for the purpose of the research according to the "Ley Organica 15/1999, del 13 de diciembre, de Protección de Datos de Carácter Personal".

The "Ley 14/2007, del 3 de Julio, de investigación biomedica" concerning medical investigations has also been strictly followed.

Informed consent has been obtained from each subject (**ANNEX 2**) after full explanation of the purpose and nature of all procedures used (**ANNEX 1**).

In order to carry out this study it was necessary to present the research protocol to the Clinical research Ethics Committee (CEIC) of Hospital Universitari Josep Trueta.

Participants have the right to access, modify, oppose or remove their personal data.

The investigators of this project declare that there are no conflicts of interest, and that they didn´t receive any economic compensation to collaborate in the study.

14 CLINICAL AND HEALTHCARE IMPACT

NAFLD is a common cause of liver disease worldwide, which warrants the attention of primary care physicians, specialists, and health policy makers. It is consider "the next global epidemic" due to its strong relationship with obesity, insulin resistance and type 2 diabetes mellitus. The incidences of these pathologies are increasing and are concerns for significant health and economic impacts of these disorders.

Furthermore, the large number of NAFLD patients with potential for progressive liver disease and cardiovascular diseases creates challenges for screening.

It is important to know more about the pathophysiology of NAFLD and its relation with other pathologies such as atherosclerosis in order to carry out new diagnostic and therapeutic methods. Further studies are needed to support this relationship between NAFLD and cardiovascular pathologies, among others, to advance research

15 FEASIBILITY

This Project has been possible thanks to the availability of the FLORINASH database, which has been the main source of all the analyzed data.

FLORINASH project generated two cohorts of several hundred obese patients from Spain and Italy, extensively characterized from a metabolic and cardiovascular point of view and biological samples were collected.

The general objective of FLORINASH was to increase, disseminate, and gain new knowledge about the biological processes and mechanisms of the host to microbiota cross talk responsible for the development of NAFLD.

FLORINASH has thus been carried out in order to address the need to find a tool to predict the risk of liver diseases such as non-alcoholic fatty liver disease (NAFLD) in the context of metabolic disease. Indeed, as an important impact of chronic metabolic disease is the progression to organ complications such as non-alcoholic hepatic steatosis (NASH), the early and accurate prediction and diagnosis of NAFLD may prevent the occurrence of NASH and liver cancer, finally reducing the cost of treatment.

Clinical data (phenotype) and samples from participants from two different European countries (Spain and Italy) served and will serve in future to generate a set of conventional and new metabolic biomarkers suitable for the prediction of NAFLD in the context of metabolic disease. As mentioned above, such metabolic biomarkers can or could be used as diagnostic tools and/or (potentially) therapeutic targets.

16 ANNEXES

ANNEX 1: INFORMATION SHEET

FLORINASH STUDY

PATIENT INFORMATION SHEET

Obesity is a chronic disease whose frequency is increasing dramatically in western countries, both among adults and children. In our country, already affects 14.5% of people. It is well known that obesity, especially severe or morbid obesity is associated with many other diseases such as hypertension, diabetes, elevated cholesterol levels and plasmatic triglycerides, cardiovascular diseases and “Fatty Liver Disease”.

We suggest participating in a European study, in which our hospital is participating and that aims to study the processes and biological mechanisms responsible for the so-called "fatty liver disease". In this way, it aims to improve prevention through diagnosis that does not mean risks to the patient (predictive diagnostic methods and markers of liver damage), effective drug design and study the role of bacterial flora in the development of this disease that can lead to liver failure and premature death.

The study will be conducted in two visits including a detailed medical history, especially collecting information about diseases associated with their obesity, anthropometric, clinical and biochemical features, a complete physical examination including a study body composition (to know your percentage of body fat), blood sample collection (in order to identify markers of liver damage), feces and urine samples (to evaluate the role of bacterial flora in the development of this disease), measuring the stiffness of their arteries by ultrasound, realization of liver ultrasound to assess the fat content of liver and a test to study the metabolism of glucose and insulin resistance will be performed; this test has to be carried out in fasting state. It is advisable to eat food rich in

sugars, flours and starches (potatoes, pasta, rice, bread) during 3 days before and avoid strenuous exercise, consume caffeine or alcohol these days.

A blood way will be put in an arm vein and an intravenous glucose and insulin solution will be continuously administered, doing blood extractions through a track lying on the other arm every 5 minutes until the end of the study (2 hours). Throughout the test it will carried out strict control of blood sugar levels.

If you have morbid obese an operation named gastric bypass will be suggested. During this operation the surgery will collect liver samples using specific tools with the aim of study histological characteristics of the fat, the inflammation and the fibrosis.

You donate a part of these samples with this aim. Samples won't be used to another purpose, and it won't become commercialize, it will be used to evaluate diagnostic capacity in a new test, and to research in more effective therapeutics targets. These samples will be used to this purpose and then destroyed.

We will give you the option of give your consent to keep your samples and be used in other studies related to the aim of this study, the research of targets to other metabolic diseases. These samples will be kept in the Biobanc of the Institute of Biomedical Research (IdiBGi) during 5 years.

You probably will not get any direct benefit from participating in this project, but will have the satisfaction of helping others in the future, because your participation may help in early diagnosis of the disease by reducing the use of other tests more uncomfortable for the patient and to design more effective therapeutic treatments.

All information collected will be analyzed and will be kept confidential and anonymously, according to current legislation on protection of personal data (ORGANIC Law 15/1999, of December 13 to protect personal data).

All data will be identified by a code and only your doctor study and colleagues will be able to relate these data with you and your medical history. Therefore your identity will not reveal to anyone except legal requirement.

Furthermore, these data in no case will contain information that directly could identify you, such as name, address, etc...

In agreement with the provisions of this legislation, you can exercise your right of access, modification, opposition and cancellation of data and destruction of samples by contacting your doctor study without giving any explanation and without imposing any detriment in your medical care.

If you want to ask any questions about the study, please contact by telephone with Dr. José Manuel Fernández-Real at the Hospital Dr.Josep Trueta phone number: 972940200, asking for 2656 extension.

Thank you so much for your collaboration.

ESTUDIO FLORINASH

HOJA DE INFORMACIÓN AL PACIENTE

La obesidad es una enfermedad crónica cuya frecuencia está aumentando de forma alarmante en los países occidentales, tanto entre la población adulta como infantil. En nuestro país afecta ya a un 14,5% de las personas. Es bien conocido que la obesidad, especialmente la obesidad grave o mórbida se asocia a muchas otras enfermedades, como son la hipertensión arterial, la diabetes, la elevación de los niveles de colesterol y de triglicéridos en sangre, las enfermedades cardiovasculares y la “Enfermedad del Hígado Graso”.

Le proponemos participar en un estudio europeo, en el que nuestro hospital participa y que tiene como objetivo estudiar los procesos y mecanismos biológicos responsables de la denominada “enfermedad del hígado graso”. De de esta manera, se pretende potenciar la prevención mediante diagnósticos que no representan riesgos para el paciente (métodos predictivos de diagnóstico y marcadores de daño hepático), diseño de medicamentos eficaces y estudiar el papel de la flora bacteriana en el

desarrollo de esta enfermedad que puede conducir a un fallo hepático y muerte prematura.

El estudio se llevará a cabo en dos visitas y consiste en la realización de una historia clínica detallada, recogiendo especialmente información acerca de las enfermedades asociadas a su obesidad, datos antropométricos, clínicos y bioquímicos, una exploración física completa que incluye la realización de un estudio de composición corporal (para conocer su porcentaje de grasa corporal), recogida de muestra de sangre (con el fin de identificar marcadores del daño hepático), heces y orina (para evaluar el papel de la flora bacteriana en el desarrollo de esta enfermedad) , medición de la rigidez de sus arterias mediante ecografía , la realización de una ecografía hepática para evaluar el contenido de grasa de su hígado y se le realizará una prueba para estudiar el metabolismo de la glucosa y su resistencia a la insulina; para la realización de esta prueba tendrá que venir en ayunas. Es aconsejable que consuma alimentos ricos en azúcares, harinas y almidones (patatas, pasta, arroz, pan) durante los 3 días previos, así como no realizar ejercicio físico intenso y no consumir cafeína o alcohol en estos días. Se le colocará una vía en una vena del brazo y se le administrará de forma continuada una solución de glucosa endovenosa y de insulina realizando extracciones de sangre a través de una vía colocada en el otro brazo cada 5 minutos hasta el final del estudio (unas 2 horas). Durante toda la prueba se llevará a cabo un control estricto de los niveles de azúcar en sangre.

Si usted presenta obesidad grave, se le propondrá además una intervención quirúrgica denominada “bypass” gástrico. Durante este procedimiento quirúrgico el cirujano recogerá muestras de hígado utilizando las herramientas apropiadas con el fin de estudiar las características histológicas de la grasa, la inflamación y fibrosis.

Usted cede parte de las muestras para el fin anteriormente expuesto. Las muestras no se utilizarán para ningún otro fin, ni se comercializarán con ellas, sino que servirán para evaluar la capacidad diagnóstica de un nuevo test y la búsqueda de dianas terapéuticas más eficaces . Estas muestras serán utilizadas con el fin expuesto y posteriormente destruidas.

Se le dará también la opción de dar su consentimiento para guardar sus muestras y ser utilizadas en estudios posteriores estrechamente relacionados con el propósito de este estudio, la búsqueda de dianas para otras enfermedades metabólicas. Estas muestras se guardarán en el Biobanco del Institut de Recerca Biomédica del IdiBGi durante 5 años.

Probablemente usted no obtendrá ningún beneficio directo por participar en este proyecto, pero si la satisfacción de ayudar a otras personas en el futuro, puesto que esta participación podría ayudar en el diagnóstico temprano de la enfermedad disminuyendo el uso de otras pruebas más incómodas para el paciente y de diseñar tratamientos terapéuticos más efectivos.

Toda la información recogida se analizará y será guardada de forma confidencial y anónima, de acuerdo con la legislación vigente de protección de datos personales (Ley ORGÁNICA 15/1999, de 13 de Diciembre de protección de datos de carácter personal).

Todos los datos estarán identificados mediante un código y sólo su médico del estudio y colaboradores podrán relacionar dichos datos con usted y con su historia clínica. Por lo tanto su identidad no será revelada a persona salvo requerimiento legal.

Además, con estos datos en ningún caso contendrán información que le pueda identificar directamente, como nombre, apellidos, dirección ,etc…

De acuerdo con lo que establece la legislación mencionada, usted puede ejercer los derechos de acceso, modificación, oposición y cancelación de los datos y destrucción de las muestras dirigiéndose a su médico del estudio sin tener que dar ninguna explicación y sin que suponga ningún perjuicio en su asistencia médica.

Si quiere hacer cualquier pregunta respecto al estudio, puede contactar telefónicamente con el Dr José Manuel Fernández-Real en el teléfono del Hospital Univeritario de Girona Dr. Josep Trueta: 972940200, pidiendo que le pasen con la extensión 2656.

Muchas gracias por su colaboración.

ANNEX 2: INFORMED CONSENT

FLORINASH STUDY

INFORMED CONSENT

Project title: "**The role of intestinal microflora in non-alcoholic fatty liver disease (NAFLD)"**

I (full name)……..…….....

I have received enough information about the study.

I could ask about the study.

My questions have been answered satisfactorily.

I have spoken with (name of investigator)……..…….....

I understand that my participation in the study is voluntary.

I understand that I can withdraw from the study:

1. At the moment I want.
2. Without giving any explanation.
3. Without incurring any difference in my medical assistance.

Thus, I agree to participate in this study.

Signature of Participant

Signature of investigator that has informed

I consent for samples to be stored and used in further studies.

Girona, at ……of…….. of 2011

ESTUDIO FLORINASH

CONSENTIMIENTO INFORMADO

Título del proyecto: "**The role of intestinal microflora in non-alcoholic Fatty liver disease (NAFLD)"**

Yo, (nombre y apellidos)……

He recibido información suficiente sobre el estudio.

He podido preguntar sobre el estudio.

Se han respondido mis preguntas de forma satisfactoria.

He hablado con: (nombre del investigador)……...

Comprendo que mi participación en el estudio es voluntaria.

Comprendo que me puedo retirar del estudio:

1. En el momento que lo desee
2. Sin tener que dar ningún tipo de explicación
3. Sin que suponga ninguna diferencia en mi asistencia médica.

Así, doy mi conformidad para participar en este estudio.

Firma del participante

Firma del investigador que ha informado

¦**Doy mi consentimiento para que las muestras sean guardadas y utilizadas en estudios posteriores.**

Girona, a ……. de …….. de 2009

ANNEX 3: CASE REPORT FORM

INCLUSION CRITERIA

1. **Aged 25 to 60 years** Yes ☐ No ☐

2. **IMC >35 kg/m^2** Yes ☐ No ☐

3. **Ability to understand study procedures** Yes ☐ No ☐

4. **Written informed consent obtained from the patient** Yes ☐ No ☐

EXCLUSION CRITERIA

1. **Systemic disease (rheumatoid arthritis, Crohn disease...)** Yes ☐ No ☐
2. **Clinical symptoms and signs of infection in the previous month** Yes ☐ No ☐
3. **Serious chronic associated illness** Yes ☐ No ☐
4. **Alcohol consumption (men: 80 gr/day ; women: 40 gr/day)** Yes ☐ No ☐
5. **Use of medications able to interfere with insulin action (metformin, glitazones, DPPIV inhibitors, sulfonylurea, insulin)** Yes ☐ No ☐

Written informed consent obtained on : _____/_____/_____

VISIT DATE: _____ / _____ / _____

DEMOGRAPHY

Date of birth: |_____|_____|_____|

(DD/MMM/YYYY)

Sex □ **Male**

□ **Female**

MEDICAL HISTORY

DESCRIPTION	Onset date	End date	Current?
			Y N
			Y N
			Y N
			Y N
			Y N
			Y N
			Y N
			Y N
			Y N
			Y N
			Y N
			Y N

FAMILY HISTORY OF OBESITY

Father □ Y □N □Unk

Mother □ Y □N □Unk

Brothers □ Y □N □Unk

TOBACCO

☐ Current smoker (at least 1 cigarette/day in the last 6 months) ☐ Ex-smoker (> 1 year)

VITAL SIGNS

Height: _____ cm Weight: _____ kg

BMI: _____ Kg/m^2

Waist circumference: _____ cm Hip circumference: _____cm

BP (seated): _____/_____ mmHg Pulse: _____ bpm

BODY COMPOSITION

Fat mass: _____ Fat free mass: _____

Performed by:

□ bioelectric impedance □ air plethysmography □ DEXA

PHYSICAL EXAMINATION

Is there any abnormality? □ Yes □ No

Specify abnormalities:

1. _____
2. _____
3. _____
4. _____

STUDY PROCEDURES

1. **INTIMA MEDIA THICKNESS :**

Date : _____/_____/_____ **IMT: _____**

2. **HEPATIC ECOGRAPHY:**

Date : _____/_____/_____ **Content of fat: _____**

Comments:

_____

_____

_____

_____

_____

_____

_____

LABORATORY

¿A blood sample has been collected in this visit? □ Y □ N *

*Reason:_____

¿Fasting?**:** □**Yes** □**No** Hours of fasting: _____

Last intake: Date: _____/_____/_____ Time: _____:_____
(DD/MMM/YYYY) (00:00-23:59)

Sample date: _____/_____/_____ Sample time: _____:_____
(DD/MMM/YYYY) (00:00-23:59)

¿ A urine sample has been collected in this visit? □ Y □ N *

*Reason:_____

Sample date: _____/_____/_____ Sample time: _____:_____
(DD/MMM/YYYY) (00:00-23:59)

¿A faeces sample has been collected in this visit? □ Y □ N *

*Reason:_____

Sample date: _____/_____/_____ Sample time: _____:_____
(DD/MMM/YYYY) (00:00-23:59)

OGTT:

75 g de glucose intake time: _____:_____

(00:00-23:59)

Glucose	**0 min:**	_____	Units: _____
	30 min:	_____	Units: _____
	60 min:	_____	Units: _____
	120 min:	_____	Units: _____
Insulin	**0 min:**	_____	Units: _____
	30 min:	_____	Units: _____
	60 min:	_____	Units: _____
	120 min:	_____	Units: _____

HEMATOLOGY

Red blood cells:	_____	Units: _____
Hemoglobin:	_____	Units: _____
Hematocrit:	_____	Units: _____
Platelets:	_____	Units: _____
Total WBC:	_____	Units: _____
Neutrophils:	_____	Units: _____
Eosinophils:	_____	Units: _____
Basophils:	_____	Units: _____
Lymphocytes:	_____	Units: _____
Monocytes:	_____	Units: _____

CHEMISTRY

HbA_{1c}: _____ Units: _____

Glucose _____ Units: _____

Cholesterol: _____ Units: _____

HDL Cholesterol: _____ Units: _____

LDL Cholesterol: _____ Units: _____

Triglycerides: _____ Units: _____

Uric acid: _____ Units: _____

Creatinine: _____ Units: _____

GOT: _____ Units: _____

GPT: _____ Units: _____

GGT: _____ Units: _____

Bilirrubin: _____ Units: _____

Total protein: _____ Units: _____

Albumin: _____ Units: _____

LBP: _____ Units: _____

Ferritin: _____ Units: _____

CRP ultrasensitive: _____ Units: _____

Erythrocyte sedimentation rate: _____ Units: _____

TSH: _____ Units: _____

Free T4 (thyroxine): _____ Units: _____

Cortisol: _____ Units: _____

CONCOMITANT MEDICATION

Trade name	Daily dose/Units	Route*	Start date	End date	Ongoing? Y /N	Indication

*1=Oral; 2= Subcutaneous ; 3=Intravenous; 4=Topic; 5=Inhalated; 6=Intramuscular; 7=Rectal; 8=Transdermic; 9=Other

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