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EASL POSTGRADUATE COURSE
METABOLIC LIVER DISEASE

50th

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VIENNA, AUSTRIA
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COURSE DIRECTORS
Jean-François Dufour, *Switzerland*
Vlad Ratziu, *France*
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EASL GOVERNING BOARD

WELCOME MESSAGE

The 2015 EASL Postgraduate Course is focused on metabolic liver disease. The EASL Governing Board selected this topic because metabolic liver disease affects a growing fraction of the population and many aspects of this disease are still intensely debated. There is no physician in clinical practice who did not have to care for an increasing number of patients with metabolic liver disease. While we now understand that this condition is associated with major changes in our lifestyle within an obesogenic environment, there are many challenges ahead in terms of understanding the multiple and complex pathogenic pathways, refining the diagnosis, optimizing non-invasive diagnostic procedures, identifying patients at risk of disease progression and those in need of specific hepatological therapies. We would like this Postgraduate Course to highlight many of these clinical practice dilemmas and to engage the audience with world-renowned experts on how to make the best management decisions in 2015 to help patients with metabolic liver disease. We are proud to have assembled an outstanding panel of speakers and hope that you will find this course stimulating, informative and useful for your everyday practice.

Jean-François Dufour
Switzerland

Vlad Ratziu
France

Herbert Tilg
Austria

WEDNESDAY, APRIL 22, 2015

Challenges in the diagnosis of NASH	11:30 - 13:30
11:30	**Q&A (QUESTIONS)** **Case presentation** Felix Brunner, *Switzerland*
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12:05	**Histological classifications in NAFLD: how and when to trust the pathologist** Pierre Bedossa, *France*
12:25	**Non-invasive diagnosis of fibrosis in NAFLD: how reliable is it?** Leon Adams, *Australia*
12:45	**Pediatric NASH: is it different and should we look for it?** Valerio Nobili, *Italy*
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14:35	**Insulin resistance: should we measure it and does it promote liver disease progression?** Elisabetta Bugianesi, *Italy*
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15:15	**Bariatric surgery: a cure for NASH?** Carel Le Roux, *Ireland*
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Extrahepatic complications of liver fat	**16:00 - 17:40**
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16:35	**NAFLD, pre-atherogenic lesions and cardiovascular events** Sven M.A. Francque, *Belgium*
16:55	**Does steatosis place patients at risk for diabetes development and progression?** Naveed Sattar, *The United Kingdom*
17:15	**Current and future insulin sensitizers in the treatment of NASH** Vlad Ratziu, *France*
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THURSDAY, APRIL 23, 2015

Carcinogenesis and NAFLD	**8:30 - 10:10**
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8:45	**Carcinogenesis and the spectrum of hepatic tumors in NASH** Augusto Villanueva, *The United States*
9:05	**Liver cancer in NAFLD: magnitude of the problem** Jean-François Dufour, *Switzerland*
9:25	**Do inflammation networks trigger NASH and drive its progression?** Herbert Tilg, *Austria*
9:45	**Impact of lifestyle on NASH (inclusive on HCC)** Ingrid Hickman, *Australia*
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Progression of liver disease in NAFLD	**10:30 - 12:10**
10:30	**Q&A (QUESTIONS)** **Case presentation** Grace Doleman, *The United Kingdom*
10:45	**Who are the NAFLD patients at risk of disease progression?** Chris Day, *The United Kingdom*
11:05	**NAFLD diabetes and alcohol: is there a safe threshold?** Stefano Bellentani, *The United Kingdom*
11:25	**The course of cirrhotic NASH: how different is it from other cirrhoses?** Arun J. Sanyal, *The United States*
11:45	**Liver transplantation for NASH** Didier Samuel, *France*
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GLOSSARY

AACR	American Association for Cancer Research
AFLD	alcoholic fatty liver disease
AKT	protein kinase B
ALD	alcoholic liver disease
ALT	alanine transaminase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
APRI	aspartate aminotransferase-to-platelet ratio index
ARFI	acoustic radiation force impulse
AST	aspartate transaminase
AUROC	area under the receiver operator characteristic curve
BMI	body mass index
CHD	coronary heart disease
CK18	cytokeratin-18
CPT	Child-Pugh-Turcotte
CV	cardiovascular
CVD	cardiovascular disease
DEXA	dual-energy X-ray absorptiometry scan
DHA	docosahexaenoic acid
DIOS	dysmetabolic iron overload syndrome
ELF	enhanced liver fibrosis
EORTC	European Organisation for Research and Treatment of Cancer
ER	endoplasmic reticulum
FFA	free fatty acid
FIB-4	Fibrosis-4 score
FLIP	fatty liver inhibition of progression
FXR	farnesoid X receptor
GGT	gamma-glutamyl transferase
GNMT	glycine N-methytransferase
HbA1c	glycated hemoglobin
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDL	high-density lipoprotein
HE	hepatic encephalopathy
HFE	human hemochromatosis protein
HIC	hepatic iron concentration
HOMA	homeostasis model assessment
HR	hazard ratio
HSC	hepatic stellate cell
IKK	I kappa B kinase
IL	interleukin
IR	insulin resistance
IS	insulin sensitivity
JNK	c-Jun N-terminal protein kinase
LAGB	laparoscopic adjustable gastric banding
LDL	low-density lipoprotein
LFTs	liver function tests
LKB1	liver kinase B1
LSM	liver stiffness measurement
LT	liver transplantation
LyPC	lysophosphocholine
MAT1A	methionine adenosyltransferase 1, alpha
MDBs	Mallory-Denk bodies
MDS	Mediterranean Diet Score
MELD	Model for End-Stage Liver Disease
MetS	metabolic syndrome
MHO	metabolically healthy obese
MRE	magnetic resonance elastography
MRI	magnetic resonance imaging
mTOR	mechanistic target of rapamycin
NAFL	non-alcoholic fatty liver
NAFLD	non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	non-alcoholic steatohepatitis
NEMO	NF-kB essential modulator
NF-kB	NF-kappaB transcription factor
NFS	NAFLD fibrosis score
NPV	negative predictive value
OCA	obeticholic acid
OGIS	oral glucose insulin sensitivity index
OGTT	oral glucose tolerance test
OLT	orthotopic liver transplant
OR	odds ratio
OSAS	obstructive sleep apnea syndrome
PC	phosphocholine
PCOS	polycystic ovary syndrome
PDGFR	platelet-derived growth factor receptor
PI3K	phosphoinositide 3-kinase
PNPLA3	patatin-like phospholipase domain containing 3 gene
PPAR	peroxisome proliferator-activated receptor
PPV	positive predictive value
PTEN	phosphatase and tensin homolog
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
RXR	retinoid X receptor
RYGB	Roux en-Y gastric bypass
SAF score	steatosis, activity, and fibrosis score
SAM	s-adenosylmethionine
SAT	subcutaneous adipose tissue
SCD1	Stearoyl-CoA desaturase 1
SFA	saturated fatty acid
SNP	single nucleotide polymorphism
SREBP1c	sterol regulatory element binding protein 1c
STAT3	signal transducer and activator of transcription 3
T2DM	type 2 diabetes mellitus
TAK1	transforming growth factor beta-activated kinase 1
TE	transient elastography
TG/TAG	triglyceride/triacylglyceride
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
UNL	upper normal limit
VAT	visceral adipose tissue
VLCFA	very long chain fatty acid
VLDL	very-low-density lipoprotein

WHO SHOULD BE SCREENED FOR NASH?

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Take home messages

- Not all patients with NAFLD will have NASH; in fact, the prevalence of NASH in unselected consecutive NAFLD patients is suspected to be below 25%.
- When evaluating a new patient with recently diagnosed NAFLD, it is essential to obtain a careful history of anthropometric and metabolic risk factors, co-morbidities, and signs and symptoms. Often, one would be able to pick up many clues with regards to disease severity.
- Depending on local resources, the initial evaluation for the possible presence of NASH may consist of applying one of many easily available risk stratification models (e.g., NAFLD Fibrosis Score, APRI, FIB-4, BARD, etc.) and/or transient elastography.
- Patients who are deemed at high risk for NASH by bedside and non-invasive criteria should be approached about a percutaneous liver biopsy, especially if they are suitable candidates for available treatments or clinical trial participation.

Introduction

NAFLD encompasses the entire spectrum of fatty liver disease in individuals without significant alcohol consumption, ranging from fatty liver to steatohepatitis and cirrhosis. NAFL is described as the presence of hepatic steatosis with no evidence of hepatocellular injury in the form of ballooning of the hepatocytes or no evidence of fibrosis. The risk of progression to cirrhosis and liver failure is minimal, but not non-existent. NASH is defined as presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning) with or without fibrosis. This can progress to cirrhosis, liver failure and rarely liver cancer.

Initial evaluation of a patient with newly diagnosed NAFLD

The majority of patients with NAFLD are identified when their liver enzymes are found to be elevated during routine blood testing. Admittedly, there are a large number of patients with normal or near normal aminotransferases but with hepatic steatosis. These patients and their physicians are unaware of the potential presence of NAFLD at this time. Hepatic steatosis is sometimes found incidentally when patients undergo imaging for related (e.g., abdominal discomfort), or unrelated reasons (e.g., kidney stone evaluation).

A key element in evaluating a new patient with NAFLD is to exclude competing and concomitant etiologies. The former includes, among others, heavy alcohol consumption, medications (e.g., tamoxifen, amiodarone), viral hepatitis (especially HCV genotype 3), and Wilson's disease. Common concomitant etiologies include hemochromatosis, autoimmune hepatitis, Wilson's disease, etc.

Table 1. Risk factors associated with NAFLD.

Conditions with established association	Conditions with emerging association
• Obesity • T2DM • Dyslipidemia • MetS*	• PCOS • Hypothyroidism • Obstructive Sleep apnoea • Hypopituitarism • Hypogonadism • Pancreato-duodenal resection • Vitamin D deficiency

*The Adult Treatment Panel III clinical definition of the MetS requires the presence of three or more of the following features: (1) waist circumference >102 cm in men or >88 cm in women; (2) triglyceride level ≥150 mg/dL; (3) HDL cholesterol level <40 mg/dL in men and <50 mg/dL in women; (4) systolic blood pressure ≥130 mm Hg or diastolic pressure ≥85 mm Hg; (5) fasting plasma glucose level ≥110 mg/dL.

Patients with NAFLD generally display a number of other metabolic and endocrine co-morbidities (**Table 1**). It is very important to systematically obtain the history of these risk factors. Although not systematically investigated, it is generally believed that the presence of multiple risk factors heightens the risk for advanced NAFLD syndrome.

Who should be screened for NASH? [1-3]

It is probably reasonable to categorize the evaluation for NASH into two tiers. The first tier involves non-invasive risk scores and transient elastography (if available) and second tier involves a percutaneous liver biopsy.

It is reasonable to consider every patient with NAFLD for tier-1 evaluation. Some methods to consider for tier-1 evaluation are:

a) Evaluation of co-morbidities: longstanding T2DM and MetS certainly increase the prevalence of NASH in patients with NAFLD.

b) Presence of rare co-morbidities such as PCOS, obstructive sleep apnoea, pan-hypopituitarism (especially growth hormone deficiency), and pancreato-duodenal resection.

c) Patients with metabolic risk factors but also receiving tamoxifen.

d) Easily calculable risk scores – NAFLD fibrosis score, FIB-4, APRI, BARD, Fibrometer NAFLD, or Hepascore. These are described in detail in Practice Guidelines [2]. They are generally comparable against each other and it probably is reasonable to choose one or two methods and apply them consistently in individual practices.

e) Transient elastography (Fibroscan) is widely available in developed countries and was recently approved in the United States (for HCV). Although M probe is associated with a high failure rate, the new machine-mandated algorithm that forces XL probe as needed appears to have overcome this. More work is needed in terms of various cut-off points for ruling-in or ruling-out advanced fibrosis. It has been suggested that LSM <7.9 kPa (M probe) or <7.2 kPa (XL probe) exclude the presence of advanced fibrosis whereas LSM >9.6 kPa (M probe) or >9.3 kPa (XL probe) establishes the presence of advanced fibrosis.

Scenarios where tier-2 evaluation by liver biopsy may be appropriate for establishing the presence of NASH:

a) Presumably, patients with LSM >9.6 kPa (M probe) and >9.3 kPa (XL probe) should be considered for the next level of liver disease staging with a percutaneous liver biopsy.

b) An anxious patient who will not be reassured until absence of NASH is firmly established with a liver biopsy.

c) Persistently elevated ALT despite attempts to lose weight and exercise – this perhaps is the most common scenario for recommending liver biopsy at a community level practice, but it is not known that persistently abnormal ALT is sufficiently predictive of the presence of NASH.

d) Tender hepatomegaly – rarely patients may exhibit tender hepatomegaly. This phenomenon appears to occur in patients with co-existing functional bowel disorders or poorly controlled diabetes.

e) Unexplained fatigue.

Percutaneous liver biopsy is required to firmly establish the presence of NASH. Below are some comments with regards to the standards for obtaining a liver biopsy:

- 16 gauge (or wider) liver biopsy needle should be used at all times.
- ≥15 mm core is required. Longer cores minimize the risk of sampling variability. Fragmented or fibrotic samples diminish histological yield.
- Transjugular needle biopsy specimens may not yield sufficient amount of tissue for firm characterization of liver histology.
- Liver histology should be reviewed by a pathologist with expertise in liver pathology.
- Pathologist should evaluate the liver biopsy in a systematic fashion and address all histological elements of NAFLD (steatosis – extent, location; inflammation – extent, location, cell type; ballooning – extent and location; fibrosis – extent, location; other features such as MDBs, Kupffer cells, iron deposition.

References

[1] Castera L, Vilgrain V, Angulo P. Noninvasive evaluation of NAFLD. Nat Rev Gastroenterol Hepatol 2013;10:666-675.

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[3] Nascimbeni F, Loria P, Ratziu V. Non-alcoholic fatty liver disease: diagnosis and investigation. Dig Dis 2014;32:586-596.

HISTOLOGICAL CLASSIFICATIONS IN NAFLD: HOW AND WHEN TO TRUST THE PATHOLOGIST?

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Take-home messages

- Liver biopsy is the only diagnostic procedure that can reliably differentiate NASH from NAFL despite its usual limitations, mainly sampling error.
- NASH is the association of liver fat, hepatocyte ballooning and lobular inflammation. In the absence of any one of these three lesions the diagnosis is NAFL.
- Although the distinction of NAFL and NASH is clinically relevant, NAFLD displays a continuous spectrum of histological lesions, a spectrum that can be reported simply with the SAF score.
- SAF score assesses, semi-quantitatively and separately, steatosis, activity and fibrosis in a simple and reproducible manner.
- The FLIP algorithm increases agreement for the overall diagnosis of NASH between observers.

Introduction

NAFLD covers a spectrum of histological lesions ranging from steatosis to a complex pattern with associated hepatocyte injury, inflammation and fibrosis. Despite the usual limitations of the liver biopsy in assessment of any chronic liver diseases, it is the only diagnostic procedure that can reliably assess these various patterns and their association, allowing the distinction between NASH and NAFL, a distinction that is essential for prognostic risk stratification [1, 2]. Therefore, whether the liver biopsy (and the pathologist) is reliable may appear as a central question in NAFLD.

When to trust the pathologist?

Histopathologic evaluation of liver biopsy samples remains central to all investigations in NAFLD. However, it is an invasive procedure that carries a low but real risk of morbidity and mortality. The main limitations and advantages of liver biopsy are summarized in **Table 1**. Thus, and considering the huge number of patients with potential NAFLD, liver biopsy should be reserved for selected patients. Unfortunately, liver biopsy may sometimes fail to provide significant information because of limitations related to the procedure itself. Indeed since the volume of a needle biopsy sample represents only a very minor fraction of the whole liver, sampling variation is a relevant issue to consider, a risk that is inversely proportional to the length of the biopsy [3]. While a 25 mm biopsy is considered optimal for assessing and quantitating detailed lesions, a 15mm long biopsy usually provides robust information for a global evaluation. These recommendations, developed in the context of chronic viral hepatitis, may be less relevant in NAFLD. Indeed in NAFLD, and contrary to chronic hepatitis, the lesions tend to show a very robust and characteristic lobular systematization affecting mainly the centrilobular zone. Since the size of a liver lobule is 0.5-1 mm, the threshold for the minimally required length of a biopsy might be lower but this needs to be formally demonstrated.

Table 1. Advantages and limitations of liver biopsy in NAFLD.

Limitations	Advantages
• Sampling error • Inter-observer variation • Invasiveness • Cost	• Reliably differentiate NASH from NAFL • Assess semi-quantitatively the severity of steatosis, activity (balooning + lobular inflammation) and fibrosis • Characterize other lesions related to NAFLD • Identify the relative liability of NAFLD in case of comorbidities • Provide prognostic factor (fibrosis)

The expertise of the pathologist is also important to consider. Indeed, the FLIP Pathology Consortium showed that concordance in interpretation between pathologists was higher when biopsies were interpreted by a group of specialized academic liver pathologists than by general pathologists [4]. However, the study shows that training with adequate histological guidelines considerably increases the robustness of interpretation, regardless of pathologist speciality and academic training. Therefore, the pathologist is reliable as long as the hepatologist (or the radiologist) provides an adequate sample.

How to interpret the biopsy?

As mentioned previously, the significant advantage of taking a liver biopsy in a patient who is clinically suspected of having NAFLD is actual confirmation (or exclusion) of NASH. In addition, and due to the high burden of the disease, comorbidities are not infrequent and the biopsy might be useful to delineate the respective contribution of each comorbidity. Finally, liver biopsy remains the recognized procedure in assessing the effect of drugs in controlled clinical trials. Indeed, liver histology was the primary endpoint in most clinical trials performed in NAFLD thus far.

Table 2. Main histological patterns in NAFLD.

Lesion type	Assessment
Steatosis	Type: macro-, medio-, microvacuole Amount: usually in % Location: zone 3, periportal, azonal, diffuse
Hepatocellular injury	Ballooning and clarification of cytoplasm Apoptotic body MDB
Inflammation	Location: portal, periportal, lobular Inflammatory cell type Extent
Fibrosis	Location: perisinusoidal, perivenular, portal Extent: focal, bridging fibrosis, annular fibrosis Architectural modification
Other	Vacuolated nuclei Megamitochondria

In NAFLD without comorbidities, the histopathological spectrum is relatively limited. Lesions should be categorized into four main groups: steatosis, hepatocellular injury, inflammation and fibrosis (**Table 2**) [5]. Correct assessment is crucial for the characterization of the severity of changes that ultimately lead to distinction between the processes considered to be non-progressive and not at risk of increased liver disease mortality (i.e., NAFL) and those with features linked to progression of liver injury (i.e., steatohepatitis, NASH). The diagnosis of steatohepatitis is based on the association of liver fat (macrovacuolar or mediovesicular steatosis of ≥5%), hepatocyte ballooning and lobular inflammation [6]. Perisinusoidal fibrosis is a useful and frequent diagnostic feature but not included formally in the diagnostic criteria of steatohepatitis. In the early stages, the pattern of injury follows a centrilobular accentuation, although, at later stages, the lobular architecture is mutilated and the zonal distribution is no longer visible. Other histological features can be seen in steatohepatitis but are not necessary for the diagnosis of NASH: perisinusoidal fibrosis, polymorphonuclear infiltrates, MDB, apoptotic bodies, clear vacuolated nuclei, microvacuolar steatosis, megamitochondria and portal inflammation. Portal inflammation is a frequent feature in pediatric NASH, but can be seen in adults and may be associated with more severe disease. When steatosis is present but lobular inflammation or ballooning are absent, the minimal requirements for steatohepatitis are not met, and the diagnosis should be NAFL (i.e. non-NASH NAFLD). The terms 'probable' or 'possible NASH' should be abandoned because they create confusion.

A final goal of liver biopsy in this setting is the semi-quantitative evaluation of the severity of injuries. Indeed, although the dichotomized diagnostic approach (NAFL *vs.* NASH) is clinically useful, it is an over-simplification that does not reflect the histological complexity of the disease. As with chronic liver diseases, NAFLD might display a continuous spectrum of histological lesions so that splitting the disease into two categories is useful but artificial. Therefore, semi-quantitative scoring system might better reflect the complexity of the histological pattern. These scoring systems are currently of limited value in common practice but are extremely useful in the context of clinical trials. The NASH Clinical Research Network (NASH CRN) from the United States and the European FLIP Pathology Consortium have both contributed towards an accurate histological evaluation of NAFLD. The NAS (NAFLD Activity Score) described by the NASH CRN is the unweighted sum of steatosis (0 to 3), inflammation (0 to 3) and ballooning (0 to 2) [7]. It is not designed to be a surrogate for the diagnosis of steatohepatitis but rather a crude evaluation of the severity of the disease, once the diagnosis of NASH has been established by the overall pathological assessment. Although the NAS is correlated with aminotransferase and HOMA values, to date there is unfortunately no demonstration of any prognostic value of the NAS [8]. While most patients with a NAS <3 and a NAS >4 are *bona fide* NAFL and NASH, respectively, there is a grey zone (NAS = 3 or 4) that includes both cases with NAFL and NASH. Consequently, its use as a histological outcome in therapeutic trials is of questionable clinical relevance.

Table 3. The components and semi-quantitative grading of the SAF score.

Feature (grade range)	Grading criteria
S: Steatosis (from 0 to 3)	<5% (S0); 5 to 33% (S1); 33 to 66% (S2); >66% (S3)
A: Activity (from 0 to 4)	Activity: the sum of ballooning and lobular inflammation Ballooning: normal hepatocytes (grade 0), clusters of hepatocytes of normal size, but with a rounded shape and pale cytoplasm (grade 1); same as grade 1 with some enlarged hepatocytes, at least 2-fold that of normal cells (grade 2) Lobular inflammation: foci of 2 or more inflammatory cells within the lobule (0: none; 1: <2 foci per 20x; 2: >2 foci per 20x)
F: Fibrosis (from 0 to 4)	None (F0); perisinusoidal or portal fibrosis (F1); perisinusoidal and periportal fibrosis without bridging (F2), bridging fibrosis (F3); cirrhosis (F4)

The SAF score has been prospectively designed by the FLIP Pathology Consortium and its use, in association with the FLIP algorithm, increases agreement for the overall diagnosis between observers [4]. It defines precisely, in a didactic and easy-to-understand form, the 3 main, cardinal histological features (Steatosis, Activity and Fibrosis) (**Table 3**). Activity is a composite score adding hepatocellular ballooning and lobular inflammation, two lesions that, in association, are supposed to sustain the development of fibrosis (each being graded from 0 to 2). Furthermore, it serves as a backbone for the diagnosis of NASH according to the FLIP algorithm. The SAF score has not yet been tested within therapeutic trials.

Staging of fibrosis relies on the Kleiner fibrosis stage [5]. Unfortunately, this system under scores perisinusoidal (pericellular) fibrosis within the lobule, which is a common pattern, particularly in patients with diabetes (**Fig. 1**). Furthermore, it does not allow the distinction between biopsies with rare or short septa from biopsies where septa are numerous (the contrary of what is done by distinguishing F2 from F3 with the METAVIR score in chronic hepatitis). This is a significant limitation since there is no clear-cut border to define what differentiates biopsies with significant fibrosis from those with advanced fibrosis. Morphometry that assesses the amount of fibrous tissue quantitatively (or collagen proportional area, CPA) might be a useful adjunct, as it is done in most clinical trials.

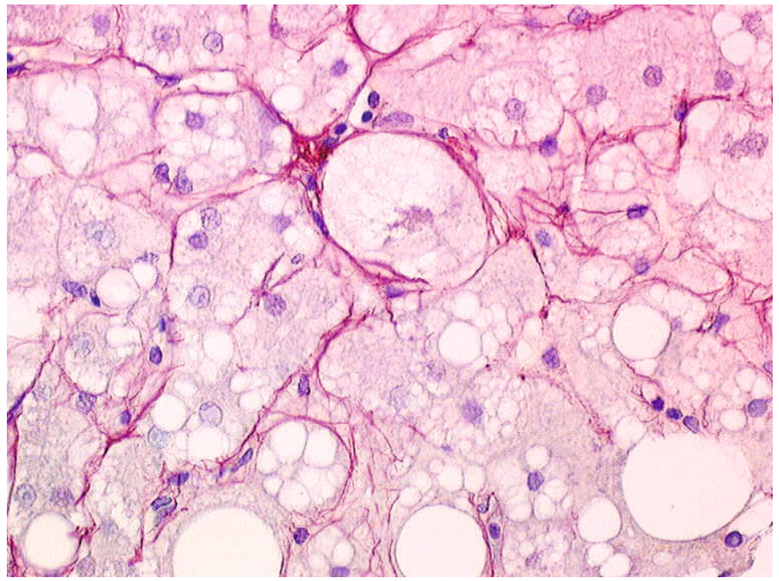

Figure 1. Typical pattern of NASH with clarification and ballooning of hepatocyte with perisinusoidal fibrosis (Sirius Red stain, x 40).

A key issue is which histological features of NAFLD predict liver disease progression and liver-related events or mortality, as these could be acceptable surrogates for therapeutic trials. Studies with large cohorts, defined histologically by a central pathologist and with long follow-up for clinical events, are necessary to answer this question. Such studies have shown that both the diagnosis of steatohepatitis and the stage of fibrosis (bridging fibrosis or cirrhosis) predict liver-related mortality [8, 9]. Because steatohepatitis most likely drives fibrogenesis, the demonstration of an independent effect of steatohepatitis from that of fibrosis can be difficult to delineate statistically because of co-linearity between the two variables.

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NON-INVASIVE DIAGNOSIS OF FIBROSIS IN NAFLD: HOW RELIABLE IS IT?

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Take home messages

- Simple and complex serum based tests have >90% predictive value for excluding cirrhosis, though are poorly predictive of cirrhosis.
- The NAFLD Fibrosis Score (NFS) and FIB-4 algorithm are clinical/serum based tests which can confirm or exclude bridging fibrosis/cirrhosis but have indeterminate values in one quarter of patients.
- Serum-based tests, including the NFS, FIB-4 and Fibrotest, predict mortality outcomes in patients with NAFLD or those with metabolic risk factors for NAFLD.
- Transient elastography is excellent at excluding advanced fibrosis and cirrhosis and has modest predictive values, although acquisition is unsuccessful in one quarter of patients.
- All tests have been developed in the hospital clinic setting without validation in the general population and have poor correlation when used as screening tools in this setting.

Introduction

Significant liver fibrosis is present in 5-10% of patients with NAFLD in the primary medical care setting. Reliable identification of these patients is important as fibrosis is the dominant histological feature that predicts outcomes and thus the need for treatment and surveillance. Furthermore, determination of fibrosis is useful to monitor disease progression or treatment response over time. The limitations of liver biopsy have led to the refinement of non-invasive techniques to predict liver fibrosis. These include simple easy-to-use clinic-based tools, serum tests or imaging-based techniques that predict fibrosis based on the physical elasticity of the liver. In general, these investigations provide information on a continuous scale as opposed to the semi-quantitative histological staging systems utilized in liver biopsies. Thus, non-invasive tests may be more sensitive to subtle alterations in fibrosis than biopsy and provide greater prognostic information.

The accuracy of non-invasive fibrosis tests is often described using the area under the receiver operator characteristic curve (AUROC), where a value of 1.0 reflects a 'perfect' test with 100% sensitivity and specificity, and a value of 0.5 reflects a test as good as chance. Although two tests may have equivalent AUROC values, they may have different sensitivity and specificity values depending upon the characteristics of the test and where the individual test cut-offs have been drawn. Typically, values at one end of the test result spectrum will have a high sensitivity and low specificity, whereas values at the opposite end of the test result spectrum will have a low sensitivity and high specificity. Test results that fall in-between often have moderate sensitivity and specificity and are not clinically meaningful, and thus comprise an 'indeterminate range'. Therefore, the majority of fibrosis tests will produce inconclusive results for a proportion of patients falling within the indeterminate range for a specific fibrosis end-point. It is also important to note that the underlying prevalence of fibrosis may influence the AUROC – the so-called spectrum effect and thus it is difficult to directly compare the accuracy of tests performed in different populations.

Predictive values

Predictive values are dependent upon the underlying fibrosis prevalence as well as sensitivity or specificity. Thus a test may be highly specific for the diagnosis of cirrhosis, but have a low positive predictive value (PPV) if the underlying prevalence (or pre-test probability) is very low. It is therefore important to realize that non-invasive test performance will vary according to the setting (and underlying fibrosis prevalence) in which they are used. Current, non-invasive tests that have been developed in tertiary hospital settings have poor agreement for the prediction of advanced fibrosis in a general population setting, although appear reliable in excluding advanced fibrosis [1].

Clinical prediction of fibrosis

Cross-sectional studies of NAFLD patients undergoing clinically indicated liver biopsies show increasing age and diabetes (odds ratio ~4) to be consistently associated with fibrosis [2]. The association with obesity and hypertension is more variable between studies, whereas dyslipidemia appears not to have a strong independent association with fibrosis. Individually these clinical factors lack sufficient accuracy to reliably predict advanced fibrosis or cirrhosis; however they may be useful to exclude advanced fibrosis, with 0/144 patients in one study having stage 3-4 fibrosis in the absence of diabetes, obesity and age ≥45 years [3].

Serum based tests

Simple, easily calculated serum-based tests include the AST/ALT ratio and the BARD algorithm. Notably, aminotransaminase levels alone are poorly predictive of fibrosis stage and tend to decline over time as fibrosis progresses. An AST/ALT ≥1.0 has modest accuracy (AUROC 0.66-0.83), reasonable specificity (84-92%), is weakly predictive of advanced fibrosis (stage 3-4) with PPVs of 26-55%, and has reported negative predictive value's (NPV) of 81-95%. The BARD score is calculated from the cumulative total of BMI ≥28 kg/m^2 (1 point), AST/ALT ratio ≥0.8 (2 points) and diabetes (1 point). Its simplicity is attractive and its strength is excluding advanced fibrosis, with a score of 0 or 1 having reasonably high NPVs (81-97%), though poor specificity (44-79%) and low PPVs (22-46%). The AST/ALT ratio (and subsequently BARD score) appear to be less accurate in patients with diabetes; the odds ratio of having advanced fibrosis with an AST/ALT ≥0.8 is 16 and 4 in non-diabetics and diabetics, respectively [4].

More complicated algorithms that combine multiple routinely-available biochemical and clinical variables have greater accuracy for predicting advanced fibrosis (**Table 1**). Algorithms that have undergone the greatest external validation, include the NAFLD Fibrosis Score (NFS; *www.nafldscore.com*) and FIB-4, which were developed to predict advanced hepatic fibrosis (defined as bridging fibrosis or cirrhosis). Comparative studies have shown these scores to be more accurate than less complicated algorithms such as BARD and the APRI [5, 6]. Both scores predict liver-related and overall mortality in NAFLD patients, although the NFS may be more discriminatory [7]. The NFS and FIB-4 have similar accuracy with AUROC for advanced fibrosis of 0.82-0.88 and 0.8, respectively. Both scores also have upper and lower cut-off points in order to maximize their diagnostic accuracy; however, this means indeterminate results will occur in one quarter to a third of patients. Both tests exclude advanced fibrosis with scores below the lower cut-off having NPVs of 88-90%. Scores above the upper cut-off are highly specific (96-98%), translating to PPVs for advanced fibrosis of 80-82%. Notably however, scores that incorporate AST and ALT become less accurate as aminotransaminase values increase.

Other tests include NAFLD Fibrometer and Fibrotest, which are proprietary. Independent validation of the Fibrotest has shown AUROC values of 0.64-0.82 for the prediction of portal based fibrosis (F2+) and AUROC of 0.86-0.89 for cirrhosis [5, 8]. Among morbidly obese individuals (BMI >35 kg/m^2), Fibrotest at a cut-off of 0.48 is poorly sensitive (8%) for significant fibrosis (METAVIR F2-4), but has excellent specificity (99.6%), translating to a NPV and a PPV of 91% and 67%, respectively, when the prevalence of significant fibrosis is 10% [9]. Fibrotest is also predictive of overall, liver and cardiovascular mortality in patients at risk of NAFLD, namely those with diabetes and dyslipidemia.

Table 1. Validated fibrosis tests in NAFLD based upon routinely-available clinical and biochemical variables. Adapted from Castera et al. [10].

Test	n	Parameters	AUROC	Cut-offs	Sens	Spec	PPV	NPV
NFS	733	IFG/diabetes, AST/ALT, Age, BMI, platelets, albumin	0.82-0.88	<-1.455 >0.676	77% 43%	71% 96%	52% 82%	88% 80%
FIB-4	541	ALT, AST, platelets, Age	0.80	<1.30 >2.67	74% 33%	71% 98%	43% 80%	90% 83%
BARD	827	BMI, AST/ALT, diabetes	0.81	2	-	-	43%	96%
APRI	576	AST, platelets	0.82	1.0	67%	81%	31%	95%
NAFLD Fibrometer	235	Glucose, ALT, AST, weight, age, platelets, ferritin	0.93-0.94		78%	96%	88%	92%
Fibrotest	267	Age, sex, bilirubin, GGT, apolipoprotein A1, haptoglobin, α2-macroglobulin	0.81-0.92	>0.30 >0.70	92% 25%	71% 97%	33% 60%	98% 89%

Values based on the prediction of advanced fibrosis. Original studies with validation cohorts presented. Sens = sensitivity; Spec = specificity.

Overall, simple serum-based tests are reasonably accurate at excluding cirrhosis with NPVs consistently above 90%. More complex serum tests with multiple covariates have higher AUROC values and are better at predicting or excluding lesser degrees of fibrosis such as F3+ (**Table 1**) [5].

Algorithms which incorporate serum measures of factors directly involved in fibrogenesis (e.g. hyaluronate) have the theoretical advantage of being more specific for fibrosis. The accuracy of these algorithms (outlined in **Table 2**) appears similar to algorithms based on multiple routine clinical and biochemical variables, however direct comparative studies are lacking.

Table 2. Validated fibrosis tests in NAFLD based upon direct markers of fibrogenesis. Adapted from Castera et al. [10].

Test	n	Parameters	AUROC	Cut-offs	Sens	Spec	PPV	NPV
Hyaluronate Type IV collagen 7S	112	Hyaluronate Type IV collagen 7S	0.80 0.82	50 ng/ml 5 ng/ml	69% 81%	83% 71%	75% 68%	84% 78%
Hyaluronate	148	Hyaluronate	0.97	42 ng/ml	100%	89%	77%	100%
ELF	192	TIMP-1, hyalularonic acid, terminal peptide of procollagen III	0.90	0.3576	80%	90%	71%	94%
Hepascore	242	Hyaluronate, α2-macroglobulin, bilirubin, GGT, age, sex	0.81	0.37	76%	84%	57%	92%

Values based on the prediction of advanced fibrosis. Studies with >100 subjects presented. Only ELF validated in a separate cohort.

Elastography based tests
Hepatic elasticity and distention reduces as hepatic fibrosis worsens. This may be quantified by measuring the velocity of a transmitted physical or sonographic impulse through the liver (shear wave elastography), or by measuring the magnitude of liver tissue distention to external pressure or internal movements such as the cardiac cycle (strain elastography).

Transient elastography (Fibroscan®)
A recent meta-analysis demonstrated modest accuracy for detecting moderate (F2+) fibrosis with sensitivity and specificity values of <80% and AUROC of 0.79-0.87. Fibroscan® has better accuracy for F3+ fibrosis (sensitivity 85%, specificity 82%, AUROC 0.76-0.98) and cirrhosis (92% sensitivity and specificity, AUROC 0.91-0.99) [11]. Correspondingly, predictive values for F2+ fibrosis are modest (PPVs 55-79%, NPVs 72-95%) but better for cirrhosis (PPVs 41-86%, NPVs 91-100%).

A limitation of Fibroscan® is acquisition failure or unreliable readings related to obesity, which occurs in one quarter of patients when using the XL probe. Studies are conflicting as to whether BMI impacts on the accuracy of Fibroscan®; however discordance between biopsy and Fibroscan® results increases at a BMI threshold of 35 kg/m^2. There is also a range of cut-offs in the literature that overlap for different levels of fibrosis (**Fig. 1**), making it difficult to determine the significance of a mid-range reading. Cut-offs should be adapted to the probe used, with readings 1.2-1.3 kPa lower with the XL probe compared with the standard M probe. Severe hepatic steatosis increases liver stiffness measurements in chronic hepatitis C infection and thus may also affect liver stiffness measurements in NAFLD.

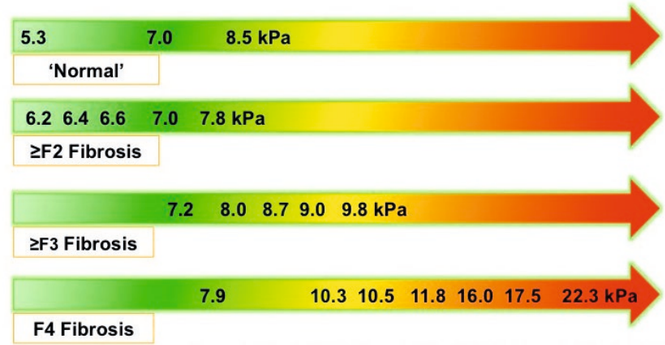

Figure 1. Recommended Fibroscan® cut-offs for different stages of fibrosis in NAFLD.

Comparative studies have shown higher accuracy, although lower specificity and PPV, with Fibroscan® when compared to non-proprietary serum-based tests such as FIB-4 and NFS [12, 13]. Specificity and PPV are increased in Fibroscan® when higher cut-offs are utilized.

Acoustic radiation force impulse (ARFI)
ARFI sonographically measures the velocity of a shear wave generated by an acoustic impulse at a single point in the liver. Only a limited number of studies have examined the accuracy in NAFLD and have shown variable accuracy for the detection of advanced fibrosis (AUROC 0.6-0.97) and cirrhosis (0.74-0.98). Similar to Fibroscan®, successful acquisition may be impacted by BMI and was unsuccessful in 20% of patients with a BMI between 30-40 kg/m^2 [14]. Further clarification of the impact of BMI, hepatic steatosis, and inflammation on accuracy as well as validation of appropriate cut-offs is required.

Real time strain elastography

Strain elastography measures the deformation of liver parenchyma following external compression with a sonographic probe or by cardiac motion. Early studies have demonstrated AUROC values for F2+ of 0.85-0.92. Different processing algorithms between studies and a lack of validation currently limit its applicability.

Magnetic resonance elastography (MRE)

MRE utilizes an external driver to produce hepatic shear waves that are detected using magnetic resonance imaging and quantified using post-sequence processing. Multiple areas of signal acquisition are likely to reduce sampling error. Only one large prospective study of NAFLD patients has been published demonstrating 2-dimensional MRE to yield high AUROC values for F2+ (0.84), F3+ (0.92) and F4 (0.89). Accuracy was not impacted by hepatic steatosis [15]. Aside from access and cost issues these findings need to be replicated in other centres prior to incorporation into clinical practice. In terms of supersonic shear imaging/real time shear wave elastography, contrast enhanced ultrasound and magnetic resonance imaging techniques, no studies dedicated to NAFLD patients have currently been published.

Monitoring fibrosis over time

Little data exists to show that non-invasive markers are dynamic and reflect changes in fibrosis over time. LSM falls by approximately 1.6 kPa 12 months after bariatric surgery, though this may also reflect improvement in inflammation [17]. Changes in different serum markers (APRI, ELF, FIB-4) over a 3.5 year period in a population-based cohort of diabetics correlated poorly with each other, suggesting caution is needed when using different tests to monitor for fibrosis change [1]. Notably, fibrosis typically progresses slowly in NAFLD (approximately 0.1 stage per year) and monitoring more frequently than annually is likely to produce false positive readings.

Combination testing

Combining two serum-based tests (e.g. FIB-4 and BARD) or Fibroscan® and a serum-based marker (FIB-4, NFS) increases accuracy, specificity and PPV, although 30-40% of NAFLD patients will have indeterminate results for one of the tests [12].

Table 3. Causes of falsely elevated readings.

Elastography	Serum based tests
• Acute hepatitis • Cholestasis • Respiration • Venous congestion • Beta-blockade • Focal liver lesions • Recent food ingestion	• Acute hepatitis • Cholestasis (bilirubin, GGT) • Hemolysis (bilirubin) • Gilberts syndrome (bilirubin) • Systemic inflammation • Recent food ingestion (hyaluronate) • Exercise (hyaluronate)

Pitfalls in fibrosis tests

Non-invasive tests are typically less accurate in the determination of mid-levels of fibrosis (e.g. Kleiner or Brunt stage 2), with AUROC levels often between 0.6-0.8 in independent validation studies. In contrast, models have greater accuracy for determining advanced fibrosis and cirrhosis, with AUROC levels often >0.9. Due to the relatively low prevalence of cirrhosis, the PPVs of biomarkers is generally modest. However, the NPVs are generally excellent (>95%), allowing reliable exclusion of cirrhosis.

Interpretation of non-invasive markers should not be performed without considering other clinical and laboratory findings as co-morbid conditions can lead to false positive or false negative results **(Table 3)**. Generally, false-positive results are more common than false-negative ones [10].

Summary

Serum-based and elastography tests accurately exclude advanced fibrosis and cirrhosis in patients with NAFLD. However, the prediction of these endpoints is modest and both types of non-invasive modalities suffer from indeterminate scores or unsuccessful acquisition in approximately one quarter of patients (**Table 4**). Serum tests are widely validated and predict future liver related morbidity and mortality in NAFLD patients, whereas elastography-based tests offer the promise of increased accuracy. Clarification of appropriate cut-offs and investigating dynamic changes over time are required.

Table 4. Diagnostic utility of non-invasive predictors of fibrosis in NAFLD.

Test	AUROC for F3-4[1]	AUROC for F4	Indeterminate % for F3-4[2]	Externally validated	Prognostic	Dynamic
Serum-based tests						
APRI	0.74	0.75	Nil	++++	++	Unknown
BARD	0.78	0.75	Nil	++++	+	Unknown
ELF	0.90	0.82	14%	+	Unknown	Unknown
FIB-4	0.86	0.86	30%	++++	++	Unknown
Fibrometer	0.94	0.94	2.5%	+	Unknown	Unknown
Fibrotest	0.80	0.86	33%	++	++	+
Hepascore	0.81	0.91	Nil	+	Unknown	Unknown
NFS	0.85	-	24-40%	++++	+++	Unknown
Elastography						
ARFI	0.98	0.98	22%	++	Unknown	Unknown
MRE	0.92	0.89	Unknown	+	Unknown	Unknown
TE	0.93	0.95	25%	+++	Unknown	Unknown

[1] AUROC values from meta-analysis or largest independent validation study if available [5, 16].

[2] The criteria for 'Indeterminate' differ between studies and are thus not directly comparable. Unsuccessful elastography acquisition included as 'indeterminate'.

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PEDIATRIC NASH: IS IT DIFFERENT AND SHOULD WE LOOK FOR IT?

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Take home messages

- NAFLD has rapidly become the leading cause of chronic hepatopathies in children. Considering the strict association between NAFLD, MetS and CVD this scenario appears particularly worrisome.
- Genetic analyses may aid the identification of children susceptible to NAFLD/NASH.
- Non-invasive tools to diagnose NAFLD in children could greatly reduce the need for liver biopsy.
- To date none of the available drugs has been shown to be effective in totally reversing NAFLD-related liver damage.
- Adequate diagnostic and therapeutic management is crucial to prevent and counteract progression of pediatric NAFLD.

Introduction: epidemiology and pathogenesis

NAFLD now represents the most frequent cause of chronic liver disease in industrialized countries in children and adolescents, as a direct consequence of the rise in childhood obesity. Although the exact prevalence of NAFLD in the pediatric setting is still unknown, recent epidemiological data indicate a prevalence of 3-10% in the general pediatric population. Prevalence increases up to >70%, with a male-to-female ratio of 2:1, in obese children [1]. The term NAFLD encompasses a broad spectrum of diverse liver conditions. The histological features of NAFLD vary from fat accumulation in >5% of the hepatocytes (simple hepatic steatosis) to NASH with necroinflammation (sometimes associated with fibrosis), which in turn can progress to cirrhosis and hepatocellular carcinoma during early adolescence.

The pathogenesis of NAFLD appears to be multifactorial, involving both genetic and environmental factors. In fact, although pediatric NAFLD is generally related to a sedentary lifestyle and hyper-caloric diet leading to a progressive increase of body mass index (BMI) and visceral adiposity, recent epidemiological, familial, and twins studies suggested a strong heritability for NAFLD [2]. Several genetic studies have demonstrated that single nucleotide polymorphisms (SNPs) in genes involved in lipid metabolism, oxidative stress, insulin signalling and fibrogenesis have been associated with a high risk for NAFLD development and progression [3]. In a recent study, Nobili *et al.* have described a multivariate logistic model based on four polymorphisms, which allowed a NASH risk score in obese children with increased liver enzymes to be extrapolated [3].

The complex interplay between genes and environment in NAFLD pathogenesis is sustained by multiple mechanisms that involve liver interactions with other organs and tissues, especially gut (the so-called 'gut-liver axis') and adipose tissue.

Diagnosis

NAFLD/NASH in children is generally asymptomatic and, therefore, the diagnosis is commonly made during a supplementary evaluation for elevated aminotransferases or hyperechogenic liver found during a routine check-up [4]. In obese children, NAFLD should always be suspected and many centres have adopted a screening program for NAFLD in high-risk individuals, particularly those presenting with features of the MetS (**Fig. 1**). The position paper by the ESPGHAN Hepatology Committee has recently delineated diagnostic criteria for pediatric NAFLD [5]. Elevated aminotransferase levels and liver hyperechogenicity deserve further evaluation and the exclusion of other causes of liver disease, because of the poor sensitivity of these tests in overweight/obese children [5]. Liver biopsy remains the gold standard for diagnosing NAFLD, distinguishing between NASH and simple steatosis and estimating the severity of liver damage, especially fibrosis.

NAFLD in children displays the same basic morphological lesions observed in adults, but the pattern of distribution of these lesions is frequently different. Hepatocellular ballooning degeneration and MDBs are only sporadically observed and portal-based chronic inflammation is predominant [6]. Based on the distinctive histological pattern of disease in children, a specific histological score (Pediatric NAFLD Histological Score - PNHS), has been validated for a better classification of children with/without NASH [7].

Non-invasive markers and imaging techniques are the first diagnostic step. Predictors of fibrosis (e.g. TE, ARFI) and serum biomarkers (e.g. ELF panel and CK18) could be adopted to reduce the need for liver biopsy [8].

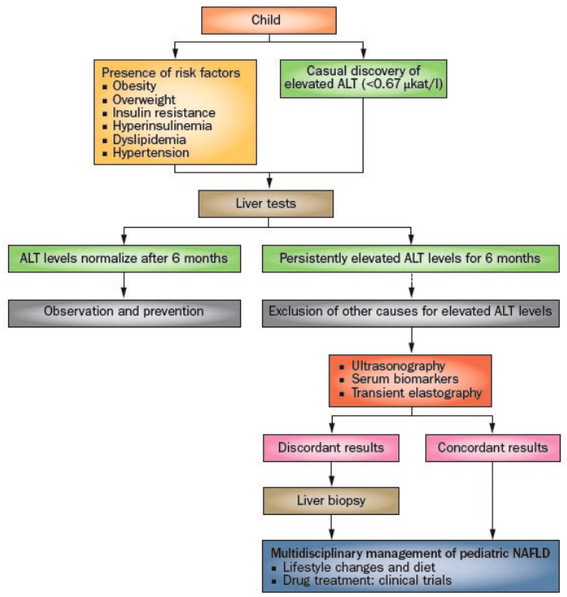

Figure 1. Management algorithm for children affected by NAFLD. *Adapted from Alisi et al. [4].*

Table 1. Completed RCTs for the treatment of pediatric NAFLD with effective drugs. Adapted from Della Corte et al. [10].

Drug	Type of study	Clinical-Trial.gov identifier	Patients	Intervention	Endpoints	Results
Vitamin E + ascorbic acid	Double blind RCT	NCT 00655018	3-20 years Biopsy proven NAFLD	Alpha tocopherol 600 IU/day + Ascorbic acid 500 mg/d	Improvement serum levels of aminotransferase, insulin-sensitivity, body weight and liver histology	Negative
TONIC	Double blind RCT	NCT 00063635	8-17 years Biopsy confirmed NAFLD ALT >60 U/L	Metformin 500 mg BID Vitamin E 400 IU BID	Reduction of 50% or less of serum ALT levels from baseline or 40 IU/L or less	Negative
Cystea-mine	Open label –preliminary study	NCT 00799578	≥10 years Biopsy proven NAFLD ALT > 60 U/L	1 gr/mg body surface area (maximum 100 mg BID)	Normalization or >50% of serum ALT levels from baseline	Positive
DHA	Double blind RCT	NCT 00885313	4-16 years Biopsy proven NAFLD	1° exptl arm: 250 mg/day 2° exptl arm: 500 mg/day	Improvement of ALT levels Improvement of serum levels of triglycerides	Negative Positive
Gluco-mannan	Double blind RCT	NCT 01553500	4-16 years Echographic evidence of hepatic steatosis	5 gr/day	Improvement in lipid metabolism Improvement in glucose metabolism	Positive (LDL-chollesterol) Negative

Treatment

Lifestyle modification represents the current first-line therapy for pediatric NAFLD, even though it is not known to improve NAFLD-associated liver damage [9]. As guidelines for the management of NAFLD in children are still lacking, the identification of effective treatments represents a challenge for pediatric hepatologists in the near future. Based on new risk factor and pathogenesis knowledge, several studies have evaluated the effects of different molecules (e.g. insulin-sensitizers, anti-oxidants, and cytoprotective agents) in the treatment of pediatric fatty liver. Several drug-based therapies (e.g. vitamin E, metformin) and dietary supplementation (e.g. VSL#3, docosahexaenoic acid) have been shown to be effective on ballooning, steatosis and inflammation, but fibrotic lesions are refractory to treatments [10]. For these reasons many clinical trials for the treatment of pediatric NAFLD have been proposed, some of which have been completed (**Table 1**), while others are still in progress (**Table 2**) (clinical trials registered on ClinicalTrial.gov as of December 2014) [10].

Table 2. Clinical Trials ongoing for the pharmacological treatment of pediatric NAFLD. Adapted from Della Corte et al. [10].

Drug	Type of study and status	Clinical-Trial.gov identifier	Patients	Intervention	Endpoints
Losartan	Double blind, RCT Recruiting	NCT 01913470	12-19 years BMI >85% ALT ≥3 UNL Biopsy confirmed NASH	0.4 mg/kg/day (max 25 mg) for one week and then increased to 0.8 mg/kg/day (max 50 mg) for 7 additional weeks	Change in ALT from baseline
Cysteamine bitartrate delayed-release (CyNCh)	Double blind, RCT Ongoing, but not recruiting	NCT 01529268	8-17 years biopsy-confirmed NAFLD (NAS >4)	600 mg/day for patients ≤65 kg 750 mg/day for patients 65-80 kg 900 mg/day for patients >80 kg	Histological endpoints: Decrease in NAS of ≥2 No worsening of fibrosis
DHA + vitamin D	Double blind, RCT Recruiting	NCT 02098317	4-16 years Biopsy proven NAFLD	DHA 500 mg/day Vitamin D 800 IU/day	Histological endpoints: Improvement in NAS score
DHA + choline + vitamin E	Double blind, RCT Ongoing, but not recruiting	NCT 01934777	4-16 years Biopsy proven NAFLD	DHA 500 mg/day Choline 400 mg/day Vitamin E 78 IU/day	Histological endpoints: Improvement in NAS score

Conclusion

NAFLD in children is a new global challenge for liver disease researchers and an important burden for health systems. During the past decade, our understanding of pediatric NAFLD in terms of epidemiology and risk factors has improved considerably, but more investigations are required to unravel its pathophysiology and to identify novel therapeutic targets. Screening for NAFLD, differential diagnosis of liver steatosis and indications for liver biopsy remain as major clinical questions for practitioners and are still debated among experts.

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NASH IN LEAN PATIENTS

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Take home messages

- NASH and advanced fibrosis occur in lean patients, albeit at a lower prevalence.
- Most lean patients with NASH have recent weight gain, other metabolic risk factors or insulin resistance.
- Since lean patients are less likely to have advanced disease, non-invasive tests of NASH and fibrosis are preferred.
- Lifestyle intervention remains the most important treatment for NASH in lean patients. Pharmacological treatment may be considered in selected cases.

Introduction

NAFLD is the most common chronic liver disease and has rapidly become an important cause of liver failure and HCC. It is strongly associated with obesity and MetS. However, a small but significant proportion of patients develop NAFLD despite normal body mass index (BMI). They are usually referred to as having lean or non-obese NAFLD. Here, we will discuss the epidemiology, clinical significance and management of lean NAFLD.

What is meant by lean?

Worldwide, BMI is used to define obesity. In western countries, the definitions of overweight and obesity are BMI of 25-30 and ≥30 kg/m^2, respectively. While easy to perform and calculate, BMI is an imperfect assessment of adiposity and cannot distinguish between muscle mass and fat mass. Besides, the pattern of fat distribution differs among races. For example, visceral obesity and metabolic complications occur at a lower BMI in Asians. Therefore, the measurement of waist circumference can provide additional information on fat distribution. Ethnic-specific definitions of obesity and central obesity have also been recommended (**Tables 1 and 2**).

Table 1. World Health Organization guidance on BMI (kg/m^2) thresholds (2004).

White Europeans	Asians	Description
<18.5	<18.5	Underweight
18.5-25	18.5-23	Increasing but acceptable risk
25-29.9	23-27.5	Increased risk
≥30	≥27.5	High risk

Table 2. International Diabetes Federation guidance on waist circumference thresholds as a measure of central obesity (2007).

Country / ethnic group	Male	Female
Europids	≥94 cm	≥80 cm
South Asians, Chinese & Japanese	≥90 cm	≥80 cm
Ethnic South and Central Americans	Use South Asian recommendations until more data	
Sub-Saharan Africans	Use European recommendations until more data	
Eastern Mediterranean & Middle East	Use European recommendations until more data	

Does NAFLD exist in lean patients?

The worldwide prevalence of NAFLD is 15-40%. However, since the diagnosis of NASH requires histological assessment, the population prevalence of NASH remains unclear and is estimated at 10-30% among NAFLD patients. In a US clinic-based study where patients received abdominal ultrasonography screening and liver biopsy was performed in the majority of those found to have fatty liver, the prevalence of NAFLD and NASH was 46% and 12%, respectively [1]. NASH was found in 30% of those with NAFLD. A Hong Kong study using proton-magnetic resonance spectroscopy found NAFLD in 27% of the adults in the general population [2]. Although liver biopsy was not performed, this study used transient elastography and five clinical prediction formulae and estimated that around 4% of the NAFLD patients in the community have advanced fibrosis or cirrhosis.

Because of the different interpretation of BMI in different populations, most NAFLD data in lean patients comes from Asia. Not surprisingly, NAFLD is more common in overweight and obese patients. However, among individuals with BMI <25 kg/m^2, 7-21% also have NAFLD (**Table 3**). The difference in prevalence can be explained by the BMI distribution in the non-obese population and differences in dietary habit and physical activities.

Table 3. Prevalence of NAFLD according to BMI (kg/m^2) [2-5].

Location	n	Prevalence in subjects with BMI <25	Prevalence in subjects with BMI ▸25
Taichung, Taiwan	3334	15%	31%
Nagasaki, Japan	1559	11%	60%
Shanghai, China	4506	21%	39%
West Bengal, India	1911	7%	32%
Hong Kong, China	922	19%	61%
Seoul, Korea	29994	13%	50%

Does NAFLD in lean patients matter?

Liver-related mortality is the third leading cause of death among NASH patients. Although NASH patients more often die of cardiovascular complications and extrahepatic malignancies than liver complications, the latter occur more commonly in NASH patients than in the general population. The important question is whether lean patients with NASH also have adverse clinical outcomes. This would determine how such patients should be managed.

In a biopsy series, leaner Asian NASH patients were found to be less likely to have advanced fibrosis and cirrhosis than Caucasians [6]. In a large Indian population-screening study using ultrasonography, computed tomography and TE, 7% and 32% of the patients with BMI <25 kg/m^2 and ≥25 kg/m^2, respectively, had fatty liver [3]. Seventy-five percent of the NAFLD patients had BMI <25 kg/m^2. Among a subgroup of NAFLD patients who underwent liver biopsy, 31% had NASH and 11% had cirrhosis.

Clinical outcome data of lean patients with NASH are limited. The incidence of HCC appears to be slightly lower for NASH-related cirrhosis than hepatitis C-related cirrhosis in Caucasians but remains significant at 1-2% per year. In less obese Asian populations, NASH-related HCC is relatively uncommon. However, the difference may not be due to BMI alone. Like all chronic liver diseases, the duration of disease determines the risk of cirrhosis and complications. In some developing countries where nutritional abundance has only occurred recently, most NASH patients only have a short duration of disease and have not had time to progress to cirrhosis and HCC.

This suggests that NASH and advanced fibrosis also occur in lean patients, albeit at a lower rate. Non-invasive tests are thus preferred to exclude the small proportion of patients with significant disease.

What causes NAFLD in lean patients?
There are two possible explanations for the development of NAFLD in lean patients. First, this may represent the milder end of the full spectrum of NAFLD/NASH. Second, patients may develop NAFLD despite normal or low BMI because of other risk factors.

Genetics
Recent genome-wide association studies have identified a number of gene polymorphisms associated with NAFLD/NASH. For example, the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene has been consistently shown to be associated with NAFLD and its histological severity. PNPLA3 hydrolyses emulsified triglycerides in hepatocytes, and the I148M substitution abolishes the enzymatic activity. As a result, the secretion of very low-density lipoproteins by hepatocytes is impaired. Interestingly, the relative effect of PNPLA3 polymorphism on hepatic steatosis is more profound in patients without MetS.

Similarly, apolipoprotein C3 (*APOC3*) gene variants have been shown to increase the risk of NAFLD in Indians [7]. Carriers of the variant alleles have hypertriglyceridemia and reduction in plasma triglyceride clearance. However, the effect of this gene variant is less apparent in subsequent validation studies in Europeans.

Taken together, genetic predisposition to NAFLD has greater effects in patients with lower metabolic burden. This partly contributes to the development of NAFLD in lean patients.

Weight gain
BMI and hepatic steatosis are both dynamic and subject to short-term changes. Among NAFLD patients with normal BMI, recent weight gain is often observed. Healthy young people can quickly develop increases in hepatic steatosis and aminotransferase levels through short-term excessive eating and sedentary lifestyle [8]. In a study using paired proton-magnetic resonance spectroscopy to measure hepatic steatosis in lean community subjects, increases in waist circumference and plasma triglycerides were associated with incident fatty liver [9].

Central obesity
BMI is an imperfect measurement of adiposity. Patients with normal BMI can have central and visceral obesity. As such, lean patients with NAFLD are often found to have greater waist circumference or waist-to-hip ratio.

Other metabolic factors
Obesity is just one component of MetS. Hyperglycemia, dyslipidemia and hypertension are also associated with NAFLD (**Table 4**) [2]. In particular, cirrhosis is uncommon in NAFLD patients with normal glucose regulation. Moreover, insulin resistance is almost universal in NAFLD patients even when plasma glucose is within the normal range. In a large retrospective study in Korea, non-obese patients with NAFLD had higher prevalence ratios for other MetS components than obese patients [4].

Diagnostic workup
The purposes of investigations in the management of NAFLD in lean patients are three-fold: establishing the diagnosis of NAFLD, screening for concomitant metabolic disorders and CVD, and assessing the severity of liver disease.

Establish the diagnosis
The diagnosis of NAFLD is usually straightforward. For practical purposes, abdominal ultrasonography is the test to detect fatty liver in most clinical settings. Fatty liver has bright echotexture, vascular blurring and deep attenuation of ultrasound signal. NAFLD can be diagnosed when fatty liver is detected and there is no evidence of an alternative liver disease. This would involve exclusion of excessive alcohol intake and consumption of drugs that may lead to fatty liver (e.g. systemic corticosteroids and tamoxifen). Further investigations to exclude other liver diseases would depend on whether the liver biochemistry is abnormal as well as the local epidemiology. For example, HCV infection, particularly genotype 3, is often associated with hepatic steatosis.

Table 4. Definition of metabolic syndrome by the International Diabetes Federation and joint societies (2009).

Measure	Categorical cut points
Elevated waist circumference	Ethnic specific definitions
Elevated triglycerides or on drug treatment	≥150 mg/dl (1.7 mmol/l)
Reduced high density lipoprotein-cholesterol or on drug treatment	Male: <40 mg/dl (1.0 mmol/l) Female: <50 mg/dl (1.3 mmol/l)
Elevated blood pressure or on drug treatment	≥130/85 mmHg
Elevated fasting glucose or on drug treatment	≥100 mg/dl (5.6 mmol/l)

However, if a patient has fatty liver together with features of advanced disease but is lean and has low metabolic burden, an alternative diagnosis should be suspected. Apart from other chronic liver diseases, hypothyroidism, hypopituitarism, hypogonadism and lipodystrophy may be considered.

Concomitant metabolic disorders and CVD
NAFLD is often referred to as the hepatic manifestation of MetS. A patient with NAFLD should therefore be screened for diabetes, hypertension and dyslipidemia. Several studies have reported a high incidence of postprandial hyperglycemia in NAFLD patients. A formal oral glucose tolerance test should be considered, especially if the fasting plasma glucose is borderline. Regardless, NAFLD is associated with increased risk of CVD; therefore, patients with cardiovascular symptoms should be promptly investigated and managed.

Disease severity
Since the pre-test probability of NASH and advanced fibrosis is lower in lean patients, liver biopsy cannot be justified unless an alternative diagnosis is suspected. Non-invasive tests are therefore preferred. These have been covered in another part of this course, so only specific points relevant to lean patients will be covered here.

The current American guidelines endorse the use of the NAFLD fibrosis score to identify patients with a higher likelihood of having advanced fibrosis or cirrhosis. The score has also recently been shown to predict long-term mortality in NAFLD patients. However, since BMI is an integral component of the score, it is unclear if calibration is needed for populations with different BMI cut-offs.

Transient elastography by Fibroscan is another population, non-invasive test of liver fibrosis in Europe and Asia [6]. Indeed, measurement failure with this technique is less likely in lean patients with NAFLD. In addition, the latest Fibroscan model allows concomitant measurement of liver stiffness and the controlled attenuation parameter (CAP). CAP measures ultrasound attenuation in the liver and can be used to estimate the degree of hepatic steatosis. Further studies are needed to establish if serial CAP measurements can accurately reflect changes in hepatic steatosis.

Management
Lifestyle modification is the cornerstone of NAFLD/NASH management. In lean patients with NAFLD, healthy diet and regular exercise are often sufficient in inducing disease remission [10]. While a weight reduction of more than 10% from baseline has the greatest effect on NAFLD, modest weight reduction of as little as 3-5% can also improve hepatic steatosis. The current American guidelines support the use of vitamin E or pioglitazone in patients with NASH and no diabetes or cirrhosis. While these two agents have been well studied using histological and biochemical endpoints, they have not been specifically evaluated in lean patients with NASH. For obvious reasons, bariatric surgery is not indicated in lean patients with NASH.

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EXCESSIVE BODY WEIGHT AND RISK OF NASH: ARE ALL OBESE PATIENTS EQUAL?

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Take home messages
- Excessive BMI is not synonymous with excessive fat and vice-versa.
- Visceral, more than subcutaneous, fat is associated with NAFLD and hepatic insulin resistance.
- Ectopic fat is usually not limited to one organ (i.e., the liver) and for this reason subjects with NAFLD have increased cardiometabolic risk.
- Adipose tissue insulin resistance is one of the main causes of ectopic fat in the liver.
- Adipose tissue insulin resistance is associated with lipotoxicity, production of ROS and cell damage.
- Saturated fats are more lipotoxic than unsaturated fats.

Introduction
Although obesity is one of the major risk factors for NAFLD and NASH, these two conditions may be present in non-obese subjects [1, 2]. Visceral fat accumulation, rather than generalized obesity, is linked to NAFLD and to MetS in general [1]. Thus, in assessing the risk or presence of NAFLD it is important to assess if a subject is obese and also to quantify fat deposition.

Assessment of obesity and fat distribution
The previous syllabus covered the definition of BMI and its limitations. In view of these limitations, a direct, more accurate measurement of fat mass should be performed (e.g. DEXA, air-displacement plethysmography, bioimpedance or body scanning procedures) [3].

Even in subjects with BMI <30 kg/m2, total adipose tissue can be up to 50% of body weight with the majority of fat accumulated as subcutaneous adipose tissue (SAT), but with accumulation also as visceral adipose tissue (VAT) (up to 10 kg or 30% of total fat) and intrathoracic fat (up to 0.5 kg) [4]. Intrathoracic fat includes mediastinal or extrapericardial fat and epicardial fat [34]. Waist circumference is a good indicator of visceral fat accumulation, although imaging techniques, such as magnetic resonance imaging or computed tomography should be used to accurately quantify abdominal fat [4]. On the other hand, the presence of epicardial fat can also be diagnosed by ultrasonography [4].

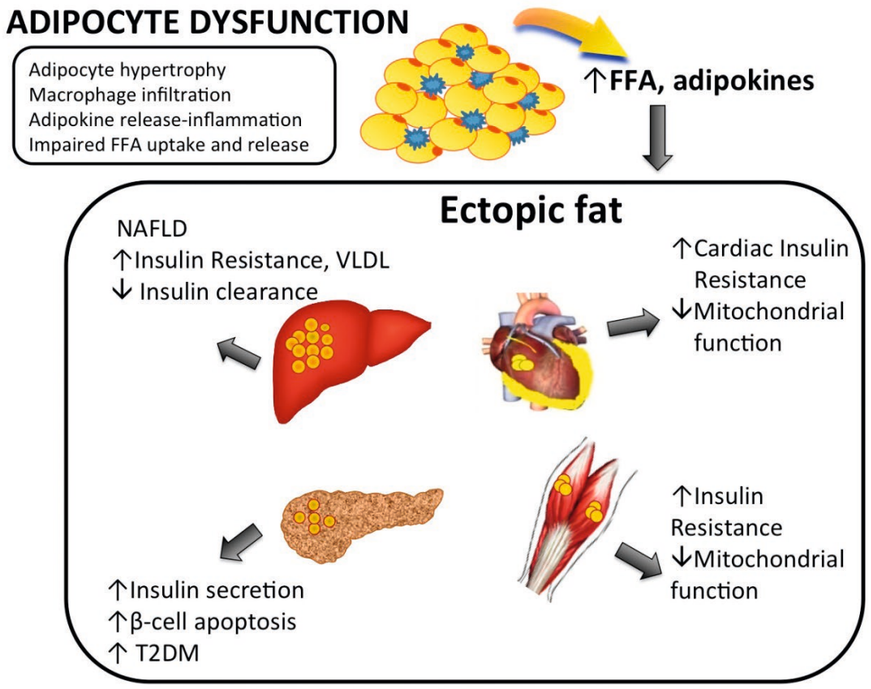

Figure 1. Hypertrophic adipose tissue is more prone to macrophage infiltration leading to adipokine release, inflammation, impaired lipolysis and lipogenesis. This results in ectopic fat accumulation and lipotoxicity in various organs. *Adapted from Morelli et al. [5].*

Excessive fat may accumulate not only in adipocytes but also inside other organs as ectopic fat. The liver is one of the sites where ectopic fat accumulates, thus determining NAFLD. Other sites include the pancreas, muscle (extra- or intra-myocellular triglycerides) and heart (intramyocardial triglycerides) [4]. Ectopic fat causes lipotoxicity, organ damage and metabolic dysfunction, often increasing the risk of T2DM (**Fig. 1**).

Metabolically healthy obese (MHO), i.e. fat and fit

Not all obese subjects are insulin resistant and/or develop NAFLD. This category has been named MHO. Their cardiometabolic risk is not as high as expected, probably because their SAT is able to expand and to capture excess fat as has been shown in overfeeding studies [6]. It has been suggested that MHO subjects do not significantly benefit from weight loss and lifestyle changes to the same extent as obese patients with metabolic co-morbidities. During over-feeding these subjects are able to store excess fat in SAT, while insulin resistant subjects tend to accumulate fat in other sites including visceral and ectopic fat. Lipid storage is regulated by the genes *DGAT2*, *SREBP1c*, and *CIDEA* expressed in SAT. In subjects that respond to overfeeding by increasing VAT, the expression of these genes is reduced [5]. For this reason, subjects with increased SAT, but low VAT are at lower risk of cardiometabolic diseases including NAFLD [4, 5].

Adiposopathy and risk of NAFLD/NASH

The release of adipokines is limited in small adipocytes. Adipocyte dysfunction appears when they become hypertrophic and are infiltrated by macrophages, promoting inflammation and the release of pro-inflammatory adipokines [5, 6] (**Fig. 1**). This phenomenon is called adiposopathy ('sick fat') [6].

Increased fat intake and a sedentary lifestyle can promote adiposopathy as well as visceral and ectopic fat accumulation [6].

Visceral fat accumulation is an independent risk factor for the development of insulin resistance and for hepatic fat accumulation. Lean subjects with NAFLD tend to have increased VAT [2, 6]. Independently of obesity, VAT is associated with alterations in both glucose and lipid metabolism [4].

The presence of large hypertrophic adipocytes has been linked to ectopic fat deposition and increased risk of metabolic dysfunction [5]. Ectopic fat in the liver (NAFLD) is frequently associated with hepatic insulin resistance, excess VLDL secretion and decreased insulin clearance [4, 5]. Pancreatic fat is associated with beta cell dysfunction and apoptosis. Intramyocellular triglycerides are associated with impaired glucose metabolism and decreased mitochondrial function. Intramyocardial fat is associated with impaired organ metabolism, increase oxidative stress and reduced mitochondrial function [4, 5].

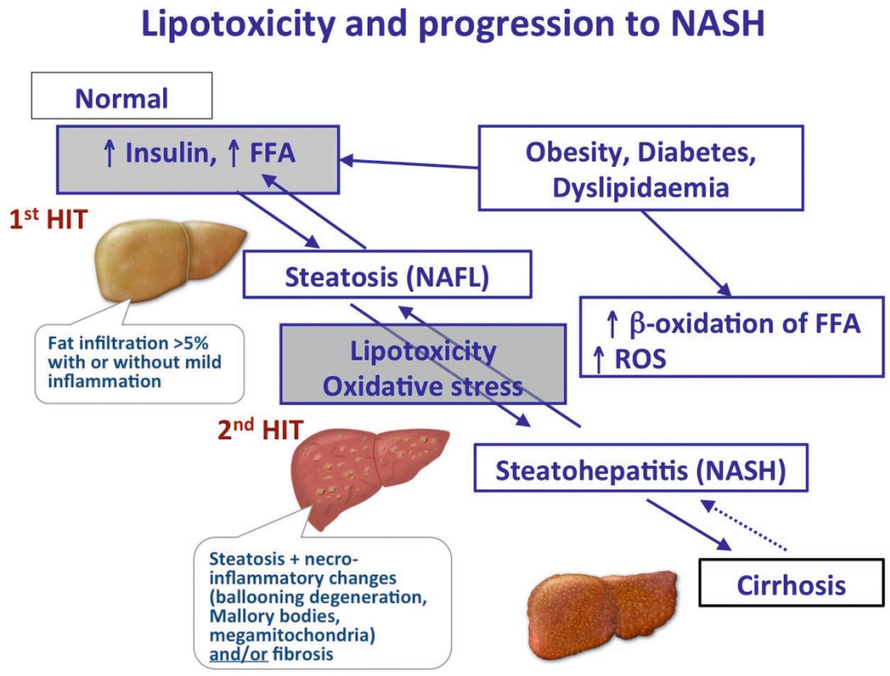

Figure 2. Lipotoxicity and progression to NASH. In the progression from steatosis to NAFLD, NASH and cirrhosis it has been proposed that obesity, dyslipidemia, diabetes and excess fat intake are the 'first hit' determining hepatic TG accumulation. A 'second hit', due to lipotoxicity and oxidative stress, could be responsible for liver damage and progression to NASH and cirrhosis.

Adipose tissue insulin resistance and lipotoxicity

Large adipocytes are more resistant to the antilipolytic effect of insulin, thus these subjects display increased lipolysis and fatty acid overflow promoting ectopic fat and NAFLD.

Insulin also promotes hepatic FFA uptake and de novo lipogenesis. FFAs are only oxidized minimally and are primarily re-esterified to triglycerides and then exported as VLDL [1]. In subjects with NAFLD, VLDL secretion is often increased but it reaches a plateau, indicating a saturable process [1]. In subjects with NAFLD, total body lipid oxidation and hepatic beta oxidation are increased [2]. We have hypothesized that one of the mechanisms that leads to NAFLD is the presence of adipose tissue insulin resistance that leads to fatty acid overflow, and the saturation of FFA oxidation and VLDL secretion that promote hepatic fat accumulation ('first hit'; **Fig. 2**) [1].

It has been proposed that steatosis progresses to NAFLD/NASH after a 'second hit' due to lipotoxicity and oxidative stress (**Fig. 2**) [1]. Lipotoxicity impairs mitochondrial function and oxidation with the production of ROS resulting in liver damage and impaired detoxification and repair abilities [1].

Not all fats are equal

In vitro data have shown that palmitate and saturated fatty acid (SFA) are known to induce lipotoxicity, mainly by producing ROS, inducing cell damage, apoptosis and cell death in various types of cells, including hepatic cells [6, 7]. Unsaturated fat (e.g., oleate) induces more steatosis with the formation of triglyceride-enriched lipid droplets and autophagy, but with a minimal effect on apoptosis [7].

Plasma and tissue metabolomic analyses revealed that several classes of lipids are altered in subjects with NAFLD/NASH [8, 9]. Subjects with NAFLD had increased plasma concentrations of triacylglycerides (TAG), FFA [1] and phosphocholine (PC) as well as increased indexes of de novo lipogenesis and desaturase activity (SCD1) [9], mainly due to hepatic and adipose tissue insulin resistance [1]. In addition, NAFLD and NASH patients exhibit a progressive decrease in the concentrations of plasma lysophosphocholines (LyPC) [8, 10], a reduction in total n-3 and n-6 polyunsaturated fatty acid (PUFA) content across most lipid classes (FFA, TAG, PC, LyPC) and reduced peroxisomal activity [9].

Metabolomic analyses of human liver tissues are limited. It has been proposed that an excess of saturated fat relative to unsaturated fat could be harmful to the hepatic metabolism. Confirming this hypothesis, it has recently been published that in subjects with the *PNPLA3* I148M allele, n-3 PUFA α-linolenic acid content is increased while several n-6 PUFAs and saturated fatty acids were decreased in the liver TAG fraction [11]. The authors found a strong inverse correlation between n-6 PUFA and TAG content independent of *PNPLA3* genotype. The *PNPLA3* I148M allele confers a predisposition to NAFLD but protection against insulin resistance [12, 13]. It is likely that different fatty acids could promote or reduce oxidative stress, since cellular models have shown that stearic acid is toxic and promotes insulin resistance while α-linolenic acid is protective [11]. Metabolomic and lipidomic studies are ongoing to search for plasma and tissue biomarkers that could predict and prevent the progression of NAFLD.

Conclusions

Subjects with NAFLD are not all obese but all tend to have visceral fat accumulation and ectopic fat in other organs. For this reason NAFLD/NASH is a risk factor for both diabetes and CVD. Visceral and ectopic fat are increased in subjects with 'low expandability' of subcutaneous fat and in those with large adipocytes that are not only hypertrophic, but also resistant to the antilipolytic effect of insulin, resulting in fatty acid overflow, inflammatory processes and eventually, adipocyte necrosis. These events generate 'signals' (release of adipokines, hormones, other unknown factors) that locally induce inflammation, recruit macrophages and increase ectopic fat accumulation, leading to lipotoxicity, reduced mitochondrial activity and metabolic dysfunction in all tissues. Among the different classes of lipids, saturated fats are more lipotoxic. Metabolomic and lipidomic studies are ongoing to evaluate plasma biomarkers to predict and prevent the progression of NAFLD.

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INSULIN RESISTANCE: SHOULD WE MEASURE IT AND DOES IT PROMOTE LIVER DISEASE PROGRESSION?

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Take home messages

- IS/IR is usually tested on glucose metabolism; several dynamic and static methods have been proposed for their quantitative assessment.
- The reproducibility of static methods depends largely on analytical variability and day-to-day variability of insulin concentrations, while this problem is generally overcome in OGTT-derived formulas.
- There is important evidence that IR promotes the progression of simple steatosis to NASH and fibrosis. The method used to measure IS/IR is critical to unveil the association between IR, hyperinsulinemia and liver damage.
- In clinical practice, this translates into the use of indices of IS/IR in the development of several algorithms for the non-invasive prediction of severe liver damage based on multiple components of the MetS.
- A better understanding of the complex mechanisms underlying the interaction between IR and liver damage remains a primary target for the proper management of NAFLD/NASH.

Introduction

NAFLD is a complex condition, ranging from simple steatosis to NASH and cirrhosis. NAFLD results from the interaction between multiple genes and social, behavioural and environmental factors. IR represents its pathophysiological hallmark and the common ground of the features of the MetS, associated with an increased risk of fat accumulation and fibrosis. Insulin signalling, via different pathways, is involved in metabolic and immune modulations, directly or indirectly affecting liver injury and the wound-healing response and possibly varying from patient to patient. An increasing number of features of the MetS, particularly T2DM and obesity, are risk factors for the presence of steatohepatitis and are important phenotypic reflections of insulin resistance. In clinical practice, this translates into the use of indices of IS/IR as screening tools to detect IR in NAFLD and in the development of several algorithms for the non-invasive prediction of severe liver damage based on multiple components of the MetS. A better understanding of the complex mechanisms underlying the interaction between insulin resistance and liver damage remains a primary target for the proper management of NAFLD/NASH.

How to measure insulin resistance

IR is typically defined as decreased sensitivity or responsiveness to metabolic actions of insulin, but this definition does not provide any insight on the type of tissue where insulin activity is measured (muscle, adipose tissue, hepatocytes, etc.) and on the substrate that is tested (glucose, lipids, proteins, etc.) [1]. From a physiological point of view, discerning the sites of IR is not trivial. IR in the skeletal muscle (or peripheral IR) is defined as a lower than expected effect of insulin on glucose disposal by the muscle, leading to hyperglycemia and compensatory hyperinsulinemia and favouring *de novo* lipogenesis in the liver. Peripheral IR is tightly linked to IR in the adipose tissue, i.e. impaired suppression of lipolysis and increased fatty acid flux from the adipocytes to other organs, including the liver. In the liver, IR leads to impaired suppression of glucose production and high glucose and insulin levels, thus setting up a vicious cycle [1, 2]. IS/IR is usually tested on glucose metabolism; however, the ability of insulin to stimulate glucose uptake (mainly in the skeletal tissue) and suppress its production (mainly in the liver) should be separately defined.

Several dynamic and static methods have been proposed for the quantitative assessment of IR (**Table 1**) [3, 4]. The glucose clamp technique remains the gold standard [5], and the validity of different methods is usually measured against that of the clamp. Techniques to directly measure IS/IR are time-consuming, expensive and often unavailable in daily routine. Therefore, more simple tests have been developed based on insulin and/or glucose levels in the fasted state alone or in combination with insulin and glucose levels at oral glucose tolerance test (OGTT) and other metabolic and anthropometric parameters [3, 4]. The reproducibility of static methods depends largely on analytical variability and day-to-day variability of insulin concentrations, since small changes in insulin result in a large error in the estimate of IR. This problem is generally overcome in OGTT-derived formulas, which include several post-load insulin measurements.

Table 1. Different methods for the quantitative assessment of insulin resistance.

Method	Parameter	Advantages	Disadvantages
Euglycemia clamp	Whole-body IS	Based on solid physiological understanding When combined with tracers it gives a comprehensive estimate of insulin effects	Complex, costly and time-consuming Not useful for epidemiological studies
Intravenous glucose tolerance test	IS	Relatively easy to perform	Complex mathematical analysis and need for computer support
OGTT-derived indices	OGIS ISI (Matsuda) ISI (Gutt) SiOGTT ISI (Stumvoll) HepIR (DeFronzo) LIRI	Based on a test used in clinical practice for diagnostic purposes Easy to perform	Based on empirical basis or complex mathematical models
Static (fasting) measurements	HOMA-IR QUICKI FIRI IGR ISI (Bennett)	Easy to quantify Low cost Useful for epidemiological studies	Low intra- and inter-laboratory reproducibility Not applicable to insulin-treated or poorly controlled diabetic patients Relatively low correlation with the ‘clamp’

Abbreviations: OGTT, oral glucose tolerance test; OGIS, oral glucose IS index; ISI, IS index; SiOGTT, IS index derived from oral glucose tolerance test; HepIR, hepatic insulin resistance index; LIRI, liver insulin resistance index; HOMA, homeostasis model of assessment; QUICKI, quantitative IS check index; FIRI, fasting insulin resistance index; IGR, insulin to glucose ratio.

Insulin resistance and liver damage

The notion that IR is closely related with liver fat accumulation is well proven, but there is important evidence that IR promotes the progression of simple steatosis to NASH and fibrosis. The method used to measure IS/IR is critical to unveil the association between IR and liver damage.

Several studies employing the glucose clamp technique have shown a close association between NAFLD and both hepatic and adipose tissue IR, as well as reduced whole-body IS (i.e. peripheral IR). Glucose disposal, a measure of whole-body IS, was reduced by 45-50% in NAFLD, together with an impaired ability of insulin to suppress endogenous glucose production, indicative of hepatic IR [6, 7]. Additionally, subjects with NAFLD exhibited a marked defect in insulin suppression of FFAs, in keeping with IR at the adipocyte level [6, 7]. Hepatic insulin-resistance correlates well with the intrahepatic triglyceride content and is a strong predictor of steatosis, independent of BMI and insulin action in liver, skeletal muscle and adipose tissues [2]. Adipose tissue IR has been repeatedly associated with the degree of liver fibrosis in NASH [6-8]. Adipose tissue IR and skeletal muscle IR are tightly linked, but the role of the latter in the onset and progression of liver damage is less clear.

HOMA-IR is calculated as the product of insulin and glucose concentration (fasting plasma insulin x fasting plasma glucose / 22.5) and is a rather crude index reflecting the final effects of hepatic and peripheral IR on fasting glucose homeostasis [9]. Since the first demonstration of IR in NAFLD, HOMA-IR has been used in epidemiological studies to demonstrate the close association between NAFLD and MetS; patients with NASH generally have more increased HOMA-IR compared to patients with simple steatosis [10]. However, this index does not provide any clue to the site of IR.

The oral glucose IS (OGIS) index [11] is calculated on the basis of glucose and insulin in the two hours following glucose ingestion (75 g), which activates the insulin–glucose homeostatic processes, and this index mostly reflects glucose uptake by muscle tissue (i.e. peripheral IR). For this reason, it correlates significantly with glucose clearance measured by the clamp technique, and is considered a more sensitive measure of IS than HOMA-IR. Severe fibrosis has been associated with decreased IS measured by OGIS in both NAFLD and chronic hepatitis C patients, independently of BMI, sex, age and MetS, and with the clustering of the clinical and biochemical features of MetS [12, 13].

Many other indices have been developed, including clinical and anthropometric parameters (e.g. age, gender and BMI), but they are not superior to the above tests and are scarcely used in clinical practice. Of note, all these surrogate indices of IR have been validated in diabetic and non-diabetic subjects against the euglycemic hyperinsulinemic clamp, but none of them has been validated in NAFLD patients by state-of-the art-techniques.

Putative mechanisms for the association between insulin resistance and liver damage

IR plays a central role in intrahepatic lipotoxic injury by allowing an excessive flow of fatty acids from adipose tissue and also by impairing peripheral glucose disposal. Supply processes include inappropriate lipolysis by insulin resistant adipocytes and increased carbohydrate precursors for *de novo* lipogenesis, secondary to peripheral and hepatic IR. Metabolites of FFAs cause lipotoxic hepatocellular injury manifested as endoplasmic reticulum stress, inflammation, apoptosis, necrosis, and dysmorphic features (e.g. ballooning and MDB formation) [14]. Reversal of adipose tissue IR by a glitazone is associated with an improvement of the necroinflammatory damage in the liver. Furthermore, hyperglycemia and hyperinsulinemia can cause an up-regulation of the connective tissue growth factor, thus promoting fibrogenesis [14, 15]. Other key players in the progression to steatohepatitis are the adipokines (adiponectin, leptin, and resistin) and several cytokines (such as TNF-alpha, IL-6 and IL-1) secreted by the adipocytes or by the inflammatory cells that infiltrate the adipose tissue in IR states. Expanded visceral adipose tissue common in IR states represents a preferential source of adipokines and cytokines potentially acting on the liver tissue [14, 15]. In conclusion, IR can act both as the first and second hit in the development of NASH. The varying outcome of the disease might be related to the relative impact of metabolic derangements, environmental conditions and host factors (e.g. the genetic and hormonal milieu), as in other conditions associated with IR, such as diabetes, hypertension, and CVD.

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DYSMETABOLIC HYPERFERRITINEMIA

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Take home messages
Dysmetabolic Iron Overload Syndrome (DIOS) is:
- defined by hyperferritinemia (500-1500 µg/l) with normal transferrin saturation and mild hepatic iron excess (50-150 µmol/g) in the setting of various features of the MetS;
- diagnosed by magnetic resonance imaging or liver biopsy;
- associated with non-alcoholic fatty liver disease in 50% of cases and with an increased risk of diabetes, cardiovascular complications, and cancer;
- easily treated by phlebotomies, but not by lifestyle modifications alone.

Introduction [1-3]
Mild hyperferritinemia is frequent in patients with metabolic abnormalities and related cardiovascular and hepatic complications. It correlates with the degree of IR as measured by the euglycemic hyperinsulinemic clamp, with the number of components of the MetS and with the decrease in serum adiponectin levels. Moreover, it is predictive of the onset of T2DM. It raises diagnostic, pathophysiologic and therapeutic issues, especially when it accounts for elevated body iron stores, coined as the dysmetabolic iron overload syndrome (DIOS).

Diagnosis
Three conditions frequently associated with MetS, chronic inflammation, excessive alcohol consumption and cell necrosis, may increase serum ferritin levels in the absence of elevated body iron stores. This must be discussed first.

It is then necessary to check transferrin saturation at least twice because of the frequency of false positive results:

- **Transferrin saturation elevated:** the diagnosis of HFE hemochromatosis is a possibility and *HFE* genotyping should be performed.
- **Transferrin saturation NOT elevated:** assess hepatic iron concentration (HIC) (normal HIC = 36 µmol/g dry weight) to determine whether body iron stores are elevated or not.
  - **HIC is assessed in the following ways:**
    - Liver biopsy: if indicated for the assessment of associated NAFLD.
    - MRI: when the device is correctly calibrated, MRI allows reliable identification and quantification of HIC in a wide range from 60 to 300 µmol/g dry weight. This technique also permits the detection of iron deposition in the spleen.
  - **Hypotheses to be discussed depending on HIC assessment:**

    Normal HIC:
    - If mild increase of serum ferritin levels (<500 µg/L; normal <400 µg/L): dysmetabolic hyperferritinemia with no iron excess is likely.
    - If significantly increased serum ferritin levels (>500 µg/L): genetic hyperferritinemia should be discussed in the absence of confounding factors such as inflammation, cell necrosis and alcohol consumption.

    Increased HIC:
    - If HIC exceeds 150 µmol/g dry weight, after ruling out a false positive (frequent due to MRI artefacts): ferroportin disease should be discussed.
    - If HIC ranges between the upper limit of normal and 150 µmol/g dry weight: DIOS is likely.

DIOS is defined as the presence of unexplained hepatic iron excess in the setting of various features of the MetS. Usually the patient is a middle-aged male with no or non-specific symptoms. Transferrin saturation is not elevated. Hepatic iron excess is mild (<150 µmol/g) and located in both hepatocytes and Kupffer cells with frequent iron deposition in spleen during MRI. NAFLD is associated in half of the cases and significant fibrosis is found in 10-15% of patients.

Pathophysiology [4-9]
Development of iron excess (Fig. 1). In DIOS, abnormalities of iron metabolism evolve according to a dynamic process with close interactions between the liver and VAT. Iron accumulates within the liver, resulting in increased production of hepcidin, the key regulator of systemic iron. At the same time, fatty acids accumulate in VAT resulting in abnormal production of cytokines, especially impairment of adiponectin synthesis, leading to IR. IR enhances hepcidin production through glucose, insulin and neoglucogenesis. This contributes to decreased hepatic iron excess, which could explain why hepatic iron excess is frequent in patients who are overweight and rare in those who are obese.

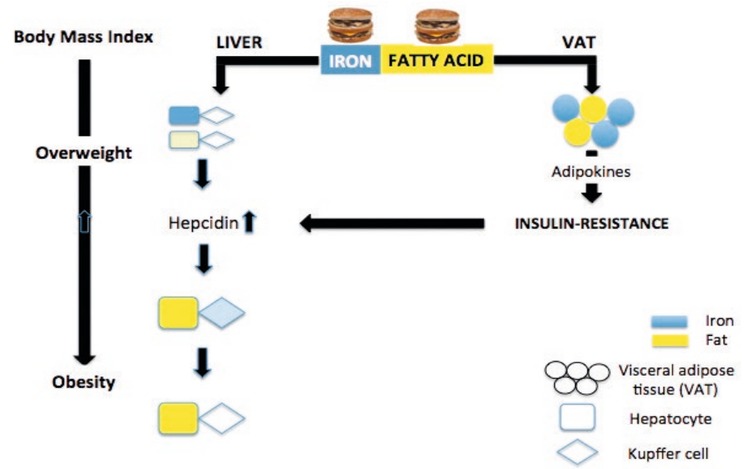

Figure 1. Schematic hypothesis for the development of DIOS.

VAT has all activated genes necessary to handle iron. VAT iron overload has been reported in overweight subjects as well as in experimental models of DIOS where a high fat diet results in a shift of hepatic iron to adipose tissue. Then, in MetS, even in the absence of identified hepatic iron overload, hyperferritinemia could be related to increased body iron stores due to iron deposition in VAT, and not to subclinical inflammation as previously thought. The production of adiponectin, the main adipokine protective against IR, is down-regulated by iron at the transcriptional level. This strongly suggests a key role of iron in the onset and/or the worsening of IR.

Consequences of iron excess. Experimental and clinical studies indicate that iron excess is associated with increased risks of cardiovascular complications, cancer, abnormal glucose metabolism and, perhaps, liver fibrosis, via the increased production of ROS.

Treatment [10]
Lifestyle modifications are mandatory with, if necessary, antihypertensive, lipid lowering and antidiabetic drugs. This results in serum ferritin normalization in less than 1/3 of cases, mainly when baseline serum ferritin levels are <500 µg/l.

Currently, there is no consensus on whether phlebotomy therapy could be beneficial for the patient. However, this type of therapy is known to improve IS in diabetic and non-diabetic subjects, and in patients with NAFLD. Furthermore, phlebotomy improves liver histology in patients with NAFLD and decreases cancer and cardiovascular risks in patients with peripheral arterial disease. Phlebotomy is also well tolerated by DIOS patients when venesections are performed every 14 days, allowing the removal of 2.5 g of iron, on average.

Large, controlled studies of phlebotomy therapy in DIOS patients with or without NAFLD are warranted. Moreover, iron may be a promising therapeutic target in such subjects.

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BARIATRIC SURGERY: A CURE FOR NASH?

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Take home messages

- Bariatric surgery is the most effective treatment for weight loss and its long-term maintenance; the most commonly performed procedures are laparoscopic gastric bypass, adjustable gastric banding and vertical sleeve gastrectomy.
- Bariatric surgery improves obesity-related comorbidities and reduces overall and cardiovascular mortality.
- Gastric bypass works by reducing hunger, increasing satiation, changing food preferences and increasing diet-induced energy expenditure.
- Adjustable gastric banding works probably through the reduction in hunger, which might be mediated through vagal signalling.
- Some of the clinical and physiological effects of vertical sleeve gastrectomy are similar to gastric bypass.
- NAFLD has been shown to improve significantly after bariatric surgery, but the evidence that NASH improves is less clear.
- Understanding the mechanisms of action of these procedures could accelerate their optimization and the development of novel, and hopefully safer, medications for obesity and T2DM.

Introduction [1-3]
The progression and pathophysiology of NASH has been covered previously in this session, suffice to say that substantial weight loss leads to an attenuation of insulin resistance and related metabolic syndrome and, concomitantly, a regression of liver steatosis. Bariatric surgery (**Fig. 1**) is the most effective treatment option for severe obesity and associated metabolic comorbidities. Several longitudinal studies have documented the marked benefit of bariatric surgery on NAFLD in close relation to the reversal of insulin resistance, but evidence for reversal of NASH is less clear.

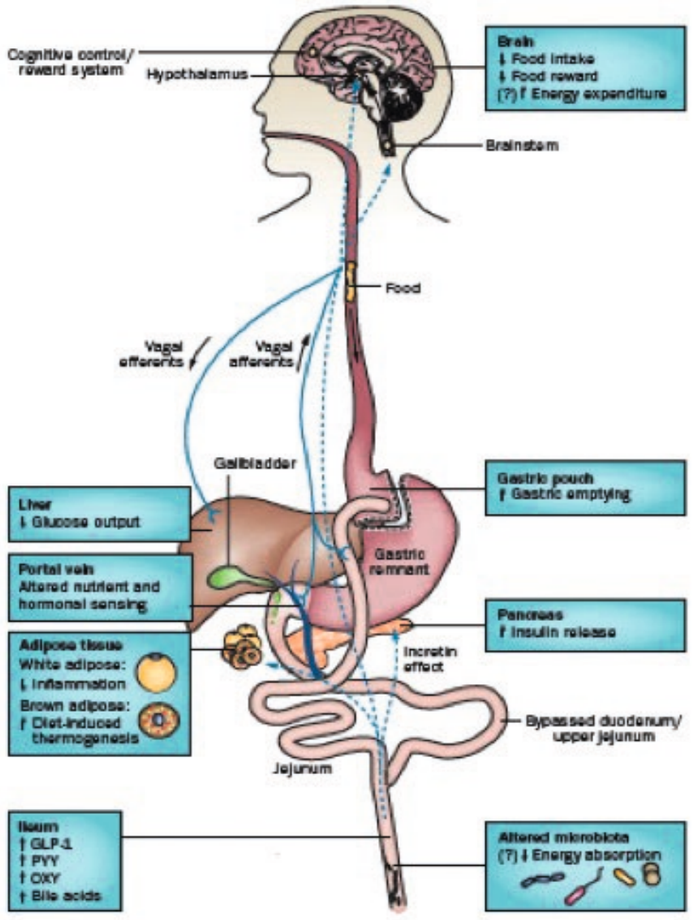

Figure 1. Mechanisms of bariatric surgery.

Effect of bariatric surgery on NAFLD and NASH

Lifestyle interventions and thiazolidinedione pharmacotherapy improve steatosis, but a weight loss of greater than 10% is usually necessary for inflammation to regress. Many retrospective and prospective clinical studies have investigated the effect bariatric surgery has on NAFLD, but no randomized controlled trial has been done. Most studies have been done in mixed populations, i.e. with and without T2DM, and investigators have reported improvements in histological examinations from liver biopsies, non-invasive ultrasonography, and plasma transaminases. The histological improvements include consistent reductions in steatosis and, in some cases, in Mallory's hyaline bodies and inflammation. However, thus far fibrosis seems more resistant to weight loss after bariatric surgery; results of the longest, largest, and best executed study so far have shown that fibrosis scores can worsen during a 5 year period after biliointestinal bypass (which as a procedure is now obsolete), Roux en-Y gastric bypass (RYGB) and laparoscopic adjustable gastric banding.

The use of serum transaminases to monitor response to treatment is questionable, on the basis of published work. Although most studies have reported decreases in liver enzymes (e.g. γ-glutamyl transpeptidase, aspartate aminotransferase and alanine transaminase) it has not been consistently shown which of these is the most reliable to monitor. Furthermore, the correlation of these enzyme measurements with histological improvements is either poor or highly variable. In the Swedish Obese Subjects study, the largest and longest case-control study of obesity interventions, the plasma concentrations of alanine transaminase decreased in the bariatric surgery group and, although concentrations of aspartate aminotransferase increased, they were still lower than those of the control group 10 years after intervention.

No data exist to prove that one bariatric procedure is superior to any of the others, although a recent study suggested RYGB is marginally better than adjustable gastric banding [1]. In the absence of conclusive mechanistic studies, correlations suggest that the lower insulin resistance and concentrations of liver pro-inflammatory and inflammatory markers could be the most likely mediators underlying the clinical improvements. The conclusion from these findings is that early intervention during the course of NAFLD, and before the development of fibrosis, could be more likely to lead to favourable outcomes, and that these might be even more pronounced in patients with T2DM or insulin resistance. Although these findings are promising, randomized controlled trials are needed that compare the effects of bariatric surgery with those of non-surgical therapies on hepatic histological appearances in patients with T2DM, obesity, or both, before NAFLD and NASH can be considered as a specific indication for bariatric surgery.

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WHY DOES LIVER FAT CONTRIBUTE TO CARDIO-METABOLIC OUTCOMES?

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Take home messages

- Both 'metabolic NAFLD' and the features of IR/MetS increase the risk of CVD, even independent of obesity.
- 'Metabolic NAFLD' and IR share a common pathophysiology, which may explain their link with CVD.
- 'Metabolic NAFLD' may be even be a better predictor of CVD as it more directly measures abnormal metabolism than the MetS.
- NAFLD patients with the PNPLA3 I148M polymorphism have steatosis but not the features of IR, implying that steatosis and IR and the risk for CVD dissociate.

'Metabolic NAFLD', MetS and risk of CVD

The MetS represent a cluster of risk factors reflecting IR [1]. It is well established that features of IR/MetS, such as fasting hyperglycemia, hyperinsulinemia, abdominal obesity, high triglycerides/low HDL cholesterol and small dense LDL cholesterol predict CVD, independently of obesity. NAFLD, diagnosed by liver enzymes, ultrasound or a liver biopsy, has also been shown in prospective studies to predict CVD, even independently of obesity (**Fig. 1**).

Cohort	N	Predictor	Follow-up years	Independent of BMI	Year[REF#]
British	7613	GGT	11.5	Yes	1995[1]
Austrian	163944	GGT	17	Yes	2005[2]
Italian Type 2 DM	2103	US	5	Yes	2005[3]
Finnish	28838	GGT	11.9	Yes	2006[4]
Dutch	1439	ALT	10	Yes	2007[5]
U.S.	3451	GGT	19	Yes	2007[6]
Italian Type 2 DM	2103	US	6.5	Yes	2007[7]
Japanese	1637	US	5	Yes	2007[8]
Mixed U.S.	2812	ALT	20	No	2008[9]
Germans	4160	GGT/US	7.3	Yes (men)	2009[10]
Chinese	612	ALT/US	1.7	Yes	2011[11]
NHANES, mixed U.S.	11371	US	14	No	2011[12]
NHANES, mixed U.S	11613	US	14	Yes	2012[13]
Mixed U.S.	2364	GGT	13.7	Yes	2013[14]

US = ultrasound-based grading
ALT: serum alanine aminotransferase
GGT: serum γ-glytamyl transpeptidase

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Figure 1. NAFLD as an obesity/MetS independent predictor of CVD events/mortality.

'Metabolic NAFLD' and the MetS share common pathophysiology

MetS and NAFLD share a common pathophysiology but also differ in many respects [2]. The liver is the site of production of two of the key components of the MetS, fasting serum glucose and VLDL, which contains most of the triglycerides present in serum. In subjects with NAFLD, the ability of insulin to normally suppress production of glucose and VLDL is impaired resulting in hyperglycemia, hyperinsulinemia and hypertriglyceridemia combined with low HDL cholesterol. The liver, once fatty, also overproduces many other markers of cardiovascular risk, such as C-reactive protein, fibrinogen, coagulation factors, and plasminogen activator inhibitor-1 [2] (Fig. 2).

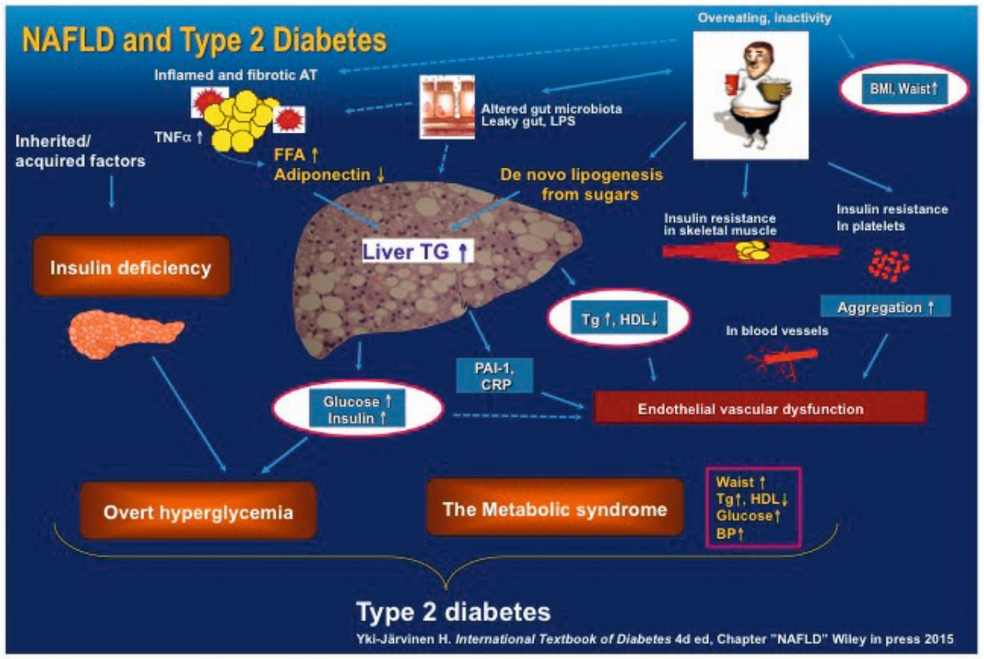

Figure 2. NAFLD and T2DM.

'Metabolic NAFLD' may even be a better predictor of CVD as it measures more directly abnormal metabolism than MetS

Increases in features of IR and markers of cardiovascular risk in NAFLD are associated with endothelial vascular dysfunction and could, in part, explain why NAFLD predicts CVD, also when adjusted for obesity [3]. On the other hand, some prospective studies have suggested that NAFLD may an even be a better predictor of the risk of CVD than MetS [3]. Whether this is because the measurement of liver fat content provides a more direct estimate of the risk of CVD than the MetS (diagnosed using ten different combinations of its five components) [1] or other mechanisms is unclear.

Dissociation between steatosis and IR

In 2008, a genome-wide association study in Hispanic, African-American, and European-American individuals showed genetic variation in PNPLA3 confers susceptibility to NAFLD. An allele in PNPLA3 (rs738409[G], encoding Ile148Met) was associated with increased liver fat, hepatic inflammation and fibrosis. This finding has subsequently been reproduced. For example, in a meta-analysis comprising 16 studies, compared with non-carriers, homozygous carriers of the variant had a 73% higher liver

fat content, a 3.2-fold greater risk of high necroinflammatory scores and a 3.2-fold greater risk of developing fibrosis [4]. PNPLA3 is predominantly expressed in the liver. *In vitro* studies with purified human PNPLA3 have shown that the wild-type enzyme hydrolyses triglycerides and that the Ile148Met substitution abolishes this activity. These data suggest that the Ile148Met substitution is a loss-of-function mutation that impairs triglyceride hydrolysis. IR is not a feature of NAFLD associated with the PNPLA3 rs738409[G], although, given the high prevalence of obesity/MetS, many subjects carrying the variant also have the MetS [2]. Subjects with NAFLD carrying the PNPLA I148M gene variant (20-50% of all subjects with NAFLD) have an increased liver fat content but a similar cardiovascular risk profile than non-carriers. This implies that the increased risk of CVD characterizing subjects with NAFLD is not due to steatosis per se. These data suggest that genotyping for the I148M variant in PNPLA3 might identify subjects who are at increased risk of hepatic but not cardiovascular complications.

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NAFLD, PRE-ATHEROGENIC LESIONS AND CARDIOVASCULAR EVENTS

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Take-home messages

- Increasing evidence points towards an increased risk of CVD in patients with NAFLD, independently of classical, metabolic risk factors.
- The pathophysiology of the link between NAFLD and CVD is complex and involves several potential mechanisms.
- It is currently unclear whether the increased risk of CVD in relation to NAFLD is confined to NASH or also applies to patients with NAFL.
- The need for screening and the impact of treatment of NAFLD on the risk of CVD are currently unknown.

Introduction

Besides the associated liver-related morbidity and mortality, it has become clear that NAFLD is also associated with an increased risk of CVD. The link between NAFLD and CVD can in part be explained by the common risk factors that they share. However, there is growing evidence that NAFLD is an etiological factor contributing to the development of CVD, independently of classical known risk factors for the latter.

NAFLD and CVD

Data is accumulating that patients affected by NAFLD have a higher risk of developing CV abnormalities, clinical CV events and even CV death. A first specific challenge in the interpretation of this data on the link between CVD and NAFLD is to distinguish between a timely correlation, simply based on underlying risk factors that are shared by both conditions, or an independent contribution of NAFLD (after correction for these shared metabolic risk factors) in the subsequent development of CVD. The latter implies a specific pathophysiological contribution of the liver affected by NAFLD to the development of CV abnormalities. Elucidating the role of NAFLD in the development of CVD therefore constitutes a second challenge, in which, besides clinical data, studies in animal models might be helpful. Finally the question of whether the role of NAFLD in the development of CVD is confined to NASH or is already present in NAFL needs to be answered. This question is particularly relevant for the treatment of NAFLD. If indeed the development of CVD is substantially influenced by NAFLD and NASH, its prevention might constitute an indication to treat NAFLD and its subtypes.

NAFLD and CVD: clinical data

Data have been recently reviewed [1-3]. The most convincing data on the role of NAFLD in CVD are those on the link between NAFLD and subclinical CHD. Both in cross-sectional and in follow-up studies, NAFLD, mostly diagnosed by ultrasound, has been shown to be an independent risk factor (after correction for classical risk factors for CHD) for: the presence or future development of

increased intima-media thickness and of impaired flow-mediated vasodilatation; the presence of carotid atherosclerotic plaques; an increased coronary artery calcium score on cardiac computed tomography; and abnormal coronary flow reserve as a marker for impaired coronary microcirculation.

In addition, clinical CHD data are emerging from large cohorts of patients, both cross-sectional and longitudinal studies as well community-based cohorts and more selected patient groups (e.g. patients with T2DM, type 1 diabetes, patients undergoing coronary angiography or patients with documented NAFLD). These data, reviewed elsewhere [2, 3], reveal that NAFLD is an independent predictor for clinical CHD, measured as the severity of the atherosclerotic lesions on coronarography or the occurrence of fatal and non-fatal CHD events. Only a few studies did not confirm the independent relationship of NAFLD with incident CHD or showed it to be confined to patients with NAFLD who concomitantly met the diagnosis of the MetS. Overall the data strongly support the independent contribution of NAFLD to an increased risk of clinically relevant CHD, even after correction for an extended set of well-established risk factors for CHD.

Several studies also showed a link between NAFLD and alterations in cardiac metabolism, structure and hemodynamic function, such as myocardial insulin resistance and mitochondrial ATP production, cardiac steatosis, myocardial hypertrophy and left ventricular diastolic dysfunction, not attributable to concomitant diabetes, obesity or arterial hypertension. The severity of these cardiac abnormalities correlated with the severity of the NAFLD. Finally, NAFLD has been associated with an increased risk of autonomic dysfunction and cardiac arrhythmias (mainly atrial fibrillation). Interestingly, recent data have shown that NAFLD is also independently linked with QTc interval prolongation, a major risk factor for ventricular arrhythmias and sudden cardiac death, which might explain in part the increased CV mortality associated with NAFLD. Finally, congestive heart failure and aortic valve sclerosis have also been linked with NAFLD independently of known risk factors.

Not all of these data are methodologically robust and most of the studies lack a liver biopsy diagnosis. However, the concept of NAFLD as being an independent contributor to the development of atherosclerosis and other functional and structural CV alterations, which subsequently lead to clinical CVD, appear to be sufficiently substantiated to integrate into the clinical approach for both the NAFLD patient and the CVD patient.

NAFLD and CVD: pathophysiological considerations

The mechanisms by which NAFLD influences the development of atherosclerosis and CVD are incompletely understood. NAFLD, T2DM, MetS and CVD share many metabolic features and risk factors, leading to the concept that they belong to a complex multi-system disease with several organ manifestations and a complex interplay between the different entities, with multiple bidirectional cause-effect relationships. The specific contribution of one entity to the others is therefore difficult to discern, and there might be substantial inter-individual variability.

The contribution of NAFLD to CVD, seen as a unidirectional cause-effect relationship, can be either indirect or direct and the potential mechanisms are summarized in **Fig. 1**.

First, as the liver is a key organ in both glucose and lipid homeostasis, it is not surprising that evidence is accumulating that NAFLD plays a role in the development of T2DM and the MetS, which are by themselves risk factors for CVD. This links NAFLD only indirectly to CVD. NAFLD has indeed been shown to contribute to the development of T2DM. Several studies, mostly diagnosing NAFLD by ultrasound or liver enzymes, have shown that NAFLD precedes and predicts the future development of T2DM independent of obesity and other factors of the MetS [4].

Secondly, the liver might also contribute directly to the development of CVD. It is clear that NAFLD is associated with an atherogenic lipid profile. In NAFLD, production of triglyceride-rich VLDL particles is increased. Insulin normally inhibits adipose tissue lipolysis (the main source of FFA flux to the liver for hepatic triglycerides synthesis) and hepatic VLDL secretion, both of which are hence increased in association with hepatic and adipose tissue insulin resistance. Subsequently, HDL-cholesterol levels fall and LDL-cholesterol levels rise, both of which are highly atherogenic conditions.

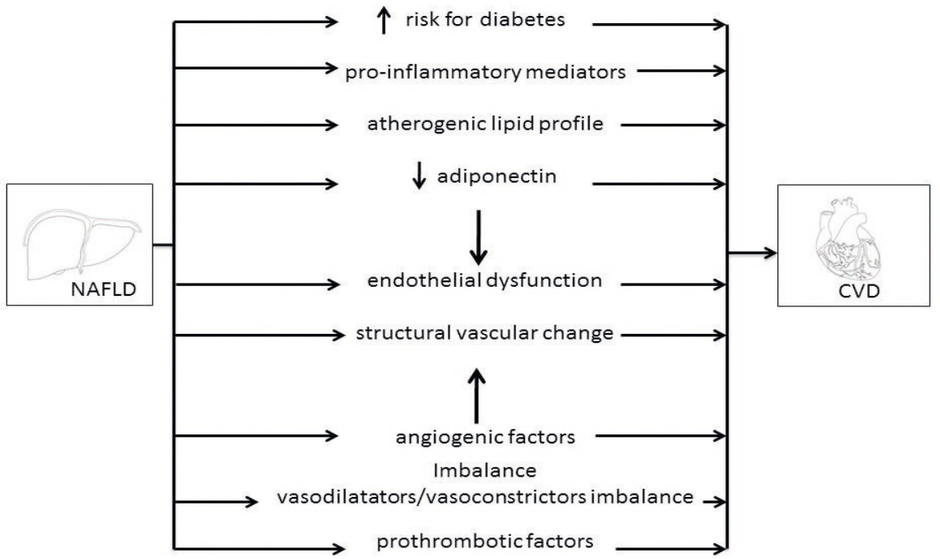

Figure 1. Schematic overview of the mechanisms that may indirectly or directly link the liver affected by NAFLD to alterations in the cardiovascular system.

Endothelial dysfunction has been shown to be an early event in the development of atherosclerosis. Several studies have recently highlighted that insulin resistance at the endothelial level occurs early in the development of NAFLD and is already present after a few days of high fat feeding, when steatosis develops but inflammation seems still to be absent [1].

Hepatic insulin resistance has been shown to be increased in severe steatosis. This is in part due to endothelial damage. An imbalance in locally produced vasodilators and vasoconstrictors has also been documented. Furthermore, steatosis also induces structural abnormalities of liver vasculature, contributing to the associated increase in intrahepatic resistance. Angiogenic factors have been shown to play a role in the intrahepatic vascular changes in cirrhosis and are also studied in NASH. Altered levels of angiogenic factors, well documented in the pathogenesis of atherosclerosis [1], have been observed in the peripheral blood of patients with NASH.

Prothrombotic factors have also been shown to play a role in the progression of liver disease. Although the liver is the main source of most of these coagulation factors, the casual role of the liver has not been proven. In particular, plasminogen activator inhibitor 1 (PAI-1) has been shown to be specifically increased in NASH.

Adiponectin, which is lower in patients with NAFLD, is another factor that might represent a link between NAFLD and CVD. Adiponectin has anti-atherogenic properties and directly affects endothelial

function. It also stimulates circulating angiogenic cells.

Inflammatory mediators can also contribute to the increased risk of CVD. NASH is associated with an increased intrahepatic production of pro-inflammatory cytokines, which are also increased systemically.

Although all these mechanisms are plausible links between the liver affected by NAFLD and the development of CVD, no studies to date have scientifically proven a cause-effect relationship. Most probably, several mechanisms are concomitantly present, and might substantially differ between patients. Further study is therefore needed to gain mechanistic insights into the pathophysiology of the NAFLD-CVD axis, with an individualized preventive and therapeutic approach as the ultimate goal.

NAFLD and CVD: NAFL or NASH?

The question of whether the role of NAFLD in the development of CVD is confined to NASH or is already present in NAFL is important. Only about 5-10% of NAFLD patients have NASH, so if the risk would be confined to NASH, this would substantially reduce the CVD burden attributable to NAFLD. This might be in contrast with the current data on the impact of NAFLD on CVD, which does not seem to fit with the relatively small number of NASH patients within the NAFLD group. The answer to this question has potential implications for the management of NAFLD patients. Indeed, if NAFL as well as NASH is associated with an increased risk of CVD, one might argue that NAFLD should be treated regardless of the presence of NASH. In this scenario, NAFL could no longer be considered as a benign condition and guidelines for the treatment of NAFLD might have to consider the treatment of NAFL, with CVD prevention as an indication.

The question, however, remains largely unanswered. The main reason is that most of the data comes from studies where no distinction is made between NAFL and NASH [2, 3]. This distinction still requires a biopsy. Series that include histology have smaller patient numbers and there is a potential to over-represent those patients with more severe liver disease. Furthermore, most of these studies have rather short mean follow-up times and methodological limitations hamper the general applicability of their results.

Nevertheless, biochemical and histological data give an indication that the risk is confined to NASH, or is at least higher in NASH patients compared to NAFL (**Table 1**). Matteoni et al. reported differences in liver-related mortality but not all-cause or other cause mortality according to the histological subtype of NAFLD [5]. Dam-Larsen et al. did not detect differences in mortality when comparing histologically-proven patients with NAFL compared to the general population [6]. More recent studies, however, consistently show CVD as more prevalent in NAFLD patients, with three out of four studies confining this risk to patients with NASH [7-10].

Although these most recent data suggest that the risk is mainly associated with NASH, or is at least more pronounced in patients with NASH compared to NAFLD, further methodologically stringent studies with long follow-up are needed to answer this question.

Treatment of NAFLD: impact on CVD?

Currently there is no approved pharmacological treatment for NAFLD. Although it can be hypothesized that improving NAFLD reduces the risk of CVD, there is currently limited data on potential changes in the risk of CVD in relation to the success of NAFLD treatment. Interestingly, two recent studies on the effects of statins on CV events demonstrated a significantly reduced CV event rate in those patients with baseline elevation of liver tests (used as a surrogate marker for the presence of NAFLD) as well as significantly improved liver tests in one of the studies [11, 12]. The cardio-protective effect of statins was less pronounced in patients with normal liver tests at baseline. Glitazones also improve CV risk, but it is unclear to what extent this can be attributed to their beneficial effect on NAFLD. Furthermore, as outlined previously, it is not clear whether the risk of CVD is increased in all subtypes of NAFLD. Therefore, no evidence-based recommendations can be formulated at present.

Nevertheless, it is recommended to screen for NAFLD in every patient with risk factors for CVD or established CVD and to screen for CVD in every patient with NAFLD. Patients should be treated accordingly with life-style modification. This recommendation is debated, as there are no data on cost-effectiveness and no pharmacological treatment when NAFLD is diagnosed. Metformin is frequently used, as it seems to have a beneficial effect on CV risk, in patients with insulin resistance although this is also debated. However, as outlined previously, metformin failed to show beneficial effects on liver histology. Other metabolic factors should be treated according to the corresponding guidelines.

Table 1. Prospective patient-based cohort studies on the risk of CHD in relation to NAFLD diagnosed by liver histology. n = number of patients, y = years.

Reference	n	Mean follow-up (y)	Histological subtypes	Comparator	Conclusion	Remark
Matteoni et al. (1999) [5]	132	18.0	Different subtypes	4 histological subtypes within the cohort	All-cause and CV mortality not different between subtypes	Increased liver-related mortality
Dam-Larsen et al. (2004) [6]	109	16.7	NAFL	General population	All-cause and CV mortality not different	
Adams et al. (2005) [7]	420	7.6	NAFLD	General population	Increased all-cause mortality	CHD 2nd cause of death
Ekstedt et al. (2006) [8]	129	13.7	NAFL/ NASH	Reference population	Increased liver-related and CV mortality in NASH	NAFL not significantly different from reference population
Rafiq et al. (2009) [9]	173	13.0	NAFL/ NASH	NAFL *vs.* NASH	CHD 1st cause of death in both NAFL and NASH	Increased liver-related mortality in NASH *vs.* NAFL
Söderberg et al. (2010) [10]	118	24.0	NAFL/ NASH	NAFL *vs.* NASH *vs.* general population	Increased CV mortality in NASH compared to NAFL and general population	No difference between NAFL and general population

Conclusion

The role of NAFLD in the pathophysiology of CV abnormalities, and hence its independent contribution to an increased risk of CV morbidity and mortality, is increasingly supported with evidence from animal models and clinical data. Whether NAFL is still to be considered benign in this regard and whether the risk is hence confined to NASH, is currently unclear, but the risk seems at least to be more pronounced in NASH patients compared to NAFL. As the role of NAFLD in CVD becomes clearer, this aspect of NAFLD should probably be incorporated in the future guidelines on its treatment indications and paradigms.

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DOES STEATOSIS PLACE PATIENTS AT RISK FOR DIABETES DEVELOPMENT AND PROGRESSION?

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Take home messages

- NAFLD is a feature of ectopic fat accumulation and is strongly linked to insulin resistance and T2DM risk factors.
- Excess liver fat is linked to both hepatic insulin resistance and increased hepatic triglyceride production. It is believed by many that liver fat is part of the pathogenesis of T2DM in many patients.
- Patients who are considered to have NAFLD should have their glycemia status checked carefully.
- Alteration in weight trajectory, and ideally some weight loss, is a key facet of the management of NAFLD.
- There is plentiful evidence that weight loss lessens risk of T2DM and improves liver fat levels in parallel with improvements in hepatic insulin resistance.

NAFLD and T2DM

NAFLD is a feature of ectopic fat accumulation and is strongly linked to insulin resistance and T2DM risk factors [1, 2]. Indeed, to diagnose NAFLD, the physician should look for a pattern whereby ALT is greater than AST (with ALT more strongly linked to insulin resistance and obesity), raised triglycerides and lower HDL-cholesterol, as well as obesity and potential abnormalities in glucose levels [1, 2]. All of these features are risk factors for T2DM and it is therefore not surprising to note that NAFLD is strongly linked to T2DM [1, 3]. Indeed, it is estimated that at least around half of all patients (more in other studies) with T2DM have NAFLD [4], whereas NAFLD as estimated by ultrasound, or raised ALT or GGT, appears to be an independent risk factor for development of T2DM [3].

Excess liver fat is linked to both hepatic insulin resistance and increased hepatic triglyceride production (which in turns leads to lower HDL- cholesterol levels) and it is believed by many therefore that liver fat is part of the pathogenesis of T2DM in many patients [5, 6]. There is also emerging evidence for excess pancreatic fat as a feature of T2DM which appears to be associated with impaired beta cell function. Interestingly, there is emerging evidence that weight loss can to some extent reverse these abnormalities [5].

Diagnostic relevance of above patterns of association

Given strong associations of NAFLD with diabetes risk, it is clear that patients who are considered to have NAFLD should have their glycemia status checked carefully [1]. This can be done using either fasting blood glucose or HbA1c for patients not fasting. Doctors should however, avoid mixing diagnostic criteria for diabetes. The finding of some derangement in glycemic status, whether it is frank diabetes or a high risk state, adds evidence for the diagnosis of NAFLD and also can help support the best management options for both the patient and the physician.

Weight change as a key therapeutic option

Given the above findings, it is clear that alteration in weight trajectory, and ideally some weight loss, is a key facet of the management of NAFLD but how to best achieve this is often unclear. Nevertheless, there is plentiful evidence that weight loss lessens risk of T2DM and improves liver fat levels in parallel with improvements in hepatic insulin resistance. A recent algorithm (**Fig. 1**) for the management of NAFLD was proposed by Sattar et al. [1]. It strongly features measures of glycemia in assessing NAFLD diagnosis and weight loss as a key therapeutic goal. Further work is needed to determine how NAFLD presence relates to diabetes progression.

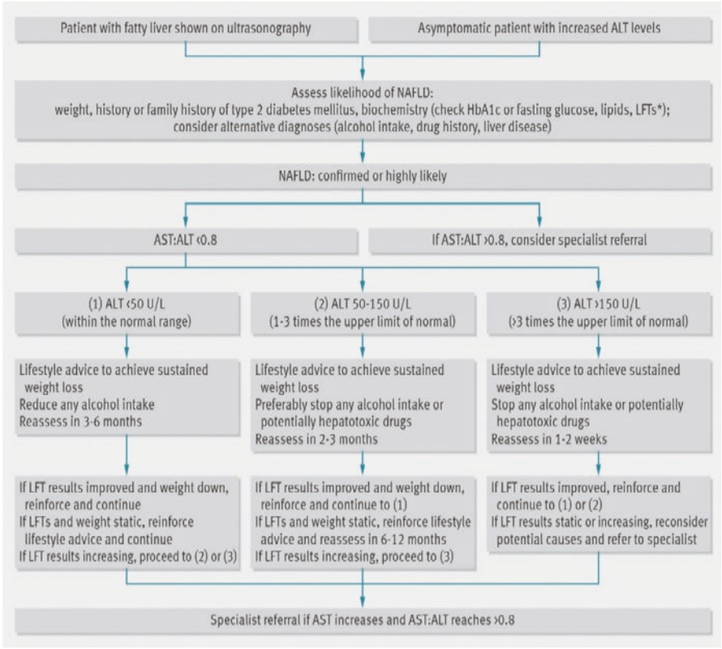

Figure 1. Proposed algorithm for diagnosis and initial management of suspected or confirmed NAFLD in primary care. *Some biochemistry laboratories only measure one of the transaminases and in such cases it will be necessary to request both ALT and AST tests in relevant patients [1].

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CURRENT AND FUTURE INSULIN SENSITIZERS IN THE TREATMENT OF NASH

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Take home messages

- IR is an almost universal finding in primary NASH. Existing studies provide proof of concept that improving IR results in both metabolic and histological improvement across the whole spectrum of steatohepatitis.
- Initial data suggested that metformin might be an ideal treatment for NAFLD but later small studies gave inconsistent results.
- Current guidelines recommend the use of pioglitazone for the treatment of steatohepatitis in patients with biopsy-proven NASH. However, the long-term safety and benefit is unknown.
- Obeticholic acid, a selective FXR agonist, has demonstrated encouraging histological results that deserve confirmation.
- The dual PPAR α and δ agonist GFT505 improves hepatic and peripheral IS, dyslipidemia, inflammatory markers and liver function tests in abdominally obese, IR patients.
- Combining insulin-sensitising agents with hepatoprotective or anti-inflammatory/ anti-fibrotic drugs could be an attractive option for future therapeutic strategies.

Introduction

NASH is associated with a significant increase in liver-related mortality as well as cardiovascular mortality. The recommended first-line therapy is non-pharmacological: weight reduction through diet, changes in lifestyle, physical exercise and limiting sedentarity. Additionally, concurrent metabolic disorders such as T2DM, hyperlipidemia, or arterial hypertension, when present, should be well controlled but their specific impact in improving the hepatic disease has not yet been determined. Occasionally, these measures result in a 7-10% weight loss, a threshold that is possibly associated with histological improvement. In patients who failed these measures or in those with advanced disease (NASH with significant fibrosis), pharmacological treatments specifically directed at improving hepatic inflammation, fibrosis and/or clearing steatohepatitis might be necessary [1]. Here we will provide an overview of data obtained so far with pharmacological agents in NASH. Most uncontrolled studies and those without histological documentation of NASH will not be mentioned. The therapeutic area in NASH is still in its infancy; therefore an urgent need exists for well-conducted, randomized controlled trials with relevant therapeutic endpoints [2].

Rationale for the use of insulin sensitizers

Insulin resistance is an almost universal finding in primary NASH. It is the main driving force behind excessive fat accumulation in the liver but may also play a role in the initiation and perpetuation of steatohepatitis and fibrosis progression. Moreover hepatic steatosis and insulin resistance potentiate

each other. A current model for the pathogenesis of NASH is centred on lipotoxicity [3], and states that the influx of fatty acids and their derivatives through the liver induces apoptosis, oxidative stress, ER stress, activation of proinflammatory pathways and ultimately liver cell injury (**Fig. 1**). The main source of free fatty acids reaching the liver is an uncontrolled release from insulin-resistant adipose tissue. Therefore, correcting IR, in particular at the adipose level, is a relevant aim and most therapeutic trials have focused on insulin sensitizers.

Metformin

Metformin is an oral biguanide approved for use in T2DM where it acts as an insulin-sensitizing agent with reduction of hepatic glucose production and increased peripheral glucose utilization [4]. Signalling is via AMP-activated protein kinase. The biguanides alter cellular bioenergetics without inducing weight gain. While initial pre-clinical (genetic models of hyper-feeding) and clinical data suggested that metformin might be an ideal treatment for NAFLD that reduces liver steatosis, inflammatory mediators and hepatic inflammation [5], subsequent small studies provided inconsistent results. In a small, randomized, 6-month trial, metformin improved glucose and lipid parameters but not hepatic histology and aminotransferases [6]. The benefit of metformin in NASH, if any, seems to be related primarily to the weight loss induced by the drug in a fraction of treated patients. The inefficacy of metformin could be explained by its very weak antisteatogenic effect and the lack of induction of circulating adiponectin compared to glitazones, although this has only been tested with medium term exposure (4 months) and not longer-term [7]. Although metformin is a safe antidiabetic drug, it is not recommended for treating NASH, as no effect on liver histology has been demonstrated. Recent data has emerged showing that metformin inhibits hepatocyte proliferation and induces cell-cycle arrest in hepatoma cell lines and also inhibits chemically-induced liver tumorigenesis in vivo. It was inferred from these observations that metformin might decrease the risk of HCC; unfortunately, the data available so far are retrospective, and the analyses do not take into account important confounders related to treatment assignment bias. No firm conclusion can be drawn regarding a putative protective effect of metformin against NASH-associated carcinogenesis.

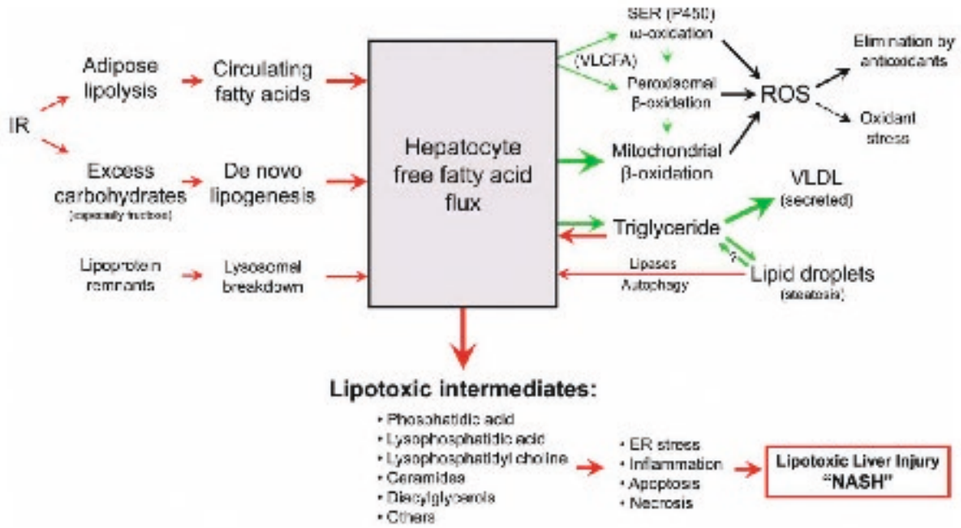

Figure 1. The lipotoxicity model of NASH. *Reproduced from Neuschwander-Tetri [3] with permission.*

Thiazolidinediones

Of all the tested drugs, glitazones are those with the best evidence-based data and also with the strongest pathogenesis-based rationale for treatment of NASH. Of major importance is the ability of glitazones to promote differentiation of insulin resistant large pre-adipocytes into small, proliferative, insulin sensitive adipocytes. Upon induction of lipoprotein lipase and of a large set of lipogenic genes, glitazones enhance fatty acid uptake and synthesis in the adipose tissue, which diverts the non-esterified FFA load towards adipocytes instead of other organs such as the liver and muscle. Ultimately, inappropriate fat storage in the latter organs is reduced, with subsequent improvement in IS despite the expansion in fat mass. Another important mechanism of action is the ability to up-regulate production of adiponectin, an insulin sensitising and antisteatogenic adipokine that increases fatty acid beta-oxidation in liver and muscle. Other actions that contribute to the reduction of IR are increased expression of AMP-activated protein kinase and up-regulation of the glucose transporter GLUT4 in muscle and adipose tissue. Finally, studies in rodents have shown that glitazones may have anti-inflammatory actions, resulting in decreased hepatic fibrogenesis in response to repetitive liver injury. Of particular interest to human disease, these studies have shown a reduction in PPARγ nuclear expression upon activation and trans-differentiation of stellate cells into fibrogenic myofibroblasts. Treatment with PPARγ agonists restored PPARγ expression and significantly alleviated the hepatic scar-forming response. PPARγ agonists also exert anti-inflammatory effects on Kupffer cells which might be indicative of direct hepatoprotective effects.

Table 1 shows the randomized controlled trials of glitazones in NASH. These trials are very heterogeneous in terms of drugs studied, dosage, treatment duration, population included, non-pharmacological associated measures and study design. The two most robust and reproducible hepatic findings are reduction of ALT and of steatosis. Maximum ALT reduction occurred by month 6 [8, 9]. The reduction in ALT levels was around 38-52% from baseline, significantly higher than for placebo (10-34%) [10, 11]. Reduction in steatosis has been documented in about one-half to two-thirds of treated patients, significantly more than with placebo in all but one trial where some patients only had minimal steatosis (5-25%) at baseline [12]. The magnitude of reduction in steatosis is usually not reported; however, with rosiglitazone the median reduction was 20%, ranging from 30-60% in the 47% of participants who were labelled responders [11]. A reduction of this magnitude is sufficient for improving hepatic and systemic insulin resistance.

Table 1. Glitazone trials with available end-of-treatment histology.

Study	Drug dose per day n	Comparator n	Treatment duration	n (drug/ comparator) for end of treatment histology	% with diabetes	Normal ALT included	Professional diet counselling*	Run-in period
Randomized controlled trials								
Sanyal, 2004 [13]	Pioglitazone 30 mg + vitamin E, n= 10	Vitamin E n=10	6 months	18 (9/9)	0	Yes	Yes	Yes (no results)
Belfort, 2006 [10]	Pioglitazone 45 mg, n= 29	Placebo n= 24, (total 55)	6 months	47 (26/21)	50 §	No	Yes	Yes
Ratziu, 2008 [11]	Rosiglitazone 8 mg, n=32	Placebo n=32	1 year	63 (32/31)	33	No	No	No
Aithal, 2008 [12]	Pioglitazone 30 mg, n=37	Placebo n=37	1 year	61(31/30)	0	Yes	No	Yes (no results)
Sanyal, 2010 [9]	Pioglitazone 30 mg, n=80	Placebo n=84	2 years	142 (70/72)	0	Yes	No	No
Open-label trials								
Caldwell, 2001 [14]	Troglitazone 400 mg, n=10	none	6 months	7	10	No	No	No
Neuscwander-Tetri, 2003 [15]	Rosiglitazone 8 mg, n=25	none	12 months	22	24	Yes	No	No
Promrat, 2004 [16]	Pioglitazone 30 mg, n=18	none	1 year	18	0	No	No	Yes

* i.e. follow-up visits with a dietician. § the remaining 50% were glucose intolerant [17]

Table 2. Outcomes of glitazone studies for inflammation, liver cell injury and fibrosis.

Author	Drug	Dose / duration	Lobular inflammation		Ballooning		Fibrosis	
			Intragroup change	**Change *vs.* comparator**	Intragroup change	**Change *vs.* comparator**	Intragroup change	**Change *vs.* comparator**
Caldwell 2001 [14]	Troglitazone	400 mg /6 mo	Improved NA	NA	NA	NA	No change	NA
Neuscwander-Tetri, 2003 [15]	Rosiglitazone	8 mg /12mo	NA	NA	Improved *P*=0.003	NA	Improved PSF	NA
Promrat, 2004 [16]	Pioglitazone	45 mg /12 mo	Improved *P*<0.001	NA	Improved *P*=0.001	NA	Improved *P*=0.04	NA
Sanyal, 2004 [13]	Pioglitazone	30 mg /6 mo	NA	NA	Improved *P*=0.01	No change	No change	No change
Belfort, 2006 [10]	Pioglitazone	45 mg /6 mo	Improved *P*<0.001	Improved *P*=0.008	Improved *P*=0.001	Improved *P*<0.02	Improved *P*=0.002	No change *P*=0.08
Ratziu, 2008 [11]	Rosiglitazone	8 mg /12 mo	No change	No change	No change	No change	No change	No change
Aithal, 2008 [12]	Pioglitazone	30 mg /12 mo	Improved *P*=0.04	No change	No change *P*=0.09	Improved *P*=0.005	Improved *P*=0.006	Improved *P*=0.05
Sanyal, 2010 [9]	Pioglitazone	30 mg /2 years	Improved NA	Improved *P*=0.001	Improved NA	Improved *P*=0.01	No change	No change

NA, statistical comparison not available; PSF, zone 3 perisinusoidal fibrosis

Improvement in necroinflammatory lesions was less consensual between studies. Ballooning improved in 32-54% of patients, more than placebo in three RCTs [9, 10, 12]. Intralobular inflammation improved in most, but not all studies [11], whereas portal inflammation was either unchanged or worse in one study with rosiglitazone [11]. Finally, no study has shown a convincing effect on fibrosis. **Table 2** shows the main histological effects of glitazones.

Needless to say, there is no consensus on the optimal duration of therapy. On one hand, a prolonged, three-year therapy did not result in additional histological improvement beyond that obtained in the first year [8]. On the other hand, glitazone-induced metabolic and hepatic effects seem to be short-lived after treatment discontinuation. Both ALT and HOMA values return to baseline starting three months after discontinuation and, in the few patients with one year follow-up biopsies, steatohepatitis recurred despite on-treatment clearance [18].

The largest RCT that tested pioglitazone in NASH was a multicentre study of low-dose pioglitazone (30 mg/day) *vs.* vitamin E (800 IU/day) *vs.* placebo in non-diabetic NASH patients over a two-year period [9]. Pioglitazone failed to achieve a statistically significant improvement of a complex composite primary end-point which included a 2-point reduction in the NAS (*P*=0.04, higher than a preset 0.025 significance level). However, secondary endpoints of elementary histological lesions, steatosis, lobular inflammation and ballooning except fibrosis were significantly improved by pioglitazone. Importantly, clearance of steatohepatitis, a major outcome with prognostic implications, occurred in 47% and 21%

of pioglitazone and placebo treated patients, respectively ($P<0.001$). Interestingly, when the analysis was restricted to patients with well-defined steatohepatitis, pioglitazone reached the primary endpoint [9]. Collectively, these data strongly suggest the efficacy of pioglitazone in NASH, thus confirming converging information from earlier trials.

It has been suggested [19], but not confirmed [20], that glitazones strongly improve adipose tissue IR which correlates with the reduction in steatosis and necroinflammation [19]. Whether this information can be used to identify responders early on during therapy is unclear but it deserves future investigation. Since most NASH patients treated with glitazones were non-diabetic, and the largest trial (PIVENS) excluded diabetic patients, current guidelines recommend the use of pioglitazone in patients with NASH but warn against the lack of substantial evidence-based data in diabetic patients. However, since these drugs are indicated for treatment of T2DM, there is considerable experience of using them in these patients. Whether their efficacy differs according to the degree of IR or the diabetic status of the patients is, at this point, entirely speculative.

The main obstacle to the widespread use of glitazones is their safety profile. Weight gain that tends to persist after discontinuation is mostly due to increased peripheral fat mass [21] and therefore seems devoid of adverse metabolic consequences. A detailed discussion on the safety of glitazones goes beyond the scope of this syllabus. Nevertheless, the use of glitazones has been severely restricted by black-box warnings based on increased cardiovascular events [22], congestive heart failure [23], bone fractures in women [24] and risk of bladder cancer for pioglitazone [25], which justified its market withdrawal in France. Recent data collected in one million T2DM individuals from six cohorts around the world did not confirm an increased risk of gall-bladder cancer [26].

Current guidelines recommend the use of pioglitazone for the treatment of steatohepatitis in patients with biopsy-proven NASH. However, the long-term safety and benefit is unknown and it should be used with caution in diabetic patients and in those with impaired cardiac function [27].

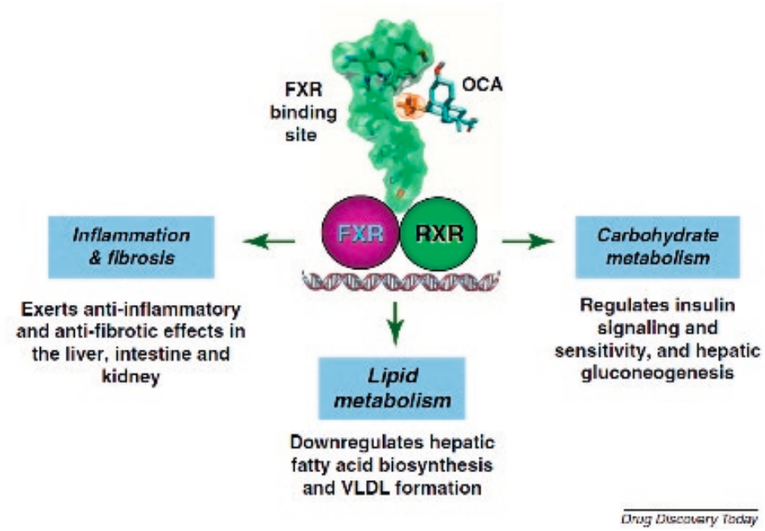

Figure 2. Metabolic and hepatoprotective actions of FXR activation. *Reproduced from Adorini et al. [29] with permission.*

The farnesoid X receptor (FXR)

Bile acids are now believed to play a crucial role in regulating liver and metabolic homeostasis. Their action is mediated through nuclear hormone receptors such as FXR and TGR5. Signalling through these nuclear receptors modulates triglyceride, glucose and cholesterol homeostasis, in addition to bile acid synthesis [28]. FXR is a member of the nuclear hormone receptor superfamily, which function as ligand-activated transcription factors upon binding of bile acids or synthetic ligands. FXR activation has a wide range of metabolic effects (**Fig. 2**). It improves both glucose metabolism (by inhibiting gluconeogenesis and glycogenolysis in the liver) and peripheral IS (in the muscle and adipose tissue). It also reduces lipogenesis by inhibiting SREBP1c and enhancing β-fatty acid oxidation. FXR-null mice develop steatosis, but also hepatic inflammation, liver cell injury and HCC that is reminiscent of human NASH. Interestingly, FXR activation has anti-inflammatory actions, partly explained by inhibition of NF-kB activation and partly by immune modulation. Since FXR agonists protect against liver inflammation and fibrosis in the methionine-choline deficient model of NASH, interest in this class of agents as a treatment of human NASH has been growing.

Several potent, synthetic FXR agonists are available. Obeticholic acid (OCA), a derivative of chenodeoxycholic acid, is a selective FXR agonist which acts as a potent metabolic regulator with antiinflammatory, immunomodulatory and antifibrotic properties [29]. A small randomized trial in T2DM patients with NAFLD showed an improvement in IS, a modest but dose-related weight loss of potential clinical relevance, a reduction in ALT levels at the lower, 25 mg dose and divergent effects on the lipid profile with a decrease in triglyceride levels and an increase in LDL cholesterol levels [30]. Recently the NASH CRN reported the results of a large phase 2b study comparing 25 mg of OCA *vs.* placebo over 72 weeks of therapy [31]. The primary endpoint was improvement in histology, as measured by a two-point reduction in a composite activity histological score without worsening of fibrosis. The therapeutic phase of the trial was stopped early partly because a pre-planned interim analysis showed that 45% (50 of 110) and 21% (23 of 109) of the OCA and placebo patients, respectively, reached the primary endpoint (relative risk 1.9, 95% CI 1.3–2.8). In terms of fibrosis score, 35% (36 of 102) and 19% (19 of 98) of the OCA and placebo patients, respectively, regressed by one stage or more. These are very encouraging data that certainly deserve confirmation in larger trials. The most concerning side effects were pruritus and an increase in LDL cholesterol. Studies are underway to fully understand the lipid changes in order to determine whether they are associated with increased cardiovascular risk or not.

Peroxisome proliferator and activator dual agonists

Peroxisome proliferator-activated receptor (PPAR)-δ, a ubiquitously expressed member of the lipid-activated nuclear receptor superfamily, has emerged as a key metabolic regulator (**Fig. 3**) [32, 33]. PPAR-δ activation results in an increase in hepatic fatty acid β-oxidation, inhibition of hepatic lipogenesis (by inhibiting maturation and translocation of SREBP1c), reduction of hepatic glucose production (mediated through activation of AMPK), and improvement in hepatic inflammation (mediated through inhibition of STAT3). PPAR-δ exerts hepatoprotective effects, in particular against lipotoxicity, an action that is mediated through a reduction in JNK phosphorylation and in the expression of multiple inflammatory cytokines [34], as well as through modulation of macrophage inflammatory activity [35]. Recently, antifibrotic effects of PPAR-δ agonists have also been described in models of liver injury [35]. Therefore, a strong rationale for PPAR-δ agonists as pharmacological agents in NASH exists.

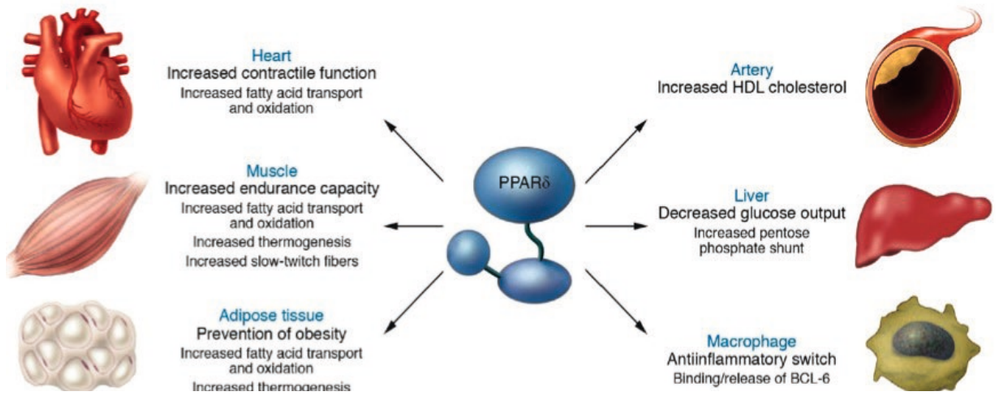

Figure 3. Therapeutic targets of PPAR-δ in the metabolic syndrome. *Reproduced from Barish et al. [32] with permission.*

GFT505 is a dual PPAR α and δ agonist that undergoes extensive enterohepatic cycling and is liver targeted [36]. Human studies performed in abdominally obese, insulin resistant patients, with or without diabetes have shown that GFT505 improves hepatic and peripheral IS, dyslipidemia, inflammatory markers and liver function tests [37, 38]. Animal data confirmed the hepatoprotective effects of GFT505 in dietary models of NASH or fibrosis with, in particular, reduction in steatosis, hepatic inflammation and pro-inflammatory genes [36]. Because genetically engineered mice lacking PPAR-α were also rescued from this phenotype, the effects carried by GFT505 were at least partly due to its PPAR-δ agonistic properties. Importantly, this compound exhibited antifibrotic properties in fibrosis models that were independent of metabolic and IR abnormalities [36], thereby suggesting universal antifibrotic potency in rodents. Based on these promising results, a large, phase IIb, randomized controlled trial is now underway in NASH patients. Earlier phase IIa studies suggest a good safety profile, in particular from the lack of evidence of PPAR-γ agonistic activity.

Conclusion

Insulin sensitizers are logical drug candidates for the treatment of NASH. Existing studies provide proof of concept that improving insulin resistance results in metabolic but also histological improvement across the whole spectrum of steatohepatitis. There are numerous regulators of insulin signalling as well as defective pathways that drive IR and also ectopic fat deposits and lipotoxic intermediates that alter insulin signalling, which can all be targeted in an effort to alleviate IR. It remains to be seen whether anti-inflammatory compounds acting at the adipose tissue level or at the hepatic level might also contribute to an improvement in insulin signalling either locally or systemically. Given the complexity of mechanisms involved in the progression of NASH, simply correcting IR may not be enough for a majority of patients. Combining insulin-sensitising agents with hepatoprotective or anti-inflammatory/anti-fibrotic drugs could be an attractive option for future therapeutic strategies.

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CARCINOGENESIS AND THE SPECTRUM OF HEPATIC TUMORS IN NASH

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Take home messages

- There is compelling epidemiological evidence of a casual link between NAFLD/NASH and HCC.
- It remains unclear what is the risk of HCC in patients with NAFLD or non-cirrhotic NASH. The role of HCC surveillance in these populations remains to be determined.
- Potential mechanisms involved in NAFLD/NASH HCC involve inflammation pathways (e.g. NF-kB), metabolic disarray (e.g. PTEN) and oxidative stress (e.g. SAM).
- In terms of chemoprevention, no drug has been able to prevent disease progression and HCC development in NASH patients. The alleged protective role of metformin needs to be confirmed in controlled studies.

Introduction

At a global scale, mortality due to liver cancer has increased by more than 50% in the last 20 years. It remains as one of the deadliest malignancies with a high ratio of mortality to incidence of 0.95. Since 2012, liver cancer is the second most common cause of death from cancer worldwide. In the US, recent data estimated that in 2014 there would be 33,190 new cases of liver cancer. Overall, patients' 5-year survival is 16%, which is second only to pancreatic cancer. Altogether, these data confirm that disease burden due to liver cancer is increasing in the US as well as worldwide. The AACR 2013 report confirms how liver cancer has become the leading cause of increased cancer mortality in the US in the last 20 years.

Different reports suggest that patients with NAFLD-HCC tend to be older, with tumors diagnosed at more advanced stages, but these assumptions are not supported by strong data. To increase the controversy, there are also studies indicating that NAFLD/NASH tumors are less aggressive compared with hepatitis-related HCC. In terms of clinical management, there is no evidence suggesting that NAFLD-HCC should be managed differently to other etiologies. Recommended management of HCC is extensively described in the 2012 EASL-EORTC guidelines [1]. In terms of early HCC detection and surveillance, guidelines recommend abdominal ultrasound every 6 months in at-risk populations. These are broadly cirrhotic patients of any etiology, and certain subgroups of patients with non-cirrhotic hepatitis B or C. Based on the available data, screening is not recommended in patients with NAFLD/NASH who haven't developed cirrhosis. Regarding prognostic prediction, the BCLC algorithm is endorsed as a general roadmap to classify patients and guide treatment decision-making. There are five treatment modalities that are recommended based on their ability to improve survival, including potentially curative options (i.e. resection, transplantation and ablation) and palliative treatments (i.e. transarterial chemoembolization and sorafenib). Following the approval of sorafenib in 2007, all phase 3 clinical trials testing new drugs in patients with advanced stages have been negative.

Also, there are no second-line options in patients that progress after sorafenib. Indeed, we lack precise knowledge of the molecular drivers responsible for tumor progression following resistance to sorafenib that could potentially guide second-line drug development. Worldwide, HCC patients are still diagnosed at intermediate-advanced stages with a median overall survival of less than two years. Also, co-occurrence of HCC with underlying cirrhosis increases toxicity to systemic agents – further restricting the therapeutic arsenal for patients with advanced HCC. In summary, despite advances in the last decade, there is still a long way to go to improve outcomes in patients with HCC.

There are several reports on the association between HCC and T2DM, including evidence from population-based studies and meta-analyses. The hazard ratio for HCC development among diabetics ranges from 2-3. Indirect evidence has also underscored the potential protective effects of metformin in HCC development. A nationwide, case-control Taiwanese study including almost 300,000 individuals found a dose-dependent reduction in HCC risk in diabetics. Proposed mechanisms of action for metformin include its ability to interfere with LKB1 via AMPK. NAFLD/NASH also interact with other etiologies to increase the risk of HCC. Data from longitudinal studies, including close to 3,000 HBV surface-antigen positive patients followed for a mean of 14 years, found an involvement of excess weight and progression of HBV-related HCC development. There are also reports suggesting a contribution of NAFLD/NASH to HCV hepatocarcinogenesis.

Molecular alterations and pathogenesis of NAFLD/NASH HCC

Numerous studies demonstrate how HCC can be classified based on common genomic traits identified through whole-genome transcriptome profiling. These studies also enabled the generation of gene signatures (i.e., combination of genes correlating with a specific phenotype) that correlate with patients' outcome [2]. Most of these reports analysed samples from resected patients with underlying liver disease due to either viral hepatitis or alcohol-related liver disease. Hence, there is limited information on the genomic portrait of NAFLD/NASH HCC. There are some data on genetic risk of NAFLD/NASH HCC. In 2008, a variant in the *PNPLA3* gene was found to be strongly associated with NAFLD. Subsequent studies suggested that this polymorphism could also be associated with NAFLD/NASH. A case-control study that included 375 individuals found an adjusted OR of 2.26 for HCC development in patients with NAFLD and the GG variant of *PNPLA3*. PNPLA3 is involved in hepatic triacylglycerol metabolism.

There is also very limited information in terms of NAFLD/NASH and candidate oncodrivers, since large deep-sequencing studies conducted so far mostly analysed viral hepatitis-related HCC. In these studies, the most frequently mutated genes affected were *TERT* promoter (60%), *TP53* (27%) and *CTNNB1* (26%) [3]. There are other less frequently mutated genes involved in chromatin remodelling [*ARID1A* (6%), *ARID2* (7%), *MLL3* (3%)], growth factor signalling [*RPS6KA3* (3%), *RAS* (2%)] and oxidative stress [*NFE2L2* (3%), *KEAP1* (3%)]. These next-generation sequencing studies also confirmed previously reported DNA copy number alterations with high-level amplifications affecting chromosome 11q13 and chromosome 6p21. Potential candidate oncogenes in these regions include *FGF19*, *CCND1* and *VEGFA*.

Mechanisms for molecular pathogenesis of NAFLD/NASH HCC

Regarding molecular pathogenesis of NAFLD/NASH HCC, several mechanisms have been proposed [4]. They can be broadly clustered in 3 major groups (**Fig. 1**):

1. Inflammation and microbiome: The association between chronic inflammation and cancer is well established. A major player in this connection is the NF-kB signalling pathway. NF-kB is a master regulator of inflammation, with a defined role in experimental HCC. *In vivo* de-regulation of essential components of this pathway including IKK and NEMO induce NAFLD [5], fibrosis and ultimately HCC. Global inhibition of NF-kB signalling protects mice from HCC development. An enrichment of progenitor-cell derived tumors in some of these models has been suggested, but this has not been confirmed in human NAFLD/NASH HCC. One of the potential mechanisms involved in hepatic inflammation is the translocation of intestinal bacteria from the gut. TLR4 and intestinal microbiota has been implicated in HCC progression and are suggested as potential new targets for chemoprevention [6]. There is evidence of a deleterious effect of gut microbiome in HSC function. In fibrotic livers, HSC are immunologically active and release mediators that promote progression from NAFLD to HCC, a phenotype related to impaired senescence. IL6 and de-regulated STAT3 signalling have also been implicated in inflammation-related HCC. In terms of inflammatory mediators of NASH-related HCC, a recent report underscored the role of intrahepatic activation of CD8+ T and NK cells to promote oncogenesis in a model of high-fat diet induced HCC. More recently, an animal model of ER stress points towards a central role of TNFα in tumor progression in NAFLD/NASH-HCC.

2. Metabolism and autophagy: Hyperinsulinemia is a frequent finding in patients with NAFLD/NASH and, as mentioned above, there are strong epidemiological links between T2DM and HCC. Insulin has a pivotal role in cell metabolism and it exerts its metabolic effects via the PI3K/AKT/mTOR pathway among others. PI3K signalling is inhibited by PTEN, which has been consistently reported as a tumor suppressor gene in human cancer. In HCC, close to 50% of cases have PTEN loss, but not due to inactivating mutations as is the case in other solid tumors. Data from animal models implicates PTEN loss in NASH and HCC. Similarly, PI3K transgenic mice also develop HCC in the context of NASH. AKT was also found activated in PDGFR transgenic mice, which developed HCC and NASH in the absence of significant fibrosis.

The peroxisome proliferation-activated receptors (PPARs) have also been implicated in the pathogenesis of NAFLD/NASH-HCC [7]. PPARs are a family of ligand-activated transcription factors with a central role in regulation of glucose and lipid homeostasis. They participate in inflammation, cell survival and differentiation through release of HDAC co-repressors. In mice, chronic exposure to PPARαagonists induces liver tumors via let-7. This miRNA is significantly repressed in HCC and modulates MYC expression. There are also experimental links between decrease activity of the farsenoid X receptor (FXR) and HCC [8]. FXR is a transcription factor that participates in metabolic homeostasis and inflammation, and there is data showing decreased FXR expression in NAFLD patients. Interestingly, liver tumors in FXR deficient mice show aberrant WNT signaling in the context of chronic liver inflammation and fibrosis.

Autophagy has also emerged as an important cellular module in carcinogenesis. It is involved in the elimination of damaged cellular components through lysosomal degradation and it is capable of inducing programmed cell death. There is evidence that links global inhibition of autophagy in the liver to steatosis, which is supported by data on impaired autophagy in experimental models of HCC. Theoretically, restoration of autophagy could be beneficial to counteract the pro-tumorigenic effects of NASH [9].

3. Oxidative stress: Fat accumulation in hepatocytes induces the generation of ROS that can interfere with mitochondrial function, induce ER stress and generate genotoxins such as lipid peroxides. Damaged DNA within hepatocytes is a potential trigger of malignant transformation and the expansion of the progenitor cell compartment. Numerous studies implicate methionine metabolism and s-adenosylmethionine (SAM) in HCC development [10]. *MAT1A*-deficient mice develop progressive NAFLD and HCC. These mice have a marked reduction in SAM levels and increased lipid peroxides in the serum, consistent with increased oxidative stress. Interestingly, *GNMT* knockout mice exhibit high levels of hepatic SAM, and they also develop NAFLD and HCC. In this case, it seems that SAM-induced epigenetic disarray due to aberrant DNA methylation may significantly contribute to malignant transformation. A combination of global DNA hypomethylation in addition to generation of ROS and nitro-active species justify these pleiotropic effects reported for hepatic SAM levels.

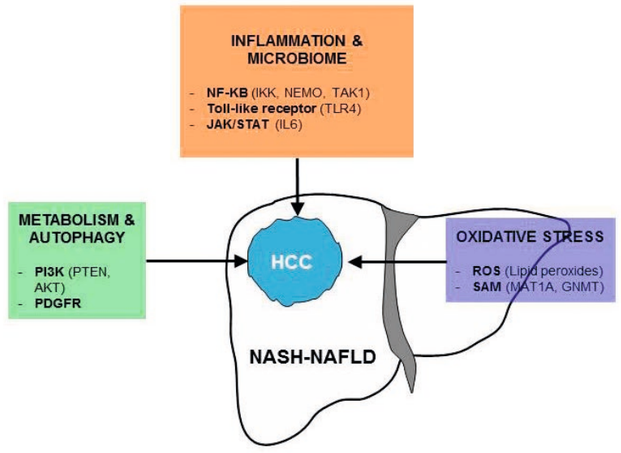

Figure 1. Summary of molecular pathogenesis of NAFLD/NASH HCC.

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LIVER CANCER IN NAFLD: MAGNITUDE OF THE PROBLEM

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Take home messages

- HCC in NAFLD is associated with cryptogenic cirrhosis, obesity and diabetes.
- HCC in NAFLD occurs often without cirrhosis.
- Cirrhotic patients with metabolic syndrome or NAFLD should undergo surveillance. In non-cirrhotic individuals, the risk of HCC development should be better stratified.
- The therapeutic algorithm used in HCC patients should be applied to patients with HCC due to NAFLD.
- The proportion of patients with HCC due to NAFLD is increasing.

HCC in NAFLD: association with cryptogenic cirrhosis, obesity and diabetes

HCC is one of the most serious complications of chronic liver disease. This is certainly the case for NAFLD. But HCC in the context of NAFLD has several specific features.

The predominant risk factor for HCC is cirrhosis, regardless of the underlying liver disease. The incidence of HCC has been determined for several etiologies. The incidence of HCC for hepatitis B-induced and hepatitis C-induced cirrhosis is estimated at 3-8%/year and 3-5%/year, respectively. In a prospective cohort of 68 NASH cirrhotic patients the 5-year HCC rate was 11%, which was 3 times lower than the 30% 5-year HCC rate observed in 69 HCV cirrhotics [1]. In a prospective US study the risk of development of HCC was similarly 2.5 fold lower in NASH cirrhotics than in HCV-cirrhotics [2].

A substantial fraction of patients with cryptogenic cirrhosis have in fact a 'burn-out NASH'. This is partly due to the fact the NASH-induced cirrhosis is not only an exclusion diagnosis (similarly to NASH which is an exclusion diagnosis), but also a default diagnosis since some of the histological features of NASH disappear when the disease reaches a cirrhotic stage. Patients with a cryptogenic cirrhosis and HCC present clinical characteristics associated with NAFLD, namely obesity and T2DM. Among 641 cases of cirrhosis-associated HCC, 44 patients with cryptogenic cirrhosis were retrospectively identified. These patients had a higher prevalence of obesity and T2DM [3]. In a French series of HCC patients undergoing surgical resection, 9% had cryptogenic cirrhosis. These patients had a higher prevalence of obesity compared to alcohol- or virus-related cirrhotics (50% *vs*. 17% *vs*. 14%, respectively) and a higher prevalence of diabetes (56% *vs*. 17% *vs*. 11%, respectively) (P<0.0001 for each) [4]. Patients with cryptogenic cirrhosis are at risk of developing HCC. In a French retrospective analysis, HCC was detected in 27% of obese patients with cryptogenic cirrhosis *vs.* 21% in hepatitis C induced cirrhosis, resulting in a similar age cumulated incidence [5].

Obesity and T2DM are both risk factors for HCC. Obesity increases mortality due to many types of cancer, but this is particularly the case for HCC. In obese individuals, the relative risk of death

from HCC is higher than for other types of cancer (e.g. 4.5 in men with a BMI of 35kg/m^2) [6]. The cumulative incidence of HCC is three times higher in patients with T2DM than in patients without T2DM [7]. There are strong pathophysiological mechanisms linking obesity, T2DM and NAFLD. It will be difficult and probably of limited clinical relevance to disentangle these three conditions to estimate their independent roles as risk factors for HCC.

HCC in NAFLD: often without cirrhosis

Beside a higher prevalence of obesity and T2DM, NAFLD patients with HCC have a lower prevalence of cirrhosis than patients with HCC and another underlying liver disease. This is an important characteristic of HCC in NAFLD which has been reported in multiple publications. In a comparison of HCC in patients with MetS and HCC in patients with chronic liver disease, the background liver was significantly more often free of significant fibrosis (F0-F2) in the former group than in the latter (65% *vs.* 26%, respectively, $P<0.001$) [8]. A study of NASH patients with HCC found that 39% and 70% of males and females, respectively, had cirrhosis [9]. In another study the proportion of cirrhotic patients was only 63% in the 596 NAFLD patients with HCC, compared with 81% of 161 autoimmune hepatitis patients with HCC, 88% of 166 primary biliary cirrhosis-patients with HCC and 80% of 1,423 alcoholic liver disease patients with HCC [10]. In a US national cohort of 1,500 patients who developed HCC from Veterans Administration hospitals, cirrhosis was less common in NAFLD-related cases (58.3%) compared with alcoholic liver disease- or HCV-related HCCs [11].

HCC in NAFLD: implications for management

The incidence of HCC in NASH cirrhosis appears to be ~2%/year [1], which is above the 1.5% suggested cut-off for the implementation of surveillance [12]. When considering patients with advanced cirrhosis the five-year cumulative incidence of HCC is 7.6%, so just in this range, and in this population, HCC is the leading cause of death, emphasising the importance of surveillance [13]. However, a significantly higher percentage of patients with NAFLD-related HCC did not receive HCC surveillance in the three years before their HCC diagnosis, compared with alcohol- or HCV-associated HCC patients. Furthermore, 62% and 78% ($P<0.01$) of NAFLD-related HCC patients and HCV-related HCC patients, respectively, received HCC-specific treatment [11]. The fact that fewer patients with NAFLD-related HCC are enrolled in a surveillance programme might be explained by the following 3 factors: 1) as reviewed above, many of these patients do not have a cirrhosis, the stage which sets the implementation of surveillance; 2) ultrasonography, the screening test, can be difficult in NAFLD patients due to their obesity; however, no alternative strategy has been adequately tested; 3) co-morbidities or advanced age makes the discovery of HCC irrelevant. This last aspect also contributes to the fact that these patients receive less HCC-specific treatments.

Since many patients with NAFLD develop HCC before reaching a cirrhotic stage, one needs to better stratify the patients at risk. This strategy should only enrol in surveillance programmes pre-cirrhotic patients at risk. Presently, there are no parameters permitting the implementation of such a strategy. It is possible it will include genetic testing, such as determination of the *PNPLA3* polymorphism. Genotype frequencies were significantly different between 100 NAFLD-HCC cases and 275 NAFLD-controls (P=0.0001), with enrichment of the rs738409 minor (G) allele. Carriage of each copy of the allele conferred an additive risk for HCC, with GG homozygotes exhibiting a 5-fold increased risk over CC. When compared to the UK general population the risk-effect was even more pronounced (GG *vs.* CC: OR, 12.19) [14].

The therapeutic options for patients with NAFLD are the same as those for patients with other underlying liver disease [15]. Resection might be more frequently considered since these patients are often not cirrhotic. Comorbidities have to be taken into account, particularly CVD and liver steatosis, which negatively impact liver regeneration. Sorafenib is the only indicated drug for HCC patients who are eligible for systemic, targeted therapy. There is currently no evidence that NAFLD-patients with HCC may benefit from such therapy. In terms of prevention, several studies have suggested that metformin lowers the risk of HCC in T2DM patients [16].

HCC in NFALD: temporal trends

With better HCV treatment it is clear that HCV-induced HCC will decline in the coming years. Roughly 8% of HCC patients have NAFLD as an underlying risk factor [11], but there is significant geographical variation in this proportion. Temporal trends also exist. HCC associated with NAFLD accounted for 35% of cases in an English referral liver unit in 2010, representing a >10 fold increase from 2000 [17]. The number of patients undergoing LT for HCC secondary to NASH increased by nearly four-fold from 2002 to 2012 (United Network for Organ Sharing registry), more than any other indication for liver transplantation in patients with HCC [18].

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DO INFLAMMATION NETWORKS TRIGGER NASH AND DRIVE ITS PROGRESSION?

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Take home messages

- Innate immunity is crucially involved in NASH pathogenesis.
- Dysregulated microbiota could reflect a starting point in the initiation of inflammation and activate innate immune signals.
- Extrahepatic tissues, such as adipose tissue, reflect a major source of inflammatory mediators.
- Inflammatory networks drive disease progression, i.e. from steatosis to fibrosis and cancer.

Introduction

NAFLD has emerged as a major health problem throughout the world. Whereas over-nutrition and obesity are crucially involved in the development of a simple fatty liver, it remains unclear why approximately 10% of all affected individuals develop the 'inflammatory' phenotype, i.e. NASH. It is increasingly recognized that soluble mediators synthesized by immune system cells (e.g. cytokines/chemokines) and adipose tissue (e.g. adipocytokines) are involved in NAFLD and its progression and also in the regulation of insulin action [1]. It has long been assumed that major triggers for the observed liver inflammation in cases of NASH might reside in the gastrointestinal tract and a close link between the intestinal microbiota and host metabolism has only recently been suggested [2]. The microbiota affects metabolic processes such as energy extraction from food, and is currently believed to contribute significantly to diseases such as obesity, T2DM, CVD and NAFLD.

Cytokines: key players in NASH

Cytokines are critically involved in the physiology of a healthy liver as well as in the pathophysiology of many acute and chronic liver diseases (**Table 1**). Production of cytokines such as IL-6 and TNFα is one of the earliest events in many types of liver injury. TNFα was the first adipocytokine found to be associated with obesity and IR. Mice lacking TNFα or the TNF receptor had improved IS in both dietary and genetic models of obesity [3]. TNFα is able to mediate many aspects of NASH. Importantly, weight loss has been demonstrated to result in a marked suppression of TNFα in the adipose tissue. Expression of TNFα and its type 1 receptor is increased in patients with NASH compared to patients with simple steatosis [4]. Small intestine bacterial overgrowth in NASH patients is associated with increased circulating TNFα levels [5]. Furthermore, certain TNFα polymorphisms are associated with susceptibility to IR, highlighting the importance of this cytokine in the interaction between fat accumulation, insulin action and inflammation in humans. An important role for TNFα is also supported by the fact that in a murine model of steatohepatitis antibody-mediated neutralization of TNFα improves liver disease [6]. Therefore, substantial evidence exists that pro-inflammatory cytokines such as TNFα are involved in the development of NASH. A link between the microbiota and development of obesity and its metabolic consequences, including NAFLD, is also becoming clearer. Clinical, but especially experimental, studies suggest that microbiotal factors are driving forces of hepatic steatosis and inflammation, involving certain toll-like receptors and induction of pro-inflammatory cytokines such as TNFα.

Innate immune signals: crucial in NASH pathogenesis

The MetS is commonly observed in obesity and is thought to develop through the interaction of various genetic and environmental factors. A complex and still poorly characterized interaction between the intestinal microbiota and the innate system may be involved in metabolic dysfunction. MetS, diabetes and obesity are characterized by low-grade inflammation, and adipocytokines play a central role. A profound effect of the pattern recognition receptor TLR5 (activated by bacterial flagellin) on structural microbial composition and its consequences for the pathogenesis of the MetS has recently been demonstrated [7]. Mice lacking the TLR5 receptor exhibit hyperphagia and develop a MetS with hyperlipidemia, hypertension, IR and obesity. Metabolic changes in TLR5 deficient mice resulted in abnormalities of the intestinal microbiota. Transfer of this altered microbiota TLR5 deficient mice into gnotobiotic mice led to MetS. These data provide not only experimental evidence that innate immune signalling is critical in the development of MetS and a fatty liver, but also suggest that alterations in the intestinal microbiota can be sufficient to induce the MetS and probably, crucially, drive the evolution of inflammation in NASH.

Table 1. Mediators of immune cells and adipocytes involved in NASH.

Cytokines and chemokines	Adipocytokines	Transcription factors	Others
TNF-α	Adiponectin	NF-kB / IKKß	Osteopontin
IL-1α/ß	Leptin	JNK-1	SAA
Gp130 family (IL-6, CNTF)	Resistin	PPARγ	CRP
IL-10	PBEF / Nampt / Visfatin	SREBP-1c	FABP-4
IL-18	RBP-4	LXR	Oxidative stress
MCP-1	IL-37	FXR	ER stress
MIP-1α/ß			iNOS
RANTES			Selectins
			ICAM-1
			VCAM-1
			TLR-4/5

Key to abbreviations: CNTF, ciliary neurotrophic factor; FABP, fatty acid binding protein; ICAM, intercellular adhesion molecule; iNOS, inducible nitric oxide synthase; LXR, liver X receptor; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RBP, retinol binding protein; SAA, serum amyloid A; VCAM, vascular cell adhesion molecule. See glossary for other definitions.

Extrahepatic tissues as major sources of inflammatory mediators: key role for the adipose tissue

The adipose tissue has been shown in many studies to reflect a major source of inflammatory mediators in cases of obesity and related disorders. In severe human obesity visceral adipose tissue in particular, but also subcutaneous adipose tissue constitute important sources of pro-inflammatory cytokines such as IL-1β,, IL-6 or TNFα. Concentrations of all these mediators exceed their liver concentrations

dramatically, suggesting that in cases of NAFLD the adipose tissue is the major cytokine source. The adipose tissue, however, is also a major source of 'beneficial', i.e. anti-inflammatory, adipocytokines such as adiponectin, the concentrations of which are decreased in obesity. Interestingly, an increase in adipose tissue expression of another anti-inflammatory IL-1 family member, namely IL-37, has been observed after weight loss [8]. Changes in the expression pattern of IL-37 resemble those for adiponectin and its expression after weight loss was almost 500-times higher in adipose tissue *vs.* liver tissue. Excessive weight loss, as achieved after bariatric surgery, dramatically reduces expression of IL-1 family members, (i.e. IL-6 and TNFα) in adipose and liver tissue, thereby potentially contributing to the improvement of IR and inflammation in NAFLD patients.

Conclusion

The concept that the intestinal microbiota plays a role in NAFLD/NASH is now strongly supported by many preclinical studies and has many attractions as it could explain very diverse aspects of these diseases. A dysbiosis could reflect an early event in patients with NAFLD and result in activation of many innate immune processes. Cytokines have emerged as major players in obesity and obesity-related disorders. They direct and control low-grade inflammation which most likely contributes significantly to disease phenotypes associated with and observed in severe obesity. Many different organs are affected by obesity and associated metabolic inflammation, including the liver, pancreas, heart and blood vessels. Therefore, multiple parallel hits might contribute to the evolution of inflammation in NASH, and both the gastrointestinal tract and the adipose tissue might reflect two major players [9]. As fibrosis is driven in most cases by inflammatory events [10], targeting inflammatory pathways and their initiating events remains a key treatment strategy in NASH.

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IMPACT OF LIFESTYLE ON NASH (INCLUSIVE OF HCC)

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Take home messages

- At least 7% body weight loss appears necessary to significantly benefit the histological features of NASH.
- The Mediterranean dietary eating pattern may improve IS and reduce steatosis in NAFLD.
- Inclusion of aerobic and/or resistance exercise in a lifestyle intervention for weight loss is important to preserve muscle mass. However, the ideal prescription of exercise (type, frequency and intensity) to reduce NASH and target muscle IR is unknown.
- There have been no studies addressing the effect of lifestyle on recurrence of HCC.
- Emerging evidence suggests a possible anti-fibrotic effect from regular coffee drinking; however, prospective controlled studies to address the mechanisms and dose effect in NASH are yet to be undertaken.

Introduction

Lifestyle intervention is a broad term used to describe the application of any number of combinations of environmental, behavioral, and motivational principles to the management of lifestyle-related health problems in a clinical setting. In studies targeted to those with NAFLD, the majority have studied obese patients and used a combination of dietary energy restriction and increased exercise. A 5-7% weight loss generally leads to improvements in steatosis and glucose control [1]. A Cochrane review of weight loss in NAFLD highlighted that heterogeneity in diet and exercise prescription (e.g. dietary composition, type, frequency and intensity of exercise programmes), short term interventions and limited histological outcome all hamper the translation of research findings into clinical prescriptions [2]. So while weight reduction through lifestyle modifications is usually recommended as a first-line treatment for NASH, the effectiveness and optimal treatment approach are yet to be determined. Specific aspects of lifestyle interventions on features of NASH such as IR, liver injury, and development and recurrence of HCC will be summarized.

Effect of lifestyle intervention on IR in NASH

Whole body IR is a key element influencing the severity of NASH. IR is identifiable across multiple tissues in people with NASH including muscle, liver and adipose tissue, with IR at each site contributing to liver damage through detrimental effects on glucose metabolism or lipid delivery [3]. The design of lifestyle interventions needs to consider how components of the intervention (diet and exercise) may independently, or in combination, target the sites and severity of IR in NASH. It has been shown previously in obesity that daily aerobic exercise (without weight loss), or calorie restriction in isolation may be ineffective at reducing muscle IR. In contrast, identical doses of exercise combined with the energy deficit can produce clinically significant (up to 60%) improvement in muscle IS [4].

The metabolic benefit of exercise alone in NASH may not be equivalent across all insulin sensitive organs. After a 6 month intervention of moderate intensity circuit exercise training (without weight loss) there were improvements in hepatic and adipose tissue IS but no effect on muscle IR [5]. Most participants were profoundly insulin resistant in their muscles at baseline, remaining one third that of healthy controls after the intervention. On the other hand, hepatic IR seems to particularly benefit from exercise irrespective of obesity, steatosis or weight change. This can occur after high intensity exercise without changes in peripheral IS [6].

The type and intensity of exercise prescription in the absence of weight loss still requires further refinement to determine which prescription will best target the severe IR associated with NASH. Given that muscle IR is likely the primary driver of progression to T2DM in this group [7], further research into the independent and combined effect of lifestyle factors on muscle metabolism is of great clinical importance.

Effect of lifestyle intervention on liver injury in NASH

Weight loss through combined diet and exercise. A randomized controlled study of a combined diet and exercise weight loss intervention demonstrated that weight reduction was positively correlated with improvement in disease severity as assessed histologically by the NAS, with improvements in steatosis seen with minimal decreases in weight. However, substantial improvements in necro-inflammation and hepatocyte ballooning was only seen in those with >7% weight loss. Of note, this histological improvement occurred without an apparent improvement in overall IR (measured indirectly by HOMA score) [8].

Exercise alone. In the first study to assess the histological effects of exercise alone on NASH, moderate intensity circuit exercise x3/week for 6 months was inadequate to improve steatosis, lobular inflammation or hepatocyte ballooning in NASH [5]. Furthermore, four months of moderate physical activity in line with general physical activity guidelines did not benefit hepatic lipoprotein kinetics in obese people with NAFLD [9]. These data contrast with other studies of exercise without weight loss that have used magnetic resonance spectroscopy to measure hepatic fat content. A number of studies have shown short term low to moderate intensity aerobic or resistance training without weight loss can result in measurable decreases in visceral adiposity and intrahepatic triglyceride content [10, 11]. The long term histological effect of these real, albeit, small changes in steatosis is of great interest.

Exercise intensity may be another important variable, with cross-sectional data suggesting an apparent protective effect of vigorous activity on the development of NASH [12]. The effects of high intensity exercise on established NASH are unknown.

The Mediterranean diet. Traditional/indigenous eating patterns may benefit obesity-related chronic diseases irrespective of changes in BMI and there has been considerable interest in the beneficial role of the Mediterranean diet on the development and progression of NASH. There is no single defined prescription of the Mediterranean diet, but rather it is a recommended eating pattern inspired by the traditional cuisines of Greece, Crete, southern France and parts of Italy. The emphasis is on plant foods such as fruits, vegetables, nuts, beans, legumes, seeds and grains, and olive oil, with moderate consumption of fish, low to moderate consumption of dairy products, predominantly as cheese and yoghurt, moderate consumption of wine (1-2 glasses per day) and low consumption of red meat and meat products.

The Mediterranean diet score (MDS) is commonly used to assess adherence to this pattern of eating and is based on the 9 dietary components typical of the traditional Mediterranean diet [13]. While there are a number of variations to the scoring system that have been developed, briefly, each study subject is assigned a score of 0 (low consumption) or 1 (high consumption) for each of the eight dietary components (excluding alcohol) relative to a study-specific and sex-specific median cut-off calculated from controls. For the alcohol component, a score of 1 is typically assigned for moderate alcohol consumption (defined for males as 2 drinks/day and for females as 1 drink/ day) and 0 for alcohol consumption above or below these values. Thus the MDS ranges from 0 (lowest adherence) to 9 (highest adherence). It is important to note that in large population studies, individuals who follow a Mediterranean diet are also more likely to engage in vigorous physical activity, have lower overall calorie intake, and are less likely to be current smokers or perform heavy activities at work [14].

In a recent cross sectional study of people with NAFLD, adherence to a Mediterranean dietary pattern was associated with less severe IR and liver disease [15]. There was an inverse correlation between Mediterranean diet score and steatosis, fibrosis and liver stiffness. Those with NASH had significantly lower adherence to the Mediterranean dietary pattern compared to those with fatty liver alone [15]. The Mediterranean diet has clear anti-inflammatory and anti-oxidant effects that could partly explain the protective effects against NASH.

In a prospective randomized cross over intervention study, 12 participants consumed a Mediterranean diet or a control diet (low fat, high carbohydrate) for 6 weeks [16]. They were permitted to drink up to two standard alcoholic drinks per day on up to 5 days per week. The Mediterranean diet did not result in weight loss but did result in a 39% relative reduction in steatosis, with significantly greater improvements in IR and fasting insulin than the control diet. This is a small study, but provides promising evidence that manipulations of dietary composition, in the absence of weight loss, may yield important therapeutic benefits.

Sugar sweetened beverages. There are plausible hypotheses for how fructose may contribute to the severity of NAFLD. Cross-sectional associations between fructose consumed in sweetened beverages and liver fibrosis have been described [17] and consuming soft drinks daily for 6 months can increase liver fat by 140% in healthy people [18], but a casual relationship with NASH remains unclear and is complicated by the independent contribution of obesity and overall energy intake. High fructose consumption in obese adolescents increases the likelihood of developing NAFLD, while this relationship is not seen in lean adolescents with similarly high fructose intake [19].

Sleep duration and quality. Monitoring and assessing sleep behaviors may be an important component of lifestyle intervention for people with NAFLD. There is mounting evidence that poor sleep quality and short sleep duration is associated with metabolic disturbances, such as IR, inflammation and obesity [20]. Cross-sectional population data has identified that short sleep duration (≤5 hours per night) and poor sleep quality (self-assessed by questionnaire) is associated with a greater risk for NAFLD compared to people who regularly sleep >7 hours per night or who report good quality sleep, respectively [21]. The presence of more severe sleep dysfunction, such as obstructive sleep apnoea syndrome (OSAS) has been associated with an increased prevalence of NAFLD. In people with NAFLD, co-morbid OSAS is associated with a two-fold greater risk for more severe disease such as NASH and advanced fibrosis, independent of age, gender and obesity [22]. There is limited data on the therapeutic potential of OSAS treatment for improving features of NASH, however, general inquiry about symptoms of OSAS and administering quick sleep questionnaires (e.g. Epworth Sleepiness Scale) when discussing lifestyle behaviours may be warranted [20].

Lifestyle intervention and cancer

One of the most worrying recent trends in patients with NAFLD is the increasing number of patients being seen with HCC, including HCC developing in non-cirrhotic patients with NAFLD. Obesity has been shown to be a risk factor for developing a range of cancers and may impact on HCC recurrence or progression. Thus a new dimension of lifestyle intervention in NAFLD relates to advice regarding HCC. Various organizations have issued dietary and physical activity guidelines for cancer survivors but all point to the lack of data after curative cancer therapies and are deemed consensus statements to inform clinical practice rather than evidence based guidelines [23-25]. The guidelines for weight loss in overweight or obese cancer survivors (based mainly on studies of breast and prostate cancer) are broad, with recommendations to achieve and maintain a healthy weight, increase plant based foods, and reduce

intake of red meat, processed meats and salt. Physical activity recommendations in these guidelines mirror those for a healthy population, generally 30 minutes of moderate to vigorous exercise at least five days per week.

While there have been no randomized controlled studies investigating the effect of lifestyle intervention after curative therapy of HCC, there are a number on weight loss interventions among overweight survivors of other cancers [26]. None have examined effects on cancer-related death or recurrence. In general, the data from these studies suggest that physical activity interventions are safe and yield improvements in fitness, strength and physical function; whereas diet interventions improve diet quality, nutrition-related biomarkers and body weight. Lifestyle intervention may mitigate adverse changes in body composition associated with cancer, such as decreased muscle mass in the setting of obesity (sarcopenic obesity). Most patients with HCC will be cirrhotic and this has implications for dietary manipulations with regards to calorie restriction, protein intake and malnutrition.

Lifestyle Intervention and HCC

Obesity, body composition and HCC risk. Obesity is associated with diabetes, steatosis, hepatic inflammation and increased oxidative stress, all of which may increase the risk of advanced fibrosis and cirrhosis. Although several studies have investigated a link between obesity and liver carcinogenesis, there is a paucity of data on whether obesity (with or without cirrhosis) is a prognostic factor in patients with NASH-related HCC who undergo curative therapy. A retrospective observational study from Japan found that obese patients (defined in that population as BMI>25) were no different to non-obese in relation to overall survival or recurrence-free survival from non-viral HCC [27].

The relationship between obesity and HCC survival is complex and more sophisticated assessment of body composition is needed to fully elucidate a link between obesity and NASH-related HCC recurrence. In addition to the proportion of overall adiposity and muscle mass, the presence or absence of cirrhosis at the time of, and after curative therapy, may significantly impact metabolism. Both aerobic and resistance exercise seems to be effective in protecting against muscle loss experienced during dieting and should be included as part of any recommendations to reduce weight after HCC curative therapy. There is also a suggestion that supplementing protein during calorie restriction may lead to reduced fat mass with preservation of muscle tissue in those at risk of sarcopenia [28]. In the absence of studies specifically targeting NASH-related HCC populations, a lifestyle intervention that combines dietary calorie restriction with exercise in order to reduce adiposity is likely to improve NASH and associated necro-inflammation. However, specific advice regarding the dose, frequency and type of exercise and weight loss prescription to reduce HCC risk in NAFLD is unknown.

Dietary patterns and HCC risk. The Mediterranean dietary pattern has been inversely related to cancer risk and the potential beneficial effects of this traditional cuisine on HCC risk is a field of growing interest. A recent study that combined two large HCC case-control datasets from Italy and Greece (HCC from all causes n=518; control n=772) demonstrated that adherence to the Mediterranean diet (defined as MDS ≥5) was associated with a 50% reduction in HCC incidence compared with MDS ≤3 [29]. The presence of cirrhosis was not included in the modelling. No individual component of the diet (such as vegetables, legumes, fish and seafood) was significantly associated with HCC risk. This suggests biological interactions between various components of the Mediterranean dietary pattern may be important. In a large dataset of 495,006 men and women participating in the NIH-AARP Diet and Health Study, it was found that red meat and saturated fat (both typically low in a Mediterranean dietary pattern) were associated with a statistically significant increased risk of HCC, while white meat appeared protective [30].

To date, epidemiological studies have used incidence of HCC from all causes and have not controlled for the influence of cirrhosis or viral hepatitis (which could account for up to 75% of cases) [29]. As non-viral HCC cases are typically rare in epidemiological datasets, it can prevent reliable risk estimates in this group. With increasing incidence of NASH-related HCC in the future, it is likely that this field of investigation will become more robust as the statistical power grows.

Coffee and HCC. Retrospective, cross-sectional studies have suggested that coffee drinking has numerous health benefits in a variety of disease states. In a recent review of cross sectional and case control studies in chronic liver disease, regular coffee consumption was associated with a lower risk of progression to cirrhosis, lower mortality rate in cirrhotics and lower rate of HCC development with an inverse association between coffee consumption and severity of NASH [31]. When analysis is restricted to non-viral cirrhosis only, the link between coffee drinking and protection from cirrhotic mortality is maintained [32]. However, in a recent small prospective observational study, the incidence of NAFLD was not related to coffee consumption [33]. Rather it was in those with established NAFLD (determined by ultrasound) that higher coffee consumption (≥3 cups per day) was associated with lower likelihood of significant fibrosis (assessed by FibroTest).

There are a number of difficulties in interpreting studies regarding health benefits of coffee drinking. Coffee is composed of >100 compounds, any of which may synergistically contribute to 'hepatoprotective' health benefits. Coffee is consumed in many different forms, e.g. filtered and unfiltered, with wide-ranging polyphenol content depending on country of origin and processing, and there is no standardized cup size. Therefore, determining the dose or exposure to 'coffee' in populations across different countries and cuisines raises difficulties. Furthermore, high coffee consumption has been linked with a number of confounding variables such as higher rates of smoking, higher sugar consumption and lower physical activity [33]. While the emerging evidence suggests a role for coffee drinking as a beneficial health behaviour in people with liver disease, blinded randomized controlled trials are needed to provide evidence for caution and/or treatment effects in patients with NASH and NASH-related HCC.

Recommendations

Physicians are encouraged to recommend lifestyle changes for patients after curative therapy for HCC on the basis of beneficial health outcomes, such as reduced steatosis, improved body composition, fitness and quality of life. However, future research is needed to inform dosing, the magnitude of effects that can be expected and the assessment of the impact of these measures on cancer recurrence and cancer related death [23].

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WHO ARE THE NAFLD PATIENTS AT RISK OF DISEASE PROGRESSION?

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Take home messages

- The presence and severity of fibrosis on liver biopsy is currently the best indicator of long-term liver outcome in patients with NAFLD, although non-invasive markers of fibrosis may prove to be as good.
- Patients with mild/moderate steatosis in the absence of any inflammation can be assumed to have a very low risk of developing fibrosis over 15-20 years. For the majority of the others fibrosis progression will be slow progressing at around 1 stage every 6-15 years.
- 1 in 5 progressors will progress more rapidly with the presence of hypertension and possibly diabetes at presentation, the factors most consistently associated with progression risk.
- A low platelet count and high FIB-4 score hold the most promise for risk stratification.
- Studies on the ability of genetic and other factors to predict the risk of disease progression are awaited with interest.

Introduction

Over the last 15 years a wealth of data has emerged on the natural history of NAFLD, addressing both the clinical course of the disease and disease progression assessed through repeat liver biopsies. This chapter will cover both types of study, focusing particularly on factors that predict disease progression, which may assist in patient risk stratification and management. NAFLD associated extra-hepatic morbidity and mortality, HCC and the course of NAFLD cirrhosis will not be discussed in any detail as they are covered elsewhere.

Long-term mortality in patients with NAFLD

Studies that have examined the overall, long-term mortality of patients with the whole spectrum of NAFLD have observed that, within 15 years of follow-up, patients with NAFLD have a 26% risk of dying, 34-69% higher than the general population of the same age and gender. In these studies, liver-related mortality was the third most common cause of death after CVD and extra-hepatic malignancy, occurring in <5% of patients [1]. Importantly, the long-term prognosis of patients with NAFLD depends to a large extent on disease stage. Thus, there is little doubt that the vast majority of patients who progress to end-stage liver disease and die a liver-related death present with, or progress to, advanced fibrosis and cirrhosis. Pooled data from long-term (~10 years) follow-up studies of NAFLD patients with advanced fibrosis and cirrhosis demonstrate a 16% mortality with 60% of the deaths liver-related compared with only ~9% liver-related in long-term (~15 years) follow-up studies of NAFLD patients without advanced fibrosis or cirrhosis [1]. Of note, cases of NAFLD-associated cirrhosis may be mis-diagnosed as the steatosis has often disappeared at this stage.

Studies in patients without advanced fibrosis/cirrhosis have also revealed that those with steatosis alone

('bland steatosis'), or with only mild inflammation or cellular injury, have no increase in overall or liver-related mortality compared with the age and gender matched general population. In contrast, patients with NASH exhibit a higher overall and liver-related mortality [2]. This is perhaps best explained by the greater propensity of patients with NASH either to have or to develop cirrhosis compared to those with simple steatosis. Therefore, in the long-term follow-up studies reported thus far, only 1% of patients with simple steatosis developed cirrhosis and died a liver-related death after a mean 15.6 years follow-up, compared with 11% of those with NASH having or developing cirrhosis, and 7.3% of those with NASH dying from a liver related cause after a similar period of follow-up.

Given these data, it would seem likely that the presence and severity of fibrosis on a NAFLD liver biopsy would be the most important histological determinant of long-term prognosis, with the difference between the prognosis of NASH and simple steatosis being due to the greater likelihood of fibrosis being present in patients with NASH compared to steatosis, rather than any adverse effect of NASH, *per se*, on prognosis. This concept is supported by a number of recent studies. A Swedish study of 118 patients with biopsy-confirmed NAFLD, followed for a median of 21 years [3], reported no difference in overall or liver-related mortality between those with definitive NASH and non-NASH (classified with the NASH CRN scoring system). In contrast, patients who died had a higher incidence of any stage of fibrosis (89%) compared with survivors (70%, $P<0.02$) and a greater incidence of fibrosis stage >2 (68% *vs.* 28%, $P<0.001$). A more recent study of 209 NAFLD patients with a median 12 years of follow-up showed the presence of NASH only correlated with liver mortality when fibrosis was included in its definition. Furthermore, when the individual histological features of NASH were analysed, only grade 3 portal fibrosis (which would include all patients with bridging fibrosis and cirrhosis) was independently associated with liver related mortality (HR 5.68, 95% CI 1.5-21.5) [4]. Further evidence of the prognostic significance of fibrosis comes from recent studies demonstrating that non-invasive scoring systems correlating with the degree of fibrosis are capable of predicting liver-related events, transplantation and death in patients with NAFLD [5].

Accepting that the presence and severity of fibrosis is the key factor determining long-term, liver-related mortality, the key question is which patients with NAFLD are most at risk of developing progressive fibrosis so they can be identified and managed appropriately? This is distinct from identifying those likely to have fibrosis at presentation, which is covered elsewhere, and starts with the premise that a histological diagnosis of NAFLD has already been made.

Histological predictors of fibrosis progression in patients with NAFLD

The best way to determine the risk of fibrosis progression in patients with biopsy-proven NAFLD is to perform repeat liver biopsies ideally after a long period of follow-up in the absence of treatment. These studies have recently been subjected to systematic review and meta-analysis [6]. This analysis identified 11 cohort studies including 411 patients with biopsy-proven NAFLD (150 with steatosis and 261 with NASH). Over 2,145.5 person-years of follow-up, 33.6% had fibrosis progression, 43.1% had stable fibrosis, and 22.3% had an improvement in fibrosis stage. The annual fibrosis progression rate in patients with steatosis who had stage 0 fibrosis at baseline was 0.07 stages (95% CI 0.02–0.11 stages), compared with 0.14 stages in patients with NASH who had stage 0 fibrosis at baseline (95% CI 0.07–0.21 stages). These findings correspond to one stage of progression over 14.3 years for patients with NAFL (95% CI 9.1–50.0 y) and 7.1 years for patients with NASH (95% CI 4.8–14.3 y) (**Figs. 1A and 1B**). When patients with stages 0 and 1 fibrosis were grouped together there was no difference between the annual progression rates in NAFL *vs*. NASH patients (0.09 stages, 95% CI 0.04–0.14, *vs.* 0.10 stages, 95% CI 0.03–0.17, respectively).

Interestingly, the proportion of fibrosis progressors who progressed from stage 0 to stage 3 or 4 fibrosis ('rapid progressors') was identical in the two histological sub-groups (17% of steatosis patients and 18% of NASH patients). Moreover, there was no association between the severity of necroinflammation and risk of progressive fibrosis in the four studies that examined it. The fibrosis progression rate did not appear to depend on the initial stage of fibrosis. Importantly, in these studies, while 90% of the steatosis patients had stage 0 or 1 fibrosis and 10% stage 2, 21% of the NASH patients had stage 2 fibrosis and 18% stage 3 and 4. This is consistent with the notion that the higher liver-related mortality in NASH *vs*. steatosis observed in some, although not all, studies reflects the higher degree of fibrosis in the NASH patients.

This analysis is supported by a more recent single centre study with 108 NAFLD patients undergoing repeat liver biopsy at a median interval of 6.6 years (range 1.3-22.6 years) [7]. The mean annual rate of fibrosis progression was 0.08 ± 0.25 stages. No difference in the proportion exhibiting fibrosis progression was found between patients with steatosis or NASH at index biopsy (37% *vs*. 43%) although all steatosis patients developing fibrosis had also developed NASH on follow-up biopsy. The NASH patients had more fibrosis at baseline than the steatosis patients, as was the case in the studies included in the meta-analysis. Interestingly, similar to an observation reported from a recent systematic review [8], steatosis patients with mild inflammation were more likely to have fibrosis progression than those with bland steatosis (60% *vs*. 24%, $P=0.07$). This finding was consistent with two of the four previous studies to have examined it [2, 9]. What's more, the bland steatosis patients who progressed had higher steatosis scores than those who didn't (2, range 2-3, *vs*. 1, range 1-2, respectively, $P=0.01$). Given these two observations, steatosis patients developing fibrosis unsurprisingly had significantly higher baseline NAS than those who did not progress (2.5, range 2-3, *vs*. 1, range 1-4, respectively, $P=0.007$).

Considering these studies together, it appears that fibrosis progression in NAFLD is generally slow, taking around eight years to progress from stage 0 to stage 1 fibrosis, although, as in other liver diseases, there is a subgroup of 'rapid progressors' who can progress 3-4 stages within 2-6 years. There appears to be no great difference in the risk of progression according to the presence or absence of NASH on baseline histology. Some evidence suggests that the lowest risk of progression is seen in patients with mild/moderate steatosis in the absence of inflammation. Given the evidence of similar rates of fibrosis progression in NASH and steatosis, it seems likely that the higher stages of fibrosis seen in patients with NASH compared to those with steatosis reflect a longer disease duration. Supporting this is the most recent study [7] where patients with NASH were nine years older than those with steatosis. In this study, 44% of the steatosis patients developed NASH after a median eight years follow-up, suggesting that NASH usually develops after steatosis. Age is a well-recognized risk factor for advanced fibrosis in multiple cross-sectional studies. These data also suggest that mechanisms related to the development of, or resulting from, the classical NASH lesions of ballooning degeneration and lobular inflammation may not be that important for fibrogenesis in NAFLD, questioning the current focus of clinical trials on resolving NASH rather than reducing fibrosis.

Figure IA

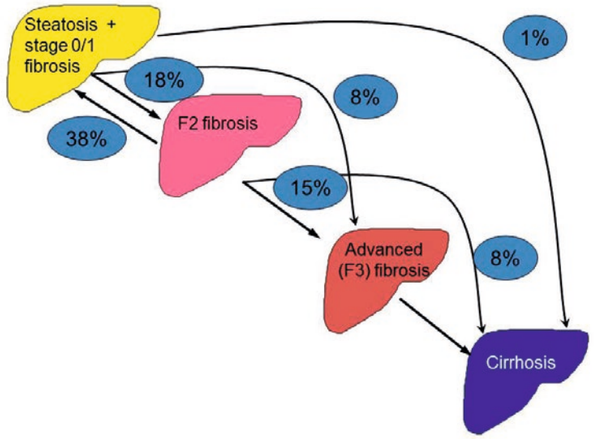

Figure IB

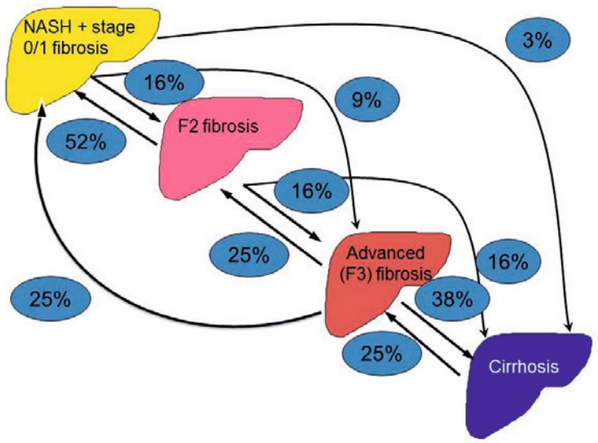

Figure 1. Fibrosis progression in patients with NAFL (1A) and NASH (1B). *Taken from data in Singh et al. [6].*

Non-histological predictors of fibrosis progression in patients with NAFLD

Many of the paired biopsy studies discussed above have also examined whether any non-histological, clinical or biochemical features can help in risk stratification of NAFLD patients into fibrosis progressors *vs*. non-progressors. In the meta-analysis of 11 cohort studies [6] the presence of hypertension (OR 1.94, 95% CI 1.00–3.74) and a low AST/ALT ratio at the time of baseline biopsy was associated with the progression of fibrosis. Features identified in some but not other studies (and therefore not significant in the meta-analysis) include age, BMI, T2DM or MetS and HOMA-IR. In the most recent study [7], fibrosis progressors had a significantly lower platelet count (P=0.04), and higher AST/ALT ratio (P=0.04) and FIB-4 score (P=0.02) than non-progressors and a non-significantly higher prevalence of T2DM (53% *vs*. 43%). At the time of follow-up biopsy, platelet count remained lower (P=0.0001) and AST/ALT ratio (P=0.01) and FIB-4 (P=0.001) remained higher in progressors than in non-progressors. NAFLD fibrosis score (P=0.001) and prevalence of T2DM was also higher in progressors.

Potential predictors of fibrosis progression in patients with NAFLD

Many other factors have been associated with susceptibility to advanced NAFLD and HCC, including genetic factors, dietary factors, caffeine and alcohol intake, gut microbiome and obstructive sleep apnoea [10]. These associations have been made exclusively by comparing the severity of NAFLD in patients with and without the particular factor, supported in most instances by biological plausibility of the association derived from animal models or other mechanistic studies. None have been studied in relation to their ability to predict the progression of NAFLD in patients with early stage disease. Although one might predict that factors associated with advanced disease in cross-sectional studies may well predict an adverse long-term outcome, this will almost certainly depend on the presumed duration of the disease. For example, if a 30 year old man with recent onset obesity presents with early stage NAFLD and has genetic and dietary factors associated with advanced disease, he is probably at higher risk of developing advanced disease than someone without these factors. Conversely, if he presents with mild disease 30 years later, still with the risk factors present, he is probably at an extremely low risk of developing advanced disease as he presumably has some protective factor(s), as yet unidentified, preventing him from developing advanced disease. Nonetheless, natural history studies including these risk factors are awaited with interest because better ways of identifying the minority of patients at risk of advanced disease are clearly needed.

Conclusions

The presence and severity of fibrosis on liver biopsy is currently the best indicator of long-term liver outcome in patients with NAFLD, although non-invasive markers of fibrosis may prove to be as good if recent results can be confirmed. At present, patients with mild/moderate steatosis in the absence of any inflammation can be assumed to have a very low risk of developing fibrosis over 15-20 years. For the majority of the others, fibrosis progression will be slow progressing at around 1 stage every 6-15 years. Just under one in five progressors will progress more rapidly, with the presence of hypertension and possibly diabetes at presentation, the factors most consistently associated with progression risk. Of the available laboratory tests, a low platelet count and high FIB-4 score hold the most promise for risk stratification. Although in need of further study, a persistently low or falling platelet count, a raised/rising FIB-4 score and new onset T2DM during follow-up may indicate the development of progressive disease. Studies on the ability of genetic and other factors to predict the risk of disease progression are awaited with interest given the urgent need to identify 'rapid progressors' for therapeutic trials and other interventions.

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NAFLD DIABETES AND ALCOHOL: IS THERE A SAFE THRESHOLD?

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Take home message

- Is alcohol a good guy or a bad guy? It depends on the dose!

Introduction

The effect of alcohol on health depends on daily intake, the means of drinking and the type of alcoholic beverage consumed. The dose-response relationship between alcohol consumption and all-cause mortality follows a J- or U-shaped curve, which suggests that all-cause mortality is reduced among those with light to moderate alcohol intake compared to those with high consumption. This effect is mainly due to a reduction in CVD, attributed to a beneficial effect on plasma lipid levels, decreased risk of thrombosis and prevention of MetS. Moreover, moderate alcohol consumption is a protective factor toward the risk of NAFLD development and it seems to enhance IS, leading to a decreased risk of MetS.

Alcohol and NAFLD

Our group was first in identifying a safe threshold level of alcohol consumption for chronic liver disease in the general population [1]. This threshold level of 30 g of alcohol/day (i.e. 3 drinks/day) is very close to that conventionally adopted (<20 g/day) to distinguish NAFLD from AFLD. Individuals who consume more than 30-50 g/d of alcohol for more than 5-10 years have a significantly higher risk of developing ALD. Not all these drinkers, however, develop liver disease. Indeed, host- and environment-related factors promote the development of ALD [2]. For example, genetic polymorphisms of alcohol dehydrogenase and their interaction with genes involved in the generation and scavenging of free radicals influence susceptibility to ALD [2]. Obesity, diabetes, IR, MetS, NAFLD, as well as chronic HCV infection increase the deleterious effects of alcohol on the liver.

NAFLD may evolve in 4-5% of the cases in NASH (although these two conditions may be unrelated according to recent views), and patients with NASH are at a high risk of cirrhosis and HCC related mortality. Patients with NAFLD are at increased risk of CVD or CHD. Indeed, they are twice more likely to die from CVD than from liver diseases. Evaluation of cardiovascular risk and management of CHD risk factors is, therefore, mandatory in these patients. As well as being associated with reduced CVD morbidity and mortality, moderate alcohol consumption also improves metabolic risk factors related to both CVD and NAFLD and is partially protective against NASH and NAFLD. Interestingly, a recent study suggests that even somewhat excessive alcohol consumption (>280 g/week, i.e. >4 drinks/day), especially in men, is likely to reduce the development of NAFLD over time [3]. Moreover, preliminary evidence suggests that the benefits of alcohol on CVD observed in the general population may extend to individuals with established NAFLD. Indeed, modest alcohol consumption has been shown to have an inverse association with carotid artery plaques and stenosis, independently from age, smoking and MetS, in men with NAFLD [4]. According to these studies, patients with NAFLD who drink no more than two to three drinks per day could perhaps be allowed to continue their drinking habits.

However, this issue is still not completely resolved. Serious concerns exist about the possible synergism between metabolic factors and alcohol in increasing the risk of HCC [5]. Another important consideration is that alcohol consumption may worsen both hypertriglyceridemia and hyperuricemia, which may be a concern in patients with NAFLD. Comparative analysis of guidelines has led to the conclusion that heavy alcohol consumption should be discouraged; meanwhile light to moderate alcohol consumption may have favourable effects on lipid metabolism and, perhaps, on liver outcomes. However, in the absence of randomized controlled trials, all guidelines advise against prescribing low to moderate alcohol consumption as a preventative / therapeutic strategy for NAFLD [6].

Alcohol and T2DM

Alcohol consumption has always been considered a risk factor for the development of T2DM. However, recent findings suggest that moderate alcohol intake might actually be associated with reduced incidence of T2DM and its vascular complications. A meta-analysis of 15 studies on 369,862 patients, followed up for an average of 12 years, concluded that subjects who consumed 6-48 g/day of ethanol exhibited a 30% reduced risk of T2DM as compared with teetotallers or consumers of >48 g/day. This effect is independent of either sex or BMI, although a lower incidence of T2DM was observed in women with a moderate alcohol intake compared with abstainers [7]. There is some evidence that moderate alcohol intake also protects against T2DM development in men [8]. Indeed, consumption of 1-3 alcoholic drinks/day was inversely related to T2DM risk (HR 0.80, 95%CI 0.67-0.94) among middle-aged and elderly Chinese men [9]. Several studies suggest that the beneficial effect of alcohol on T2DM development may be at least in part mediated by body fat distribution in the European population [10].

Current guidelines specific to diabetic patients are rather cautious because of the calories and body-weight issues, as well as some untoward metabolic and vascular effects of alcohol. In brief, such guidelines indicate that if a diabetic adult chooses to drink alcohol, his/her daily intake should be kept consistently modest (for women 10-13 g/d, i.e. one drink/day or less, and for men 20-25 g/d, i.e. two drinks/day or less). To reduce the risk of nocturnal hypoglycemia in patients on insulin or insulin secretagogues, alcohol should always be ingested with food.

Putative biological mechanisms

In observational studies, alcohol consumption of 10-30 g/day (one to three drinks/day) is associated with lower fasting insulin concentrations and lower markers of IR, both under fasting and after glucose load. On the other hand, a negative effect has been found on blood pressure, which increases proportionally to the amount of alcohol consumed. Most intervention studies suggest that a consumption of 25-40 g ethanol /day (up to 4 drinks/day), improves lipid profile and IS as well as serum adiponectin in menopausal women, while in men it seems to have no influence on IS and body fat distribution and it may even promote hepatic steatosis. Of interest, liver histology rather than lipidemic profile is associated with cardiovascular risk in NAFLD and other cirrhotic and non-cirrhotic liver disease of varying etiology. These findings are in agreement with recent evidence suggesting that NAFLD is the precursor rather than a mere 'manifestation' of the MetS.

Conclusion

Available data do not enable physicians to suggest that patients with NAFLD and/or T2DM should be encouraged to drink alcohol to reduce the risk of progression of their disease. Likewise, it remains uncertain whether patients with NAFLD/T2DM should be discouraged from drinking alcohol in cases where they consume moderate amounts (namely <20/30 g/daily). Further studies are eagerly awaited to address these clinically relevant questions.

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THE COURSE OF CIRRHOTIC NASH: HOW DIFFERENT IS IT FROM OTHER CIRRHOSES

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Take home messages

- NASH progresses to cirrhosis in 15-20% of subjects.
- Multiple features of the MetS, abnormal ALT, increasing obesity and age, along with increasing inflammation, ballooning and having some fibrosis, are risk factors for progression.
- The natural course of compensated NASH cirrhosis can be long; however, once decompensation occurs the mortality is high.
- HCC can occur in NASH both in the presence and absence of cirrhosis.
- NASH is projected to become the leading indication for liver transplantation and also the leading etiology of HCC.

Introduction

The pathophysiology, characteristics and public health importance of NAFLD and NASH are covered elsewhere in this course. This syllabus focuses on the cirrhosis-linked outcomes in NASH and their relationship to other common chronic liver diseases.

Rates of development of cirrhosis

Most cross-sectional studies have about 20% of subjects with bridging fibrosis or cirrhosis. Approximately 15-20% of subjects with NASH have been reported to progress to cirrhosis [1]. However, the available literature is limited by its largely retrospective nature, the small number of subjects in individual studies and ascertainment bias in many cases. The NIDDK NASH Clinical Research Network has provided the first prospective data on progression of NASH. These data come from the longitudinal database (which has selection bias because follow-up biopsies were not performed in all subjects in a protocol-mandated manner) and from the control arms of the PIVENS and FLINT clinical trials (where such biases did not exist, but bias due to participation in a clinical trial cannot be excluded). In the largest study with paired biopsies from the CRN cohort, a total of 375 subjects were studied [2]. Whereas those without fibrosis at the initial biopsies did not progress to advanced fibrosis, an increasing number of subjects with stage 1 and 2 disease, respectively, progressed to bridging fibrosis or cirrhosis. Elevated ALT, presence of the MetS and the presence of some fibrosis as well as the severity of inflammation were independent markers of progression. The PNPLA3 SNP associated with NAFLD is also associated with both steatohepatitis and fibrosis stage [3]. The recently discovered *TM6SF2* gene mutation has also been linked, controversially, to more advanced disease [4].

Outcomes of cirrhosis related to NASH: a prospective study

Several small studies have provided data on the outcomes of cirrhosis due to NASH. The largest study included 152 subjects with NASH related cirrhosis who were followed prospectively in a protocol-driven manner for over 10 years [5]. In this study, the control population was an age, gender and race-matched population of subjects with cirrhosis due to HCV who were seen concurrently. All subjects provided informed consent to participate in a study to examine the natural history of their disease. Cirrhosis was defined by liver histology in all cases. Cirrhosis was attributed to underlying NASH by (1) histologic features of steatohepatitis; (2) an absence of clinically significant alcohol consumption (40 gm/week assessed clinically) and (3) negative tests for alternate causes of cirrhosis. In the presence of cirrhosis, steatosis with varying combinations of cytologic ballooning, MDBs and inflammation were used as histologic evidence of concurrent NASH. With progression towards cirrhosis, central to central and central to portal bridges develop, distorting the hepatic lobular architecture. Zone III, pericellular fibrosis is therefore difficult to define in subjects with cirrhosis and was not considered an independent criterion for NASH and cirrhosis. A nurse and a physician independently interviewed the patient and labelled the condition to be 'non-alcoholic' if the weekly alcohol consumption was <40 gm/week. These strict criteria were chosen based on the available literature when the study was initiated and to exclude the confounding effects of moderate alcohol consumption.

Mortality. Subjects with compensated cirrhosis have an approximately 3.5-4% risk of mortality annually. This is related to increased risk of cardiovascular events, clinical decompensation with sepsis and multi-organ failure and HCC [5]. This risk is slightly but statistically significantly lower than that seen in those with compensated cirrhosis due to HCV infection. These data have been corroborated in another independent cohort [6]. Subjects with NASH related cirrhosis have a significantly higher rate of cardiovascular mortality compared to HCV related cirrhosis. Once cirrhosis progressed from a compensated to a decompensated stage, i.e. CPT stage B or C, the mortality was similar to that in the literature for other causes of decompensated cirrhosis such as HCV. BMI and CPT score were independent predictors of mortality. When MELD was included in the model, BMI was no longer significant. This was driven mainly by creatinine as virtually all subjects with a creatinine >2 mg/dl died without a liver transplant. A creatinine of 1.65 was the best single predictive laboratory parameter of mortality (**Fig. 1**) [5].

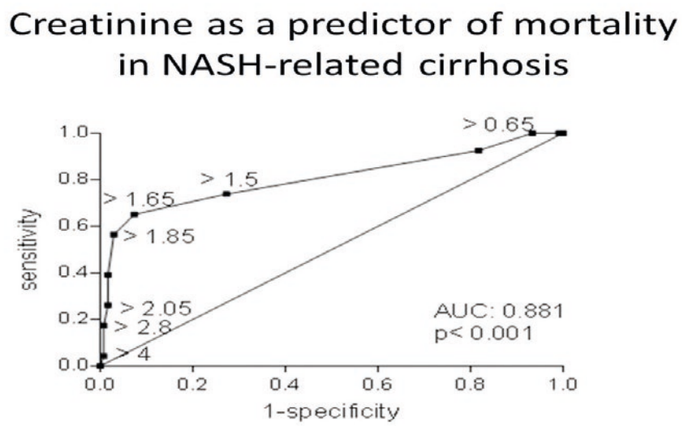


Figure 1. Creatinine as a predictor of mortality in NASH-related cirrhosis.

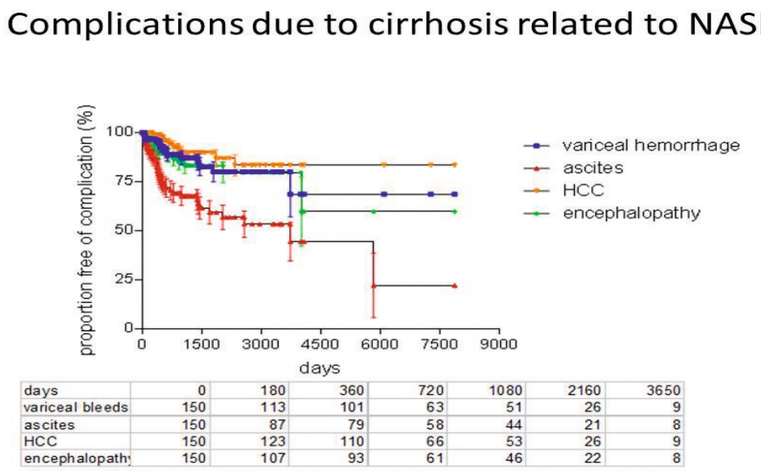
Development of liver failure. Hepatic decompensation and liver failure were defined in several ways: (1) development of ascites, variceal hemorrhage or hepatic encephalopathy; (2) progression from CPT stage A to B or a 2-point increase in score; (3) decline in liver function (worsening hyperbilirubinemia, hypoprothrombinemia), and (4) development of HCC.

1. Development of ascites variceal hemorrhage and hepatic encephalopathy.

In subjects with NASH related cirrhosis, ascites is the first clinical feature of decompensation. It is also the most common complication of cirrhosis (**Fig. 2**) [5]. It occurs however at a slower rate than in those with HCV related cirrhosis. The future rates of development of hepatorenal syndrome were similar to those in HCV related cirrhosis once ascites developed.

The rates of development of varices were similar in those with NASH related cirrhosis and HCV related cirrhosis [5]. The rates of development of variceal hemorrhage were also not significantly different. Variceal hemorrhage was the least common complication of cirrhosis in this population. This may be related to the aggressive use of primary prophylaxis in those who were identified to have varices and reflects national trends of declining rates of variceal hemorrhage. The presence of varices was independently predicted by MELD score and low platelet counts in those with NASH-related cirrhosis.

The rates of development of hepatic encephalopathy (HE) were intermediate between those for ascites and variceal hemorrhage. No deaths were directly attributable to HE in the absence of sepsis. The MELD score was independently associated with the development of HE (**Fig. 2**).



days	0	180	360	720	1080	2160	3650
variceal bleeds	150	113	101	63	51	26	9
ascites	150	87	79	58	44	21	8
HCC	150	123	110	66	53	26	9
encephalopathy	150	107	93	61	46	22	8

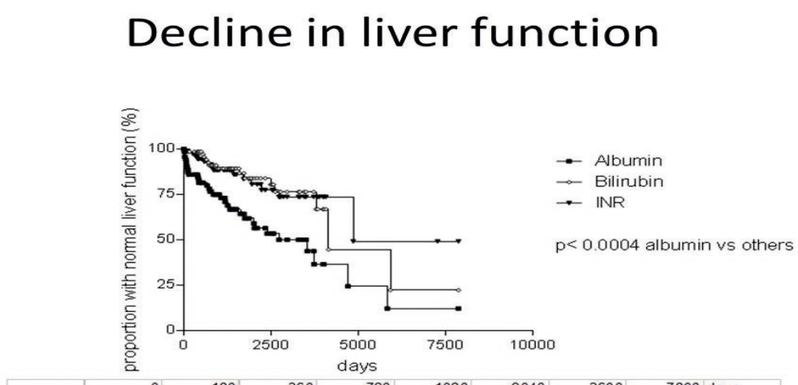
Figure 2. Complications due to cirrhosis related to NASH.

2. Progression of CPT score.

Subjects with NASH decompensate at a somewhat slower rate than those with HCV related cirrhosis with respect to a 2-point worsening of their CPT score [5]. The lower rate of development of ascites drives much of this difference. In those with NASH related cirrhosis, hypoalbuminemia and development of ascites were the principal causes of a 2 point or greater increment in CPT scores.

3. Decline in liver function.

Hepatic synthetic dysfunction develops over time in a majority of subjects who have compensated NASH related cirrhosis. Albumin declines early and is usually the first laboratory test to become abnormal. An albumin <3.5 gm/dl was also the most common laboratory evidence of hepatic synthetic dysfunction (Fig. 3). A decline in albumin levels also heralds clinical decompensation (unpublished personal observation).



	0	180	360	720	1020	2040	3600	7800	days
Albumin	74	73	60	49	41	23	7	1	
Bilirubin	72	72	71	60	51	29	10	1	
INR	74	74	70	61	50	29	10	1	

Figure 3. Decline in liver function.

4. Development of HCC.

HCC developed in about 7% of subjects over 10 years in those with compensated cirrhosis due to NASH. These rates are lower than in those with HCV related cirrhosis [5, 6]. HCC can also occur in the absence of cirrhosis in subjects with NASH and accounts for up to 50% of HCC due to NASH [7]. Recently, NASH related HCC has been identified to be the second most common etiology for HCC that requires liver transplantation [8]. The risk factors for the development of HCC in the absence of cirrhosis in those with NASH remain to be fully elucidated. The consumption of modest amounts of alcohol (below the threshold above which steatohepatitis cannot be called non-alcoholic) in subjects with NASH has been linked to the risk of HCC in a retrospective analysis [6].

Areas of future research
Several areas of future research are warranted. These include variances in the outcomes of NASH related cirrhosis in different parts of the world, the factors contributing to the development of clinically meaningful outcomes in those with NASH related cirrhosis, development of predictive models, particularly for HCC, and eventual development of strategies to prevent the complications described above that drive liver related mortality. Furthermore, data on the impact of an anti-fibrotic strategy on cirrhosis related outcomes are eagerly anticipated.

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LIVER TRANSPLANTATION FOR NASH

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Take home messages
- NASH is becoming the second indication for LT in The United States.
- In contrast, NASH represents <5% of the indications in Europe.
- NASH-related HCC is a growing indication for LT.
- NASH is frequently associated with comorbidities, in particular obesity and diabetes.
- Morbid obesity is associated with increased perioperative morbidity.
- NAFLD recurrence is observed in 50% of patients at two years.
- Long-term outcome is similar to other indications.

Introduction
Worldwide, the main indications for LT are HCV cirrhosis, alcoholic cirrhosis and HCC. In East Asia, HBV-related or HCC-related cirrhosis are the main indications for LT. LTs for decompensated HBV cirrhosis have decreased over the last five years thanks to improvements in antiviral therapies. Moreover, direct-acting antiviral agents will impact the number of patients requiring LT for HCV decompensated liver cirrhosis. The main indications for LT in Europe are HCV cirrhosis, alcoholic cirrhosis and HCC. We may expect to see a shift in the indications for LT within the next five years among the causes of cirrhosis and the causes of HCC.

NASH
The disappearance of some of the histological features of NASH in the end stages of the disease can make diagnosis difficult, particularly in the absence of a well-documented medical history.
Furthermore, the diagnosis of alcoholic cirrhosis may be overestimated in Europe. Indeed in many cases, coexistence of lesions of NASH and of alcoholic steatohepatitis (ASH) are potential causes of cirrhosis in patients who have a regular moderate drinking and predisposing factors for NASH. The impact of NASH in Europe may therefore be underestimated. Several groups have combined patients with NASH-related cirrhosis and patients with cryptogenic cirrhosis who have predisposing factors of NASH to better evaluate the true incidence of the disease. The same applies to HCC, where the cause of cirrhosis is sometimes unclear. In Europe, NASH remains a confidential cause for LT, representing <5% of the indications. Between 2004 and 2013 in The United States, new LT waitlist registrants with NASH increased by 170% (from 804 to 2174) and in 2013, NASH became the second-leading disease among LT waitlist registrants, after HCV [1].

HCC and NASH
HCC represents 22% of the LT indications in The United States and >30% in France. Patients with NASH-related cirrhosis are at risk of HCC. In addition, it seems that HCC can develop in NASH even in the absence of cirrhosis. In The United States, 49% of HCC cases are due to HCV, followed by NASH (13%), which represents a 4-fold increase between 2002 and 2012 [2].

Characteristics of patients with NASH on the waiting list
In The United States, the rate of HCC among patients on the waiting list was 21% and 24% in patients with NASH and HCV, respectively, while it was only 7% in ALD patients. The mean MELD score at inscription was higher in ALD patients (19.1) than in HCV patients (15.7) or in NASH patients (16). In contrast, the mean BMI (31.6 kg/m2) and the prevalence of diabetes (46%) were higher in NASH patients than in patients with other liver diseases.

Specific aspects of candidates for NASH-related LT
This higher rate of comorbidities in NASH patients may explain their higher rate of mortality on the waiting list in The United States. Furthermore, the rate of perioperative complications is higher in obese patients with wound dehiscence, ventral hernia and longer hospital stay, particularly in those with a BMI of >40. In patients with very high BMI, particularly morbid obesity, LT might be contraindicated. Contraindication may be solely due to BMI although the severity of associated comorbidities can also be important. In some centres, physical exercise, and in some cases, treatment of obesity is recommended as preparation for LT. However, surgical treatment of obesity (e.g. sleeve gastrectomy, surgical bypass) might be difficult or impossible in patients with end-stage liver disease. Recently, temporary gastric balloon to decrease the patient's BMI prior to LT have been proposed.

Outcome post-LT [3]
Perioperative outcome. As previously mentioned, the rate of postoperative complications seem higher in most series of patients with obesity due to an increase in wound healing deficiencies, respiratory complications, and length of hospital and ICU stay after surgery. However, in patients with NASH at three years post-LT there was no difference in survival compared with other indications.

Long-term outcome. Whatever the indications of LT, it appears that long-term complications, such as major weight gain, obesity, diabetes and arterial hypertension are frequent, with MetS as a source of cardiovascular complications in the long-term. The rate of de novo NAFLD and NASH was 20% and 10%, respectively, in a single centres series.
In one series the recurrence of NAFLD on the graft has been reported as >50% within the first two years post-LT and 100% at five years. The impact of potential graft loss is still in evaluation.

Conclusion
LT for NASH is a growing indication. This trend is particularly marked in The United States and has to be evaluated in Europe in the coming years. NASH is a growing indication in both decompensated liver diseases and in HCC. Higher perioperative morbidity can be observed in patients with NASH, particularly in those with severe or morbid obesity. Despite recurrence of NAFLD or NASH on the liver graft, medium-term survival seems identical to other causes of LT. Strategies to correct MetS and obesity before LT have to be implemented. Prevention and treatment of dysmetabolic syndrome after LT will be essential to prevent NAFLD and NASH recurrence and to decrease cardiovascular related complications.

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NOTES

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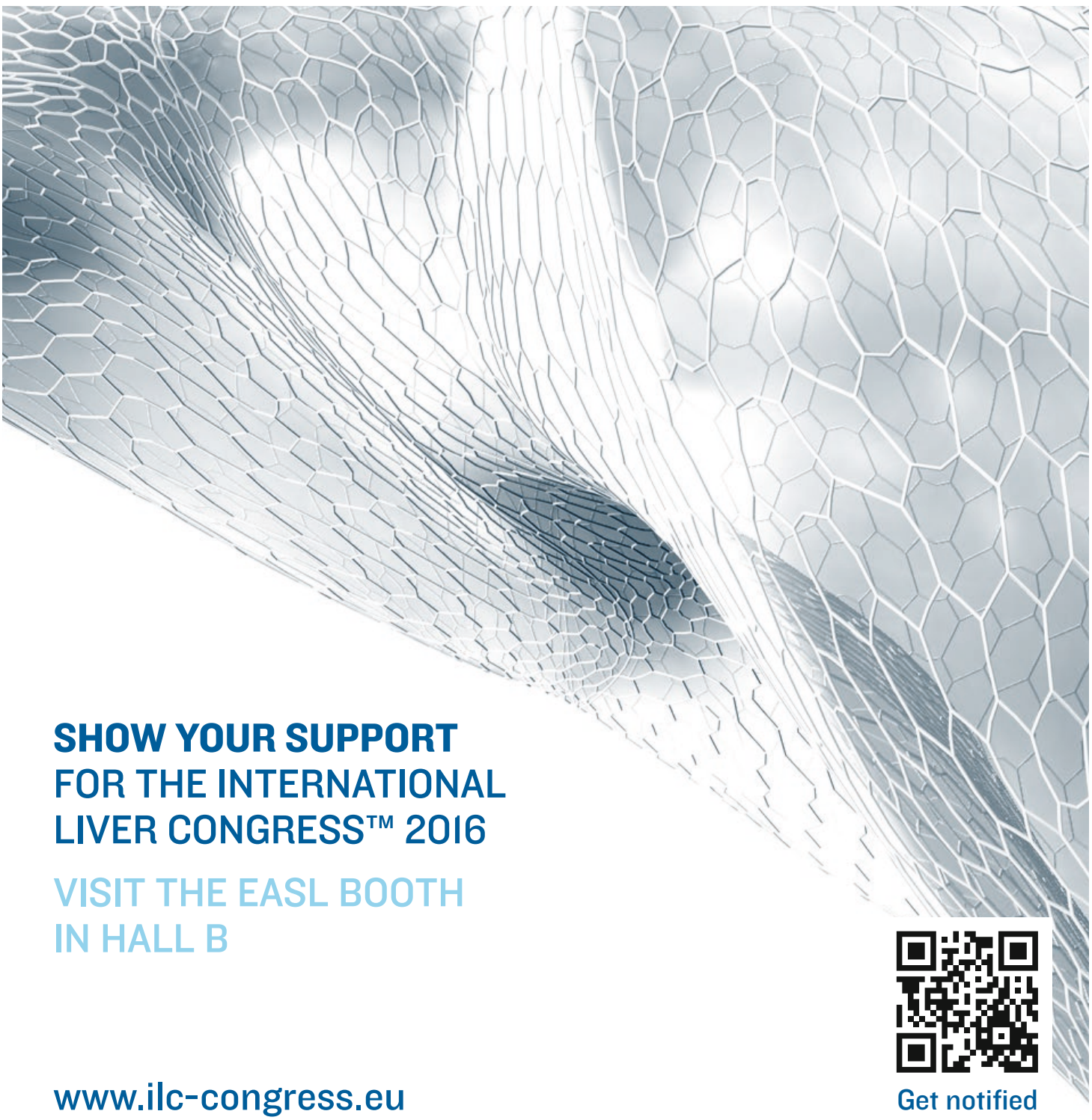

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