UNIVERSITÀ
DEGLI STUDI
DI PADOVA

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CURRICULUM: Clinical Methodology, Metabolism, Endocrinology, Nephrology and Exercise
SERIES **XXXIV**

NON-ALCOHOLIC FATTY LIVER DISEASE IN OBESITY AND ALSTRÖM SYNDROME, AN ULTRA-RARE GENETIC MODEL FOR METABOLIC DISEASE

Thesis written with the financial contribution of Progetti di Rilevante Interesse Nazionale (PRIN) from the Italian Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) grant number 2017L8Z2EM to Prof. Roberto Vettor.

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ABSTRACT

Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) is one of the main comorbidities in patients with obesity (PwO). Alström syndrome (ALMS) is an ultra-rare monogenic disease representing a model of metabolic disease characterized by obesity, type 2 diabetes mellitus (T2DM), early incidence of NAFLD and multi-organ fibrosis. Although liver biopsy is considered the gold standard for NAFLD diagnosis, it is not recommended for screening in the general population and repeated follow-up. Thus, the usage and validation of imaging techniques coupled with non-invasive tests (NITs) are strongly required. Bariatric surgery is the most effective therapy for obesity and several studies showed that it can improve NAFLD. We study PwO before and after surgery weight loss analysing NAFLD outcomes by 2D-Shear Wave Elastography (SWE) and different NITs in a short (6 months, V6m) and long-term (4 years, V4y) follow-up. Moreover, we evaluated NAFLD and NITs in patients with ALMS (PwA), analysing the different contribution of metabolic and genetic alterations in NAFLD progression.

Methods

34 PwO, 18 PwA genetically characterized, and 25 controls were enrolled and underwent a complete clinical evaluation before and after bariatric surgery. Anthropometric data, biochemical parameters, inflammatory markers and fibrosis indexes [Fibrosis-4 Index (FIB-4), AST on ALT ratio (AST/ALT), AST-to-platelet ratio index (APRI)] were collected. In PwO Enhanced Liver Fibrosis test (ELF), the caspase-cleaved fragments of keratin 18 (M30 K18) and Fibroblast Growth Factor-21 (FGF21) levels were measured. Liver fibrosis and steatosis was quantified using SWE, by the estimation of liver stiffness (LS) and comparing liver and kidney parenchyma, respectively.

Results

Weight loss (WL) was 23.84 ± 6.10 % at V6m and 24.77 ± 6.82 % at V4y. A parallel reduction in blood glucose and triglycerides level was observed. AST/ALT ratio ($p < 0.001$) and APRI ($p = 0.042$) early improved. However, FIB-4 displayed an opposite trend with a slight improvement at V6m and a significant worsening at V4y ($p=0.002$). The marker of apoptosis M30 K18 was reduced only at V4y ($p=0.034$). ELF did not correlate with other markers nor change after WL. FGF21 level was reduced at V4y ($p=0.009$). The prevalence of severe steatosis in PwO decreased over time with a corresponding increase of mild steatosis (χ^2 $p=0.031$). LS was reduced both at V6m and at V4y ($p= 0.008$).

LS ($p < 0.001$) and steatosis ($p=0.013$) were higher in PwA compared with controls. In PwA LS correlated with FIB-4 ($r = 0.590$, $p = 0.012$) and age ($r = 0.505$, $p = 0.032$), while the degree of steatosis was associated with triglyceride levels ($r = 0.504$, $p = 0.032$). LS showed an increasing trend in patients affected by metabolic comorbidities (obesity and T2DM) and displayed a significant correlation with waist circumference ($r = 0.624$, $p = 0.012$), insulin resistance index ($r = 0.670$, $p = 0.004$) and glycated haemoglobin ($r = 0.715$, $p < 0.001$). Lastly, we described a new pathogenic variant of exon 8 in *ALMS1*.

Conclusion

WL obtained by bariatric surgery displayed favourable short-term and long-term effects in PwO and NAFLD. SWE and B-mode ultrasound scan represent promising tools to accurately evaluate early liver fibrosis and steatosis in PwA (adults and children) and in PwO during follow-up.

M30 K18 seems the more promising fibrosis markers and FGF21 reduction could play a role in long-term improvement of liver fibrosis and steatosis in PwO.

PwA displayed enhanced steatosis, an early-increased age-dependent LS that is associated with obesity and T2DM but also strictly linked with genetic alterations suggesting that *ALMS1* could be involved in liver fibrogenesis. Our study underlines the utility to investigate ultra-rare diseases as a model of common diseases to identify new pathogenic pathways, novel therapeutic targets and the best diagnostic tools for complications.

1.0. INTRODUCTION

1.1. OBESITY

1.1.1. The size of the problem

Obesity is considered a noncommunicable disease (NCD), a term that can be referred to the most important chronic non-infective diseases (a group of conditions which includes diabetes mellitus, cardiovascular diseases and tumors), globally recognized as the main cause of premature death and disability.

Obesity was considered only as a risk factor of the other NCDs for a long time. Today it is known to be a complex, multifactorial, chronic-relapsing disease, with a chronic mild-moderate inflammatory state (1).

World Health Organization (WHO) in 1998 showed concern about the growing trend of obesity spread, first using the word "epidemic" to underline its global impact (2). Today, after more than twenty years, body weight excess afflicts over two billion people, around 30% of the global population. In 2017 the Global Burden of Disease Group stated that since 1980 obesity prevalence has doubled in over 70 countries, with a tendency to constant increase in most of the remaining nations. Regarding pediatric population, in 2017 UNICEF declared that "in the last 15 years there has been no progress in combating the rate of overweight (3)".

Despite the progress made in the field of medical research and sanitary policies, obesity keeps being a major global public health problem. It is defined a global health emergency by WHO, which promotes its prevention through surveys implementation in order to monitor its evolution over time, knowledge of its determinants, research and implementation of interventions and a continuous multisectoral and multidisciplinary evaluation process (4).

1.1.2. Definition

WHO defines obesity and overweight as an abnormal or excessive fat accumulation in relation to lean mass, to the point that it causes a serious health risk (5). Body Mass Index (BMI) is an anthropometric parameter used to estimate fat mass. BMI is the ratio of body weight (expressed in kg) to square height (expressed in m^2).

WHO uses BMI as follows:

BMI	Nutritional status
< 18.5 kg/m2	Underweight
18.5–24.9 kg/m2	Normal weight
25.0–29.9 kg/m2	Pre-obesity
30.0–34.9 kg/m2	Obesity class I
35.0–39.9 kg/m2	Obesity class II
≥ 40 kg/m2	Obesity class III

These parameters are used for adults. The use of percentile scales, which take age and sex of the subject into account, is recommended for pediatric and adolescent individuals (6).

BMI is routinely used in clinical practice, epidemiological studies and fitness industry. It represents a standardized screening tool, simple and inexpensive, based on the evidence of a linear relationship between BMI values and the onset of complications related to overweight and obesity (7). However, it is important to consider that this method offers an inaccurate and approximate measure of body fat accumulation (8). First of all, it estimates body mass as a whole, with no difference between lean and fat mass (in other words, it isn't an adiposity index). Not every subject with a BMI exceeding normal weight has an excessive fat mass (i.e. athletes with a significant proportion of muscle mass). Fat mass quantity is also variable based on sex, age and ethnic group.

- Women have a higher percentage of body fat and subcutaneous fat compared to men having the same BMI and age.
- Aging provides a physiological increase of fat mass and a reduction of lean mass, even with no BMI variations.
- Asian subjects have a higher body fat percentage compared to Caucasic ones having the same BMI (9). Based on these remarks, WHO suggests using lower cut-offs to identify subjects risk

of complications in Asiatic population (a BMI of 23 kg/m2 to define overweight and 25 kg/m2 to define obesity) (10).

Another limit of using BMI is represented by the fact that it cannot estimate body fat distribution, so that it doesn't allow to distinguish central obesity (visceral fat excess or *android habitus*) from peripheral obesity (subcutaneous fat excess mainly in the lower part of the body or *gynoid habitus*). The clinical importance of visceral fat excess lies in the fact that it constitutes the proportion of fat mass mostly involved in the increased risk of complications, such as cardiovascular disease, stroke, diabetes, hypertension (11). Some recent studies even suggest that a certain amount of subcutaneous fat, especially in the lower body (gluteal-femoral region and legs), may in fact have a protective effect against cardiovascular risk, which is typically increased in patients with obesity (12). Waist circumference (WC) is another anthropometric index that can be used to estimate visceral fat excess. WC is measured in cm, halfway between the inferior rib and the anterior superior iliac spine. Based on this parameter, central obesity is defined by WHO as a WC greater than 94 cm for men and 80 cm for women (11). The *International Diabetes Federation* suggested to differentiate these cut-offs based on ethnic groups, considering lower values for Asian population (90 cm for men and 80 cm for women).

Methods to estimate body fat: advantages and limitations

Table 1 shows a brief list of some of the most used methods to measure and quantify mass body composition. BMI and WC belong to the anthropometric indexes group.

Table 1. *Tools available to determine body composition (Modified from (13))*

Tools available to determine body composition
Anthropometry: the measurement of the physical properties of the human body, including BMI, waist circumference, waist-to-hip ratio and skinfold thickness.
Bioelectrical impedance analyses: determines the electrical impedance, or resistance to the flow of an electric current, through the body; this parameter can then be used to calculate an estimate of total body water. On the basis of the constant hydration of fat-free mass, total body fat can be calculated.
Densitometry - Underwater weighing: determines body density by measuring the mass per unit volume of the body; density is used to estimate the total body fat.

- Air displacement plethysmography: based on the same principle as underwater weighing, but uses air displacement rather than water immersion.
Imaging-based methods - Dual-energy X-ray absorptiometry (DEXA): a widely used method to study bone mineral density, from which the total body fat can also be estimated. - Computerized Tomography (CT) and Magnetic Resonance Imaging (MRI): mainly used to estimate abdominal fat in obesity research.

New non-invasive methods have been elaborated to measure body composition and fat distribution *in vivo*, such as densitometry, bioelectrical impedance analyses, Dual-energy X-ray absorptiometry (DEXA), Computerized Tomography (CT) and Magnetic Resonance Imaging (MRI) (14). However, they are not always available in every medical center, they can be expensive or time consuming and they may present other problems which make them not so frequently used. For these reasons, BMI and WC still play an important role in the initial assessment of fat deposits in the obese patient, albeit the mentioned above limits (8).

1.1.3. Epidemiology

Data from WHO

Obesity is defined by many Authors as the epidemic of 21st Century as it has a growing prevalence rate (13). Data from WHO show that the current number of people affected by obesity has tripled since 1975. According to the statistics available at the time this thesis is being written (5):

- In 2016 more than 1.9 billion adults in the world were overweight (39% of the population), of which 650 million were obese (13% of the population) (5).
- In the same year, 340 million children and adolescents aged between 5 and 19 were overweight or obese (5).
- Considering children under 5 years old, in 2020, 39 million children were overweight or obese (5).

Unlike past decades, during which overweight and obesity were considered a public health problem in industrialized countries, today they have a strong impact in middle and low-income Countries as

well, especially in urban areas. The number of overweight children under 5 years of age in Africa has increased by 24% since the year 2000. In addition, more recent data indicate that in 2019 almost half of the world's pediatric population belonging to the same age group and having a higher BMI than standard was Asian (5).

According to WHO, obesity and overweight cause a larger number of premature deaths compared to the ones caused by malnutrition, except in some areas of Asia and Sub-Saharan Africa. This data is linked to the evidence of a substantial increase in the risk of developing metabolic diseases (e.g. type 2 diabetes and liver steatosis), cardiovascular diseases (myocardial infarction, hypertension and stroke), diseases of the musculoskeletal apparatus (osteoarthritis), neurological diseases (e.g. Alzheimer's), depression and certain types of cancer (e.g. breast, ovary, prostate, liver, kidneys and colon) (16). It has been estimated that for every increase of 5 units of BMI above 25 kg/m^2, mortality from all causes increases by 29%, mortality from cardiovascular causes increases by 41%, while diabetes-related mortality increases by 210% (6). Obesity is the world's second avoidable cause of cancer after smoking (17).

Obesity also reduces quality of life and increases morbidity. For example, load arthropathy, one of the most frequent complications of excessive weight accumulation, is among the main causes of disability and early retirement (16).

Epidemiology data in Italy

According to data of the year 2019, collected in the "Rapporto Osservasalute 2020" written by the National Health Observatory, overweight and obesity are widely present in the population in Italy (17).

- Overall, 46.3% of 18-year-olds are overweight. Of these, 1 out of 10 are affected by obesity (10.9%)
- There are considerable differences in regional comparison (there is a difference of almost 10 percentage points between the region with the highest incidence of overweight people and the region with the lowest incidence). The highest prevalence of people over the age of 18 and over with obesity is found in Molise (15.0%), Basilicata (13.7%), Puglia (12.8%) and Abruzzo (12.7%), while the regions with the lowest prevalence are Provincia Autonoma di Bolzano (7.7%), Sardinia (7.9%) and Provincia Autonoma di Trento (8.8%).

- Analyzing the period of time from 2001 to 2019, there is an increase, though not linear, in the prevalence of obesity.
- As the age increases, the percentage of population in a condition of excess weight increases. The most critical age group weight-wise is the one between 65 and 74: 45.3% of the population in this age group is overweight and 15.6% is affected by obesity.
- The prevalence of excess weight is greater in men than in women. The 35-44 age group has the highest difference between overweight men and women and the 45-54 and 60-64 age groups are those with a highest difference between genders for people with obesity (17).

The impact on economy and public health: the burden of obesity

In 2019, the Organization for Economic Cooperation and Development (OECD), which currently includes 36 countries and of which Italy is one of the founding members, published the report "The Heavy Burden of Obesity. The Economics of Prevention"(18). The data collected on the prevalence of obesity in the Countries included in the OECD follow the trend of those declared by the WHO: more than half of the population of 34 out of 36 countries is overweight and almost one in four people is affected by obesity.

Overweight and obesity are linked to a reduction in life expectancy which, depending on the country, varies from 0.9 to 4.2 years (**Figure 1**). If incidence rates continue to rise at the same rate as in recent years, in 2050, 92 million premature deaths will be attributable to the complications of obesity.

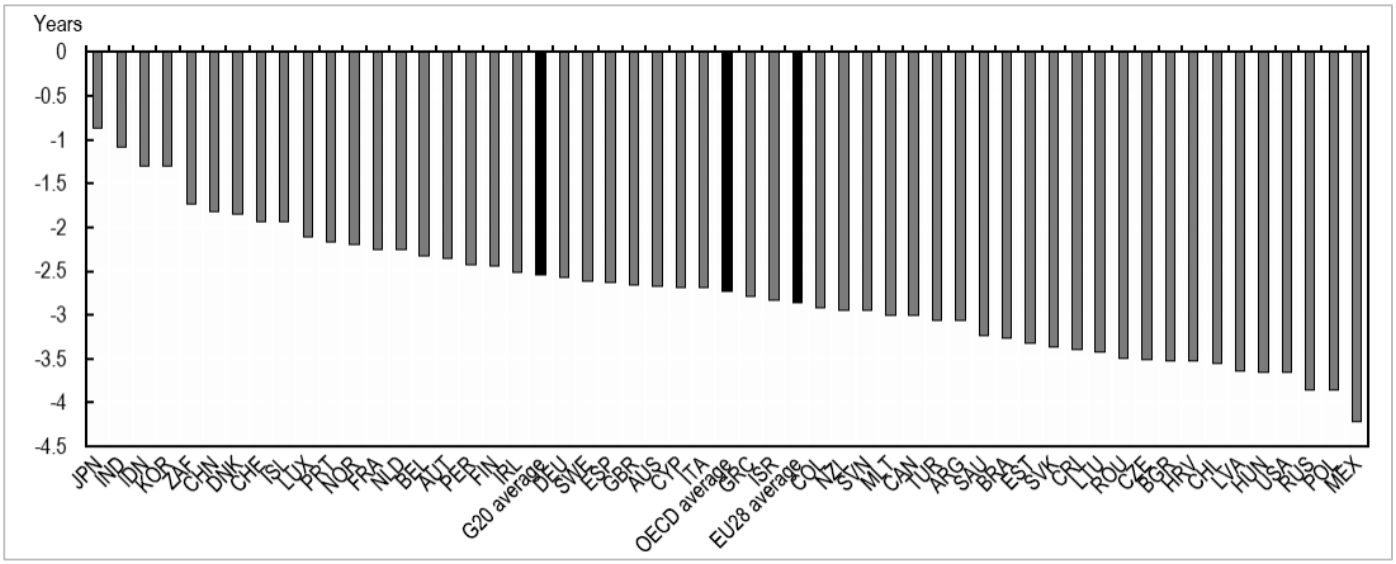

***Figure 1**. Impact of excess weight on the reduction of life expectancy (in years) in OECD countries: projections for the years 2020-2050 (OECD. The Heavy Burden of Obesity: The Economics of Prevention, 2019).*

It is estimated that OECD countries spend 8.6% of their healthcare systems' budget on overweight-related and obesity-related diseases (18).

Therefore, ready and effective policies are needed, aimed at preventing and combating the factors that cause the onset of overweight and obesity and their complications.

1.1.4 Pathophysiology

Obesity is the result of the complex interaction between environmental factors (economic, psychological and social factors), genetic and individual predisposition, which, combined to varying degrees, lead to an imbalance between income and energy expenditure which ultimately results in excessive accumulation of adipose tissue (16).

Energy homeostasis is regulated by maintaining a balance between energy supply and expenditure. The first is provided by the intake of food and drink, while the second is the result of the sum of the energy used at rest (necessary to support physiological processes) and the energy used during exercise (19). If the intake exceeds the expenditure, the excess of energy must be stored. The vast majority of this process takes place in the adipose tissue. In the event of a negative balance, however, energy reserves are consumed to ensure the necessary needs for survival (19).

Although useful for teaching purposes and for a first assessment of the patient with obesity, this concept of energy balance is reductive and does not consider many factors that can influence variations. Moreover, a model that only takes into account the simple equation between incoming and outgoing calories implies that the success of an obesity prevention and treatment program is only subordinate to the patient’s willingness in maintaining a healthy lifestyle for a sufficient period of time. This concept not only feeds the stigma towards patients with obesity (20), but is also scientifically incorrect. It has been demonstrated that weight loss induced by caloric restriction can set in motion some mechanisms able to recover weight, such as the increase of the search for food and the reduction of energy expenditure, that can last a long time (even years), until the body fat deposits have returned to their initial levels. This phenomenon has been observed in both those with normal weight and those with obesity, suggesting that the weight recovery, frequently found in the latter as a result of a low-calorie diet, is part of a physiological response, aimed at conserving the

body's energy reserves (21). Therefore, before reviewing the various factors influencing weight accumulation, it is appropriate to outline the main physiological mechanisms regulating food intake by the central nervous system (CNS) and how fat tissue stores energy reserves and manages excess energy.

HYPOTHALAMIC CONTROL CIRCUITS

The system that regulates energy homeostasis resides in the CNS and is the result of thousands of years of evolution. In our ancestors, such a system, suited to respond adaptively to long periods of migration accompanied by poor availability of food, was essential for survival. It was also important for mechanisms to be developed and passed on to future generations, in order to research and select the best food resources (avoiding toxic foods, for example) (22). The center that regulates food intake and the maintenance of body mass in humans was identified in the hypothalamus more than 70 years ago by researchers. More recent genome-wide association studies (GWAS), have shown that most genes associated with maintaining BMI encode for proteins expressed in CNS (22). Food intake is regulated by a highly complex system, consisting of redundant but extremely efficient neuronal circuits.

Signals from the external world are conveyed through sight and smell. The choice and identification of a food also uses the information stored during previous experiences and, therefore, may also involve auditory and somatosensory inputs (23), thus generating anticipatory responses (22). Once placed in the mouth, the foods arouse gustative and mechanical sensations, determining in deciding whether to proceed with the ingestion of the food. Ingested substances are further processed in the gastrointestinal tract and its variety of taste receptors which, together with numerous chemo- and mechanocectors, collect information on the volume and composition of food (22). Signals collected are conveyed to the CNS through the vagus nerve and blood stream with neurotransmitters and hormonal mediators. Many organs and systems dialogue with the CNS, playing a crucial role in food research and choice (23). The key structure within the CNS, involved in the regulation of hunger and satiety mechanisms, is the hypothalamus. It contains three regions which, according to Sternson and Eiselt, are *the three pillars for the neural control of appetite* (24).

1. The first is the arcuate nucleus (ARC). ARC is located in the middle-basal hypothalamus, near the median eminence, circumventricular organ, rich in fenestrated capillaries through which the hormonal and nutritious signals afferent from the periphery, reach the hypothalamus (25).

There are two groups of neurons that are functionally distinct and antagonistic in ARC:

- Neuropeptide Y (NPY) and the Agouti-related peptide (Agrp) releasing neurons, with orexigenic action. When excited, in response to the unsaturated energy deficit during fasting, Agrp/NPY neurons activate the search for food and transmit signals that result in unpleasant sensations associated with hunger.
- Proopiomelanocortin (POMC) releasing neurons, with anorexigenic action. Food ingestion promotes the splitting of POMC into α-Melanocyte-Stimulating Hormone (α-MSH) within neurons, which then interacts with melanocortin receptors 3 and 4 (MC3/4R) of neurons downstream. These receptors, although widely distributed in different areas of the CNS, have a higher density in the paraventricular nucleus (PVN) of the hypothalamus, which is responsible for modulating sympathetic messages to peripheral organs and for secreting numerous neuropeptides. Injuries to PVN may cause hyperphagia and obesity, underlining the important role in inhibiting hunger (25). POMC neurons also project to other regions and nuclei of the hypothalamus (e.g. the dorsomedial and ventromedial hypothalamus) which reach extra-hypothalamic circuits achieving an integrated response to different situations of energy accumulation or expenditure.

2. Lateral hypothalamus (LH) is mainly responsible for the realization of actions and behaviours aimed at the research and ingestion of food. It is also responsible for the feelings of emotional gratification associated with eating (24).

3. Neurons related to Calcitonine Gene Related Peptide (CGRP) are in the parabrachial nucelus (PBN) and are activated by signals deriving from eating, thus inducing satiety (24).

These three systems are activated sequentially. AgRP neurons are excited by hormonal peripheral signals that indicate an energy deficit and mediate the drive for food. Their activity decreases drastically when the desired food is identified or accessed. The consumption of the meal is guided by the circuits associated with the LH activated by of hunger and by positive feedback mechanisms in which the rewarding aspect of appetizing foods stimulates a greater consumption. This feedback is counteracted and turned off by the circuit located in the PBN (24).

Other brain circuits involved in the control of appetite are the mesolimbic system, with the ventral tegmental area and nucleus accumbens, and the nucleus tractus solitarii, regulating the hedonic aspects of the search for food (23). Various hormones participate in the regulation of appetite and

energy expenditure, such as insulin, leptin, ghrelin, GLP-1, peptide YY3-36, cholecystokinin etc., forming the so-called gut-brain axis (25). A recent study by Beutler et al. showed that, in mice with high-calorie diet-induced obesity, a high fat diet (but not carbohydrates and proteins) is related to the attenuation of the Agrp/NPY neuron response to external stimuli and GI tract regulator signals, including those mediated by ghrelin and CCK. A subsequent weight loss would restore sensitivity to external signals, but not responsiveness to modulation by hormones of GI origin. These findings reveal that obesity triggers an important dysregulation of the hypothalamic circuits, which is only partially recovered with the weight loss induced by diet. If they were to be confirmed by further research in humans, such new acquisitions would contribute to the understanding of the mechanisms responsible for the difficulty in maintaining the weight loss long term (26).

THE ADIPOSE TISSUE

The adipose tissue (AT) has been considered for a long time a simple deposit of lipids, functionally passive. Conversely, nowadays it is recognized as a complex organ with endocrine function. It belongs to the class of connective tissues and plays a key role in regulating energy homeostasis, modulating food intake, thermogenesis, glycemic metabolism, insulin sensitivity, etc.

AT depots are classified according to their anatomical location in Visceral Adipose Tissue (VAT), that is correlated to a higher cardiovascular risk, and Subcutaneous Adipose Tissue (SAT) which seems to display a cardioprotective role, according to some studies (12,29). It should be noted that the abdominal SAT can be further subdivided into two sub-types, with specific transcriptional and metabolic profile:

• the superficial SAT, functionally similar to SAT of the gluteal-femoral region

• the deep SAT, with a metabolic risk profile much more similar to VAT (29).

The main cells of the adipose organ are the mature adipocytes, responsible for the storage of energy deposits in the form of triglycerides. Besides these, numerous other cell types are present in the hepatic stroma: pre-adipocytes, fibroblasts, cells implicated in the inflammatory processes (macrophages, lymphocytes and eosinophils), microvessel cells and the adipose stromal/stem cells (ASCs) (15). ASCs have been isolated and characterized from both mice and humans proving their capacity to give rise to different lineages of mesenchymal origin in vitro and in vivo. In particular several researchers contribute to identify their surface markers and tissue localization by means of

gold standard techniques such as cytometry, IHC and IF (30). ASCs belong to the Mesenchimal Stem Cells (MSC) population and have been compared with Bone Marrow and Umbilical –derived stem cells especially for their immunomodulatory properties in the clinical trial setting. In humans, the combination of a few robust markers (CD34+/CD31-/CD45-) allows to identify and quantify the ASC population ex-vivo to study its role in different pathological conditions (31). Several studies, mainly conducted on animals, evidence a possible hierarchical relationship between adipogenic stem cells and suggest the existence of white, brown and beige precursors as well as depot-specific ASCs. Recent studies and new single-cell analysis underline the heterogeneity of AT progenitors describing new regulatory subpopulations controlling the AT expansion (ASC reg) (32).

AT contains three histological types, each of which is characterized by different functions and expression profile (15) (summarized in **Table 2**):

• White AT (WAT) with subcutaneous (sWAT) and visceral (vWAT) anatomical location. It is characterized by more modest vascularization and innervation than brown TA. The main function of WAT is to deposit lipids in the form of triacylglycerol (TAG), which are mobilized by lipolysis when the energy demand increases. In fact, fatty acids can be supplied by diet or produced by de novo lipogenesis (DNL) (33). DNL is partially performed in the liver and it is activated upon chronic increase in blood glucose levels or prolonged adrenergic stimulation (34).

• Brown AT (BAT): it constitutes only 4.3% of the total adipose organ and it is predominantly located in the supraclavear and interscapular regions (15). BAT is involved in the so-called *chill-free thermogenesis*, a very important process in maintaining body temperature and regulating the body's fat content. Its cells are rich in mitochondria (this is the reason for the brown color) and express the Uncoupling Protein 1 (UCP1) in the mitochondrial inner membrane with fundamental role in thermogenesis. The main UCP1 function is the decoupling of ATP synthesis in the mitochondrial oxidative processes thus leading to the dissipation of energy in the form of heat (35).

• Beige AT represents an inducible form of BAT (15). It is characterized by adipocyte cells dispersed in clusters within the WAT having WAT morphology but expressing UCP1 (36). They seems to have a distinct origin maybe originating from WAT precursors or mature adipocytes in response to a number of stimuli (cold exposure, catecholamines, exercise, drugs such as thiazolidinediones, etc.) by a process called "browning or beiging" (28).

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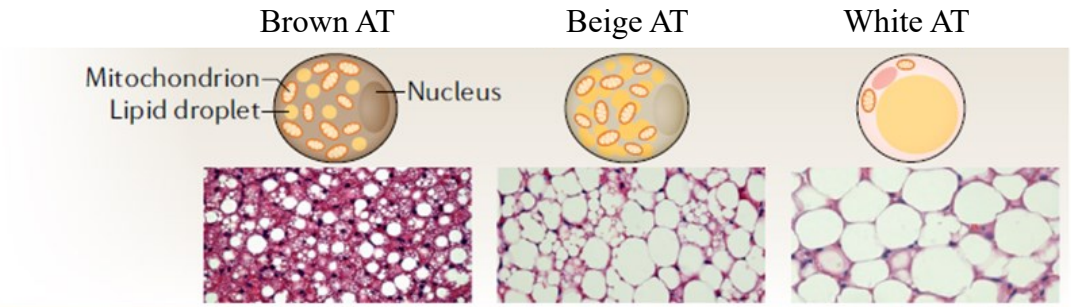


	Brown AT	Beige AT	White AT
Function	Thermogenic	Thermogenic	Energy storage
Mitochondria	High	High	Low
Lipid droplets	Multilocular	Multilocular	Unilocular
Location	Interscapular BAT	Sporadic in WAT (more in subcutaneous WAT)	WAT depots
Developmental origin	Embryonic	Adult (highly inducible)	Adult

Table 2. *Main differential characteristics of different types of adipose tissue (AT). Modified from (29).*

Most adipose cells produce and secrete a large number of cytokines (adipokines) that mediate a number of autocrine, paracrine and endocrine effects both locally and remotely. The composition of this secretion of adipose tissue depends on its regional distribution. For example, adipose stem cells located in the VAT typically secrete high levels of pro-inflammatory factors (IL-6 and C-C-chemokine ligand 2 or CCL2) and low levels of anti-inflammatory mediators (adiponectin and IL-10) (15). In this regard, in recent years there has been a growing interest in the perivascular visceral adipose tissue (PVAT), whose mesenchymal cells, through the secretion of a number of pro-inflammatory agents, appear to be directly implicated in cardiovascular complications related to obesity (37,38).

On the other hand, adipokines produced by brown and beige adipose tissue, also called batokines, exert their action by improving systemic metabolism because they act on a series of organs and tissues (liver, pancreas, bone tissue, CNS). For example, serum levels of Fibroblast growth factor-21 (FGF-21), produced predominantly by the liver and (at some level) by BAT, are typically increased in patients with obesity and have a protective role in the development of insulin resistance and the development of type 2 diabetes mellitus (T2DM), possibly promoting thermogenesis in BAT and beiging of WAT, thus resulting in improved carbohydrate and lipid homeostasis (35). This explains the remarkable plasticity of TA and its ability to adapt in numerous physiological and pathological situations.

Obesity is characterized by the so-called *adipose disease*, adipose tissue dysfunctions responsible for the metabolic complications that are observed in patients with obesity (39). In particular, it is important to consider the adiponiche structure, referring to anatomical and functional unit that regulates ASCs homeostasis and behavior in AT. The adiponiche works as a specialized and finely tuned microenvironment that contributes to the quiescence of ASCs, maintaining their stemness, regulating their proliferation and differentiation in order to support physiological adipocyte turnover and maintain energy balance (40). A proper adiponiche (lean adiponiche, **Figure 2**) working in all its components is the main regulator of AT expansion and remodelling: damage in one or more elements of the adiponiche results in a dysfunctional milieu deeply correlated to the pathological alterations described in AT of patients with obesity and metabolic complications (obese adiponiche, **Figure 2**).

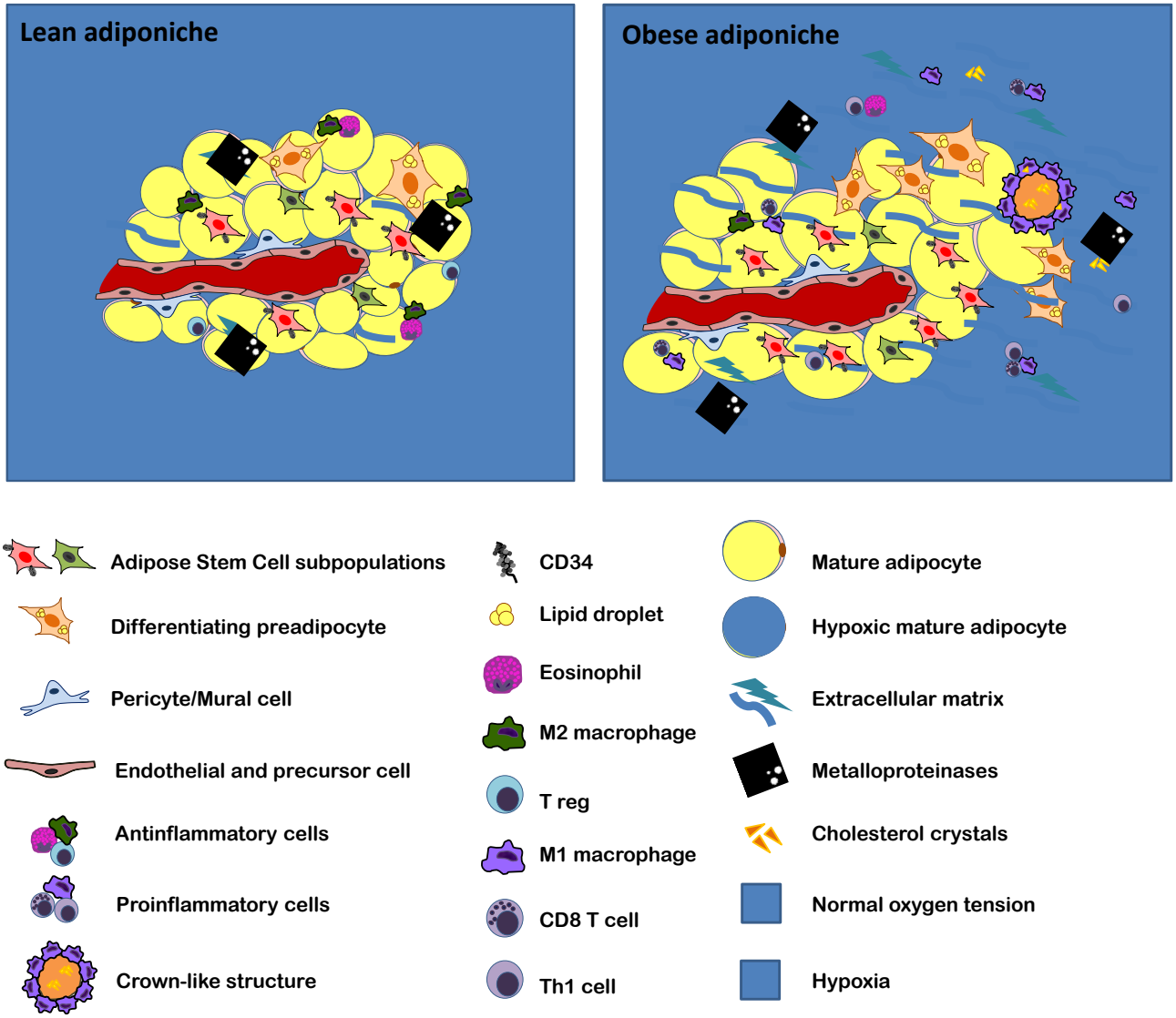


Figure 2. *Lean and obese adiponiches. In lean adipose tissue (lean adiponiche) different adipose stem cell (ASC) subpopulations (represented by different colors – pink and green) provide physiological adipocyte turnover. CD34+ ASCs (Figure 2) are thought to reside at perivascular sites. Few inflammatory cells are present, represented mostly by M2 macrophages, regulatory T cells (Tregs), and eosinophils that display anti-inflammatory activity. During weight gain (obese adiponiche), AT grows both through hypertrophy and hyperplasia, and proinflammatory cells infiltrate the niche, leading to an increase in the M1/M2 macrophage ratio, T lymphocytes (CD8+ and type 1 T helper cells, Th1), and natural killer cells, and to a decrease in anti-inflammatory cells. Moreover, hypoxia develops because of the increased distance between hypertrophic adipocytes and vessels, increased cell oxygen consumption, and an imbalance between tissue growth and oxygen supply. Consequently, extracellular matrix deposition increases, leading to tissue fibrosis. Pyroptosis of adipocytes is seen in obese AT that triggers and stimulates macrophage infiltration accompanied by the formation of crown-like structures. Degenerating adipocytes display typical morphological features such as cholesterol crystals (Courtesy of Dr G. Milan (40)).*

The main determinant of the adipose mass is the number of adipocytes that is defined during childhood and maintained in the adult life, even following a substantial loss of weight (33). Adipose cells are also characterized by a relatively slow turnover (about 10% of the total is renewed every year (41)).

In response to an excessive caloric intake over time, the adipose tissue has the extraordinary ability to expand through hyperplasia (increase in number) or hypertrophy (increase in size) of its adipocytes. In obesity, the expansion of the adipose tissue, especially in the visceral area, is achieved both these ways (**Figure 3a**) (33).

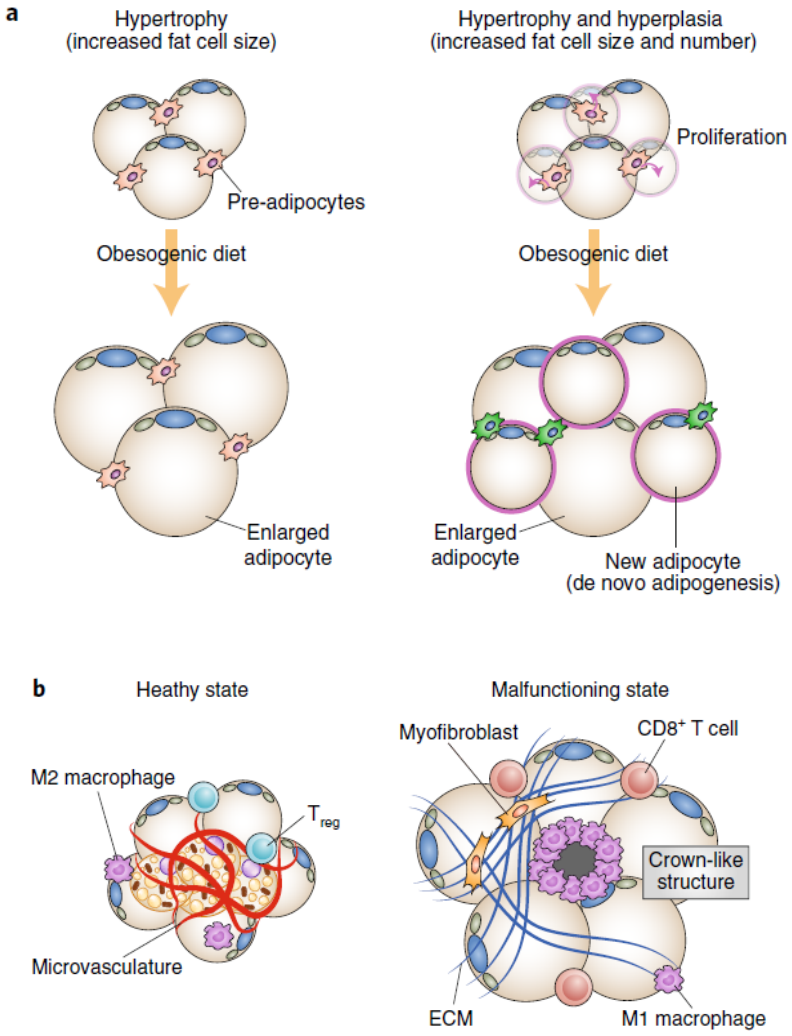


Figure 3. *Expansion types of adipose tissue (a); Normally working adipose tissue (on the left) and tissue with adiposopathy (right)(b)* (*Modified from (33)*).

Hyperplasia, achieved through the mobilization of the progenitor cells, is considered a process of benign adaptation, while hypertrophy is linked to the presence of dysfunctional adipocytes and lipid overloads and low-grade inflammation at the expense of the adipose tissue (15) (**Figure 3b**). The phlogistic process of the adipose tissue is characterized by a predominance of macrophages with phenotype M1 (pro-inflammatory) compared to those with phenotype M2 (anti-inflammatory), in

response to the cytokines produced by Th1 lymphocytes in the inflammatory infiltrate. M1 macrophages are also implicated in regulating the expansion and ectopic deposition of adipose tissue, through the production of platelet-derived growth factor-β (PDGF-β) that activates pericytes and promotes new angiogenesis. In the pathological expansion of adipose tissue, a rarefaction of the capillary network is also observed, which leads to reduced nutrient intake and hypoxia aggravating adipocytes dysfunction and insulin resistance and promoting fibrosis (15). Fibrosis and qualitative changes in the extracellular matrix appear to play an important role in amplifying and supporting structural changes in the adipose tissue. In addition, clinical studies have shown a positive association between subcutaneous WAT fibrosis and the development of insulin resistance and T2DM in patients with obesity. The degree of fibrosis in the sWAT also seems to have an inverse correlation with the effectiveness of the results obtained by bariatric surgery in terms of loss of weight (33).

The mechanisms described above are associated with imbalances in the adipokine secretion and changes in the lipidic profile with increased levels of free fatty acids (FFA), diacylglycerol and ceramides. The excessive lipids accumulate as adipose tissue in ectopic areas (e.g. liver, heart and skeletal muscle) where the lipotoxicity of these compounds contributes to the development of the complications related to obesity.

As already mentioned, the adipose tissue secretes a series of substances with autocrin, paracrin and endocrine activity called adipokines: leptin, adiponectin, resistin, visfatine, chemerin, etc.

Leptin, already mentioned at the beginning of this paragraph, with regard to its role in the central regulation of appetite, also has other important functions. For example, it can promote the activation of the macrophage/monocyte system and natural killer cells (NK), stimulating their proliferation, phagocytosis, and the release of free oxygen radicals by neutrophils. It is mainly produced by WAT adipocytes and its levels are determined mainly by the amount of fat mass and the size of the adipocytes (27). It is considered a potential marker of obesity-related complications. Its blood levels were found to be related to atherosclerotic disease, nephropathy, and diabetic neuropathy (27).

Adiponectin is a 28 kDa protein with a similar structure to tumor growth factor (TGF) or tumor necrosis factor (TNF). In vivo and in vitro studies have shown that it has anti-atherogenic and anti-inflammatory effects. An increase in its serum levels was observed in response to endothelial damage, while it seems that its levels are reduced in hypertensive disease and diabetic nephropathy (27).

Figure 4 provides a summary of the steps involved in the genesis of the metabolic complications described above.

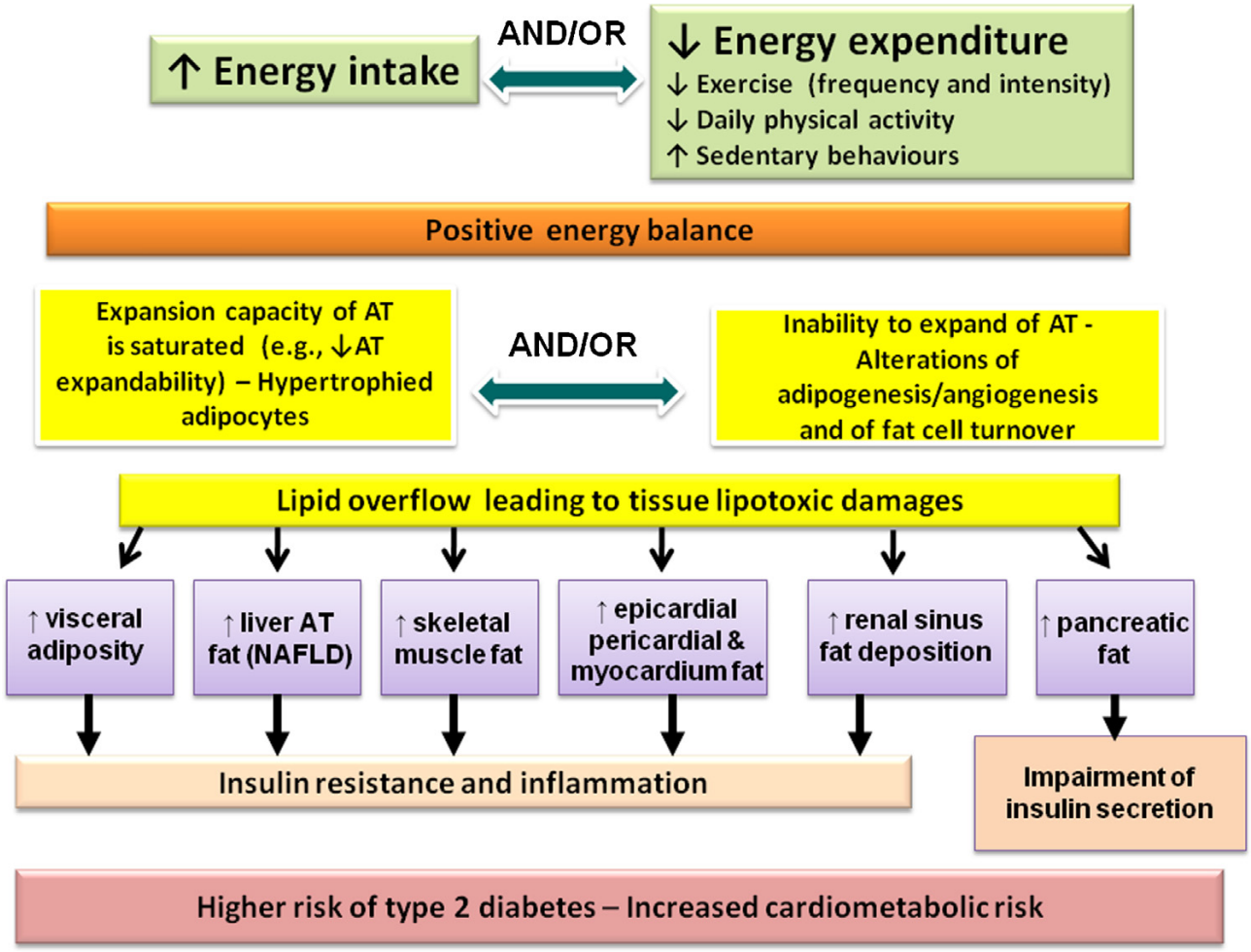


Figure 4. *Dysfunctional expansion of TA leads to the development of adipose disease (characterized by low-grade inflammation, hypoxia, alteration of angiogenesis and insulin resistance) resulting in lipotoxicity in various organs and development of metabolic complications associated with obesity. TA: adipose tissue; NAFLD: liver steatosis; T2DM: diabetes mellitus type 2 (Modified from (42)).*

Although most pathophysiological processes leading from adipose tissue pathology to obesity are covered, it is still unclear what are the determinants that regulate the different expansion and metabolic activity of the various areas of adipose tissue and why some individuals (albeit in a clear minority), even having excessive fat accumulations, are free from metabolic complications (thus defining the *metabolically healthy obesity*) (43). Research in this field in recent years has focused more and more on the importance of the role of mesenchymal cells in determining how the adipose tissue expands in response to different pathogenetic factors. Recent studies have shown that the function of adipose stem cells should be considered not as an entity in its own right, but as the result

of a mutual interaction with other cellular structures and extracellular components. Together, these structures form the so-called *niche of the adipose tissue*, a functional unit consisting of its own microenvironment and within which the regulation of homeostasis and the function of stem cells takes place. In this perspective, the development of obesity and the metabolic complications linked to it could be reconsidered as the result of a dysfunction of the niche of the adipose tissue, rather than a generic pathology of the adipose tissue (40).

FACTORS INFLUENCING THE DEVELOPMENT OF OBESITY

As already mentioned, obesity is the result of the complex interaction between genetics/epigenetics, lifestyle, individual and environmental factors. A positive, albeit modest, daily energy balance can also contribute over time to weight gain (13).

ENVIRONMENTAL FACTORS

- **Socio-economic and cultural factors**

In the last century, overweight was considered a symbol of economic comfort, beauty and health. In some cultures and societies these aesthetic canons are still valid (e.g. some Pacific islands) and can influence individual choices, thus leading to a greater incidence of obesity in these areas (16).

Data collected in the WHO European Region indicate a higher prevalence of overweight and obesity "among socially disadvantaged communities with lower levels of income, education and access to care" (44). In an interesting review of the scientific literature of 2019, *An et al* analyze the relationship between the globalization process and the epidemic spread of obesity, defining a conceptual model, consisting of three possible pathways: economic globalization, the spread of new technologies and social globalization. This model is in line with most analyses in the scientific literature of the phenomenon (3,45,48). Economic globalization has led to market deregulation with reduced prices, new supermarket chains and fast-food restaurants. As a result, there has been an increase in the supply of foods with a high content in simple sugars and fats at reduced prices. In addition, the advent and spread of new technologies in homes and workplaces, together with an increase in motorization, has allowed the reduction of manual work and an increase in sedentary behavior. Finally, on a social level, globalization has promoted the spread of a western lifestyle, encouraging consumers' preference for high-calorie foods (45).

- **Pollution**

Several studies have shown a certain correlation between pollution (especially the one given by oxides of nitrogen, ozone and atmospheric particulates PM_{10} and $PM_{2.5}$) and the risk of obesity and cardiovascular diseases related to metabolic imbalances. In addition, there is an association between pollution and a greater severity of cardiovascular disease in people with obesity than in patients having a normal weight (47). Studies in the pediatric population show the same pollution-obesity correlation and indicate the most susceptible age group being the one between 5 and 10/11 years old. The pathogenetic mechanism seems to involve the modification of the adiponectin levels and has negative effects on the cholesterol metabolism (48). A recent general population study in South Korea found a positive correlation between environmental concentrations of heavy metals (cadmium, lead and mercury) and risk of development of obesity and Nonalcoholic Fatty Liver Disease (NAFLD) in adulthood (49).

- **Infective factors**

The hypothesis that an increased susceptibility to infections can contribute to the development of obesity arises from the observation of a reduced immune response to some vaccines. One of the viral agents investigated in this regard is adenovirus AD-36, which has been found to be able to stimulate the proliferation of adipocytes and the consequent weight gain. Moreover, in the population with obesity compared to normal weight population, antibody titer against AD-36 were found to be significantly higher. However, in light of current scientific evidence, viral infections do not appear to play a predominant role in the epidemic spread of obesity (21).

GENETIC AND EPIGENETIC FACTORS

Studies within families with increased prevalence of obesity, homozygous twins and GWAS studies have estimated the genetic contribution to the development of obesity between 40 and 70% (50). In addition to this, genetic variants associated with higher probability of high BMI values have been identified. The latter involve more than 1,000 loci but contribute only by 6% to BMI variability. To date, their role in the pathogenesis of obesity remains unknown. However, it has been observed that in the substantia nigra and the insular cortex (involved in neuronal systems mediating reward and motivation), there is a greater expression of genes located near these loci (50).

Alongside oligo- and polygenic forms there are also monogenic forms of obesity, which are quite

rare. They involve about 5% of obese patients in the western world and are more frequent in inbred marriages. They are characterized by a single mutation or a single genetic event (e.g. a chromosomal anomaly) and may be part of a syndromic picture (e.g. Alström Syndrome, Prader-Willi syndrome, Bardet-Biedl syndrome) or present as isolated forms, although the distinction between these two possibilities is not always so marked. The pathogenesis of these forms involves a group of about ten genes (e.g. LEP, LEPR, POMC, MC4R, etc.) that have the common characteristic of coding for proteins involved in the hypothalamic regulation of appetite (leptin and its receptor, for example) (50). In addition to the variations in the sequences coding the various genes, genetic-environmental interaction also plays a very important role in establishing the individual susceptibility to accumulating excessive fat storage. In this context, epigenetics (the science that studies the regulation of changes in gene expression in the absence of changes in nucleotide sequences) is able to offer a better explanation of the complex reasons that led to the pandemic spread of obesity (51). Epigenetic modulation is achieved through DNA methylation, histone modification, microRNA regulation and, simplifying, they are translated into different "packaging" (windings) of the DNA that from time to time can promote or silence gene expression in the tissues (52). These changes can be inherited by later generations, but are nevertheless reversible and extremely sensitive to the influence exerted by external (e.g. diet and physical activity) or internal (genetic, hormonal mediators) factors (13).

Metabolic imprinting

Longitudinal studies have shown that critical periods exist in neonatal, childhood and adolescence, during which the interaction between genetic/epigenetic factors and environmental influences can define and modify the metabolic program of an individual, thus favoring the development of obesity (13). In the prenatal period (especially in the first 20 weeks of gestation) an excess of maternal weight represents a risk factor for the development of the child's overweight. A high birth weight positively correlates with the risk of developing obesity throughout life, while a low weight in the new-born correlates with an excess of fat mass, regardless of BMI, in adolescence. Breastfeeding appears to have protective effects against overweight. A rebound of early adiposity (see Figure 11 and its explanation) in childhood has been shown to be one of the factors predisposing in adulthood to the risk of excessive accumulation of subcutaneous adipose tissue and high values of BMI and WC (13). Unfortunately, a high percentage of children with obesity (71%, if they are severely affected) retain their status in the adult age (53).

THE INTESTINAL MICROBIOME

The gut microbiome is composed of a set of micro-organisms (bacteria, Archaea, viruses and others) with a mainly symbiotic behavior with the host. The number of cells that compose it is far greater than that of the whole organism, which is why some authors define it as the "second human genome" (54). The intestinal flora performs several functions: it promotes the digestion and absorption of nutrients, participates in the synthesis of essential aminoacids and vitamins and has a very important role for the proper functioning of the immune system and metabolism (55). The majority of bacterial species resident in the GI tract are *Firmicutes* (60-80%) and *Bacteroidetes* (15-25%), while other types are present in a minority share (e.g. *Proteobacteria, Actinobacteria, Verrucomicrobia*) (56).

Although the relationship between dysbiosis and the development of metabolic diseases (such as T2DM and obesity) has been established for some time, to date there is no precise qualitative and quantitative definition of the typical flora of the healthy individual, also because the composition of the microbiome is characterized by a great metabolic flexibility susceptible to intra- and inter-individual variability. Studies on homozygous twins have shown that the GI flora may undergo considerable variations under the influence of different genetic and environmental factors (in particular dietary habits), the use of drugs (e.g. prolonged antibiotic therapy), etc. (54).

In subjects with obesity, variations in normal microbial flora have been observed both qualitatively (e.g. an altered ratio between *Firmicutes* and *Bacteroidetes* in favor of the former) and quantitatively (reduction in the number of species) (57). The pathogenetic mechanisms that bind dysbiosis to the development of obesity (schematized in Figure 12) are complex and involve the regulatory signals that are part of the gut-brain axis (58), fundamental in the modulation of energy homeostasis. A diet rich in animal fats seems to favor the increase of intestinal permeability resulting in increased levels of endotoxins (especially lipopolysaccharides or LPS) in the systemic circulation, which contribute to obesity-related inflammation and insulin resistance. Moreover, some bacterial species (in particular Firmicutes (59)), have a high ability to influence the digestion of certain types of nutrients, thereby increasing caloric absorption and fat deposition. It has been hypothesized that the microbiome may inhibit the fasting-induced adipose factor (or FIAF), a central mediator in the regulation of lipid metabolism, resulting in increased triglyceride deposition in adipocytes. Finally, the bacterial flora is able to modulate mitochondrial oxidation of fatty acids, ketogenesis, insulin resistance and glucose uptake, lipogenesis and the synthesis of triglycerides and cholesterol (60). In recent years, many species have also been identified as having a probable protective effect against obesity. For example,

the *Akkermansia muciniphila (Verrucomicrobia)* has proved to be able to interact with the metabolism of the host, contributing to the maintenance of intestinal integrity and favoring eubiosis in addition to the already known function of regulating the production of intestinal mucus (59).

Studies in this field open new scenarios of investigation and development of therapies, based on the modulation of the bacterial flora and its functions in patients with obesity.

LIFESTYLE

As already discussed, an excessive accumulation of TA develops when the caloric intake exceeds the energy expenditure of an organism. It would therefore be logical to think that the factors that determine obesity are very simple to detect in the increased consumption of food and in a sedentary lifestyle. This is only partially true: several individual and environmental factors can affect the introduction of calories and energy expenditure, both in qualitative and quantitative terms. E.g. food choices and physical activity are often dictated, not only by genetic factors and individual susceptibility, but also by family habits, cultural imprinting and economic possibilities.

As already mentioned regarding the influence of environmental factors, globalization with a rapid spread of a "Western Lifestyle" is the most important reason for the increase in the incidence of obesity in the last 50 years. Socio-cultural changes have had a negative impact especially in three areas: the quality of the diet, the sharp drop in physical activity and the dysregulation of sleep-wake rhythms.

- **Diet**

According to the World Obesity Federation Position Statement published in 2017, "Food is the most important environmental risk factor for the development of obesity" (61).

Western lifestyles have led to increased consumption of low-cost, processed foods, high in simple sugars and saturated fats, and relatively low levels of fiber, minerals, and vitamins. Such availability of food, coupled with the continuous media advertising of the food industry, contributes to the stimulation of the search for high palatability and calorie-rich foods. Scientific literature agrees that "wrong" food choices are not so much dictated by lack of goodwill as by the involvement of neural circuits, the processing of sensory signals and emotional and cognitive control of nutrition, located in the cortico-mesolimbic system and in close connection with the hypothalamic nuclei of the hunger and satiety center (62). In addition to the increase in the amount of food introduced and its caloric

content, it is important to remember that the quality of macronutrients (proteins, carbohydrates and fats) also influences the behavior of the food search. E.g. a high-fat meal has a much lower satiety-inducing capacity than a high-protein meal (19). By contrast, studies have shown that a diet involving a balanced consumption of macronutrients (consisting of 50-55% of carbohydrates with a lipid share below 30% and a protein content of 0-20%) is associated with a reduced risk of developing obesity (13). Finally, an interesting review analyze the role of changes in lifestyle and diet on the dysregulation of normal physiological processes involved in the development of obesity (63). In Western Countries, diet rich in unsaturated fats and simple sugars has effects not only on the increase in energy balance, but it also promotes low-grade systemic inflammation, dysbiosis, dyslipidemia and insulin resistance, thus contributing to the development of metabolic complications associated with obesity (63). This makes it possible to acknowledge the importance of dietary interventions and dietary habits, which cannot be separated from the knowledge of the complex interaction with the environment in programming therapeutic objectives for the patient with obesity.

- **Physical activity**

Also physical activity is strongly influenced by socio-economic and cultural factors (e.g. type of transport used to go to school/work, use of video games and the PC that forces people to sit for many hours, type of employment). Regular physical activity promotes the reduction of fat mass and the increase of lean mass. Increasing muscle mass stimulates both total energy expenditure and Resting Energy Expenditure (REE) (64). The resistance exercise promotes the uptake and oxidation of FFA by the skeletal muscle, thus preventing its accumulation, responsible for the development of insulin resistance and the state of chronic inflammation, characteristic of obesity. Strength exercises, on the other hand, increase the REE even in the 24 hours following their performance, probably stimulating muscle hypertrophy. Finally, recent studies show that some products of the energy metabolism of skeletal muscle (e.g. lactates) are able to influence positive changes in the gut microbiome (64).

- **Sleep-wake rhythm**

The control of nutrition is under the influence of the sleep-wake cycle and circadian rhythms. The dysregulation of these rhythms linked, for example, to night work shifts and a small amount of sleep,

is associated with the development of obesity, pre-diabetes and diabetes and dyslipidemia (6). The mechanisms by which insufficient qualitative and quantitative sleep can influence the metabolic state of the individual are still being studied. Sleep reduced to 4 hours/night for a period of 5 consecutive days has been found to be sufficient to activate a systemic immune response by lymphocytes and the subsequent production of cytokines (e.g. IL-6, IL-1β, IL-17). Several studies confirm that chronic sleep deprivation in a modern society active 24 hours a day promotes the state of chronic inflammation and insulin resistance. Sleep debt, moreover, can lead to increased levels of leptin, thus inducing changes in the circuits that regulate the center of satiety at the hypothalamic level. It has been observed that this dysregulation also involves the hedonic aspects of nutrition: individuals who stay awake at night (for work reasons, for example) tend to choose foods with higher fat content. Finally, the stress caused by lack of sleep can also affect eating behavior, thus triggering a vicious circle that further contributes to the imbalances just described (63).

1.1.5. Complications

As already discussed in paragraph 1.1.4. obesity is characterized by high levels of FFA, inflammatory cytokines and intermediate products of lipid metabolism even in organs other than adipose tissue, thus contributing to the development of insulin-resistance and various metabolic complications that may involve numerous organs and systems (**Table 3**) (62).

Table 3: *Obesity Complications (Modified from (19)).*

Neurological	Intracranial hypertension, cerebrovascular disease, paresthesic meralgia
Cardiovascular	Coronary heart disease, hypertension, peripheral venous disease, varicose veins
Respiratory	Obstructive Apnea Syndrome, central sleep apnea, pulmonary embolism
Metabolic	Insulin resistance, Type 2 Diabetes Mellitus, dyslipidemia
Gastrointestinal	• Hepatic: Non-Alcoholic Fatty Liver Disease, cirrhosis, hepatocellular carcinoma • Gallbladder stones • Gastro-Esophageal Reflux Disease • Colon cancer • Hernias
Renal	Glomerulopathies
Gynecological	Menstrual irregularities, Polycystic Ovary Syndrome, endometrial carcinoma, reduced fertility, stress urinary incontinence
Urological	Hypogonadism, prostate cancer
Breast	Breast cancer
Musculoskeletal	Osteoarthritis, back pain, gout
Dermatological	Lymphedema, cellulite, venous stasis in the lower limbs, intertrigo
Psychiatric	Mainly depression

Compared to a normal adult, a patient with BMI 40kg/m2 has an increased risk of developing T2DM (OR,7.37; 95% CI, 6.39-8.50), hypertension (OR, 6.38; 95% CI, 5.67-7.17), hyperlipidemia (OR, 1.88; 95% CI, 1.67-2.13), asthma (OR, 2.72; 95% CI, 2.38-3.12), arthritis (OR, 4.41;95% CI, 3.91-4.97) (6).

In addition to the listed comorbidities, the most recent publications highlight another fact that emerged during the current pandemic from SARS-cov-2 started in December 2019 and that at the time this work was written was responsible for almost 5 million deaths (65): obesity was found to be an independent risk factor and a predictive factor of negative prognosis in patients who developed COVID-19 (66). Observations on patients hospitalized for acute respiratory syndrome, related to SARS-infectionCov-2, showed that patients with obesity need more frequent ventilation assistance and hospitalization in intensive and semi-intensive care wards than those with normal weight (67). The pathophysiological mechanisms responsible for this phenomenon are still to be clarified, but it seems that the increased expression of some receptors by adipose tissue in the patient with obesity (e.g. ACE2 and DPP4) may promote an increase in the viral load in the host and a more severe course of the disease (68).

1.1.6. Therapy

Therapy based on the initial classification of the patient with obesity includes an accurate history, objective examination and the appropriate laboratory analysis.

In the history collection, particular attention should be paid to diseases that may predispose to the development of obesity (e.g. hypothyroidism, Cushing's syndrome, trauma and CNS tumors that may involve the hypothalamus) and any medical treatment that may affect weight gain (e.g. antidepressant, antiepileptic, corticosteroid drugs). Family history of obesity, the history of the patient's weight gain, eating habits and physical activity should be investigated, any previous attempts to lose weight and environmental and cultural factors that may have impacted on the failure to meet targets in terms of weight loss (69).

The objective examination (**Table 4**) and laboratory tests should be aimed at identifying CV risk factors (hypertension, dyslipidemia, diabetes) or other obesity-related medical conditions such as obstructive sleep apnea syndrome (OSA) and osteoarticular problems (69).

***Table 4**. Some signs and symptoms that can be observed during the objective examination in the patient with obesity.*

System/organ	Possible finding	Associated comorbidities
Head/neck	Gibbo	Hypercortisolism
	Facies lunaris	Hypercortisolism
	Poor display of soft palate and uvula	OSA
CardioVascular	Tachycardia, irregular rhythm	FA
	Valvular murmurs	Heart failure
Lung	Wheezing	Bronchial reactivity
Abdomen	Hepatomegaly	NAFLD
	Accumulation of abdominal adiposity	Insulin resistance
Gonads	Vaginal atrophy	Menopause
	Testicular hypotrophy	Hypogonadism
Nervous system	Reduced distal sensitivity (tactile, thermal, vibratory)	T2DM
Musculoskeletal	Increase in joint space	OA
	reduction of joint excursion	OA
	Proximal muscle weakness	Hypercortisolism
Skin	Erythematous rash and skin folds	Candidiasis
	Fibromi penduli	Insulin resistance
	Hyperpigmentation of the skin folds	Acanthosis
	Hirsutism	nigricans
	Acne	PCOS
	Strie rubre	PCOS
	Edema	Hypercortisolism
		Heart failure

OSA: Obstructive apnea syndrome; FA: atrial fibrillation; PCOS: polycystic ovary syndrome; OA: osteoartrite; NAFLD: non-alcoholic hepatic steatosis; T2DM: Diabetes mellitus type 2. (Modified from (70)).

During the first examination, measurements should also be made to calculate BMI (weight and height), WC to assess the risk of visceral obesity and neck circumference to assess the risk of sleep apnea (men > 43 cm, women > 41 cm) (69).

1.1.6.1. Changing lifestyle

According to the guidelines of the Italian Society of Obesity (SIO) (69) therapy should be aimed to correcting of eating habits and to a physical rehabilitation program that takes into account the clinical conditions of the patient. The goal of such interventions must be the reduction of the 10% of the initial weight (in 4-6 months), which is deemed adequate to correct the morbidity due to the excess of adipose tissue. In patients with Grade III obesity, greater weight loss is recommended, but such weight loss has been found difficult to maintain in the long term.

The calorie restriction must be customized according to the characteristics of the energy expenditure in the individual patient. In general, the SIO guidelines recommend that this restriction should be between 500 and 1,000 calories and that it should not be less than 1,300 calories per day for outpatients.

In the education of the patient, attention should also be paid to the composition in terms of macronutrients (carbohydrates, proteins and fats) which seem to play an important role in maintaining the weight loss (13). For a balanced diet a daily calorie intake distributed as follows is recommended (69):

- Carbohydrates: 50 - 55% of the total energy.
- Proteins should provide 15% of the total daily energy.
- The lipid content must not exceed 30% (saturated fats must constitute no more than 10%).

In addition, the consumption of alcohol and sugary drinks should be discouraged, due to their high caloric content and poor satiating power.

The weight loss is greater when a low-calorie diet is associated with physical activity (with a dose-dependent effect) (69). Also according to the SIO guidelines:

- With less than 150 minutes per week of aerobic exercise of moderate intensity the weight reduction is minimal;
- 150-250 minutes per week implies a modest reduction (2-3 kg in 6-12 months);
- 250-400 min per week help achieve a weight drop of about 5-7.5 kg in 6-12 months.

Before starting a patient with obesity to a physical exercise program it is important that a careful cardiovascular, pneumological and orthopedic assessment is carried out.

In addition to lifestyle modification, for optimal first-line management of obesity, a therapeutic education program should also be set up in order to increase the patient's awareness of their condition and the therapeutic measures put in place to counteract it and promote the therapeutic alliance.

1.1.6.2. Pharmacologic therapy

Pharmacologic therapy for patients with obesity is indicated if first-line therapies prove insufficient. However, the guidelines stress the need for it to be part of a comprehensive program that includes diet and physical activity. The pharmacological approach finds indications in subjects with BMI ≥ 30 Kg/m^2, or in subjects with BMI ≥ 27 Kg/m^2 that present other risk factors or other diseases related to

obesity. **Table 5** gives a brief description of the main characteristics of drugs used in the treatment of obesity (71–73).

The active ingredients currently on the market include (73):

- Central appetite suppressants: Phentermine/Topiramate, Naltrexone/Bupropion, Setmelanotide.
- Drugs acting peripherally: Orlistat
- Mixed action drugs (central and peripheral): Liraglutide

The drugs listed above are all approved by the Food and Drug Administration (FDA). The European Medicines Agency (EMA), however, has not approved Phentermine/Topiramate, while it has qualified Setmelanotide as "orphan teraphy"(73).

At present, the drugs approved in Italy for the treatment of obesity and overweight in adults are Orlistat, Liraglutide, Naltrexone/Bupropion (72).

Drug	Target and mechanism of action	Effect	Dosages and routes of administration	Most frequent side effects
Fentermina/ Topiramate	Phentermine: transporter SLC6A2 Topiramate: unclear mechanism	Appetite suppression and increased energy consumption.	Extended-release oral capsules (11.25-69 mg; 15–92 mg; 3.75–23 mg; 7.5–46 mg).	Paresthesia, dry mouth, constipation, dysgeusia, insomnia, irritability and alopecia.
Naltrexone/ Bupropion	Naltrexone: opioid receptor antagonist in the CNS. Bupropion; norepinephrine and dopamine inhibitor	Reduced appetite and increased energy expenditure	Tablets with prolonged release (8-90 mg).	Nausea, constipation, vomiting, dizziness, dry mouth, drowsiness.
Setmelanotide	MC4R receptor agonist of melanocortin	Increased sense of satiety after a meal with reduced overeating.	10 mg, subcutaneous injection.	Hyperpigmentation, reaction on injection, nausea and headache.
Liraglutide	GLP-1 receptor agonist	Simultaneous reduction of the feeling of hunger and hunger desire for consumption of food	Maximum dose 3 mg/day, subcutaneous injection.	Nausea, diarrhea, constipation, digestive difficulties. slight increase in heart rate (reversible with discontinuation of treatment).
Orlistat	Inhibitor of gastric and pancreatic lipases, diacylglycerol-lipase and αβ- hydrolases	Reduced absorption of dietary fats by the digestive tract, resulting in increased fecal excretion.	Oral capsules; 120 mg on prescription; 60 mg as an over-the-counter product. Maximum dose: 120 mg three times daily.	Gastrointestinal disorders, cramps, flatulence, fatty and oily stools, fecal incontinence. Reduction of the absorption of vitamins A, D and E

Table 5. *Some of the main features of the drugs used in the treatment of obesity (Modified from (73))*

1.1.6.3. Bariatric surgery

Bariatric surgery represents the approach able to obtain the best results in terms of weight loss in the long term, compared to the previously discussed conservative therapies (74,75).
According to the guidelines of the Italian Society of Obesity and Metabolic Diseases Surgery (SICOB), the current indications to bariatric surgery refer to the severity of obesity and the potential reversibility of the clinical picture and are addressed to patients with;

- BMI ≥ 40 kg/m^2, in the absence of any other comorbidity;
- BMI ≥ 35 kg/m^2, with comorbidities among those classically considered to be associated with obesity, including T2DM resistant to medical treatment (76).

The surgical approach is contraindicated in these cases (74,75):

- Absence of a verifiable period of medical treatment;
- Patient unable to participate in a prolonged follow-up protocol or unable to take care of himself and without adequate family and social support;
- Psychiatric pathologies (psychotic disorders, severe depression, personality disorders and eating behavior), alcoholism and drug addiction;
- Diseases with reduced life expectancy.

The surgeries can be grouped into three main categories, according to their mechanism of action:

1. Mechanical restrictive surgeries in which a mechanical obstacle to the transit of food is created, such as the Adjustable Gastric Bandage (BGR)
2. Interventions with restrictive (mechanical) and functional (anorectic) action: Laparoscopic Sleeve Gastrectomy (LSG) and Roux-en-Y Gastric By-pass (RYGB).
3. Interventions with only malabsorptive action, such as the Mini Gastric By-pass (MGB) which consists in the creation of a small gastric pocket of about 60 ml excluded from the remaining stomach and connected to the small intestine by means of terminal-lateral anastomosis at a 200 cm distance from the duodenum. The MGB is also called Gastric By-pass with single anastomosis (SAGB) or with unique anastomosis (OAGB).

The most commonly used procedures globally are LSG (45.9% of cases), RYGB (39.6%) and BGR (7.4%), illustrated and described in **Figure 5**.

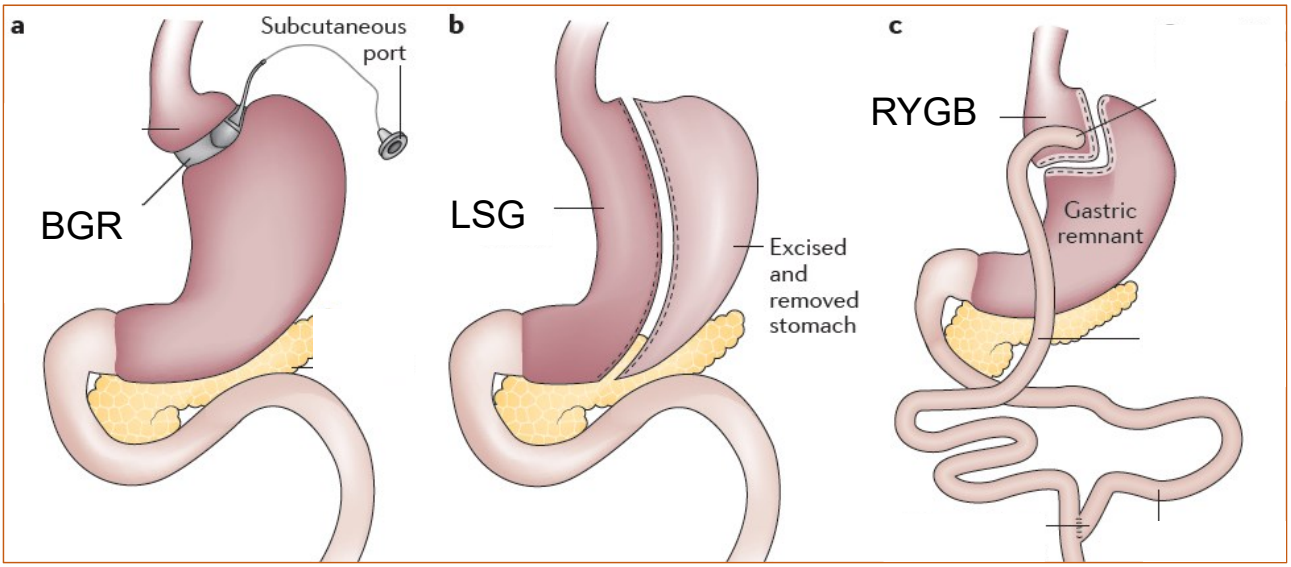


***Figure 5**. a) Adjustable Gastric Bandage (BGR): Mechanical restrictive intervention in which an adjustable circular silicone prosthesis is placed around the upper portion of the stomach; a small "neo-stomach" is created (about 30 ml) that will accommodate the food coming from the esophagus; b) Laparoscopic Sleeve Gastrectomy (LSG): vertical resection along the great curvature of about 4/5 of the stomach with complete removal of the gastric fund; c) Roux-en-Y Gastric By-pass (RYGB): the stomach is completely divided, so as to obtain in the upper portion a very small "pocket" (20-30 ml) that is anastomized with the small intestine. A second anastomosis is performed between the food loop and the bilio-pancreatic loop. (Modified from (13)).*

Compared to RYGB, LSG has a lower incidence of nutritional deficits and complications. Possible short-term complications of LSG are: bleeding, stenosis, reflux, and vomiting. In the long term it is possible that the portion of the stomach saved expands resulting in a reduction of the restriction (75). In some cases, re-Sleeve Gastrectomy may be required, always laparoscopically as the first choice (76).

Perioperative mortality for bariatric surgery procedures is currently around 0.03%-0.2% and is in clear improvement compared to the past (77).

1.1.7. The benefits of weight loss in patients with obesity

The target of weight loss in patients with obesity or overweight with comorbidity is at least 5-10% in the various guidelines. This target is based on the improvements observed in patients in terms of complications such as T2DM, dyslipidemia, hyperglycemia, osteoarthritis, stress incontinence, GERD, hypertension and PCOS. The lipid profile, blood sugar and blood pressure values may be further improved if the weight drop is greater. To observe regression or improvement in the clinical picture of NAFLD and OSA, a weight loss of more than 10% is often required (**Figure 6**) (78).

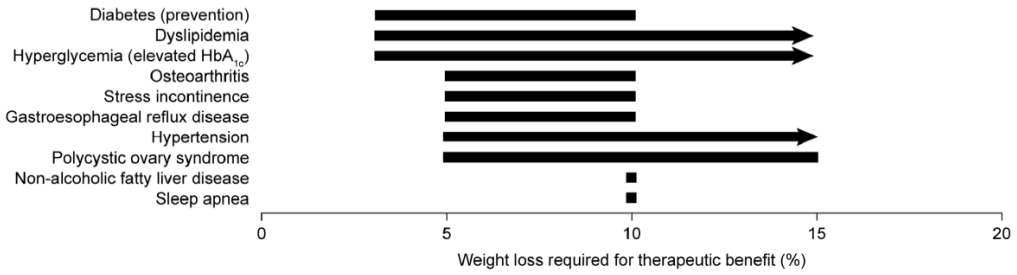


Figure 6. *Benefits of modest weight loss (5-10%). The lines show the percentage of weight loss that demonstrated the respective benefit on the different co-morbidities related to obesity (Modified from (78)).*

As already mentioned, bariatric surgery has proven to be the most effective therapy in obtaining weight loss and in controlling obesity-related complications. In particular, randomized and observational studies have shown its superiority over medical therapy alone in improving blood glucose control, improving hypertension and dyslipidemia, lowering the risk of cancer occurrence (77).

In addition, a recent controlled prospective study published in the NEJM by *Carlsson and coll* covering a period of time of over 20 years, showed that life expectancy in patients undergoing bariatric surgery is on average 3 years higher than those who have followed medical therapy (79).

1.2. ALSTRÖM SYNDROME

1.2.1. Definition and epidemiology

Alström syndrome (ALMS; OMIM #203800) is a monogenic ultra-rare disorder and was described for the first time in the 1959 by the Swedish psychiatrist Carl-Henry Alström et al. (80). Thereafter, more than 900 patients with ALMS have been reported worldwide. ALMS has a prevalence of 1: 1000000 inhabitants and was caused by mutation in the *ALMS1* gene. The prevalence of ALMS is higher in ethnically or geographically isolated populations, where consanguinity is more common (81). *ALMS1* is on chromosome 2p13, that consists of 23 exons and encodes a predicted 461.2-kDa protein of 4169 amino acids. Three alternative splice variants of ALMS1 have been described with different tissue-specific expression and functions (82, 83). Most of the pathogenic variants clustered in exons 8 (51%), 10 (16%) and 16 (17%), which are considered "mutational hotspots" (84–87). To date, over 268 pathogenic variants have been involved in ALMS (85, 86) of which 90% are nonsense or frameshift changes (insertions or deletions) that could be translated in truncated or non-functional proteins. ALMS patients with mutations only in exon 8 appeared to have delayed and milder renal complications, perhaps due to tissue-specific expression of different splice isoforms (88). There is interfamilial and intra-familial clinical variability in the phenotypes, which may be explained by the effects of genetic and/or environmental modifiers (89).

1.2.2. Diagnosis

ALMS is characterized by a wide spectrum of progressive and highly variable disease symptoms (84, 90, 91). Delay of onset of some of the characteristic features (i.e., type 2 diabetes mellitus, T2DM, hepatic dysfunction and renal disease) makes early differential diagnosis very difficult in young children. Thus, diagnosis of ALMS is confirmed by identification of the biallelic pathogenic variants in ALMS1 gene (84, 87). Diagnostic criteria based on age progression of cardinal clinical features: infants through 2 years, children aged 3–14 years, and adolescents/adults over 15 years (**Table 6**) (84). The atypical presentation in some cases suggests that the diagnostic criteria for ALMS may need to be broadened to include patients with a mild phenotype (89, 92).

1.2.3. Clinical features

ALMS is a multisystem, progressive disease. First symptoms usually occur during the first year of life either with cone-rod dystrophy with nystagmus and/or transient dilated cardiomyopathy with congestive heart failure (DCM/CHF). Retinal dystrophy is a major and consistent manifestation

Table 6. Diagnostic criteria for Alström Syndrome.

	Birth – 2 years^	3–14 years	15 years – adulthood
Proof*	2 *ALMS1* mutations	2 *ALMS1* mutations	2 *ALMS1* mutations
Minimum diagnosis requires	(a) 2 major criteria or (b) 1 major and 2 minor criteria	(a) 2 major criteria or (b) 1 major and 3 minor criteria	(a) 2 major and 2 minor criteria or (b) 1 major and 4 minor criteria
Major criteria	- ALMS1 mutation in 1 allele and/or family history of ALMS - Vision (nystagmus, photophobia)	- ALMS1 mutation in 1 allele and/or family history of ALMS - Vision (nystagmus, photophobia, diminished acuity, if old enough for testing: cone dystrophy by ERG)	- ALMS1 mutation in 1 allele and/or family history of ALMS - Vision (history of nystagmus in infancy/ childhood, legal blindness, cone and rod dystrophy by ERG)
Minor criteria	- Obesity - DCM/CHF	- Obesity and/or insulin resistance and/or T2DM (history of) DCM/CHF - Hearing loss - Hepatic dysfunction - Renal failure - Advanced bone age	- Obesity and/or insulin resistance and/or T2DM (history of) DCM/CHF - Hearing loss - Hepatic dysfunction - Renal failure - Short stature - Males: hypogonadism Females: irregular menses and/or hyperandrogenism
Other variable supportive evidence	- Recurrent pulmonary infections - Normal digits - Delayed developmental milestones	- Recurrent pulmonary infections - Normal digits - Delayed developmental milestones - Hyperlipidemia - Scoliosis - Flat wide feet - Hypothyroidism - Hypertension - Recurrent UTI - Growth hormone deficiency	- Recurrent pulmonary infections - Normal digits - History of developmental delay - Hyperlipidemia - Scoliosis - Flat wide feet - Hypothyroidism - Hypertension - Recurrent UTI/urinary dysfunction - Growth hormone deficiency - Alopecia

^Diagnostic criteria in children should be re-evaluated when patient grows older. *If two mutations are found, confirm one inherited from each parent. ALMS, Alström Syndrome; ERG, electroretinogram; T2DM, type 2 diabetes mellitus; DCM/CHF, dilated cardiomyopathy with congestive heart failure; UTI, urinary tract infections (Modified from (84)).

(incidence 100%) of the disease leading to visual impairment that is often severe and can lead to blindness before the age of 20 (87). The differential diagnosis includes other causes of retinal dystrophies such as Leber's Congenital Amaurosis, Achromatopsia, Bardet-Biedl or Early Onset Cone rod dystrophy. Hearing impairment is the second most common manifestation of ALMS and characterised by progressive bilateral sensorineural hearing loss. No abnormality is detected during newborn screening, but deafness has been diagnosed as early as the age of 1 and 70% before the age of 10 with lifetime risk of 100% (84,87,90). In most cases, the initial DCM/CHF improves and patients may remain stable for many years (84,87,89,90). However, in about 15%, a recurrence of DCM/CHF occurs during adolescence or adulthood. In those without infantile cardiomyopathy, de novo disease develops in approximately 1 out of 5 patients (87,89, 90). There is evidence on post-mortem and on non-invasive cardiovascular imaging of coarse replacement and diffuse interstitial fibrosis in the heart. It is not known whether this is direct consequence of the abnormal ALMS1 protein or a result of the metabolic derangements that are associated with ALMS (87, 90). A range of clinical features have been frequently reported in ALMS and include recurrent otitis media, short stature in adulthood, kyphoscoliosis, urinary incontinence or retention, hypothyroidism, empty sella, male hypogonadism and female hyperandrogenism, gastrointestinal dysfunction, delayed developmental milestones (84,87,89), epilepsy, buccofacial apraxia (92) and autistic-spectrum behavioral abnormalities (88–91). The effect of dual sensory loss could result in significant anxiety and depression. Pregnancy is a possible rare event in ALMS women (89), as a case in our center (not published). There is a higher prevalence of upper and lower respiratory tract infection in ALMS, mainly in children. Restrictive lung disease is frequently described, and it can be due to extrapulmonary restriction from obesity or less commonly kyphoscoliosis, sometimes in combination with alveolar and interstitial fibrosis (84, 87, 89). Renal disease in ALMS is common, starts early and progresses with age leading to advanced Chronic Kidney Disease at a young age. Patients with Advanced Kidney Disease should be evaluated for possible early kidney transplant (84, 87, 89, 93). Early childhood obesity with hyperphagia, insulin resistance (IR), T2DM, hypertriglyceridemia, and progressive liver dysfunction are common findings (84, 87, 89, 90), described in detail in "Metabolic complications" paragraph and represented in **Figure 7**.

Figure 7. *Patients with Alström Syndrome, 30 years, BMI 40,3 Kg/m*^{2}

1.2.4. Pathophysiology

ALMS1 encodes a large ubiquitously expressed protein that is associated with the centrosome and the basal body of primary cilium (94, 95). The centrosome is the major microtubule-organising center in most animal cells and has roles in regulating cell cycle transitions and cytokinesis. This particular location of *ALMS1* to the ciliary basal body suggests its contribution to ciliogenesis and/or normal ciliary function (94-96), or centriolar stability (97). Primary cilia are small organelles that extend from the surface of the majority of mammalian cells. The cilium structure involves a microtubule-based axoneme enclosed by a sheath of plasma membrane, which is assembled and maintained by a microtubule motor protein-driven process termed intra-flagellar transport. Cilia have cell-specific functions working as odorant receptors (olfactory neurons), mechanoreceptors (kidney), or have a key role in light detection and photo-transduction (retina). Cilia also regulate embryonic development and fertility in males (98). In kidney epithelial cells, shear stress stimulates lipophagy by primary cilia, contributing to the production of fatty acids and providing mitochondrial substrates to generate ATP through β-oxidation (99). Among the ciliopathies associated to defective primary cilium, Bardet–Biedl syndrome (BBS) overlaps with ALMS. A role for BBS and ALMS1 proteins in the regulation of Notch was evidenced. Loss of *ALMS1*, enhances Notch activation and the accumulation of receptor in late endosomes, but does not disrupt recycling (100). An important role for G protein couple receptors and calcium signaling defects was suggested both in ALMS and BBS (101). Patients with ALMS develop systemic severe fibrosis, which is independent from diabetes or end-stage organ

failure (90). Zulato et al. (102) reported an excessive ECM production and a failure to eliminate myofibroblasts as key mechanisms in ALMS derived dermal fibroblasts in association with a delayed cell cycle (**Figure 8**). Butler et al. (103) obtained similar results by gene expression studies in ALMS lymphoblasts. The primary cilium has a significant role in energy homoeostasis. Mutations in genes that compromise ciliary structure or function, in either humans or mice models, cause monogenic or oligogenic forms of obesity, T2DM and metabolic defects (104). A direct role of *ALMS1* in glucose homoeostasis via the GLUT4 trafficking pathway in adipose tissue (AT) was evidenced by our group (105) (**Figure 9**). In the Alms1GT/GT and fat aussie (foz/foz) mouse models, hyperinsulinemia develops early, and pancreatic islets show beta cell proliferation (106, 107). Otherwise, zebrafish Alms1 mutant failed to respond to glucose challenge, and showed overall reduced β-cell numbers, with higher apoptosis and reduced cell proliferation. These events are associated to an excessive insulin secretion probably due to membrane depolarization in β-cells (108, 109). Alms1GT/GT mice have aberrant insulin signaling either downstream or independent of AKT signaling, before the increase of body weight and circulating insulin levels. A 50% reduction in GLUT4 protein in subcutaneous AT and visceral fat in Alms1GT/GT mice was observed (105). In addition, Alms1GT/GT AT displayed an altered location of GLUT4 in the basal state and a reduced translocation of GLUT4 to the PM when insulin stimulated, both in vivo and in vitro. In agreement, selective reactivation of Alms1 gene in AT of an ALMS conditional knockdown mice model (Almsflin/flin;Adipo-Cre+/−) restored systemic insulin sensitivity and glucose tolerance (110). ALMS1 could regulate glucose transport through the actin cytoskeleton, which plays an important role in insulin stimulated GLUT4 transport. Disturbances in actin architecture and transferrin receptor trafficking in fibroblasts derived from patients with ALMS were reported. In addition, ALMS1 interacts with Actinin 4 (ACTN4) and other members of the cytoskeleton-associated recycling or transport complex (111). In muscle, ACTN4 has been associated with GLUT4 trafficking and its knockdown impaired the transporter localization to the PM after insulin stimulation (112). The ALMS mouse model foz/foz displays hyperphagia before weight gain (107) and a reduced number of hypothalamic primary cilia, suggesting a role for ALMS1 protein in their structural maintenance. A normal ciliary localization of Sstr3 and Mchr1, two proteins involved in hypothalamic appetite regulation and localised specifically to the primary cilium, was reported in foz/foz mouse brain sections. However, the reduced number of cilia could impact on the overall signaling capacity stemming from Sstr3/Mchr1/AC3 and other signal transduction proteins (113). Poekes et al. found that foz/foz mice exacerbated diet-induced obesity is due to a combination of hyperphagia and

reduced energy expenditure, the latter being due to a defective brown adipose tissue (BAT) diet-induced non-shivering thermogenesis (114). In fact, foz/foz mice BAT showed a reduction of mitochondrial, noradrenaline, UCP-1 content and of D2 and β3 adrenergic receptors (110). *ALMS1* is expressed in mouse pre-adipocytes and downregulated during adipogenesis (111). Its disruption induced hypertrophic adipocytes and increase lipogenic enzymes expression in AT (105). ALMS1 mRNA levels are upregulated in growth arrest conditions, in response to serum starvation but not to contact inhibition (116).

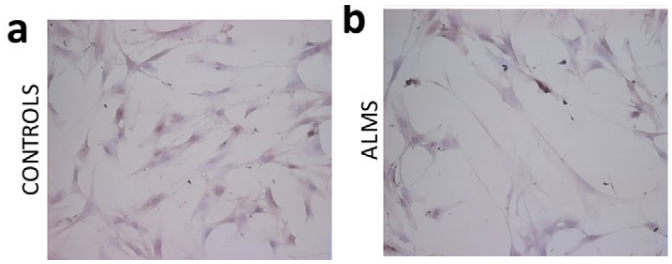


Figure 8. *Fibroblasts of healthy control (a) and (b) ALMS patient, grown on standard tissue culture (2D cultures) coverslips and stained with hematoxylin-eosin (magnification 206), Courtesy of Dr E. Zulato (102).*

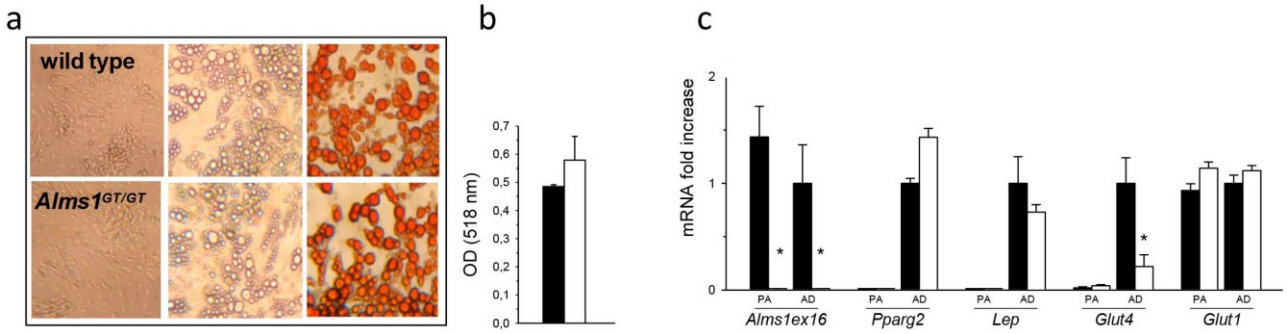


Figure 9*. In vitro characterization of pre-adipocyte adipogenic potential and adipocyte insulin responsiveness from 6-week-old Alms1GT/GT mice. (a) Representative pictures of in vitro adipogenic differentiation from wt and Alms1GT/GT SAT pre-adipocytes (326magnification). Pre-adipocytes (left) were grown in adipogenic medium until fully differentiation (middle) and then stained with Oil Red-O (right). (b) Spectrophotometer ODs from Oil Red O staining of in vitro differentiated adipocytes (n = 3) are reported as mean values 6SEM. (c) mRNA expression of reported genes in pre-adipocytes (PA) and mature adipocytes cell cultures (AD) from wt (black bars) and Alms1GT/GT (white bars) mice was normalized to Rn18s content, reported as arbitrary unit mean ratio 6SEM and expressed as fold change with respect to wt AD, arbitrarily set as 1 for each transcript. *p,0.05 wt vs. Alms1GT/GT. Courtesy of Dr. F. Favaretto (105).*

1.2.5. Metabolic complications

Individuals with ALMS have a high prevalence of metabolic syndrome (117). The metabolic complications could manifest in the first years. In 182 ALMS patients, hyperinsulinemia usually developed between ages 18 months and 4 years. T2DM was diagnosed as early as age 5 years, with

a median age at onset of 16 years. Eighty-two per cent of patients older than 16 years were diabetic (90). The severity of IR is more than 10 times that of BMI-matched patients with obesity (117). Reduced insulin-stimulated glucose disposal and hyperinsulinemia have been observed in patients as young as 1 year of age and can appear before the start of obesity in children, often evolving to T2DM during childhood, with variable age of onset (88, 89, 117). Acanthosis nigricans is common in ALMS and may precede diabetes. Fasting hyperinsulinemia, C peptide elevation and T2DM were confirmed in other cohorts (116–119). Both IR and β-cell failure are the two determinant factors responsible for the development of glucose metabolism alterations in ALMS (116, 117). The progression to overt T2DM is mostly due to a progressive failure of β-cell insulin secretion without any further worsening of IR with age (117). The severity of IR was more than five times that of equally patients with obesity (116). Obesity is one of the cardinal features of ALMS (87, 91, 117). ALMS is characterized by fat accumulation in subcutaneous instead of visceral regions (87). In a large cohort of ALMS patients, childhood obesity occurred in 98% of patients. Although birth weight was within the reference range, rapid weight gain was observed within 2–36 months (87, 90). Obesity in some patients moderated with the onset of other clinical complications (89, 120). Resting energy expenditure was comparable in patients with ALMS and controls but the hyperphagia score trended higher, suggesting that higher intake rather than the lower metabolic rate is probably the primary driver for obesity (116). Obesity-related syndromes, such as Prader–Willi syndrome (PWS), are characterized by hyperphagia. A higher number of coding and non-coding RNA expression disturbances (both upregulated and downregulated) were seen in ALMS in comparison to PWS and males with non-syndromic obesity probably reflecting the complex multiorgan pathology of ALMS (103). Such alterations seem disproportionate to age, BMI and duration of T2DM (116, 121). Most patients had moderate to severe hypertriglyceridemia with normal total cholesterol levels (90, 116, 117, 122). There were cases of acute pancreatitis related to very high hypertriglyceridemia (90). Liver involvement in ALMS was firstly described by Connolly et al. in 1991 (123), with pathological findings of chronic active hepatitis in a child. Four other ALMS patients with evidence of liver disease during the third decade of life were described by Awazu et al. (124). Acute liver failure in a patient 8-year-old was reported by Quiros-Tejeira et al. (125) and liver fibrosis with HCC was demonstrated by Morgan et al. (126). It was described another case of a patient with ALMS, 19-year-old, who showed up at the emergency department with oesophageal varices bleeding, despite no previous signs of hepatic cirrhosis (127). An evaluation from all over the world reporting hepatic and gastrointestinal findings in 97 patients with ALMS revealed severe elevation in liver transaminase levels in 92% of the patients, while

hepatomegaly and splenomegaly were observed since the age of 8 years. Biopsies proved hepatic steatosis, inflammation, bridging fibrosis, and cirrhosis in 16 subjects and esophageal and gastric varices were shown in 14 patients (90). Very recently, the first liver transplant in a 19-year-old male patient with ALMS has been described, with a positive follow-up at 2 years (128). Transient elastography (TE) was the first tool developed to quantify liver fibrosis by measuring mechanical shear wave propagation through the liver parenchyma and recently Gathercole et al. used this method in patients with ALMS (122). In our recent study we evaluated liver fibrosis quantifying liver stiffness by shear wave elastography (SWE) and steatosis using ultrasound sonographic (US) liver/kidney ratio in 18 patients with ALMS, analyzing the contribution of metabolic and genetic alterations in NAFLD progression (129).

1.2.6. Therapy

Currently, no therapy is known for ALMS other than managing the underlying systemic diseases. Consensus clinical management guidelines for ALMS have been recently published by a group of international experts and patient associations with the aim to support equitable care and the standard of therapy for ALMS patients (87). When possible, ALMS subjects and families should be referred to a center of expertise and followed by a multidisciplinary team (MDT). MDT clinics in the UK have improved treatment for patients and knowledge to care givers in many aspects including patient satisfaction, continuity of care and education (130). The most important therapy consists in the prevention or the treatment of the metabolic complication. An increasing number of individuals with ALMS live long enough for the dyslipidaemia and T2DM to result in coronary artery disease, and fatty liver to progress to cirrhosis (87,131). Therefore, lifestyle changing, aerobic exercise and diet induced weight loss are highly recommended as primary treatment for ALMS patients with T2DM and obesity (84,87,91,92). T2DM, dyslipidemia and obesity should be treated according to the guidelines of wider population. Younger individuals rarely necessitate insulin, but some patients require insulin in very-high doses and long term. Many ALMS patients benefit from insulin-

sensitising agents such as metformin and/or thiazolidinediones and/or dipeptidyl peptidase 4 inhibitors (87,92). GLP-1 receptor agonists may be considered both to treat T2DM and obesity (87,92). The management of hypertriglyceridemia includes search and treatment of secondary factors such as obesity, metabolic syndrome, T2DM, hypothyroidism, chronic liver and kidney disease. Some patients responded to a low-fat diet combined with statins and nicotinic acid (132). Other medications include omega-3 fatty acids and fibrates. Recently, the FDA and EMA granted orphan drug designation to PBI-4050 for the treatment of ALMS. Currently, PBI-4050 is under investigation in a phase 2, single-centred, single-armed, open-labelled study of the safety, tolerability and effects on biomarkers in 12 subjects with ALMS in the UK for a duration of 24–48 weeks (133). PBI-4050, a synthetic analogue of a medium-chain fatty acid, is a new dual G protein-coupled receptor GPR40 agonist/GPR84 antagonist that exerts antifibrotic activity in several models of fibrosis (134). These data suggest that PBI-4050 also has the potential to treat ALMS fibrosis. Setmelanotide, the new melanocortin-4 receptor agonist, induced a sustainable reduction in hunger and substantial weight loss in patients with defects in the gene encoding proopiomelanocortin or leptin receptor deficiency (135). Basket trials are ongoing to determine the effect of setmelanotide on weight, hunger and other factors in patients with rare genetic disorders of obesity including ALMS, and confirm efficacy and safety (www.clinicaltrial-gov).

1.3. NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic Fatty Liver Disease (NAFLD) has recently been recognised as the most widespread form of chronic liver disease globally, with a prevalence of 25% in the adult population (136).

Hepatitis in the context of liver steatosis was first described by Tharel in 1962. Later, Ludwig et al. from the Mayo Clinic introduced the term Non-alcoholic Steatohepatitis (NASH) in 1980. Until then it was generally believed that liver steatosis or non-alcoholic fatty liver (NAFL) was a benign condition and that patients with obesity-related morbidity had far more significant health problems to keep under control (137). Although less than 10% of NAFLD patients develop complications, it is one of the primary causes of mortality related to liver disease, liver cancer and liver transplant needs, due to its high prevalence (136).

1.3.1. Definition and epidemiology

NAFL is defined by the presence of more than 5% hepatocyte steatosis, associated with metabolic risk factors (in particular, obesity and T2DM) and in the absence of other chronic liver diseases and/or excessive alcohol consumption (30 g/day for men and 20 g/day for women) (136).

Clinically and histologically, the disease comprises a continuous spectrum of conditions (**Figure 10**) ranging from simple steatosis (NAFL), to non-alcoholic steato-hepatitis (NASH), cirrhosis and liver cancer, in the most advanced stages (138):

- NAFL is characterized by the presence of steatosis involving > 5% of the hepatic parenchyma.
- In NASH (found in about 20% of patients with NAFL), in addition to steatosis, there are also degeneration, lobular inflammation and ballooning of hepatocytes (which are swollen, with light and rarefied cytoplasm and hyperchromic nucleus).
- Over time, a proportion of patients with NAFL/NASH can progress to cirrhosis and from it about 10% progress to hepatocellular carcinoma (HCC).

The prevalence of NAFLD is considerably variegated, in relation to the pattern of detection used in the various geographical areas.

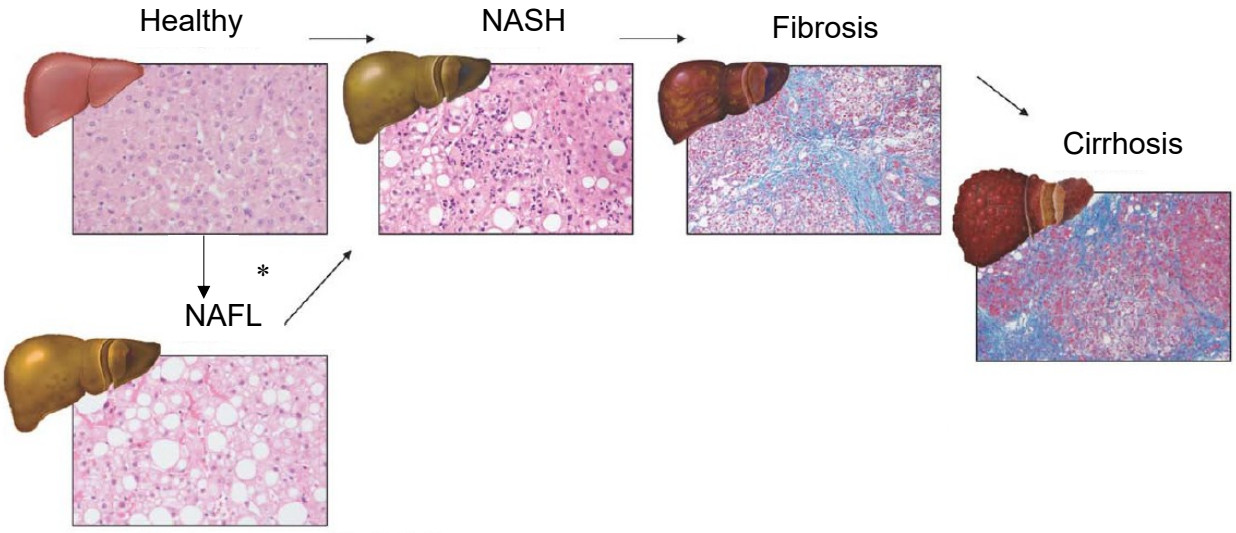


Figure 10. *Histological characteristics of the pathologies included in the Non-alcoholic Fatty Liver Disease. The asterisk (*) indicates the possibility of regression from fibrosis. NAFL, non-alcoholic fatty liver. NASH, non-alcoholic steatohepatitis (Modified from (138)).*

The average global prevalence of NAFLD in adults is 25%, with higher values in the Middle East and South America (32% and 31%, respectively) (139). Given the close pathogenic correlation between liver steatosis and metabolic disorders, the prevalence observed in the population of patients with T2DM is 40-80% and it is 30-90% among those suffering from obesity (140). With regard to the older population, a higher incidence of NAFLD and NASH was observed, partly due to increased comorbidity in older patients (e.g., hypertension, dyslipidemia and T2DM) and partly because of a longer duration of the disease. Studies show mixed results in the prevalence of NAFLD and NASH between men and women. However, a higher trend in fibrosis and higher transaminase values (AST and ALT) has been observed more frequently in men. The prevalence of NAFLD in children is estimated at 7.6% in the general population and 34% in children with obesity (141). Prevalence in Europe follows the global trend (24%) (142).

There are only few studies on the incidence of NAFLD in the general population. A study, conducted for a follow-up period of 8.5 years, estimated with ultrasonographic methods an incidence of 18.5/1,000 per year in the general population in Italy (143). In the coming years, the incidence is expected to rise steadily in some countries (China, France, Germany, Italy, Japan, Spain, the United Kingdom and the USA), resulting in an increase in cases of HCC and hepatic disease-related mortality (142).

Direct estimation of the prevalence of NASH alone in the general population is more difficult, as it requires the execution of a biopsy, which is invasive and cannot performed on all patients. Data collected from post-mortem biopsies on livers of transplanted patients and liver donors showed a prevalence of 1.4 to 15% in these groups. Indirect epidemiological studies have shown a prevalence of 3% - 6% of NASH in adults (138,139).

A recent study conducted by Golabi et al. based on the analysis of data from the National Vital Statistics System, showed that the main causes of death in patients with NAFLD in the USA were the hepatic complications of the disease (45.8% of cases) followed by the cardiovascular ones (10.3%) and extrahepatic cancer (7.0%). Among the latter, the most frequently found were lung cancer, colorectal cancer, and pancreas cancer (144).

1.3.2. Pathophysiology

The strong association between NAFLD and diseases of metabolic origin (such as obesity, T2DM, insulin resistance) falls within the very definition of this disease, so as to be defined by many Authors "the manifestation of metabolic syndrome in the liver" (145-147).

Metabolic syndrome (MetS) is characterized by a many cardiovascular risk factors (such as visceral obesity, hypertension, dyslipidemia and altered glycemic profile) and its definition varies slightly between scientific organisations (148) (the most frequently used definitions are listed in **Table 7**).

In patients with severe obesity that also have the characteristics of Mets, the prevalence of NAFLD, measured with imaging techniques, is around 90-95% and over a third of these presents NASH to a histological examination. Increases in BMI and WC are also associated with an increased risk of liver disease progression, especially in older patients, probably due to the fact that the presence of NAFLD turned out to be more associated with the accumulation of visceral fat, rather than subcutaneous fat (149). This accumulation, as already mentioned in paragraph 1.1.4. , involves an increase in lipolysis, worsening insulin resistance and the release of several pro-inflammatory and pro-fibrogenic mediators that participate in promoting the development of NAFLD and accelerate its progression (149).

***Table** 7. The three most commonly used definitions for the Mets. NCEP: ATP III: National Cholesterol Education Program: Adult Treatment Panel III; IDF: International Diabetes Federation. Note that the definition of the IDF differs from that of NEP:ATPIII substantially for the more restrictive criteria with respect to the WC. (Modified from (148)).*

WHO 1999	Presence of insulin resistance or glucose > 6.1 mmol/L (110 mg/dl), 2 h glucose > 7.8 mmol (140 mg/dl) (required) along with any two or more of the following: 1. HDL cholesterol < 0.9 mmol/L (35 mg/dl) in men, < 1.0 mmol/L (40 mg/dl) in women 2. Triglycerides > 1.7 mmol/L (150 mg/dl) 3. Waist/hip ratio > 0.9 (men) or > 0.85 (women) or BMI > 30 kg/m2 4. Blood pressure > 140/90 mmHg
NCEP (National Cholesterol Education Program) ATP3 2005	Presence of any three or more of the following: 1. Blood glucose greater than 5.6 mmol/L (100 mg/dl) or drug treatment for elevated blood glucose 2. HDL cholesterol < 1.0 mmol/L (40 mg/dl) in men, < 1.3 mmol/L (50 mg/dl) in women or drug treatment for low HDL-C 3. Blood triglycerides > 1.7 mmol/L (150 mg/dl) or drug treatment for elevated triglycerides 4. Waist > 102 cm (men) or > 88 cm (women) 5. Blood pressure > 130/85 mmHg or drug treatment for hypertension
IDF (International Diabetes Federation) 2006	Waist > 94 cm (men) or > 80 cm (women) along with the presence of two or more of the following: 1. Blood glucose greater than 5.6 mmol/L (100 mg/dl) or diagnosed diabetes 2. HDL cholesterol < 1.0 mmol/L (40 mg/dl) in men, < 1.3 mmol/L (50 mg/dl) in women or drug treatment for low HDL-C 3. Blood triglycerides > 1.7 mmol/L (150 mg/dl) or drug treatment for elevated triglycerides 4. Blood pressure > 130/85 mmHg or drug treatment for hypertension

For several years, in the past, the most likely pathogenic hypothesis on the onset and development of NAFLD was the so-called "two-hit hypothesis". According to this theory, a first event ("first hit"), consisting of the accumulation of lipids in the hepatocytes would increase the vulnerability of the

liver, making it in fact more susceptible to factors that, at a later stage, would be responsible for promoting liver damage, inflammation and fibrosis ("second hit") (147). Today this theory is replaced by the "multiple-hit hypothesis", which considers the complexity of the pathogenesis of NAFLD. According to this theory, not one or two, but a series of factors (genetic background, insulin resistance, lipotoxicity, innate immunity activation, intestinal microbiome, diet and lifestyles, etc.) contribute to the progression of liver disease (150) (**Table 8**).

Table 8. *Factors associated with the onset and progression of NAFLD. Bold: factors considered to have a protective effect on the development of the disease. (Modified from (136)).*

Comorbidities	Genetic factors	Environmental factors
• T2DM • Insuline resistance • Dyslipidemia • Obesity • Hypertension • Hypopituitarism	• PNPLA3 • TM6SF2 • GCKR • MBOAT7 • **HSD17B13**	• Fructose • Cholesterol • Alcool • **Physical exercise** • **Coffee**

The pathogenic processes involved in the genesis of NAFL can be seen as a continuum with major metabolic diseases: obesity, Mets and T2DM (**Figure 11**) (146).

A number of environmental and genetic factors are responsible for weight gain and fat accumulation. Exceeding the capacity of expansion of adipose tissue (explained in detail in paragraph 1.1.4.) leads to an increase in circulating FFA and fat storage in the visceral and ectopic environment. FFA storage in the skeletal muscle promotes insulin resistance (IR), inhibiting insulin-mediated glucose uptake. IR also involves the liver, in which production of glucose, lipogenesis de novo, release of VLDL and dyslipidemia (resulting in increased atherogenic risk) are promoted. In addition, the accumulation of triglycerides and toxic metabolites in the liver promotes cell damage, apoptosis and fibrosis. Hepatocytes affected by these dysfunctions synthesize and secrete dipeptidyl-peptidase or dipeptidyl peptidase 4 (DPP4), which in turn promotes fat tissue inflammation and worsening IR.

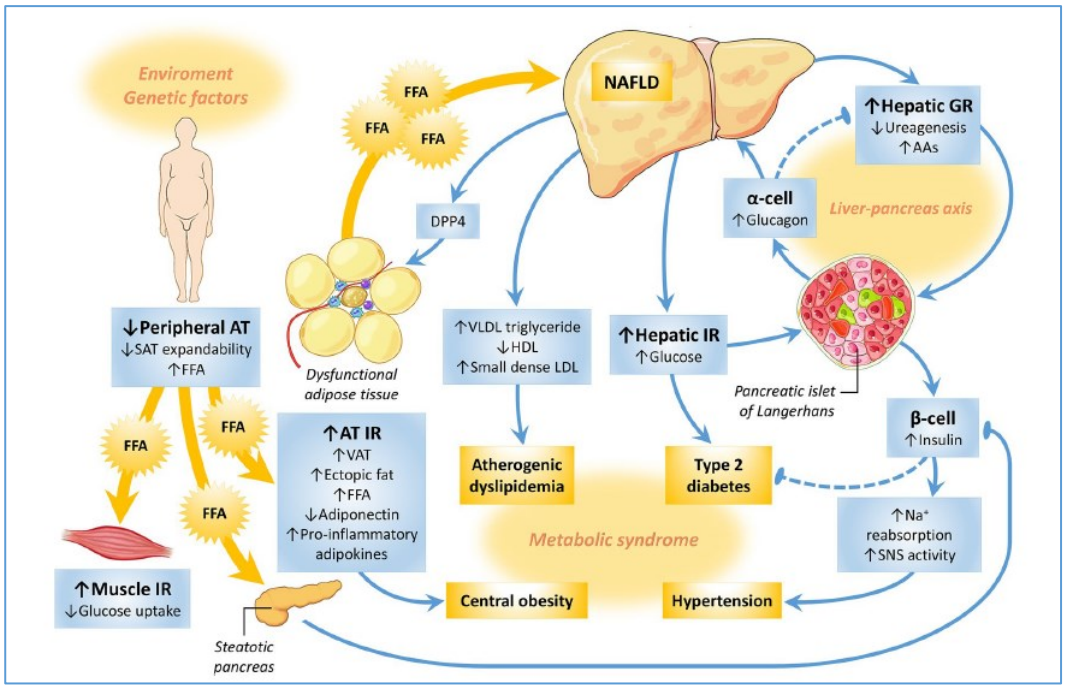


Figure 11. *NAFLD pathogenesis. GR: glucagon resistance; SNS: sympathetic nervous system; DPP4: dipeptidil- peptidase 4 (Modified from (146)).*

The role of the pancreas: the "liver-pancreas axis"

Recent studies have shown that the role of the pancreas in the pathogenesis of NAFLD is not limited to dysregulation in the production of insulin alone, but that there is a real liver-pancreas axis involved in the development of chronic liver disease (146). The deposition of FFA in the pancreas exerts a lipotoxic effect, causing β cell dysfunction that promotes hyperglycemia and the development of diabetes. However, the increased fat deposit in the liver also promotes the resistance of the liver itself to the action of glucagon, thus reducing ureagenesis and causing an increase in the circulation of amino acids. The latter in turn stimulate the pancreas to the compensating production of glucagon, ultimately establishing a vicious circle in which the hyperglucagonemia leads to a further increase in the liver release of glucose (146).

The resulting state of IR and hyperinsulinemia can also contribute to increased sodium reabsorption and sympathetic SN hyperactivation resulting in elevated blood pressure (146).

Role of the gut microbiome: the "gut-liver axis"

The gut-liver axis has long been recognized as one of the major players in the development of NAFLD. Nutrient signals, along with other information from the intestine, reach the liver via portal

circulation. The slow rate of blood flow within the liver sinusoids allows interaction between signals derived from the intestine, hepatocytes and immunity cells residing in the liver. When a dysfunction of the intestinal barrier occurs (with increased permeability of the intestine), the passage of products of bacterial origin occurs, such as lipopolysaccharides (LPS), which reaching the liver through the portal circulation are able to promote inflammation and liver fibrosis (characterized by increased extracellular matrix production). In addition, animal studies have recently shown that alterations of the intestinal microbiome (dysbiosis) also represent one of the risk factors for the development of NAFLD (145).

This close correlation between metabolic diseases and NAFLD has sparked an interesting debate in the scientific world about a reassessment of its definition during the last decade. In 2020 a group of experts published an article in the Journal of Hepatology expressing a consensus statement regarding the most appropriate use of the term "metabolic dysfunction associated-liver disease" or MAFLD and proposing positive criteria for the diagnosis of MAFLD (**Figure 12**) in opposition to the definition of NAFLD in terms of exclusion of alcohol related, infectious, genetic and autoimmune causes of liver disease (151).

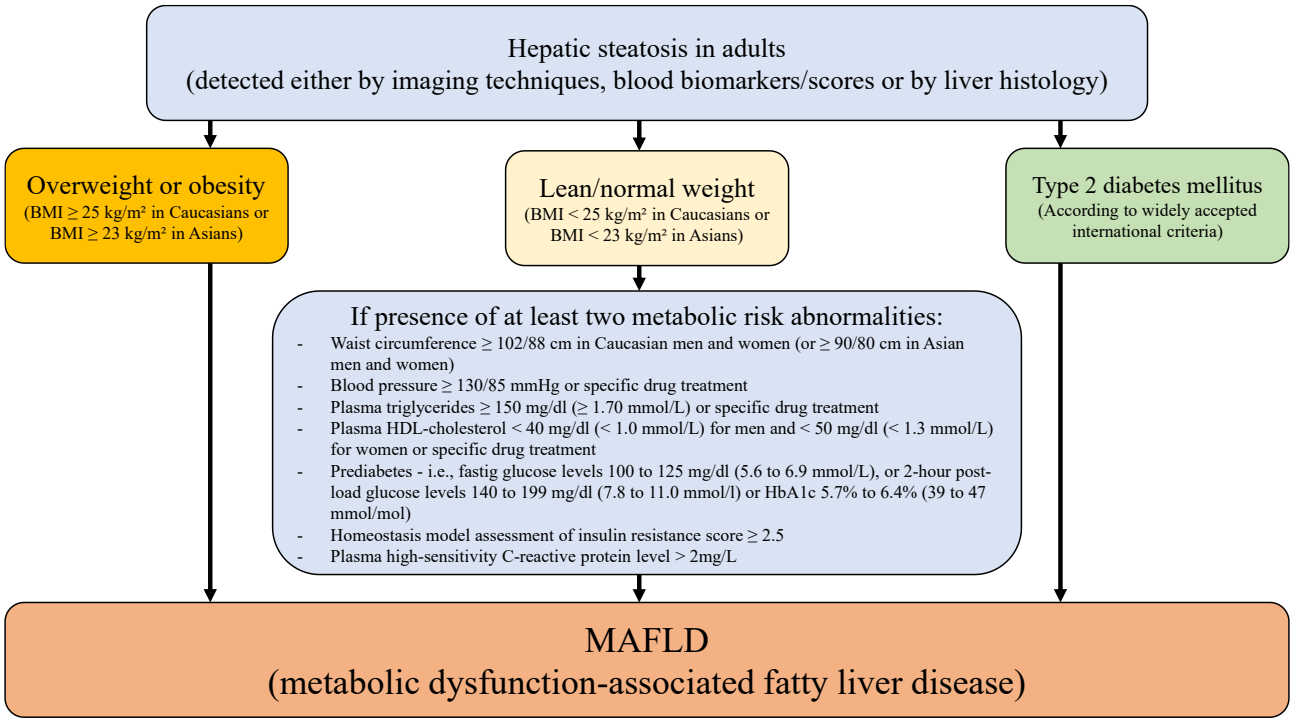


Figure 12. *Flow chart of positive diagnostic criteria of MAFLD (Modified from (151)).*

From a clinical point of view, a definition of NAFLD based on positive diagnosis criteria offers the undisputed possibility of stratifying patients who are affected in more homogeneous disease progression risk groups (152) and also allows to include normal patients with an increased metabolic risk factors, despite not being affected by obesity or diabetes (147). On the other hand, further studies are needed to resolve the critical issues that this new definition offers, such as the epidemiological discrepancies detected between the prevalence of patients classified as NAFLD and those defined as suffering from MAFLD (152) and in the pediatric population.

As far as the natural history of the disease is concerned, NAFLD consists of a heterogeneous group of conditions, in each of which a different contribution of the promoting factors may determine different degrees of progression over time. In most cases it manifests itself as a chronic, stable liver disease with no tendency to progression. However, a proportion of patients experience fibrosis and an increased risk of developing complications related to the more advanced stages of NAFLD and the onset of HCC.

The complexity of the physiopathological processes described above highlights the need for early identification of patients at risk of progression in order to prevent the development of NASH and cirrhosis and their associated complications. In this regard, several studies have shown that, in patients with NAFLD, the presence of fibrosis, especially in advanced stages, is a prognostic marker linked to increased morbidity and mortality for liver causes and in general (136). Fibrosis can develop both in NAFL and in NASH. However, the speed of its progression turned out to be different, comparing these two forms. A meta-analysis of Singh et al., based on studies performed on biopsy findings, quantified this difference in temporal terms: the evolution of a fibrosis stage (starting from F0 as a base) to the next (for example, from F0 to F1 or from F1 to F2) is on average 7.1 years for NASH and 14.3 years for NAFL (153).

These data further underline the need for effective methods for the earliest and most accurate diagnosis of NAFLD patients in order to achieve a correct treatment approach and the most appropriate follow-up, evaluated on a case-by-case basis.

Extrahepatic complications of NAFLD

In addition to complications related to liver disease, patients with NAFLD have an increased risk of developing CV diseases (which represent the main reason of mortality from all causes), dyslipidemia,

T2DM, chronic kidney disease, tumors (in addition to HCC, also colorectal cancer), OSA and post-operative complications in major surgeries (141,154).

1.3.3. Diagnosis

In most cases, patients with NAFLD are asymptomatic or have non-specific symptoms, such as fatigue, sleep disturbance or discomfort at the right upper quadrant of the abdomen. Liver enzymes, transaminases (ALT, AST) in particular, should always be evaluated for a correct diagnostic approach of liver disease. However, it should be taken into account that they offer poor reliability in identifying NASFL/NASH, as they have a low AUC (between 0,6 and 0,7) (141). At present there are no disease-specific markers and the diagnosis of NAFLD requires the exclusion of other causes of chronic liver disease (**Table 9**). The most frequent clinical finding is hepatomegaly (155). In more advanced stages, the disease may present with signs of portal hypertension. For a complete assessment of the patient, possible conditions that may occur in association with NAFLD, such as polycystic ovary syndrome (PCOS) (associated with hyperandrogenism, in young women), OSA and psoriasis should also be investigated (155).

Table 9. *Some of the most frequent causes of secondary hepatic steatosis (non-NAFLD) (Modified from (156)).*

Macrovesicular steatosis	**Microvesicular steatosis**
Excessive alcohol consumption ➢ 40 g/day in males ➢ 20 g/day in females	Reye's syndrome
Drugs: amiodarone, tamoxifen, methotrexate, corticosteroids, other	Drugs: valproic acid, antiretrovirals
Hepatitis C (genotype 3 of HCV)	Steatosis associated with pregnancy, HELLP syndrome
Wilson's disease	Inborn errors of metabolism: Lysosomal acid lipase deficiency (Wolman's disease in children, cholesteryl ester storage disease in adults), other
Lipodystriphies	
Parenteral nutrition	
Subacute or severe caloric deprivation	

1.3.3.1. Liver biopsy: the gold standard

Guidelines of the European Association for the Study of the Liver (EASL) (157) and the American Society for the Study of Liver Diseases (AASLD) (158) agree that liver biopsy is the only method capable of diagnosing NASH and distinguishing it from simple steatosis (NAFL).

Biopsy is recommended in all patients with NAFLD who have an increased risk of NASH and/or advanced fibrosis. The patients considered at high risk of NASH are those having the characteristics of MetS, with high levels of transaminases (with a high ratio of AST/ALT in particular), aged > 60 years and of Hispanic ethnicity. The results of the direct observation of the hepatic parenchyma allow to distinguish between NAFL, NAFL with inflammation and NASH and to describe the possible presence of fibrosis (predictive factor of primary importance in chronic liver disease) (146).

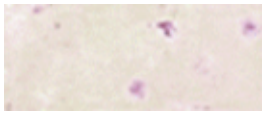
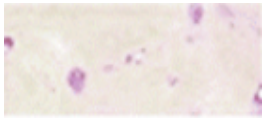
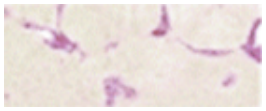
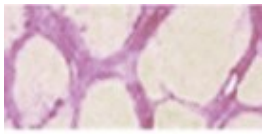
Most scientific literature describes fibrosis according to the criteria of NASH CRN, based on the Metavir classification, a scoring system originally developed for the histological description of hepatitis C (138) (**Table 10**).

Table 10. *Classification of disease activity by NASH-CNR system (NASH Activity score, NAS) (Modified from (156)).*

Steatosis (%)	Lobular inflammation	Ballooning
0: <5	0: No	0: No
1: 5-32	1: <2 foci	1: mild
2: 33-66	2: 2-4 foci	2: abundant
3: >66	3: >4 foci	

However, there are several histological classifications elaborated to quantify fibrosis (examples are Ishak and Metavir, **Table 11**).

Table 11. *Histological staging of fibrosis, according to the Ishak and Metavir classification, in comparison (Modified from (159)).*

Appearance	Ishak stage: categorical description	Ishak	Metavir
	No fibrosis (normal)	0	F0
	Fibrosis expansion of some portal areas ± short fibrous septa	1	F1
	Fibrosis expansion of portal areas ± short fibrous septa	2	F2
	Fibrosis expansion of most portal areas with occasional portal to portal (P-P) bridging	3	
	Fibrosis expansion of portal areas with marked portal to portal (P-P) bridging as well as portal to central (P-C)	4	F3
	Marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis)	5	
	Cirrhosis, probable or definite	6	F4

The quantification fibrosis degree constitutes a key moment of the histological examination, seen its close relationship (and absolutely independent from the other histological characteristics) with global and liver-related mortality in NAFLD patients.

In addition, histological examination is able to provide information on the state of disease activity, obtained from the NASH activity score or NAS (**Table 12**) and represented by a numerical score from 0 to 8 (156).

Table 12. *Stages of fibrosis according to the CRN Fibrosis Staging Classification. (Modified from (156)).*

Stage	**Degree of fibrosis**
0	Without fibrosis
1a	Mild perisinusoidal fibrosis (zone 3)
1b	Moderate perisinusoidal fibrosis (zone 3)
1c	Periportal/portal fibrosis exclusively
2	Fibrosis zone 3 + periportal/portal
3	Fibrosis bridging
4	Cirrhosis

Despite its undeniable advantages, the liver biopsy has some limitations that make it an unsuitable method for large-scale screening of the population or for a follow-up of patients with NAFLD (136,160,161):

- It was originally meant to be a useful tool for the etiological diagnosis, rather than the staging of hepatic fibrosis;
- It provides an instant view of a dynamic disease;
- It is not free from sampling errors (the disease does not affect the liver in a homogeneous way) and intra- and inter-observational variability (using semi-quantitative scoring system, it requires to be done by experienced personnel);
- It is an invasive method and presents possible complications (e.g. hemorrhage in 0.3 % of cases, haemobilia and mortality in 0.01 % of cases)

For these reasons, whenever possible, the use of non-invasive tests (NITs) is preferred.

1.3.3.2. Non-invasive test

According to the 2016 EASL guidelines, non-invasive markers of hepatic fibrosis should be used to reduce the need for liver biopsy (162):

• to identify the risk of NAFLD among individuals with increased metabolic risk in the context of primary care;

• in order to identify those with worse prognosis (e.g. advanced NASH) in the context of secondary and tertiary care;

• to monitor disease progression;

• to predict response to therapeutic interventions.

Theoretically and ideally, a marker of hepatic fibrosis should (163):

- Be liver-specific, easy to measure and readily available
- Provide early diagnosis with high accuracy and prognostic elements for the patient
- Correlate with extracellular matrix deposits and reflect longitudinal fibrosis progression/regression changes
- Be validated (producer independent) in relation to different etiologies of liver disease
- Not affected by physiological changes (sex, age, dietary habits, exercise, habitus, circadian variations)
- Save additional invasive investigations or complex diagnostic tests

As it can be easily imagined, none of the markers currently available has all of these features. However, some offer valuable help in the screening of chronic liver disease, identifying patients at risk of progression of the pathology with good sensitivity and specificity.

Classification

The NITs can be divided into two large groups:

1. Serum biomarkers;

2. Liver Stiffness measurement (LS), with ultrasound sonography (US) which provides indirect estimation of fibrosis in the liver using various imaging techniques.

SERUM BIOMARKERS

Table 13 summarizes the main serological markers used in the non-invasive assessment of NAFLD.

Table 13. *Available serological markers, subdivided according to information on various aspects of chronic liver disease.*

STEATOSIS	SteatoTest Hepatic Steatosis Index (HIS) Fatty Liver Index (FLI)
NASH	**Markers of inflammation** Ferritin, C-reactive protein (CRP), Adipokines, tumor necrosis factor α (TNF-α), Interleukin (IL)-6, IL-8 Leptin, resistin, visfatin
	Markers of oxidative stress Oxidized low-density lipo-protein, malonaldehyde, and thiobarbituric acid reactive substances
	Markers of apoptosis Caspase-cleaved fragments of the intermediate filament keratin 18 (M30 K-18)
	Marker of metabolic stress Fibroblast Growth Factor (FGF)-21
	Complex models
FIBROSIS	Fibrosis-4 index (FIB-4) AST on ALT ratio (AST/ALT) AST to Platelet Ratio Index (APRI) Enhanced Liver Fibrosis test (ELF) NAFLD fibrosis score (NFS)
	Complex models

Markers of steatosis

In simple steatosis, normal levels of transaminases can often be observed, and they begin to show fluctuating levels only as the disease progresses. Identifying NAFLD at its early stages for early therapeutic intervention could prevent the possible progression of the disease to cirrhosis and reduce the risk of HCC. Among the most used tests for this purpose are (164):

- SteatoTest (2005): it is a validated test, with reasonable accuracy for the assessment of moderate to severe steatosis
- Fatty Liver Index (FLI) (2006): it evaluates steatosis on a scale from 0 to 100. It showed good accuracy in determining steatosis in studies compared to liver ultrasound (165).

Markers of NASH

Although at present there is a lack of specific treatment options for NASH, its identification has a prognostic importance. The proposed biomarkers are based on the physiopathological stages of the

disease progression, as described in Section 1.1.4.: death from hepatocyte apoptosis, oxidative stress, and inflammation (164):

- **Markers of inflammation**

  Ferritin and high-sensitive C-reactive protein (hs-CRP) are nonspecific markers of NASH-associated inflammation. Also several adipokines and cytokines have shown a certain correlation with steatohepatitis. For example, low levels of adiponectin and high levels of tumor necrosis factor α (TNF-α) appear to be associated with a more significant liver damage.
- **Markers of oxidative stress**

  Oxidative stress contributes to the pathogenesis of NASH, but it is difficult to detect markers related to it because of their volatile nature. The results on the substances studied to date have given mixed results.
- **Markers of apoptosis**

  One of the main consequences of ongoing apoptosis of NAFLD is the activation of the caspase cascade (mainly the caspase-3 pathway) that cleavages a series of substrates, including Caspase-cleaved fragments of the intermediate filament keratin 18 (M30 K-18), one of the main sources of intermediate filaments in the hepatic parenchyma. Studies have shown that serum K-18 levels correlate with histological changes and reflect the severity of NASH (166). It was also observed that the association of K-18 with other biomarkers, such as the Fibroblast growth factor-21 (FGF-21), shows an increase in the positive predictive value (PPV) of 82% and a negative predictive value of 74% (161) for NASH.
- **Marker of metabolic stress: FGF-21** is part of a family of 22 proteins involved in a number of metabolic functions. It is mostly produced by hepatocytes, stimulated by the increase of the circulating FFA, and in smaller proportion by other cells (adipocytes, skeletal muscle cells and pancreatic β cells). FGF-21 was first discovered in the CNS, in which it can act by regulating circadian rhythms, and stimulate the pituitary-adrenal hypothalamus axis to cortisol production which can induce hepatic gluconeogenesis (167). Today its role in different tissues has been extensively studied (**Figure 13**): FGF-21 exerts various anti-inflammatory, antidiabetic and anti-hyperlipidemic actions, making it a potential target for the development of new therapies to be used in pathologies on a metabolic base (167).

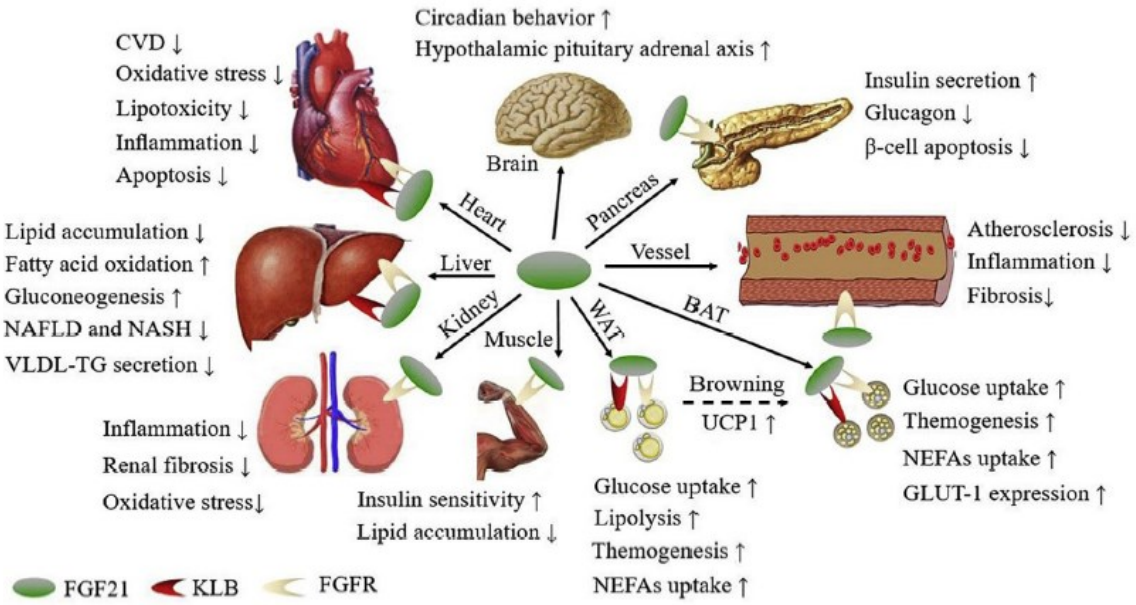


Figure 13. *Relationship between FGF21 and different organs and tissues. FGF-21: fibroblast growth factor; FGFR: FGF-21 receptor; KLB: β-klotho protein; WAT: white adipose tissue; BAT: brown adipose tissue; UCP1: uncoupling protein-1; CVD: cardiovascular disease; TG: triglycerides; VLDL: very low density lipoprotein; GLUT-1: glucose-1 transporter; NEFA: non-esterified fatty acids (Modified from (167)).*

There are also complex models designed to predict disease progression (e.g. NASH tests, NASH diagnostics, etc.), but many of them require further validation to prevent the use of routine (166).

Markers of fibrosis

Major scores for the determination of fibrosis in patients with NAFLD include (161):

- **Fibrosis-4 index (FIB-4):** used to assess the severity of fibrosis, is calculated according to the formula:

  $$(\text{Age} \times \text{AST}) / (\text{Platelet count} \times \sqrt{\text{ALT}})$$

  FIB-4 offers the possibility to predict fibrosis with different values of PPV and NPV, depending on the proposed cut-offs. The cut-off of 1.3 predicts advanced fibrosis with a sensitivity of 85% and a specificity of 65% and is the most used with values of 36% for PPV and 95% for NPV. For people over 65 years of age, different cut-offs are recommended (168).

- **AST on ALT ratio (AST/ALT)** was calculated dividing the AST to ALT concentrations (U/L) (138).
- **AST-to-platelet ratio index (APRI):** Developed for HCV-related fibrosis, it calculates the ratio of AST to platelets. From recent meta-analyses it appears to have a fair sensitivity for the advanced stages of disease (severe and advanced fibrosis and cirrhosis) (138).
- **Enhanced Liver Fibrosis test (ELF)** incorporates three serum markers involved in the synthesis and breakdown of extracellular matrix: hyaluronic acid (HA), N-terminal propeptide of collagen type III (PIIINP), and tissue inhibitor of metalloproteinase 1 (TIMP-1) (169).
- **NAFLD fibrosis score (NFS)**: is calculated according to a formula that includes a series of parameters (age, BMI, presence of impaired fasting blood sugar/diabetes, AST/ALT, platelets, albumin). Values < -1.455 are predictive of low risk of advanced fibrosis, while values > 0.675 predict a high risk of F3-F4 (138).

MEASUREMENT OF THE LIVER PARENCHYMA STIFFNESS (LS)

The various LS available are largely based on the method of US elastography, an imaging technique that evaluates the elasticity of tissues, that being the tendency of the tissue to resist the deformation imposed by force applied on it and the ability to resume its original form after the cessation of the stimulus.

According to the different characteristics, these techniques (**Figure 14**) can be classified into:

- **"Strain imaging"** techniques: they measure tissue deformation (strain), determined by the pressure exerted by the probe resting on the surface of the body. They don't provide information on the extent of the deformation force applied, illustrating only the elasticity of the tissue through the constitution of a map (elastogram) (170).
- **"Shear wave imaging (SWI)"** techniques: they create a dynamic stress on the tissue, produced by a mechanical vibration (as in 1D transient elastography, TE) or an acoustic radiation force (ARFI). The latter is the basis of the ARFI investigation methods and the shear wave elastography (SWE) (170). SWI also offer a quantitative measurement of the image and are more sensitive in detecting diffuse pathology of the hepatic parenchyma (such as that found in NAFLD).

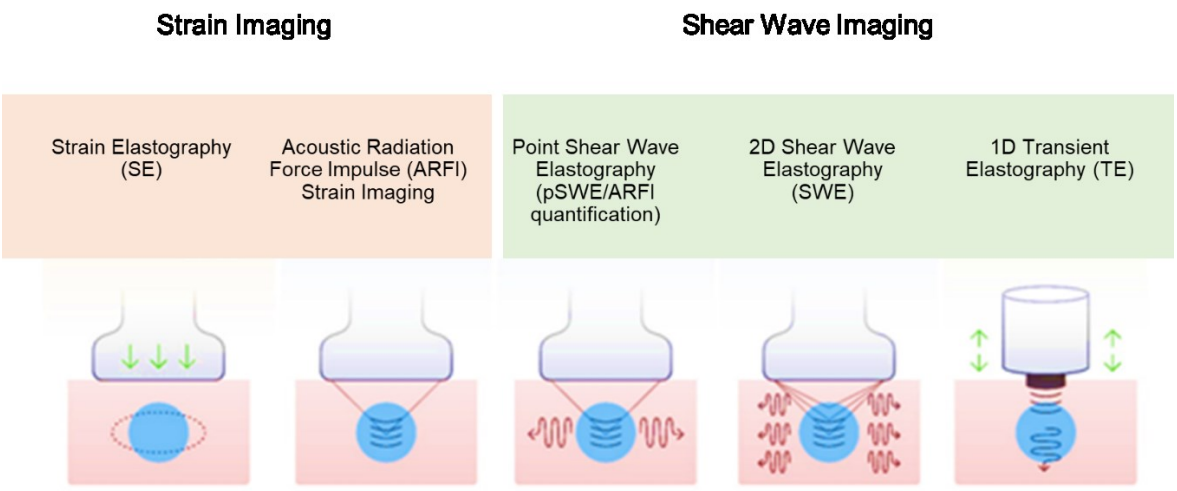


Figure 14. *Illustration of the main elastographic techniques (Modified from (170))*

Transient elastography

Transient elastography (TE) (or Fibroscan®) was the first ultrasound-based elastographic method to be developed and marketed for the e valuation of chronic hepatopathies.

It is performed without a direct guide of imaging (171). It uses a 50 MHz ultrasound transducer mounted on the axis of a piston, which generates a frequency pulse of 50 Hz and a width of 2 mm that propagates through the subcutaneous tissue and liver. The velocity of propagation is measured by an ultrasound transducer and correlated through the Young module (also called module of elasticity) to the rigidity of the tissue, which is expressed in kPa (172). The probe is placed in the intercostal spaces of the upper right quadrant of the abdomen, with the right arm in maximum extension. The evaluation area is fixed and depends on the type of probe used (pediatric, adult, XL). the accuracy of the results depends on the choice of the appropriate probe (171). At least 10 measurements are required for the validity of the exam, with a success rate of 60% and an interquartile range (IQR) of < 30% (163). The Area Under A Receiver Operating Characteristic (AUROC, used for estimating the accuracy of diagnostic tests) was 0.87-0.98, according to literature. The probability of obtaining a correct classification is 85%-94% for cirrhosis (stage F4) and 57%-90% for advanced fibrosis (stage F3). However, there is considerable overlap between the intermediate stages of hepatic fibrosis (171).

Point Shear Wave Elastography

Point shear wave elastography (pSWE) uses short-life acoustic pulses emitted with a 2.6 MHz frequency to induce compression in liver tissue, which generates shear waves that propagate perpendicularly to the direction of the pulse. The shear waves are recorded using ultrasound (US) in a small region of interest (ROI) of 5 x 10 mm that the operator can locate at his choice within the area explored (172). The monitoring and estimation of shear waves (measured in kPa or in m/s) is done using the real-time B-mode ultrasound image. Imaging also makes it possible to identify and avoid large vessels and bile ducts during the measurement (170). Scientific studies have shown that p-SWE may be more reliable than TE in the Liver Stiffness measurement, showing similar, if not greater, accuracy in the identification of hepatic fibrosis (170).

Two-dimensional shear wave elastography (SWE)

In this method, a series of focused ultrasound pulses generates plane shear waves within the examination tissue, the waves propagate transversely and are recorded by an ultrasonic system. The propagation rate of shear waves is used to measure (kPa or m/s) the stiffness of the tissue under examination (172).

This technology allows to examine a circular ROI measuring a few cm^2, positioned at the operator's own discretion according to the ultrasound image in B-mode. Stiffness information are provided in real-time (1 frame per second) within the ROI, by displaying a color map in which, conventionally, the blue color represents a greater elasticity and the red color represents a greater rigidity of the tissue. The ultrasound image in B-mode and the color map overlaid on it help the operator choosing the location of the ROI within which the stiffness will be measured (172) (**Figure 15**).

The great advantage of SWE is that, in addition to the B-mode examination and the speed maps of the shear waves, it offers the possibility to indirectly estimate the visco-elastic properties and the steatosis, thus allowing a multiparametric assessment of the hepatic parenchyma to be performed by:

- The construction of dispersion maps that, by calculating the slope with which the shear waves are dispersed, give an estimate of the visco-elastic properties of the organ under examination, quantified in (m/sec)/kHz. Preliminary clinical studies state that the measurement of the visco-elastic characteristics of the liver may provide information on necrotic-inflammatory changes and the deposition of FFA in the liver (173).

• Attenuation Imaging (ATI) coefficient evaluates the attenuation of the sound pulse (measured in dB/cm/MHz) and is thought to offer an excellent quantitative estimate of the liver steatosis (174).

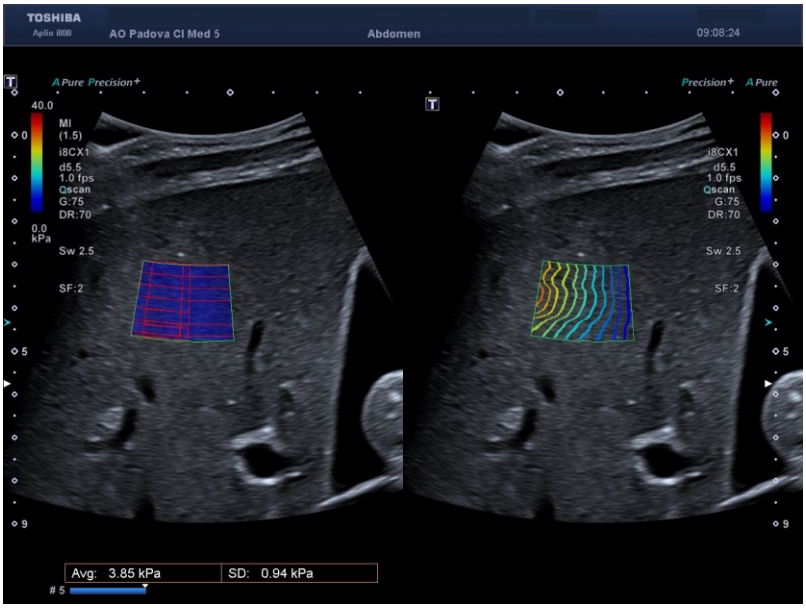


Figure 15. *Image obtained during the exam with shear wave elastography (SWE). The small rectangle represents the region of interest. It should be placed at least 1.5-2.0 cm below the hepatic capsule. On the right, the shear wave velocity measurement is displayed. (Courtesy of Prof. G. Bombonato).*

NITs limitations

The main limitations of non-invasive fibrosis diagnosis techniques in NAFLD/NASH are summarized in **Table 14**. A recent work by Patel and Sebastiani states that the great advantage of the NITs currently available is to avoid unnecessary and risky repetitions of biopsies. However, they have diagnostic limitations that should always be taken into account, interpreting the results, before making any clinical decisions (163).

Table 14. *Limitations of the main methods of non-invasive diagnosis of hepatic fibrosis. (Modified from (163)).*

Type of limitation	**Serum biomarkers**	**Transient elastography (VCTE)**	**Shear wave elastography**	**MRE**
Technical limitations	Not Liver specific	Requires training and experience for validated quality criteria No B-mode image and unable to select liver region of interest	Requires dedicated US training Quality criteria not yet validated Unable to compare reported parameters of shear wave speed (range 0.5-4.4 m/s) or Young's modulus (2-150 kPa) between US devices, VCTE, or MRE	Requires specialised technician or radiologist
Discrimination of adjacent fibrosis stages	No	No	No	No
Performance for intermediate fibrosis stage	Poor	Overlapping LSM range	Limited data	Overlapping LSM range
Cost and availability	Patented marker panels not readily available and costly	Not widely reimbursed Access concerns in resource limited practices	Not readily available outside specialised centres	Costly Not available outside dedicated radiology centres
False positivity	Haemolysis. Gilbert's disease. cholestasis, immune thrombocytopenia, inflammation. age, exercise, non-fasting	Acute hepatitis, inflammation, non-fasting, exercise. hepatic venous congestion. inflammation or infiltration, alcohol excess, cholestasis, steatosis, portal vein thrombosis	Left vs. Right hepatic lobe, acute hepatitis, hepatic inflammation or infiltration, non-fasting, exercise, right heart failure, extrahepatic cholestasis, breathing cycle (end-expiration vs. end-inspiration)	Inflammation, cholestasis, hepatic venous congestion, postprandial state, and right heart failure
Failure	Indeterminate "grey zone" scores in 30-50% for simple markers (NFS, APR1, FIB-4)	Higher failure rates than serum tests: operator inexperience, narrow intercostal	Higher failure rates than serum tests: BMI, tissue depth >2-3 cm below skin surface	Higher failure than serum tests: waist circumference/ BMI. claustrophobia, iron deposition,

		space, body habitus, ascites		massive ascites, higher field strength (3 T vs. 1.5 T)
Thresholds	Variable for simple markers across aetiologies	Variable across aetiologies	Not validated across aetiologies	Vary between gradient-recalled echo vs. echo planar imaging, 2D vs. 3D acquisition. 40 vs. 60 Hz, and across aetiologies
Differentiation between simple steatosis and	No	No	No	No
NASH Follow-up of dynamic fibrosis changes	No	No	No	No

APR1: AST-platelet ratio index; AST: aspartate aminotransferase; BMI: body mass index; CLD: chronic liver disease; FIB-4: fibrosis-4; LSM: liver stiffness measurement; MRE: Magnetic resonance elastography; NAFLD: non-alcoholic fatty liver disease; NFS: NAFLD fibrosis score; US: ultrasound; VCTE: vibration-controlled transient elastography.

1.3.4. Therapy

NASH represents a complex and multifaceted pathology, with different risk factors and metabolic complications. At present there is no specific therapy that guarantees NAFLD resolution. The first-line treatment is lifestyle modification through a balanced diet and exercise, similar to those implemented in the patient with obesity and explained in detail in section 1.1.6. In addition, patients with NASH should abstain from or limit alcohol consumption, which is associated with progression of liver lesions and reduction of the possibility of resolution of the disease (175). **Table 15** shows the therapeutic approach in patients with NAFLD/NASH.

Exercise exerts its beneficial effect on pathology, reducing the fatty acid content in the (regardless of weight), reduces insulin resistance and can change the de novo synthesis of FFA in liver (175).

Table 15. *Therapeutic approach in patients with NAFLD/NASH (American Association for the Study of Liver Diseases). (Modified from (175))*

	NAFLD	Suspected NASH	Biopsy-proven NASH	NASH cirrhosis
Obtain baseline liver function tests including CBC, transaminases (AST/ALT), bilirubin, alkaline phosphatase, creatinine, INR	✓*	✓	✓	✓
Medical optimization of comorbid conditions:	✓	✓	✓	✓
Control of type 2 diabetes, hypertension, and dyslipidemia				
Cardiovascular optimization				
Statin therapy as indicated by ACC/AHA guidelines				
Intensive lifestyle modification with goal of 7%-10% weight loss	✓	✓	✓	✓
Caloric restriction				
Aerobic exercise regimen				
Minimize alcohol use	✓	✓	✓	✓
Minimize added fructose intake	✓	✓	✓	✓
If patients are unable to achieve weight loss goal and may be otherwise eligible, refer for bariatric surgery evaluation	May consider, particularly for patients with BMI >35 or BMI >30 and type 2 diabetes	✓	✓	✓
Consider pioglitazone for patients with or without diabetes (30 mg/d)			✓	
For patients without diabetes, consider vitamin E (800 IU/d)			✓	
Consider eligibility for clinical trial participation		✓	✓	✓
Initiate screening for hepatocellular carcinoma per AASLD guidelines				✓
Initiate screening for esophageal varices per AASLD guidelines				✓
Consider evaluation for liver transplant if clinically decompensated				✓

Abbreviations. AASLD: American Association for the Study of Liver Diseases; ACC/AHA: American College of Cardiology and American Heart Association; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; CBC: complete blood cell count; INR: international normalized ratio; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis.

*Check marks indicate that the treatment strategy should be used.

Among drug therapies, vitamin E (an antioxidant) and pioglitazone (a thiazolidinedion) have been shown to induce improvements in randomized trials.

The main aspect of the treatment is the modification of CV risk factors, which is why the guidelines also recommend the prescription of statins in high-risk patients (175).

2.0. AIM

Among the complications of obesity, NAFLD occupies a prominent place, showing a much higher prevalence (90-95%) than in the general population (about 25%). Moreover, Alström syndrome (ALMS) is an ultra-rare monogenic disease representing a model of metabolic disease characterized by insulin-resistance, T2DM, obesity, early incidence of NAFLD and multi-organ fibrosis.

Consequently, in patients with ALMS and in patients with obesity, it is important to be able to readily identify NAFLD to set up an appropriate therapeutic and follow-up program, to prevent its evolution towards the more advanced stages (NASH, fibrosis and HCC) and reduce mortality and morbidity.

Among the therapies of obesity, bariatric surgery has proven to be the most effective method in obtaining and maintaining weight loss and, consequently, in improving the metabolic profile and complications related to obesity. However, few data are available in the literature from longitudinal studies on the influence of weight loss on the course of NAFLD in the short and long term in patients with obesity. The purpose of our study is to determine the short- and long-term effect of weight loss reaching by bariatric surgery on the progress of NAFLD, in patients with obesity in a prospective assessment, using non-invasive methods (laboratory analysis, serum markers and ultrasound scan with SWE).

3.0. METHODS

3.1. Patients and controls

3.1.1. Patients and controls (1)

This is a prospective study started on August 2017 and finished in November 2021. Thirty-four patients with obesity were recruited at the Centre for the Study and Integrated Treatment of Obesity, Padua University Hospital and evaluating before surgery, 6 months year and 4 years after surgery. Patients underwent a multi-disciplinary baseline evaluation according to a standard clinical protocol and were assigned to surgical treatment following current international guidelines (176). The baseline (visit 0, V0) evaluation was completed within 6 months before surgery and before the beginning of a 4-weeks very low-calorie diet prescribed immediately before surgery. After surgery, patients were regularly seen for medical and nutritional management at least once a year, and a complete clinical re-evaluation including medical examination, laboratory analysis and liver ultrasound with SWE was scheduled in all patients after 6 months (V6m) and 4 years (V4y). Patients received a nutritional plan: a balanced hypocaloric diet providing an energy deficit of around 500 kcal per day with about 25–30% of energy from fat, 50–55% from carbohydrates and 20% from protein. Patients also received physical activity prescriptions (at least 150 min per week of moderate intensity physical activity). At V6m a patient dropped out. In addition, at V4y, other 4 dropouts occurred. For this reason, we were able to collect data of 29 patients. LSG is performed as the first-choice procedure at our centre except for patients affected by severe gastro-oesophageal reflux disease (where mini gastric bypass is usually performed). LSG was performed by the same surgical team, as we described previously (177).

Specific exclusion criteria were diagnosis of cancer in the previous 5 years, infection with hepatotropic viruses, excessive alcohol consumption, auto-antibodies indicative of autoimmune hepatitis, genetic hemochromatosis, a current infection, predictor signs for cirrhosis or HCC at the US, and use of hepatotoxic drugs.

All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ' Padua Ethical Committee for Clinical Research ' (2892P, 11/06/2015).

The control group was recruited for comparing results from ultrasound scan. The demographic characteristics and the ultrasound parameters recorded in this control group are described in **Table**

16. The mean measurements of the left hepatic lobe in the control population were 9.00 ± 1.24 cm. The diameter of the spleen was 9.95 ± 1.39 cm.

Table 16. *Demographic, anthropometric and ultrasound scan parameters of the control group.*

Ultrasound scan control group (n= 135)	
Age (years)	47.88 ± 14.40
Male/Female	65/70
Body Mass Index (kg/m^2)	22.37 ± 2.64
Left hepatic lobe (cm)	9.00 ± 1.24
Liver Stiffness (kPa)	4.13 ± 0.58
Spleen (cm)	9.95 ± 1.39

The mean values of the elastography measured with the 2D-SWE (Shear Wave Elastography or SWE) were 4.13 ± 0.58 kPa, compatible with a population without evidence of liver disease, comparing the data obtained with those in literature (mean cut-off values for fibrosis at stage F2 from 5,7 to 8,3 kPa, depending on the studies) (170,173). At present there are no cut-off studies for mild fibrosis degrees. However, as already mentioned above (paragraph 1.2.3.) the great advantage of 2D-SWE ultrasound is to offer a non-invasive method to identify patients with moderate to severe fibrosis (170) in a population with a high prevalence of chronic liver disease (NAFL and NASH), in order to ensure a prompt start to the most appropriate diagnostic-therapeutic pathways to reduce the risk of evolution of the pathology.

3.1.2. Patients and controls (2)

Eighteen patients who fulfilled clinical diagnostic criteria for ALMS (84,87) and carried *ALMS1* pathogenetic variants were enrolled at the Internal Medicine 3, Padua University Hospital, in the period between 2017 and 2019. Patients underwent a multi-disciplinary evaluation, and a complete medical history was taken (nutritional aspects, physical activity, smoking and drinking habits, drug and medications, past and current medical conditions). The diagnosis of T2DM, hypertension and metabolic syndrome was performed according to recent guidelines. We recruited 25 healthy

volunteers as US controls with a negligible daily alcohol consumption. Control subjects had normal weight (BMI 22.4 ± 4.5 Kg/m2) and were age-matched with the patients with ALMS (age 28 ± 8 years). The study was conducted in accordance with the Declaration of Helsinki and approved by the Local Ethics Committee (Prot. n. 2371P); in-formed written consent was obtained by each patient.

3.2. Genetic analysis

Patients with ALMS were genetically determined. Genomic DNA, obtained by QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) extraction from the peripheral blood of all ALMS patients, was amplified using a standard PCR protocol with HotStarTaq Master Mix Kit (QIAGEN) using primer sequences firstly for "hot spot" regions of ALMS1 (exon 8, 10, and 16) and, if negative, for all other exons (1–7, 9, 11–15, 17–23). Amplicons were purified with Illustra ExoProStar (GE Healthcare, Chicago, IL, USA), sequenced using BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by the 3130xl Genetic Analyzer (ThermoFisher Scientific). Coding regions and exon–intron boundaries were analyzed; primers sequence and conditions are available on request. Sequences were compared to the GenBank reference sequence NM_015120.4 for ALMS1 using Clustal Omiga, a freely available tool (178). Pathogenic variants of ALMS1 identified by genomic sequencing were described according to the guidelines of the Human Genome Variation Society (HGVS) reported by den Dunnen et al. (179) and were validated using VariantValidator.org_v0.1.3 (180). The new variant described in the present study was submitted to the Euro-WABB online database, a locus-specific database (in the Leiden Open Variation Database format) listed by the Human Genome Variation Society in the Locus Specific Mutation Databases LSDBs (www.HGVS.org) (86).

3.3. Anthropometric measurements

BMI was calculated as weight (kg) divided by height-squared (m^2). Height was measured to the nearest 0.01 m using a stadiometer. Body weight was determined to the nearest 0.1 kg using a calibrated balance beam scale. Waist circumference was assessed using a tape measure. Weight loss percentage (WL%) was calculated as follows: 100% × (weight V0 – weight V1) / weight V0. Weight gain percentage (WG%) was calculated as follows: (weight V5 – weight V1) / weight V0.

3.4. Biochemical assessment and non-invasive test

For each patient, we measured fasting plasma glucose (FPG), basal insulin, C-peptide, lipid profile ((total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-cholesterol) and low-density lipoprotein-cholesterol (LDL-cholesterol), triglycerides (TG)), platelets, serum creatinine, ALT, AST

and gamma glutamiltrasferase (GGT). All biochemical blood analyses were performed with a standard diagnostic kit according to WHO First International Reference Standard: fasting glucose (Glucose HK Gen.3, Roche Diagnostic, USA), insulin, Tumor Necrosis Factor-α (TNF-α), Interleukin-6 (IL-6) (IMMULITE 2000 Immunoassay, Siemens Healthcare GmbH, Germany), hs-CRP (CardioPhase High Sensitivity C-Reactive Protein, Siemens Healthcare), Thyroid Stimulating Hormone (TSH) (Elecsys TSH, Roche Diagnostic GmbH, Mannheim, Germany), vitamin D3 (Diasorin Liaison XL LAS, Saluggia, Italy), Leptin (Leptin-RIA-CT, Mediagnost, Germany). We were not able to collect leptin values at V4y. Full blood count (platelets) was measured by flow cytometry (Sysmex Europe GmbH, Germany), serum lipids by spectrophotometer (Roche Diagnostic, USA) and urea, serum creatinine, ALT, AST, GGT titers were assayed by enzymatic method with the addition of pyridox-al-5-phosphate in compliance with IFCC reference methods (181). The insulin-resistance index was indirectly estimated using the homeostasis model assessment (HOMA) as follow: (fasting serum insulin (μU/ml) x fasting plasma glucose (mmol/l))/22.5 (182). HOMA was not calculated in patients with insulin treatment.

The Fibrosis-4 index (FIB-4) was calculated using the following formula: (age (years) x AST)/(platelet counts (x 109/l) x ALT1/2) (138, 168). A low cutoff point (<1.30) and a high cutoff point (>2.67) best discriminate between the absence and presence of advanced fibrosis, respectively (138).

AST on ALT ratio (AST/ALT) was calculated dividing the AST to ALT concentrations (U/L) (138). The references ranged from, according to our laboratory, 10 to 35 U/L for AST, 7 to 35 U/L for ALT.

The AST-to-platelet ratio index (APRI) was calculated as AST (U/l)/(upper limit of normal)/platelet count (x109/l) x100 (138).

The caspase-cleaved fragments of the intermediate filament keratin 18 (M30 K18), indicative of apoptosis, was quantified by M30 Apoptosense® ELISA (PEVIVA, VLVbio AB, Sweden). The values of K-18 proposed in the original work presenting it as a marker of liver fibrosis are 145 U/L (126-190) (166). In a 2017 meta-analysis by He et al. the cut-off values for patients developing NASH are 121.6-380.2 U/L (0.60-0.95 sensitivity and 0.60-0.97 specificity) (183).

The Enhanced Liver Fibrosis test (ELF) was quantified by ADVIA Centaur XP system (kindly provided by Siemens Healthcare Italy). ELF values between 7.7 and 9.7 suggested moderate fibrosis and values ≥ 9.8 are indicative of severe fibrosis.

Fibroblast Growth Factor-21 (FGF21) levels were measured in serum samples by ELISA (BioVendor, Czech Republic). The reference values of the Central Laboratory of the Padova University Hospital are:

- in the 21-49 age group: 192,7 ± 128, 2 pg/ml for men and 173,5 ± 148,8 pg/ml for women
- in older age groups (up to 85 years of age): 298.7 ± 227.5 pg/ml for men and 322.3 ± 237.3 pg/ml for women.

Two cut-offs are suggested in literature for the diagnosis of NASH: 126 and 578 pg/ml with a sensitivity and specificity > 90% for the diagnosis of NASH but with a modest PPV (0.59-0.78) and NPV (0.49-0.60). The measurement of CK-18 followed by that of FGF-21 increased the NPV to 74% and the PPV to 82% (161).

The dosage of M30 K18, ELF and FGF21 were possible only in 26 out 29 patients.

3.5. Ultrasound scan

All patients were referred for an ultrasound scan (US) (Canon Medical System, Aplio i800, probe i8CX1, US band between 4 MHz and 6 MHz) of the abdomen in order to detect the presence and the degree of hepatic steatosis and to assess LS. Each US was performed by the same sonographer of the Internal Medicine 5 at Padua University Hospital. Patients fasted from midnight of the day scheduled for the scan. SWE measurements were performed on the right lobe of the liver, through intercostal spaces with the patient lying in the supine position with the right arm in maximal abduction. Measurements were performed at least 1.5 to 2.0 cm beneath the Glisson capsule to avoid reverberation artifacts. The mean value of 5 consecutive measurements was used for statistical analyses. In part 1 the degree of steatosis (0 to 3) was measured by B-mode US, by comparing the echogenicity of the hepatic parenchyma and that of the right kidney cortical (ordinal grade of steatosis). The different grades were attributed as follows:

- Grade 0: absence of steatosis (normal echogenicity; there are no differences between echogenicity of the hepatic and cortical parenchyma of the kidney)

- Grade 1: mild steatosis (with a slight increase in the hepatic parenchyma echogenicity compared to the renal cortical, without significant attenuation of the ultrasonic beam in depth)

- Grade 2: moderate steatosis (increased echogenicity of the hepatic parenchyma associated with attenuation of the ultrasonic beam in depth, but with the diaphragm still clearly visible along the deep contour of the liver and indistinguishable vessel walls)

- Grade 3: severe steatosis (great increase in liver echogenicity with important attenuation of the ultrasound beam in depth; the vascular detail, the portal bifurcation to the hilum and the diaphragm are not visible).

In the part 2 liver/kidney method (L/K) was used, consisting in the comparison between the echogenicity intensity measured in the liver (region of interest, approximately 1.2 x 1.2 cm) and in the right kidney cortex, sampled at the same depth to reduce the attenuation bias of the two different organs (184,185). Echo intensity analysis of digitized B-mode images was performed using the software Horos®. To determine the presence of steatosis we considered a cut-off value of 1.6 calculated as mean value (1.2) ± 2 SD (0.2) in our control group. Patients with signs of portal hypertension at the US underwent to esophagogastroduodenoscopy and esophageal varices were classified according to the Japan Society for Portal Hypertension (186). All the measures were performed in accordance with the Italian Association for the Study of the Liver (AISF) (187).

3.6. Statistical analyses

Statistical analyses were performed using the SigmaPlot v.14 (Systat Software, Adalta, Arezzo, Italy). All variables were tested by Normal Test (Shapiro-Wilk test) and Equal Variance Test (Brown-Forsythe). For the analysis of variables in the three times (V0, V6m and V4y) One Way Analysis of Variance was used when Normality Test and Equal Variance Test had been passed (data are presented as mean values ± standard deviations) or, if not, with Friedman Repeated Measures Analysis of Variance on Ranks (data are presented as median value (25th-75th percentile)). The analysis of variables in the three groups (normal glucose tolerance, prediabetes and diabetes) were tested by One Way Analysis of Variance or Kruskal-Wallis One Way Analysis of Variance on Ranks. To isolate the groups that differ from the others, a multiple comparison procedure (Bonferroni t-test or Tukey test) was used. The T- test or Kruskal-Wallis Sum Ranks test was used for the analysis of two groups. The Chi-square test test was carried out for categorical variables. Pearson's correlation coefficient (r) and the relative p values were calculated to analyse simple linear correlations between two variables. In all analyses, the p values were two-sided and a p value lower than 0.05 was considered statistically significant.

4.0. RESULTS

PART 1. Short- and long-term effects of bariatric surgery on Non-Alcoholic Fatty Liver Disease in patients with obesity.

4.1. Clinical and biochemical characterization before (V0), 6 months (V6m) and 4 years (V4y) after bariatric surgery

Out of 34 patients, 29 patients were at the end enrolled in the study. Comorbidities present in the selected patients were as follows: hypertension (n = 18), dyslipidaemia (n = 13), prediabetes (n = 12), T2DM (n = 9), OSA (n = 7), and functional impairment (n = 11). Clinical and biochemical characteristics of the population are resumed in **Table 17**.

WL at V6m was 23.84 ± 6.10 % and was maintained at V4y (24.77 ± 6.82 %) with a corresponding improvement of glycaemic profile, triglycerides, HDL and transaminases (AST and GGT). An initial improvement, followed by a slight re-increased it was observed in WC values. TC and LDL values did not improve. Bile acids remained unchanged at V6m and increased significantly 4 years later, although in the normality range. Unspecific index of inflammation (IL-6 and TNFα) did not present a statistically significant improvement although we described a decreasing trend for hsCRP. Leptin values showed the same trend of BMI. Full blood count (red blood cells, white blood cells and platelets) remained in the normal range during the follow-up even if we evidenced a significantly decrease. The same was for creatinine values. Vitamin D3 did not increased in a significantly way even if patients took an oral supplementation after surgery. TSH showed an important reduction at V4m.

Regarding non-invasive index of fibrosis, we highlighted an improvement of AST/ALT ratio and APRI. On the other hand, FIB-4 values showed an opposite trend with a slight improvement at V6m followed by a statistically significant worsening at V4y. The marker of apoptosis (M30 K18) showed an improvement only at the long-term follow-up ($p = 0.034$) (**Figure 16A**). ELF, as a result of the measurement of the three indices (HA, PIIINP and TIMP1), showed different trend for each patient and the median values were substantially unchanged over the three times (**Figure 16B**). Out of 26 patients, 7 presented values ≥ 9.8, suggesting severe fibrosis at baseline. On the other hand, considering HA and PIIINP we highlighted a statistically significant worsening of the former and an improvement of the latter at V4y (HA increased and PIINP decreased) (**Table 17**).

Table 17. *Anthropometry, biochemical variables and non-invasive test in 29 patients at baseline, 6 months and 4 years after sleeve gastrectomy.*

n = 29	Before (V0)	6months (V6m)	4 years (V4y)	p
Age (years)	51.48 ± 8.69	–	–	
Sex (M/F)	10/19	–	–	
Weight (kg)	120.5 ± 23.6	91.7 ± 19.2 *	89 ± 19.2 *	< 0.001
BMI (kg/m^2)	43.74 ± 7.39	33.13 ± 6.26 *	31.90 ± 5.00 *	< 0.001
WC (cm)	124.6 ± 15.6	104.3 ± 16.6*	108.6 ± 15.6 *	< 0.001
WL%	–	23.84 ± 6.10	–	
WG%	–	–	- 1.49 ± 6.65	
TWL%	–	–	24.77 ± 6.82	
FPG (mmol/L)	5.7 (5.4-6.4)	4.8 (4.3-5.3) *	4.9 (4.6-5.4) *	< 0.001
Insuline (mU/L)	14.6 (10.5-25.7)	9.3 (5.2-11.0)	8.8 (5.4-14.2) *	< 0.001
HOMA	3.9 (2.5-7.4)	2 (1.0-2.6) *	2.0 (1.0-3.2)	< 0.001
TC (mg/dl)	183 ± 45	188 ± 40	196 ± 50	0.993
HDL (mg/dl)	49 ± 11	50 ± 9	64 ± 14	0.819
TG (mg/dl)	116 (92-153)	88 (74-119)	75 (56-97) *	< 0.001
LDL (mg/dl)	108 ± 41	115 ± 35	114 ± 46	0.615
AST (U/L)	24 (18-28)	18 (16-20) *	19 (16-22)	0.045
ALT (U/L)	30 ± 10	19 ± 10	17 ± 6	0.407
GGT (U/L)	20 (15-27)	11 (9-19) *	12 (10-17) *	< 0.001
Bile acids (µmol/L)	0.69 (0.38-1.2)	0.63 (0.49-1.0)	1.0 (0.7-1.4)■	0.014
IL-6 (ng/L)	2.3 (1.9-3.8)	2.3 (1.9-3.0)	2.2 (1.9-3.4)	0.159
TNFα (ng/L)	8 (6-9.2)	8.1 (6.1-9.2)	7.8 (5.6-19.2)	0.740
hsCRP (mg/L)	5.81 ± 3.66	3.14 ± 3.08	2.18 ± 2.73	0.720
Leptin (µg/L)	36.2 ± 17.0	15.4 ± 15	–	< 0.001
WBC (x 10^6/mm^3)	7.51 ± 1.88	6.00± 1.78 *	5.84 ± 1.44 *	0.001
RBC (x 10^6/mm^3)	4.78 ± 0.30	4.68 ± 0.29	4.50 ± 0.28 * ■	0.001
PTL (x 10^3/mm^3)	265 ± 81	246 ± 72 *	249 ± 76	0.012
Creatinine (µmol/L)	70 (63-79)	68 (60-74) *	65 (56-77)	0.004
vit. D (nmol/L)	41.0 ± 16.9	65.4 ± 29.4	64.2 ± 23.6	0.680
TSH (mU/L)	1.66 (1.30-2.33)	1.48 (1.01-2.00) *	1.48 (1.01-2.00)	0.002
FIB-4	0.96 (0.58-1.10)	0.91 (0.70-1.37)	1.01 (0.80-1.37) *	0.002
AST/ALT	0.82 (0.64-0.88)	1.02 (0.82-1.2) *	1.22 (0.91-1.44) *	< 0.001
APRI	0.1 ± 0.04	0.08 ± 0.03*	0.09 ± 0.03	0.042

n = 29	Before (V0)	6months (V6m)	4 years (V4y)	p
M30 K-18 (U/L)	116.6 (79.6-222.2)	93.9 (58.8-125.5)	67.3 (57.6-111.6) *	0.034
ELF	9.1 (8.6-9.8)	9.3 (8.8-9.7)	9.2 (8.6-9.9)	0.816
HA (ng/ml)	35.1 (19.3-78.9)	41.4 (25.9-70.5)	46.4 (23.9-101.5) *	0.048
PIIINP (ng/ml)	9.6 (8.3-11)	9.8 (8.5-11.8)	7.6 (6-9-8.6) ■	0.028
TIMP1 (ng/ml)	217.5 (193.8-247.4)	213-1 (192.1-239.8)	216 (202-236)	0.832
FGF-21 (pg/ml)	187.4 (134.2-409.3)	169.8 (126.8-439.6)	136.5 (79.8-237.0) *	0.009

*M: male. F: female. BMI: Body Mass Index. WC: waist circumference. WL%: Percent Weight Loss. WG%: Percent Weight Gain. FPG: Fasting plasma glucose. HOMA: Homeostasis model assessment-insulin resistance index. TC: total cholesterol. HDL: High Density Lipoprotein -cholesterol. LDL: Low Density Lipoprotein -cholesterol. TG: triglycerides. ALT: alanine aminotransferase. AST: aspartate aminotransferase. GGT: gamma glutamyltransferase. hsCRP: high-sensitivity C-Reactive Protein. IL-6: interleukin-6. TNFα: Tumor Necrosis Factor α. vit. D: vitamin D3. TSH: Thyroid Stimulating Hormone. WBC: White Blood Cells. RBC: Red Blood Cells. PTL: platelets. FIB-4: Fibrosis-4 index. APRI: AST to Platelet Ratio Index; M30 K-18: caspase-cleaved fragments of the intermediate filament keratin 18. ELF: Enhanced Liver Fibrosis test. HA: hyaluronic acid. PIIINP: procollagen III amino-terminal peptide. TIMP1: tissue inhibitor of matrix metalloproteinase 1. FGF-21: fibroblast growth factor-21. Statistical analysis was performed with One Way Repeated Measures Analysis of Variance when Normality Test (Shapiro-Wilk) and Equal Variance Test (Brown-Forsythe) have been passed (and data are presented as mean values ± standard deviations) or, if not, with Friedman Repeated Measures Analysis of Variance on Ranks (and data are presented as median value (25th-75th percentile)). p values were corrected for multiple hypothesis testing, Bonferroni or Tukey Test (*versus V0, ■ versus V6m). We were not able to collect leptin at V4y.*

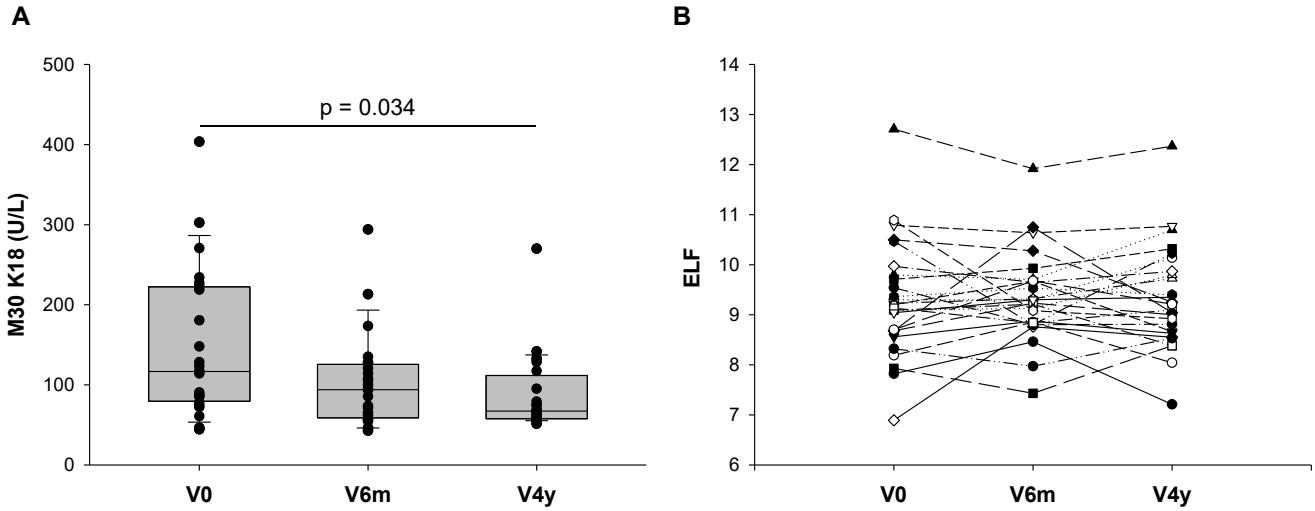


Figure 16. *Caspase-cleaved fragments of the intermediate filament keratin 18 (M30 K18) values, indicative of apoptosis* ***(A)*** *and Enhanced Liver Fibrosis test (ELF) values indicative of fibrosis* ***(B)*** *at baseline (V0), 6 months after bariatric surgery (V6m) and 4 years after bariatric surgery (V4y).*

FGF-21 levels decreased over time and in a statistically significant way only at the end of the follow-up (p = 0.009, **Figure 17**).

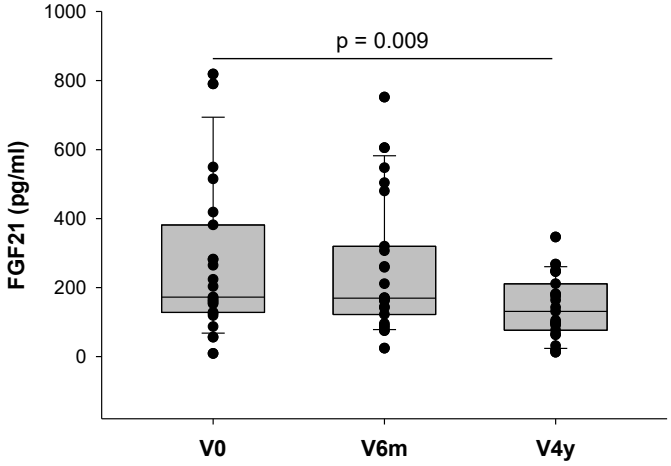


Figure 17. *Fibroblast Growth Factor-21 (FGF21) levels, indicative of metabolic stress) at baseline (V0), 6 months after bariatric surgery (V6m) and 4 years after bariatric surgery (V4y).*

We described a correlation between FIB-4 and APRI and AST/ALT in the three timepoint ($p < 0.001$). M30 K-18 correlated only with APRI ($r = 0.409$, $p < 0.05$) and AST/ALT ($r = -0.434$, $p < 0.05$) at baseline and not with FIB-4 ($r = -0.069$, $p = 0.750$). Interestingly FIB-4 correlated with BMI ($r = -0.414$, $p = 0.028$), but M30 K18 did not. M30 K18 correlated at baseline with HOMA ($r = 0.537$, $p = 0.015$). ELF correlated only with FIB-4 at the end of the follow-up ($r = 0.451$, $p = 0.024$).

FGF-21 correlated with HOMA at baseline ($r = 0.431$, $p = 0.045$) and with triglycerides both at baseline ($r = 0.612$, $p = 0.002$) and at V4y ($r = 0580$, $p = 0.004$). FGF-21 values at V4y inversely correlated with TWL% ($r = -0.474$, $p = 0.022$).

We divided patients according to their glycaemic profile before bariatric surgery (patients with normoglycemia: N, with prediabetes: preDM and with type 2 diabetes mellitus: T2DM). FIB-4 levels at baseline were different with normoglycemic displaying the lowest levels (0.77 ± 0.36), preDM showing intermediate levels (0.82 ± 0.23) and T2DM the highest values (1.14 ± 0.51), $p = 0.066$. Similarly, we described the same trend at V6m (N: 0.90 ± 0.4, preDM: 0.94 ± 0.38, T2DM: 1.35 ± 0.54, $p = 0.060$). None difference we seen at V4y: N: 1.10 ± 0.75, preDM 1.02 ± 0.81, T2DM 1.05 ± 0.77, $p = 0.958$. The same trend was seen for FGF-21 (N 144.5 (44-184.9), preDM 241.7 (146.4-797.2), T2DM (273.2 (163.2-409.3), $p = 0.118$) and M30-K18 values (N 100.7 ± 44.6, preDM 160.2

± 109.6, T2DM 165.6 ± 91.2), at baseline. M30 K-18, ELF and FGF-21 did not statistically significant differed among the three groups.

4.2. Evaluation of liver stiffness and hepatic steatosis at V0, V6m and V4y

The morphological ultrasound showed a significant decrease of the left liver measurement at V6m and was substantially maintained at V4y ($p < 0.001$) (**Table 18**). The bipolar spleen diameter did not display a difference in the follow-up ($p = 0.419$) (**Table 18**).

At baseline, out of 29 patients, 10 did not displayed steatosis, and 19 had steatosis, evaluated by B-mode US. Prevalence of steatosis did not change at V6m or V4y. Nevertheless, severe steatosis significantly decreased at V6m with a corresponding increase of mild and moderate steatosis and a further reduction of moderate and severe steatosis at V4y (χ^2 p=0.031) (**Table 18** and **Figure 18).**

Table 18. *Ultrasound parameters in 29 patients at baseline, 6 months and 4 years after sleeve gastrectomy.*

n = 29	**Before (V0)**	**6 months (V6m)**	**4 years (V4y)**	**p**
Left Liver (cm)	11.9 ± 3	9.2 ± 1.8*	9.4 ± 1.4*	p < 0.001
Spleen (cm)	10.7 (10-12.2)	10.5 (10-11.3)	10.8 (9.4-11.6)	p = 0.419
Steatosis (n)				p = 0.031
no	10	11	10	
mild	5	5	11	
moderate	4	10	6	
severe	10	3	2	
Liver Stiffness (kPa)^	7.2 (6.4-8.1)	5.8 (4.3-7.7)*	5.2 (4.4-7.7)*	p= 0.008

*Statistical analysis was performed with One Way Repeated Measures Analysis of Variance when Normality Test (Shapiro-Wilk) and Equal Variance Test (Brown-Forsythe) have been passed (and data are presented as mean values ± standard deviations) or, if not, with Friedman Repeated Measures Analysis of Variance on Ranks (and data are presented as median value (25th-75th percentile)). p values were corrected for multiple hypothesis testing, Bonferroni or Tukey Test (*versus V0). ^n = 26, outliers' values were excluded from the analysis.*

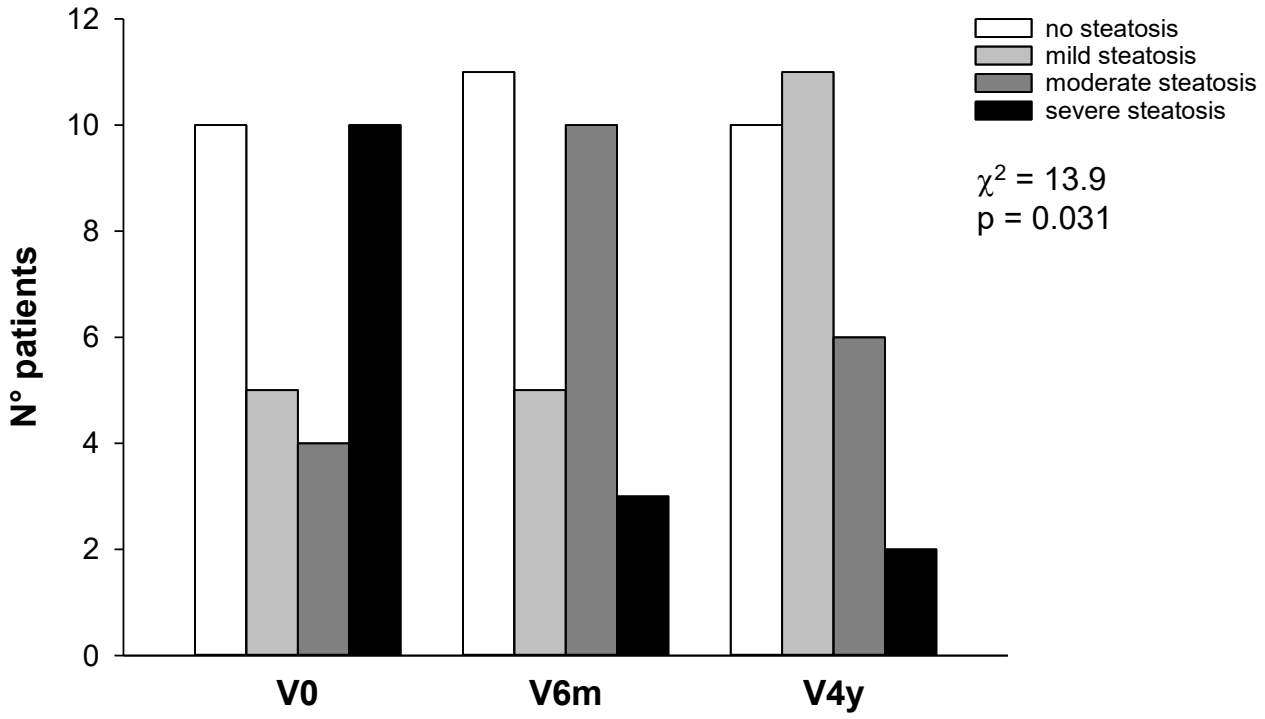


Figure 18. *Number of patients having no steatosis (white bars), mild steatosis (light grey bars), moderate steatosis (medium grey bars) and severe steatosis (black bars) at baseline (V0), 6 months after bariatric surgery (V6m) and 4 years after bariatric surgery (V4y).*

The degree of hepatic steatosis correlated with weight in all the timepoints (V0: r = 0.371, p = 0.047; V6m: r = 0.507, p = 0.005; V4y: r = 0.664, p < 0.001).

Liver stiffness, measured by SWE method, presented a statistically significant reduction 6 months after and was maintained at 4-year follow-up (p= 0.008) (**Table 18 and Figure 19A**). Dividing patients in subgroups according to the presence of steatosis, liver stiffness showed a progressive reduction from patients with steatosis in V0 to patients without steatosis at V4y, although there was not a statistically significant difference between patients with or without steatosis at the three timepoints: V0, steatosis 7.4 (6.8-8.2), no steatosis 6.4 (6-8.2), p = 0.246; V6m, steatosis 6.4 (4.2-7.7), no steatosis 4.9 (4.3-7.8), p = 0.829; V4y, steatosis 5.7 (4.6-7.9), no steatosis 5 (4-6), p = 0.161 (**Figure 19B**).

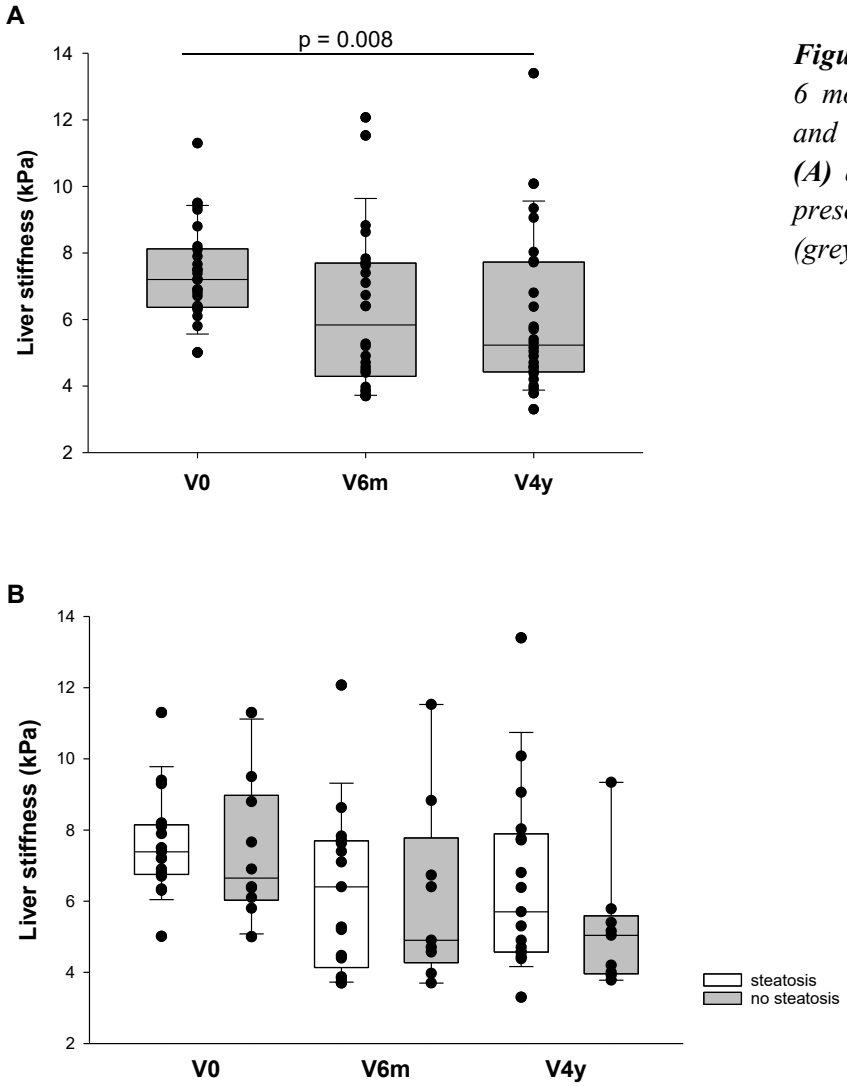


Figure 19. *Liver stiffness at baseline (V0), 6 months after bariatric surgery (V6m) and 4 years after bariatric surgery (V4y)* ***(A)*** *and Liver stiffness according to the presence (white bars) and the absence (grey bars) of steatosis* ***(B)****.*

Considering the TWL%, we described a significant difference in the liver stiffness values at V4y between patients who reached a loss < 20% (10.9 (5.5-17.6), n = 6) and patients who obtained > 20% (5.3 (4.4-7.7), n = 23), p = 0.090, even if without reaching the statistical significance (**Figure 20**). Importantly, the three patients with outlier values of liver stiffness excluded from V0-V6m-V4y comparison presented all a TWL < 20%.

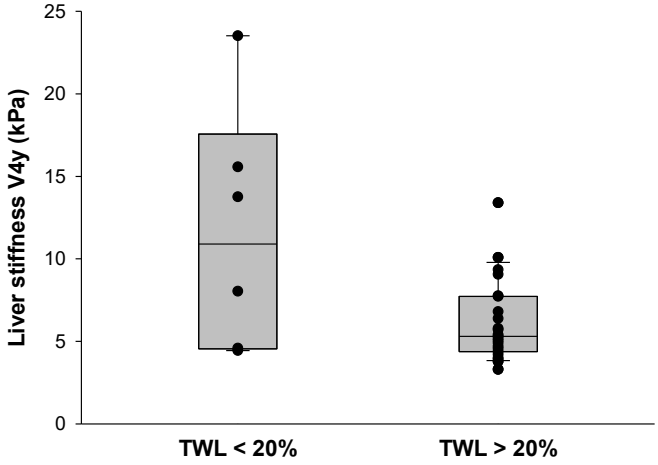


Figure 20. *Liver stiffness at 4 years after bariatric surgery (V4y) in patients with a total weight loss (TWL) % < 20 % (n = 6) and in patients with a TWL > 20 % (n = 23).*

Liver stiffness correlated with WC in all the timepoints (V0: r = 0.654, p = 0.011; V6m: r = 0.534, p = 0.027; V4y: r = 0.627, p < 0.001). Liver stiffness values measured by SWE did not correlate with the serum markers of fibrosis and FGF-21 values.

We divided patients according to their glycaemic profile before bariatric surgery and the prevalence of steatosis was higher in T2DM (n = 8 out of 9) compared with N and preDM at baseline (p = 0.175). Furthermore, we noted that patients with T2DM presented highest values of liver stiffness in the three time, even if without reaching the statistical significance: V0, N 6.9 ± 1.7, preDM 7.4 ± 1.1, T2DM 7.8 ± 2, p = 0.454; V6m, N 5.5 (3.7-7.7), preDM 5.3 (4.6-6.7), T2DM 7.4 (4.5-7.8), p = 0.668; V4y, N 5.2 (4-8.1), preDM 4.9 (4.2-6.8), T2DM 6.4 (4.6-8), p = 0.414.

PART 2. Liver Fibrosis and Steatosis in Alström Syndrome: A Genetic Model for Metabolic Syndrome

4.3. Clinical, biochemical and genetic characterization of patients with Alström syndrome

Clinical evaluation and biochemical parameters of the 18 patients with ALMS are reported in **Table 19**.

Table 19. *Anthropometric characteristics and biochemical parameters in 18 patients with Alström syndrome. Data are presented as the mean values ± standard deviations when the normality test (Shapiro–Wilk) and equal variance Test (Brown–Forsythe) were passed or, if not, as the median value (25th–75th percentile).*

	Patients with Alström Syndrome ***n*** **= 18**
Sex (M/F)	7/11
Age (y)	24 ± 11
Weight (kg)	63.9 ± 12.7
BMI (Kg/m^2)	27.1 ± 4.3
WC (cm)	86 ± 10
Platelets (×10^9/l)	215 ± 77
FPG (mg/dl)	4.2 (3.8-6.2)
Insulin (mU/l)	17.7 (10.8-36.7)
C-peptide (µg/l)	3.6 (2.2-6.2)
HOMA	3.5 (2-6.3)
Hb1Ac (mmol/mol)	34 (32-40)
TC (mg/dl)	163 ± 49
HDL (mg/dl)	39 (36-51)
LDL (mg/dl)	100 ± 30
TG (mg/dl)	114 (61-168)
ALT (U/l)	39 (25-73)
AST (U/l)	29 (21-46)
GGT (U/l)	42 (19-61)
Urea (mmol/l)	5.4 (4.6-6.8)
Creatinine (µmol/l)	63 (59-78)
eGFR (ml/min/1.73m^2)	117 ± 33
FIB-4	0.49 (0.34-0.92)
APRI	0.36 (0.22-0.71)
ALT/AST	1.23 (1-1.69)

M: male. F: female. BMI: body mass index. WC: waist circumference. FPG: fasting plasma glucose. HOMA: homeostasis model assessment-insulin resistance index. Hb1Ac: glycated hemoglobin A1c. TC: total cholesterol. HDL: high-density lipoprotein-cholesterol. LDL: low-density lipoprotein -cholesterol. TG: triglycerides. ALT: alanine aminotransferase. AST: aspartate aminotransferase. GGT: gamma glutamil transferase. eGFR: estimated glomerular filtration rate. FIB-4: Fibrosis-4 Index. APRI: AST-to-platelet ratio index. ALT/AST: ALT on AST ratio.

All patients were genetically characterized and all *ALMS1* variants identified were predicted to cause premature protein truncation; thus, they can be considered true pathogenic variants. We were not able

to identify the second *ALMS1* pathogenic vari-ant in 3 out of 18 ALMS patients (17%). We described a new pathogenic variant of *ALMS1* in exon 8: c.2611_2614delTTCT p.(Phe871Ilefs*10), a deletion of four nucleotides causing a frameshift and predicting a truncated protein of only 871 amino acids (aa) compared with the wild type spanning 4169 aa (**Table 20**).

Table 20. *Description of ALMS1 pathogenic variants in patients with Alström syndrome. Pathogenic variants of ALMS1 identified by genomic sequencing were described as c.DNA variants in respect to the Reference Sequence NM_015120.4 according to the guidelines indicated by the Human Genome Variation Society (HGVS) described by den Dunnen et al. (179) and were validated using VariantValidator.org_v0.1.3 (180).*

			GENOTYPE Allele 1	
ID	**Variant**	**Exon**	**Protein**	**Reference**
1	?	?	?	
2	c.7304_7305delAG	8	p.(Glu2435Valfs*7)	Marshall 2015
3*	c.1046G>A	5	p.(Trp349*)	Nasser 2018; Weisschuh 2016
4*	c.1046G>A	5	p.(Trp349*)	Nasser 2018; Weisschuh 2016
5	c.2164A>T	8	p.(Lys722*)	Marshall 2015
6#	c.3019dupA	8	p.(Arg1007Lysfs*15)	Marshall 2015
7#	c.3019dupA	8	p.(Arg1007Lysfs*15)	Marshall 2015
8	c.1568dupT	8	p.(Ser524Lysfs*13)	Marshall 2015
9§	c.3425C>G	8	p.(Ser1142*)	Marshall 2015
10§	c.3425C>G	8	p.(Ser1142*)	Marshall 2015
11	c.4937C>A	8	p.(Ser1646*)	Marshall 2015
12	c.2041C>T	8	p.(Arg681*)	Dassie 2021
13+	c.3425C>G	8	p.(Ser1142*)	Marshall 2015
14+	c.3425C>G	8	p.(Ser1142*)	Marshall 2015
15	c.6486_6489delAACT	8	p.(Thr2163Lysfs*4)	Marshall 2015
16	c.10557dupT	16	p.(Pro3520Serfs*5)	Dassie 2021
17	c.3251_3258delCTGACCAG	8	p.(Ala1084Aspfs*3)	Marshall 2015
18	c.3425C>G	8	p.(Ser1142*)	Marshall 2015

			GENOTYPE Allele 2	
ID	**Variant**	**Exon**	**Protein**	**Reference**
1	c.11313_11316delTAGA	16	p.(Asp3771Glufs*20)	Marshall 2015
2	c.10975C>T	16	p.(Arg3659*)	Marshall 2015
3 *	c.1046G>A	5	p.(Trp349*)	Nasser 2018; Weisschuh 2016
4 *	c.1046G>A	5	p.(Trp349*)	Nasser 2018; Weisschuh 2016
5	c.11313_11316delTAGA	16	p.(Asp3771Glufs*20)	Marshall 2015
6 #	c.10830_10831insC	16	p.(Arg3611Glnfs*7)	Marshall 2015
7 #	c.10830_10831insC	16	p.(Arg3611Glnfs*7)	Marshall 2015
8	**c.2611_2614delTTCT**	**8**	**p.(Phe871Ilefs*10)**	**NEW**
9 §	c.3425C>G	8	p.(Ser1142*)	Marshall 2015
10 §	c.3425C>G	8	p.(Ser1142*)	Marshall 2015
11	c.11703delA	18	p.(Lys3901Asnfs*8)	Marshall 2015
12	c.5135T>G	8	p.(Leu1712*)	Marshall 2015
13 +	?	?	?	
14 +	?	?	?	
15	c.6486_6489delAACT	8	p.(Thr2163Lysfs*4)	Marshall 2015
16	c.11580dupT	17	p.(Ile3861Tyrfs*7)	Dassie 2021
17	c.6731delA	8	p.(Asp2244Valfs*24)	Marshall 2015
18	c.9379C>T	10	p.(Gln3127*)	Marshall 2015

*For each variant, the first description in the reference column is reported; the new variant (NEW) described in the present study is indicated in bold and was submitted to the Euro-WABB online database, a locus-specific database (in the Leiden Open Variation Database format) listed by the Human Genome Variation Society in the Locus Specific Mutation Databases (LSDB) (www.HGVS.org). Each patient was indicated by a specific identification number (ID); the symbols *, #, § and + identify siblings.*

The prevalence of overt T2DM in patients with ALMS was 44% (8/18), the prevalence of obesity was 28% (5/18), hypertension was present in 33% (6/18) and the prevalence of metabolic syndrome was 56% (10/18).

Nine patients out of eighteen (50%) showed an increase in transaminases (AST and/or ALT) above the normal range; the three patients (17%) with signs of portal hypertension (splenomegaly or/and esophageal varices) during the US and the esophagogastroduodenoscopy are described in detail in **Table 21**. All these three patients presented the criteria for the diagnosis of metabolic syndrome.

Table 21. *Clinical and biochemical evaluation of the three patients with Alström syndrome and signs of portal hypertension.*

Patients ID (Symbols)	Hb1Ac (mmol/mol)	BMI (Kg/m²)	ALT (U/L)	AST (U/L)	GGT (U/L)	Steatosis (L/K)	LS (kPa)	FIB-4	Portal hypertension signs
ID 1 (Δ)	66	33.32	24	25	46	1.02	9.7	2.44	Esophageal varices (F3), splenomegaly
ID 2 (○)	36	26.12	51	55	79	1.14	6.7	3.13	Esophageal varices (F2), splenomegaly
ID 5 (□)	105	32.58	62	86	835	1.16	13.2	1.83	Esophageal varices (F1)

Symbols are used in Figure 21A–B, 23A and 24A–B. ID: identification number of patients with ALMS reported in Table 20. All three patients had a history of type 2 diabetes mellitus and presented the indicated values of glycated hemoglobin 1Ac (Hb1Ac). BMI: body mass index. ALT: alanine aminotransferase. AST: aspartate aminotransferase. GGT: gamma glutamil transferase. L/K: sonographic hepatorenal ratio. LS: liver stiffness. FIB-4: Fibrosis-4 Index. F1: straight, small-caliber varices; F2: moderately enlarged, beady varices; F3: markedly enlarged, nodular or tumor-shaped varices (according to the Japan Society for Portal Hypertension (186)).

4.4. Evaluation of liver stiffness and hepatic steatosis

LS was significantly higher in patients than in controls (5.3 (4.1–6.5) versus 3.7 (3.3–4.2); $p < 0.001$) (**Figure 21A**), also excluding the three patients with signs of portal hypertension ((ID 1, 2 and 5, described in Table 20 and Table 21, white symbols) (4.8 (4–6.2) versus 3.7 (3.3–4.2); $p = 0.002$). Patients with ALMS displayed L/K values higher than controls (1.6 (1.2–2) versus 1.3 (1–1.4); $p = 0.013$) (**Figure 21B**).

Patients with signs of portal hypertension (ID 1, 2 and 5) described in Table 20 and Table 21 displayed highest LS values and lowest L/K values (**Figure 21**).

LS was significantly correlated with AST values ($r = 0.52$, $p < 0.05$) but not with ALT ($r = 0.340$, $p = 0.164$) or ALT/AST ratio ($r = 0.142$, $p = 0.563$). Interestingly, LS significantly correlated with FIB-4, the validated non-invasive score for detecting advanced fibrosis ($r = 0.590$, $p = 0.012$) (**Figure 22A**), and weakly with APRI, a similar index of fibrosis, ($r = 0.45$, $p = 0.067$). Surprisingly, we found that LS was correlated with age only in patients with ALMS ($r = 0.505$, $p = 0.032$) (**Figure 22B**) and not in controls ($r = 0.215$, $p = 0.299$).

We found a lack of association between steatosis, estimated by L/K, and transaminases values. On the contrary, L/K values were significantly correlated with TG levels ($r = 0.504$, $p = 0.032$) (**Figure 22C**),

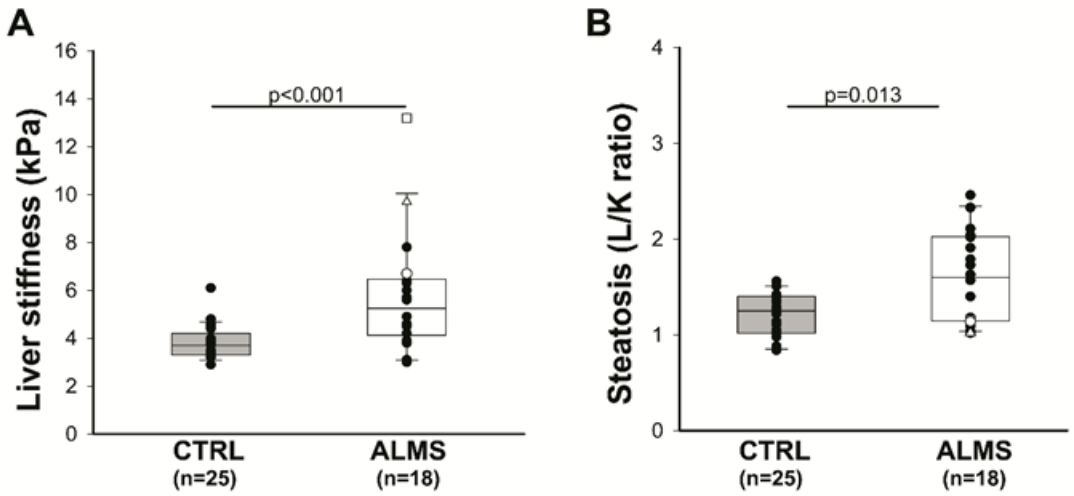


Figure 21. *Liver fibrosis and steatosis in patients with Alström syndrome. Liver stiffness was evaluated by shear wave elastography* ***(A)*** *and liver steatosis was quantified by the sonographic hepatorenal ratio (L/K)* ***(B)*** *in patients with ALMS and in controls (CTRLs). Results are presented as a box plot, with 25th and 75th percentiles and median values. ID 1 (white triangle), ID 2 (white circle) and ID 5 (white square) patients had clinical signs of portal hypertension and are described in detail in Table 21. Statistical analysis was performed using the Mann–Whitney U-test.*

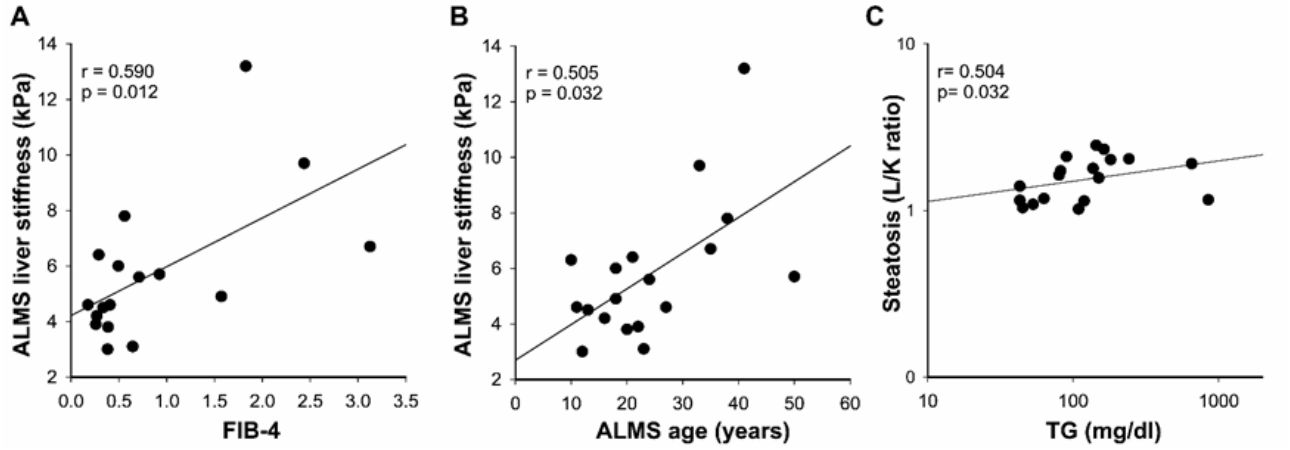


Figure 22. *Correlation analysis of the liver stiffness and the sonographic hepatorenal ratio in patients with Alström syn-drome (ALMS). The simple correlations between liver stiffness and Fibrosis-4 Index (FIB-4)* ***(A)****, liver stiffness and age* ***(B)*** *and steatosis evaluated by sonographic hepatorenal ratio (L/K) and triglycerides (TG) levels* ***(C)*** *were performed by Spearman's correlation in the 18 patients with ALMS. Data are reported into logarithmic scale in* ***(C)****.*

Lastly, we divided patients according to the presence of steatosis determined by L/K cut-off (1.6) calculated in our control group (CTRL, **Figure 21**) identifying liver steatosis in 10 out of 18 patients with ALMS (56%). LS did not significantly differ between these two subgroups (no steatosis 4.6 (3.1–6.7) versus steatosis 5.3 (4.4–6.1), p = 0.845] (**Figure 23A**). Moreover, we did not find any

correlation in patients with ALMS, between LS and steatosis estimated by SWE and L/K, respectively (r = –0.065, p = 0.792) (**Figure 23B**).

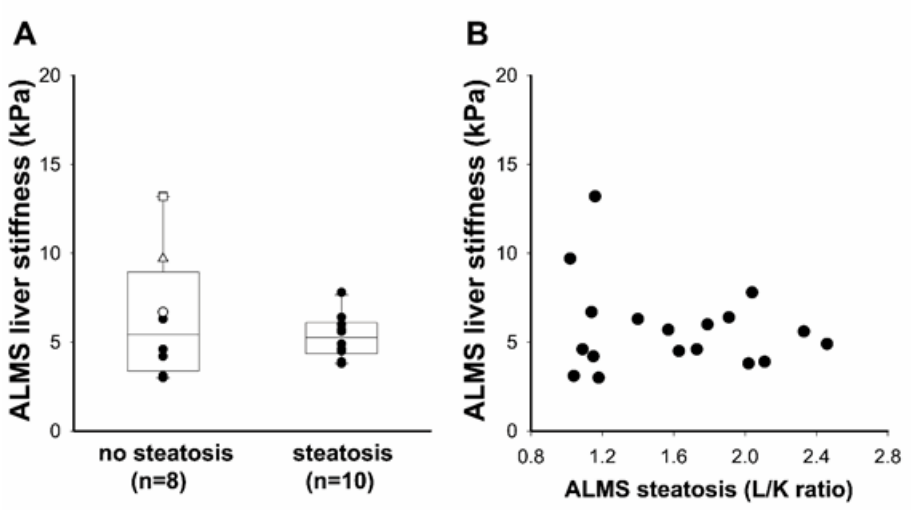


***Figure 23**. Relationship between liver stiffness and liver steatosis in patients with Alström syndrome. **(A)** Liver stiffness was evaluated by the shear wave elastography in patients with ALMS divided into two subgroups on the basis of the sonographic hepatorenal ratio (L/K) cut-off value (no steatosis, with L/K < 1.6) and steatosis (with L/K > 1.6). Results were presented as a box plot, with 25th and 75th percentiles and the median values. ID 1 (white triangle), ID 2 (white circle) and ID 5 (white square) patients had clinical signs of portal hypertension and are described in detail in Table 21. Statistical analysis was performed using the Mann–Whitney U-test. **(B)** The simple correlation between liver stiffness, evaluated by the shear wave elastography, and liver steatosis, evaluated by sonographic hepatorenal ratio (L/K), was performed by Spearman's correlation in patients with ALMS.*

4.5. The role of comorbidities: obesity and T2DM

We divided patients with ALMS into subgroups according to the presence of obesity (BMI ≥ 30 Kg/m2) and T2DM and analyzed the distribution of LS values. LS was not significantly increased in ALMS patients with obesity compared with normal weight ALMS patients even if we showed an increasing trend (6 (4.8–11.5) versus 4.6 (3.9–6.4), p = 0.126) (**Figure 24A**). Likewise, LS showed an increasing trend in patients with T2DM compared with patients with normoglycemia, but the difference did not result in statistical significance [6.2 (4.9–9) versus 4.6 (3.6–5.9), p = 0.062) (**Figure 24B**).

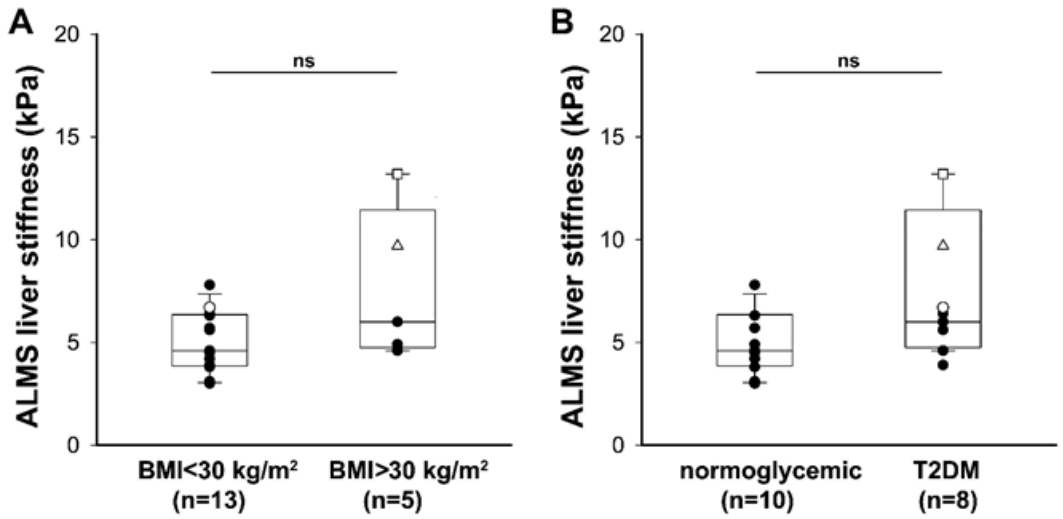


Figure 24. *Liver stiffness and metabolic complications in patients with Alström syndrome. Liver stiffness was evaluated by the shear wave elastography in patients with ALMS divided into subgroups according to the presence of obesity* ***(A)*** *and type 2 diabetes mellitus (T2DM)* ***(B)****. Data are reported as a box plot with 25th and 75th percentiles and median values. ID 1 (white triangle), ID 2 (white circle) and ID 5 (white square) patients had clinical signs of portal hypertension and are described in detail in Table 21. Statistical analysis was performed using the Mann–Whitney U-test.*

Nevertheless, LS showed significant correlations with waist circumference (r = 0.624, p = 0.012; n = 15) (**Figure 25A**), HOMA (r = 0.670, p = 0.004; n = 15) (**Figure 25B**) and Hb1Ac (r = 0.715, p < 0.001; n = 14) (**Figure 25C**) in patients with ALMS.

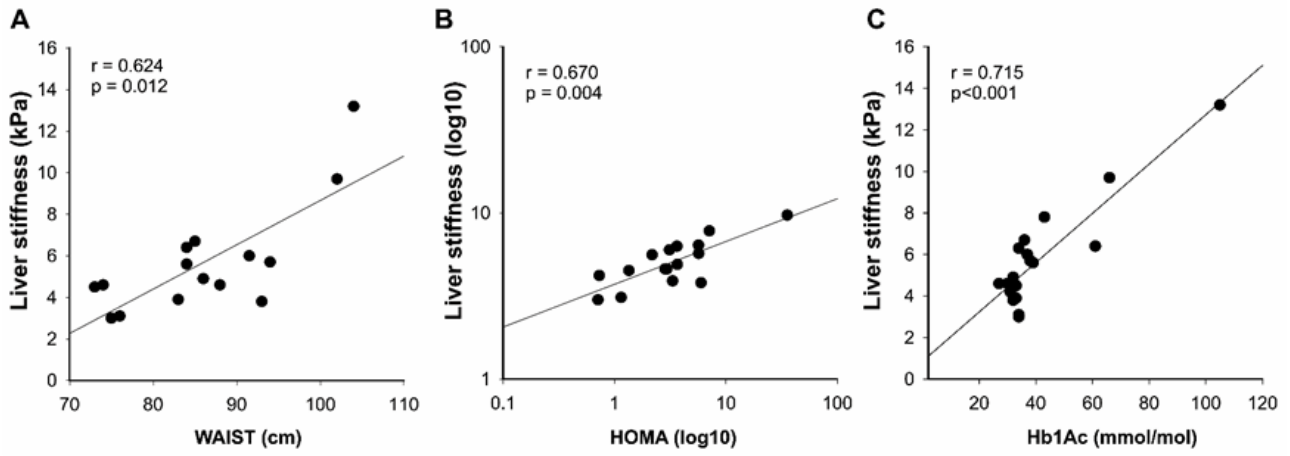


Figure 25. *Correlation analysis of the liver stiffness and biomarkers of metabolic complications in patients with Alström syndrome. The simple correlations between liver stiffness, evaluated by the shear wave elastography, and waist* ***(A)****, (n = 15) and homeostasis model assessment (HOMA)* ***(B)*** *(n = 15) and glycated hemoglobin 1Ac (Hb1Ac)* ***(C)*** *(n = 14) were performed by Spearman's correlation in the indicated patients with Alström syndrome. Data were transformed into logarithmic values in* ***(B)****.*

5.0. DISCUSSION

PART 1. Short- and long-term effects of bariatric surgery on Non-Alcoholic Fatty Liver Disease in patients with obesity.

Several studies showed that weight loss has a favourable effect towards liver disease in patients with obesity. Among the different therapeutic approaches, bariatric surgery has demonstrated to have the greatest impact on weight loss (75). Several data investigating the efficacy of bariatric surgery on NAFLD has been obtained from RYGB, reporting its beneficial effect on NAFLD and NASH (188, 189), in short and long term. Other data, although less, also confirmed the role of LSG in ameliorate NAFLD (177, 190-192).

To date liver biopsy represents the gold standard for the diagnosis of NAFLD (158, 162). However, as explained in the latest update of the EASL guidelines (157), it is not suitable for the follow-up of a liver disease with high prevalence as NAFLD, because it is poorly tolerated by patients and since it is not free from risk of complications, and it could result in an unfavourable risk-benefit ratio. For this reason, EASL itself recommends in the screening and serial follow-up of patients with obesity the use of NITs (serological markers, complex scores, and imaging procedures) to rule out NAFLD and decide the need for further investigation. This approach is useful because it avoids unnecessary serial biopsies and at the same time identifies patients who should be started on the most appropriate therapy, depending on the case, to reduce complications, morbidity and mortality in patients at risk of disease progression.

Up to now, few data in literature focus on the short- and long-term effect of bariatric surgery on the progression of NAFLD in patients with obesity, carried out using NITs.

In our study, we followed up 34 patients for 4 years after bariatric surgery to record the possible effects on NAFLD of weight loss in the short and long term. At V6m the weight decreased significantly and maintained at V4y. Thus, there was no weight regain and weight loss occurred much faster in the first six months and then was maintained. Similar results were found in the literature after a 4-year follow-up (193).

Analysis of the lipid profile revealed a significant reduction only for TGs in the long term. We showed similar results in a comparative study of the effects of weight loss after LSG versus MBP on lipid profile (194), in which we demonstrated that LSG did not ameliorate the cholesterol profile, except for TG. Furthermore, AST and GTT decreased over time. Inconsistent results are available regarding

transaminases trend after bariatric surgery (177, 195, 196). It is worth to note that raised liver enzymes, assumed as surrogate indexes of NAFLD, were reported only in 21% of cases with obesity and did not correlate with obesity class (158, 197). A strong improvement in the glycaemic profile was also observed with reduction in FPG and HOMA in the short term and their maintenance in the long term, as a meta-analysis showed previously (198). Unspecific index of inflammation and bile acids did not significantly change. Leptin, produced in proportion to the amount of TA, showed as expected a reduction at 6 months after surgery, with a trend like that of BMI. To resume, we confirmed that bariatric surgery can reduce the values of the most important participants in the pathophysiology of NAFLD.

Among serum markers, we also evidenced both a short and long-term improvement in APRI and AST/ALT ratio values (195), whereas FIB-4 followed an upward trend. If we considered age before surgery, we highlighted again a statistically significant difference between V0 and V4y (data not shown), with FIB-4 values worsened. Thus, we suggested that FIB-4 values may be useful in the diagnosis process, and not in the follow-up, as already showed in a retrospective study on patients undergoing bariatric surgery (199). Moreover, FIB-4 inversely correlates with BMI. This paradoxical effect is already described in the scientific literature for patients with obesity presenting with severe fibrosis. In fact, obesity has been shown to be a protective factor in decompensated liver cirrhosis (200). Also, ELF values did not help in monitoring NAFLD process during the follow-up, although this marker it is considered important for the diagnosis (138,169), but probably its use should not be recommended in real life study with a small sample size. Significant reductions are observed in M30 K-18 in the long term but not in the short term. Probably, since M30 K-18 is a marker of apoptosis, during the first period following bariatric surgery, the surgery-related inflammatory stress could interfere with the decrease of this marker. Since M30 K-18 correlates with histological changes and reflect the severity of NASH (166), this result confirms its use in the follow-up of patients after bariatric surgery.

Dividing patients by their metabolic impairment into N, preDM, and T2DM, FIB-4 levels at baseline and at V6m were different in subgroups, with an increasing trend from N to preDM to T2DM. At 4y the difference disappeared probably because FIB-4 levels were higher in all patients. M30 K-18, ELF did not statistically significant differed among the three groups. Given the increased risk of the presence of fibrosis in patients with NAFLD and T2DM (139, 149, 151, 154, 158, 162, 201, 202), it is possible that the lack of difference seen in M30 K-18 and ELF may be due to the small sample size.

According to some authors, FGF21 is a potential biomarker to diagnose NAFLD. The up-regulated FGF21 level in NAFLD is believed to have a protective effect against the lipotoxicity of fatty acids and oxidative or endoplasmic reticulum stress (203). In our study, FGF-21 showed a statistically significant reduction only in the long term. It was demonstrated that FGF21 levels were reduced after energy-restricted treatments and severely increased after bariatric surgery, independently of the weight reduction magnitude, insulin sensitivity, or ketosis. Therefore, FGF21 appears to be a marker of severe nutritional stress (204). A systematic review and meta-analysis showed that RYGB surgery significantly increased FGF-21 levels, whereas, in studies with follow-up duration ≥ 1 year, FGF-21 levels decreased significantly (205). Accordingly, at short time, FGF-21 should be only considered as a marker of metabolic stress. Furthermore, FGF21 was found to be increased in patients with obesity, T2DM and MS and the plasma FGF21 level is correlated with intrahepatic triglyceride content and grade of hepatic steatosis (206). Consensually, we showed a correlation with plasma TG and with HOMA, two important participants in the pathogenesis of NAFLD. Moreover, TWL% inversely correlated with FGF-21 values at V4y, highlighting the role of the WL in ameliorating the inflammation state.

A progressive statistically significant improvement in the degree of steatosis was observed and was related to the weight loss. The same trend was seen for the size of the left lobe of the liver. Liver stiffness values, studied with SWE, significantly improved. Moreover, dividing patients into presence and absence of steatosis, we saw that fibrosis, quantified as liver stiffness, was greater in patients with steatosis and progressively reduced, both in patients with steatosis and in those without. In addition, the degree of steatosis correlated with weight and liver stiffness was related with WC, confirming that weight and especially visceral fat are implicated in the progression of the NAFLD. Furthermore, presence of steatosis was higher in T2DM compared with N and preDM, as we previously demonstrated (177) but without statistical significance, probably due to the small sample size of the subgroups. Moreover, patients with T2DM displayed higher values of liver stiffness, in parallel to the results from serum markers of fibrosis. Again, also using US as a NITs to monitor fibrosis, we demonstrated that WL determine a significant reduction of fibrosis, measured as liver stiffness, since patients with a WL < 20% showed values greater than patients obtaining a WL > 20% at V4y.

Liver stiffness values measured by SWE did not correlate with the serum markers of fibrosis and FGF-21 values. On the other hand, both AST/ALT, APRI, M30 K18 and FGF-21, liver stiffness improved after bariatric surgery. This improvement may depend to the degree of WL, particularly for

liver stiffness. Indeed, SWE is a recent technique that has shown excellent results in studies, but still requires large population studies to define universally accepted cut-off values (170-173).

The trend of the serum markers and liver stiffness detected by SWE corresponds to the resolution of NASH observed at 1 year after bariatric surgery in biopsies from 84% of patients in a recent study (190). Furthermore, in the same paper fibrosis began to decrease by 1 year after surgery and continued to decrease until 5 years ($p < 0.001$).

PART 2. Liver Fibrosis and Steatosis in Alström Syndrome: A Genetic Model for Metabolic Syndrome

ALMS displays severe metabolic phenotypes involving different organ and tissues and can be regarded as a genetic model for obesity, type 2 diabetes, NAFLD and metabolic syndrome.

In particular, the early and quick progression towards cirrhosis in patients with ALMS makes the evaluation of hepatic fibrosis an important matter for diagnostic assessment, follow-up, and therapy of this ultra-rare fibrotic disease.

This is the first study that, considering the improvements in imaging techniques, adopts SWE to assess the degree of fibrosis and the L/K to quantify the liver steatosis in ALMS. We genetically characterized 18 patients with ALMS describing a new pathogenic variant of exon 8 in ALMS1 and performed in this cohort imaging analysis, biochemical assessment, and expert clinical evaluation. We showed that the LS and the L/K was significantly higher compared with the controls, suggesting an increased liver fibrosis and steatosis. In fact, both SWE and L/K demonstrated a good applicability and diagnostic performance (171-173,184,185) even if they have never been tested for the diagnosis of liver fibrosis and steatosis, respectively, in patients with Alström syndrome, compared with liver biopsy.

The TE was the first tool developed to quantify liver fibrosis by measuring mechanical shear wave propagation through the liver parenchyma (138), recently used in patients with ALMS (122). However, several studies have demonstrated that the SWE is more accurate in the assessment of liver fibrosis compared with TE (138,207), both in adults and in children (208).

The high potential of SWE to detect liver fibrosis was enhanced by the correlation between LS and FIB-4 we observed in patients with ALMS (138). Conversely, we did not find any correlation between LS and other serum biomarkers such as ALT/AST ratio and APRI; however, these two well-known scores for fibrosis had a lower accuracy and sensitive compared to FIB-4 (207).

Interestingly, we found that LS was correlated with age only in patients with ALMS and not in controls. In the general population, both FIB-4 and LS display an age-dependent increase, respectively, for age greater than 65 (209) and 54 years (210), but this was not the case for our cohort, given all patients were <50 years old. Thus, we could hypothesize that patients with ALMS seem to be older than their age in respect to the fibrotic evolution.

Taken together these results suggest the early appearance of hepatic fibrosis and its rapid progression in patients with ALMS and indicate FIB-4 as a reliable tool to predict fibrosis which is strongly related to LS also in ALMS. The combination of FIB-4 and SWE could improve the prediction of long-term outcomes in patients with suspect advanced fibrosis.

We estimated a 56% of steatosis in patients with ALMS by the L/K, which is considered a useful method to diagnose and grade hepatic steatosis (184,185). L/K values were significantly correlated with TG levels, confirming TG's involvement in NAFLD pathogenesis also in ALMS. However, we did not find any relation between LS or L/K ratio and ALT levels, and it could be explained by the normal ALT levels of patients with NAFLD also during disease progression. Furthermore, although elevated aminotransferases should raise suspicion for NASH, normal levels should not be used to exclude NASH (158).

To study any relation between steatosis and fibrosis in ALMS, we divided patients according to the L/K cut off (steatosis and non-steatosis subgroups), and we did not find any difference in their LS values. This interesting result suggests that alterations of the *ALMS1* gene could act as an independent determinant of multi-organ fibrosis (84,89,102). In other words, the NAFLD pattern reported in ALMS could be characterized by a predominant predisposition to fibrosing steatohepatitis, beyond the presence of fatty acids infiltration in the liver. The progression from steatosis to fibrosis was enhanced when we considered the features of the three case reports with signs of portal hypertension: it is worth noting that all patients presented high levels of LS and none displayed steatosis. We can assume that the initial presence of steatosis was replaced very early by fibrosis, partly by common mechanisms of NAFLD progression and partly by the specific role of dysfunctional ALMS1 protein in liver cells.

According to the multiple-hit hypothesis, diet, environmental and genetic factors, together with IR, obesity and low-grade inflammation, describe the pathogenesis of NAFLD and the risk of progression to inflammation and fibrosis (NASH) or persistence in a stable stage of disease (NAFLD) (150). Thus, the prevalence of obesity (28%), T2DM (44%) and metabolic syndrome (56%) in our ALMS cohort

has been considered as an additional factor linked with the development of NAFLD/NASH besides the genuine effect of the genetic disease per se. LS increased consensually with the presence of metabolic complications even if the difference did not result significant, probably due to the small sample size of the subgroups. In fact, LS showed a significant correlation with waist circumference, HOMA and Hb1Ac, supporting the strong association be-tween liver fibrosis and metabolic complications (158,162) also in ALMS. Furthermore, among patients with ALMS and also among our population, has demonstrated an increased prevalence of hyperphagia, which represents probably the primary driver for obesity and its complications (87,89). It is worth noting that the three cases with worse prognosis for liver disease all suffered from T2DM and metabolic syndrome, while two out of three were affected by obesity.

At present, there are no predictive parameters which could be used to evaluate the progression from NAFL to NASH and advanced fibrosis as well as to explain why some patients with ALMS develop serious liver disease while others do not. The pathogenesis of NASH-related fibrosis in the general population is not well known and even more in ALMS. We previously showed, in fibroblast primary cultures obtained from patients with ALMS, that both an excessive extracellular matrix production and a failure to eliminate myofibroblasts could represent key mechanisms (102).

In *Alms1* mutant (foz/foz) mice it has been demonstrated that bone marrow-derived macrophages (BMMs) contributed to the hepatic macrophage accumulation (211). Moreover, the activation of the AP1 transcription factor c-JUN in the pathologic fibroblasts has been described as a possible unified mechanism of apparently different fibrotic disease (212). Recently, Geberhiwot et al. showed that patients with ALMS dis-played insulin resistance at different tissue levels (adipose tissue, liver and skeletal muscle) compared with BMI-matched controls providing some evidence, in a new mouse model, that adipose tissue could represent the main driver for metabolic dysregulation in ALMS (110). Thus, several mechanisms could work together, and further studies will be required to link *ALMS1* loss of function with metabolic alterations of patients with ALMS.

6.0. CONCLUSION

The description of the liver disease in patients with ALMS may help to the study of the most common NAFLD. Patients with ALMS displayed an early increased liver stiffness dependent on age, associated with T2DM and obesity, but in addition linked to specific genetic alterations. Patients with ALMS displayed also increased steatosis, which was correlated with TG levels but not with the degree of fibrosis. Thus, the classical NAFLD progression from steatosis to fibrosis does not completely explain the liver disease in ALMS and we could suggest the involvement of *ALMS1* deficiency. Thus, a better knowledge of ALMS1 protein function in liver fibrogenesis could expand the pathways involved in NAFLD/NASH present also in more common metabolic diseases. Both SWE and FIB-4 appeared a promising tool to study the presence of fibrosis and their association could represent an important NIT to monitor patients with this rare disease in the follow-up and to accurately evaluate the response to new promising treatments against liver fibrosis (134,135).

Indeed, ALMS is a model of T2DM, metabolic syndrome and NAFLD, useful to study the progression of these comorbidities in a more common disease, such as obesity. In patients with obesity, we showed that weight loss obtained by bariatric surgery is effective in significantly reducing M30 K18, AST/ALT ratio, APRI, FGF-21, the degree of steatosis and liver stiffness. Since liver stiffness, steatosis and FGF-21 correlated with weight loss, we can hypothesize that is the weight loss the principal factor determining the improvement of NAFLD. Finally, FIB-4 seems not to be important to follow up patients after weight loss, probably because its strong correlation with advanced fibrosis.

As a limitation of this study, we have not been able to correlate NITs results with the histologic pattern or the degree of fibrosis with a pathological scoring system because we did not carry out liver biopsies both in patients with obesity and in patients with ALMS. However, biopsy is not an ideal test to propose and repeat many times during the follow-up, especially for pediatric patients (157,158,162); therefore, it could be very useful to find a reliable non-invasive method for assessing hepatic fibrosis (157,207). Secondly, another limitation is the small simple size of patients with obesity, particularly for the subgroups accordingly metabolic impairment. Nevertheless each patient was completely characterized (anthropometric, sonographic and biochemical parameters).

In conclusion, our study showed that SWE examination is a promising diagnostic tool to predict liver fibrosis stage that could further be reinforced by the concomitant evaluation of the FIB-4 in patients

with ALMS and with M30 K18, AST/ALT ratio, APRI and FGF-21 in patients with the more common obesity. Our study underlines the utility to investigate ultra-rare diseases as a model of common diseases to identify new pathogenic pathways, novel therapeutic targets and the best diagnostic tools for complications.

William Harvey said, regarding on the relevance of rare disorders in medical research: *"Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows tracings of her workings apart from the beaten paths; nor is there any better may to advance the proper practise of medicine than to give our minds to the discoverer of the usual law of nature, by careful investigation of cases of rarer forms of disease (London 1657)"*.

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