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Diabetes & Metabolism

Diabetes & Metabolism 34 (2008) 633

Editorial

J. Girard, S. Halimi

Inserm U563-CNRS UMR 8140, Université Paris Descartes, Institut Cochin, Paris, France.

Patients with nonalcoholic fatty liver disease, viral hepatitis C, hemochromatosis and alcoholic liver disease have increased risk of type 2 diabetes. Moreover, patients with type 2 diabetes and liver disease are at risk of severe liver disease, cirrhosis, liver failure and hepatocellular carcinoma. Awareness of type 2 diabetes as a significant risk factor for liver injury may improve diagnosis and intervention to minimize the progression of chronic liver diseases. Our understanding of the links between type 2 diabetes and the development and progression of chronic liver diseases has benefited mainly from retrospective studies but prospective studies are needed to fully evaluate the cause and the effect of type 2 diabetes in liver injury. Identifying the mechanisms by which liver disease increased the prevalence of type 2 diabetes and whereby type 2 diabetes increase liver disease severity could offer new insights into the treatment of chronic liver disease, including the role of weight reduction and pharmacological interventions with insulin sensitizers. Insulin resistance and altered β-cell function are usually present. The role of increased proinflammatory cytokines and reduction of protective cytokines, hyperinsulinemia and hyperglycemia in the activation of hepatic stellate cells and stimulation of collagen production are prime focuses in research in this area.

The present symposium was co-organized by ALFEDIAM *(Association de Langue Française pour l'Étude du Diabète)* and AFEF *(Association Française pour l'Étude du Foie)* for trying to answer a number of these questions. First, the definition and the description of natural history of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) and the mechanisms of nonalcoholic hepatic steatosis will be described. Then, role of insulin resistance and of inflammation in these pathologies will be discussed. Finally the pharmacological treatments of NAFLD and NASH and viral hepatitis will be described.

The aim of this symposium was to offer the possibility for diabetologists and hepatologists to meet and to discuss some problem of common interest. This supplement of *Diabetes and Metabolism* contains the summaries of most of the conferences presented at this symposium and we hope this will be of help to your clinical practice.

Jean Girard,
Past President of ALFEDIAM
Serge Halimi,
President of ALFEDIAM

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Definition and natural history of metabolic steatosis: clinical aspects of NAFLD, NASH and cirrhosis

L. Serfaty[a, b*], M. Lemoine[a, b]

[a]*Service d'Hépatologie, Hôpital Saint-Antoine, 184, rue du Faubourg Saint-Antoine, 75571 Paris cedex 12, France.*
[b]*Inserm UMR S 893, UPMC, Paris, France.*

Abstract

Metabolic steatosis or non-alcoholic fatty liver (NAFLD) is the most common cause of chronic liver injury in Western countries. Histological signs of necroinflammation, indicating the presence of non-alcoholic steatohepatitis (NASH), are present in 20-30% of cases. While steatosis on its own has a benign course, NASH may be associated with fibrosis and may progress to cirrhosis, terminal liver failure and hepatocellular carcinoma. NAFLD is closely associated with the metabolic syndrome, its prevalence reaching 50-90% in obese patients. The clinical impact of NAFLD has been demonstrated in large cohort studies by the overprevalence of cirrhosis and hepatocellular carcinoma in obese and diabetic patients. In terms of survival, liver disease is the third most common cause of mortality in patients with NAFLD. When associated with other causes of liver disease such as alcohol consumption or hepatitis C infection, metabolic steatosis may be a major risk factor for disease progression.
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Résumé

Définition et histoire naturelle de la stéatose métabolique (NAFLD) : stéatose non alcoolique (NASH) et cirrhose. Aspects cliniques

La stéatose métabolique ou stéatose non alcoolique (NAFLD en anglais) est la première cause d'hépatopathie chronique dans les pays industrialisés. Des signes histologiques de nécro-inflammation, qui définissent la stéatohépatite non alcoolique (NASH en anglais), sont présents dans 20 à 30 % des cas. Alors que la stéatose pure est d'évolution bénigne, la NASH peut être associée à des lésions de fibrose et évoluer vers la cirrhose, l'insuffisance hépatique et le carcinome hépatocellulaire. La NAFLD est étroitement associée au syndrome métabolique, sa prévalence pouvant atteindre 50 à 90 % chez les patients obèses. La gravité clinique de la NAFLD a été démontrée par le surrisque de cirrhose et de carcinome hépatocellulaire dans de larges cohortes de patients obèses ou diabétiques. En termes de survie, une maladie hépatique est la 3e cause de décès chez les patients atteints de NAFLD. Lorsqu'elle est associée à d'autres causes d'atteinte hépatique comme la consommation excessive d'alcool ou l'infection par le virus C, la stéatose métabolique est un facteur de risque majeur de progression de la maladie.
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Keywords: Metabolic steatosis; NAFLD; NASH; Cirrhosis; Hepatocellular carcinoma; Metabolic syndrome; Obesity; Diabetes; Insulin resistance; Unexplained cytolysis; Review.

Mots clés : Stéatose métabolique ; NAFLD ; NASH ; Cirrhose ; Carcinome hépatocellulaire ; Syndrome métabolique ; Obésité ; Diabète ; Insulinorésistance ; Cytolyse inexpliquée ; Revue.

Non-alcoholic fatty liver disease (NAFLD) includes a spectrum of liver injuries that mimics alcohol-induced liver disease, yet affects individuals who are not heavy drinkers. NAFLD is likely to be the most common cause of chronic liver disease in many countries and may also potentiate liver damage induced by other agents, such as alcohol or the hepatitis virus. The natural history of NAFLD is uncertain, and there is skepticism as to its clinical importance, given the discrepancy between the high prevalence of NAFLD in the population and the low prevalence of clinically significant liver disease.

*Corresponding author.
E-mail Address: lawrence.serfaty@sat.aphp.fr

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1. Definition

Seen in the majority of patients with the metabolic syndrome, NAFLD—or metabolic steatosis—is now considered a manifestation of the syndrome [1]. Liver biopsy remains the gold standard for detecting and staging fatty liver disease as steatosis alone, which has a benign course and steatohepatitis or non-alcoholic steatohepatitis (NASH), which may be associated with fibrosis, and progression to cirrhosis and hepatocellular carcinoma (HCC). NASH was first described in 1980 by Ludwig *et al.* in 20 patients at the Mayo Clinic over a 10-year period [2]. These patients had histological evidence of alcoholic hepatitis on liver biopsy, but no history of alcohol abuse.

Classically, patients with NAFLD have slightly elevated liver enzyme values, deny excessive alcohol consumption, and have negative serological tests for viral hepatitis, autoimmune liver disease and congenital causes of chronic hepatitis. NAFLD is strongly associated with the metabolic syndrome, especially obesity and type 2 diabetes. In obese patients, the prevalence of NAFLD has ranged from 50% to 90% [3,4]. Obesity may also increase the risk of NAFLD after exposure to particular insults, such as alcohol-related liver problems. Bellentani *et al.* found ultrasound evidence of fatty liver in 46% of non-obese and 95% of obese heavy drinkers, demonstrating that obesity doubles the prevalence of alcohol-induced fatty liver disease [5]. As for diabetes, it is now established that insulin resistance may play a major role in the pathogenesis of NAFLD [1]. Consistent with this hypothesis, mild insulin resistance is very common in the earliest stages of NAFLD, and more severe insulin resistance (as in type 2 diabetes) correlates with more advanced stages of NAFLD [1].

2. Prevalence

Epidemiological studies are difficult to carry out as no single blood test, imaging study or histological parameter is 100% sensitive or specific for NAFLD. The prevalence of NAFLD in European and Japanese population-based studies is estimated to range from 14% to 21% [5,6]. In a US population-based study, NAFLD was the most likely cause of unexplained abnormal liver enzymes: 27% of adults had elevated AST, ALT or GGT levels, and 79% of those cases could not be explained by other common causes of liver disease, suggesting that NAFLD could represent approximately 30 million people in the US alone [7].

In patients with unexplained elevated liver enzymes, fatty liver was demonstrated in 20-30%, and steatohepatitis with varying degrees of fibrosis was seen in an additional 15-30% [5,8,9]. Thus, NAFLD accounts for around 70% of cases of 'cryptogenic' chronic hepatitis in the general population. The prevalence of NAFLD is even higher in obese and diabetic populations, demonstrated by liver biopsy in up to 90% of patients with cryptogenic hepatitis [3,4]. However, these diagnostic criteria probably underestimate the true prevalence of NAFLD. It has been shown that some patients with NAFLD have normal aminotransferase levels [10]. Moreover, patients with other types of liver disease may also have NAFLD, which can influence the outcome of those other diseases. It is now established that steatosis and steatohepatitis are frequently seen in chronic hepatitis C infection, and are major independent risk factors for progression to cirrhosis [11]. Therefore, positive tests for viral hepatitis do not entirely exclude a diagnosis of NAFLD.

3. Clinical impact

Only limited data are available on the natural history of NAFLD. Several distinct histological appearances have been identified in the natural course of this chronic liver disease: fatty liver alone; steatohepatitis; steatohepatitis with fibrosis; and cirrhosis [12]. It has also been noted that the development of cirrhosis is associated with fatty disappearance.

Cross-sectional studies of NAFLD indicate that most patients have a fatty liver alone, and it is now accepted that such patients rarely progress to steatohepatitis or fibrosis over time. In one longitudinal study, repeat liver biopsies in patients with fatty liver alone showed no progression to steatohepatitis over a 10-year period [13]. These data were further confirmed by other studies [14]. In contrast, progression from fatty liver alone to steatohepatitis was only noted in one patient following liver transplantation [15]. Morbidly obese individuals with a fatty liver alone who undergo rapid weight loss following bypass surgery have also been reported to develop steatohepatitis [16].

At the time of diagnosis, about 30-40% of patients with NASH have advanced fibrosis, whereas 10-15% have established cirrhosis [12]. Established risk factors for advanced fibrosis in patients with NASH are mainly age, obesity and diabetes [8]. Conversely, epidemiological surveys have clearly demonstrated an overprevalence of cirrhosis and HCC in obese and/or diabetic patients [17-20]. Indeed, a long-term follow-up involving more than 800,000 US veterans showed that type 2 diabetes doubled the risk of chronic non-alcoholic liver disease or HCC, and that HCC incidence correlated with the duration of diabetes (Fig. 1) [20].

Assessment of the rate of progression of fibrosis in NASH patients is limited by the fact that all studies are retrospective, and few patients have undergone repeat biopsies during follow-up. In one study, six out of 13 patients showed

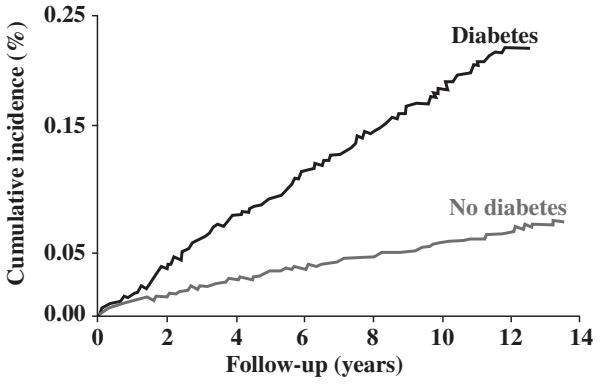

Fig. 1. The cumulative risk of hepatocellular carcinoma among veterans according to the presence of diabetes (adapted with permission from El-Serag *et al.* [20], © 2008 Elsevier Publishing, Inc.).

progression of fibrosis and one patient developed cirrhosis after a median duration of 4.5 years [14]. Similarly, five out of 13 patients developed increasing fibrosis over a mean follow-up of 3.5 years [21]. In yet another report, 132 patients with NAFLD were divided into four categories based on their liver histology: 1) fatty liver alone; 2) fat plus lobular inflammation; 3) fat plus ballooning degeneration; and 4) fat plus ballooning plus either Mallory's hyaline or fibrosis. Cirrhosis was present in four of 19 patients in group 3 and in 14 of 26 in group 4 [22].

Cryptogenic cirrhosis, which represents around 10% of cirrhosis, is probably related to NASH progression in the majority of cases, given the significantly higher prevalence of metabolic factors such as obesity and diabetes compared with other chronic liver disease [12,23]. In patients with cryptogenic cirrhosis, longitudinal studies have shown that the rate of complications such as decompensation or HCC was similar to the rate observed in patients with HCV-related cirrhosis [24].

The precise risk of mortality in patients with NAFLD is not known. In a study of 30 patients with NASH followed-up for more than 10 years, the five-year survival was only 67% and the 10-year survival was 59% [25]. Although the overall mortality was not significantly different from that of an age- and gender-matched population, liver-related mortality was higher. In another retrospective series, liver-related mortality was 7/54 over 18 years of follow-up in those with fatty liver, ballooning degeneration and Mallory bodies or perisinusoidal fibrosis [22]. Although most patients with NASH without bridging fibrosis or cirrhosis have a very low risk of death up to 5-10 years from the time of diagnosis, those with more advanced disease are at a higher risk of death as a consequence of NASH. A recent long-term follow-up showed that, in 420 patients with NAFLD, survival was lower than that expected in the general population (standardized mortality ratio, 1.34; 95% CI, 1.003-1.76; p= 0.03), and liver disease was the third most common cause of death, after cardiovascular disease and malignancy [26] (Fig. 2).

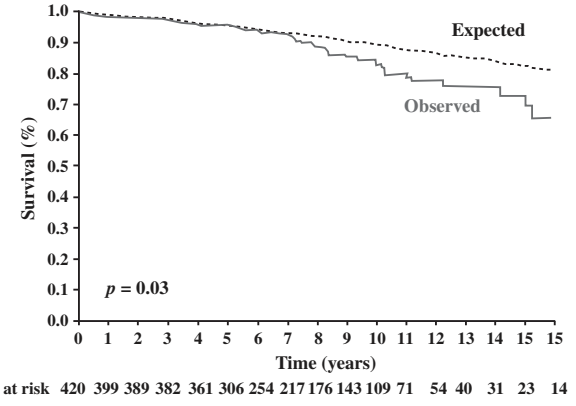

Fig. 2. Overall survival of patients diagnosed with NAFLD compared with the general population of the same age and gender (adapted with permission from Adams *et al.* [26], © 2008 Elsevier Publishing, Inc.).

4. Conclusion

NAFLD, or metabolic steatosis, is likely to represent the leading cause of chronic liver disease in Western countries, given the extensive prevalence of obesity and type 2 diabetes in those populations. Progression of disease is low, and only a minority develops into cirrhosis or HCC. However, given its high prevalence in the general population, NAFLD is soon likely to become the leading cause of cirrhosis and HCC in the developed countries. At present, cryptogenic cirrhosis and its complications are the second most common indication for liver transplants in the US. When associated with other frequent causes of chronic liver disease, such as alcohol consumption or HCV infection, metabolic steatosis may be a major factor in disease progression. This highlights the urgent need for diagnostic markers and efficient treatments for patients with NAFLD [27].

Conflicts of interest: The authors have none to declare.

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Definition and natural history of metabolic steatosis: histology and cellular aspects

V. Paradis*, P. Bedossa

Pathology Department, Beaujon hospital Clichy, 110, bd Général Leclerc, 92118 Clichy cedex, France; & Inserm U773 Paris

Abstract

In patients with diabetes and metabolic syndrome, liver changes may be observed on histology that are characterized as non-alcoholic fatty liver disease (NAFLD). The NAFLD spectrum covers a variety of histological features, including steatosis, necroinflammation and fibrosis. Although steatosis usually follows a benign course, steatohepatitis is prone to progress to fibrosis and cirrhosis. Establishing the degree of severity of liver lesions, the main endpoint of the disease, can identify patients at risk of disease progression. This may be achieved by liver biopsy. For that purpose, a scoring system for both activity (grade) and fibrosis (stage) is available with good reproducibility. In addition to the commonly seen histopathological patterns of lesions, additional changes are reported in patients with diabetes, including glycogenic hepatopathy and hepatic hepatosclerosis.
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Résumé

Définition et histoire naturelle de la stéatose métabolique. Aspects histologiques et cellulaires

Des modifications morphologiques du parenchyme hépatique sont rapportées chez les patients diabétiques et les patients atteints de syndrome métabolique. Ces lésions sont variées (stéatose, stéatohépatite et fibrose) et entrent dans le cadre de lésions de stéatopathie métabolique. Alors que la stéatose est une lésion bénigne, potentiellement réversible, les lésions de stéatohépatite, qui associent des lésions de souffrance hépatocytaire et d'inflammation, peuvent évoluer vers la fibrose, voire la cirrhose. La sévérité des lésions hépatiques, un des facteurs pronostiques de la maladie, peut être établie sur la biopsie hépatique, en particulier à l'aide d'un score histologique fondé sur l'évaluation du grade d'activité et du stade de fibrose. En parallèle de ces lésions associées au syndrome métabolique, d'autres anomalies morphologiques (hépatopathie glycogénique et hépatosclérose diabétique) ont été rapportées chez les patients diabétiques.
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Keywords: Steatosis; Steatohepatitis; Fibrosis; Diabetes; Metabolic syndrome; Review.

Mots clés : Stéatose ; Stéato-hépatite ; Fibrose ; Diabète ; Syndrome métabolique ; Revue générale.

1. Introduction

In patients with diabetes and metabolic syndrome, the liver may display damages typical of the spectrum of non-alcoholic fatty liver disease (NAFLD). Given the significant increase in patients with features of the metabolic syndrome, the growing prevalence of NAFLD is expected [1]. Although liver disease is most often benign, it is nevertheless the third most common cause of death in patients with NAFLD, following cardiovascular diseases and malignancy [2,3]. The NAFLD spectrum covers a variety of histological features, including steatosis, fibrosis and necroinflammation. Although steatosis is a benign condition that usually does not progress to more severe liver disease, steatohepatitis (NASH) is a risk factor for the development of cirrhosis, end-stage liver failure and hepatocellular carcinoma [4,5]. The main objective of this review is to describe the pathological appearances of NAFLD and the specific features associated with diabetes.

*Corresponding author.
E-mail Address: vparadis@teaser.fr

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2. Liver pathology in NAFLD

2.1. Basic pathological features

Steatosis is defined as triglyceride accumulation in hepatocytes, and a minimum excess overload of at least 5-10% of hepatocytes is considered significant steatosis [6]. In NAFLD, steatosis is usually macrovesicular and most often located in the centrolobular area [7] (Fig. 1 and 2). Hepatocyte ballooning, a feature denoting cellular injury, is characterized by enlarged, swollen hepatocytes with or without Mallory's hyaline in the cytoplasm (Fig. 3) [6]. Balloon cells are often closely associated with steatotic hepatocytes in the perivenular areas in perisinusoidal fibrosis. Lobular inflammation is usually mild, typically composed of mixed inflammatory cells, including mononuclear and polymorphonuclear leukocytes. Portal inflammation may be present, but with no specific characteristics, and mainly in obese pediatric populations [7]. As the disease progresses, liver fibrosis may occur. Indeed, natural history studies suggest that fibrosis progression occurs in approximately 35% of patients over 3-6 years, and up to 12% of patients will progress to cirrhosis over 8-10 years [8,9]. The characteristic pattern of fibrosis that distinguishes steatohepatitis from other forms of chronic liver disease is the initial deposition of extracellular matrix in the perisinusoidal area of lobule zone 3 (Fig. 4). In addition, periportal fibrosis with the formation of fibrous septa, leading to bridging fibrosis and cirrhosis, may eventually develop (Fig. 5). Finally, additional features may be reported in the context of NAFLD, including megamitochondria, granular iron pigmentation within hepatocytes and glycogenated nuclei (Fig. 6).

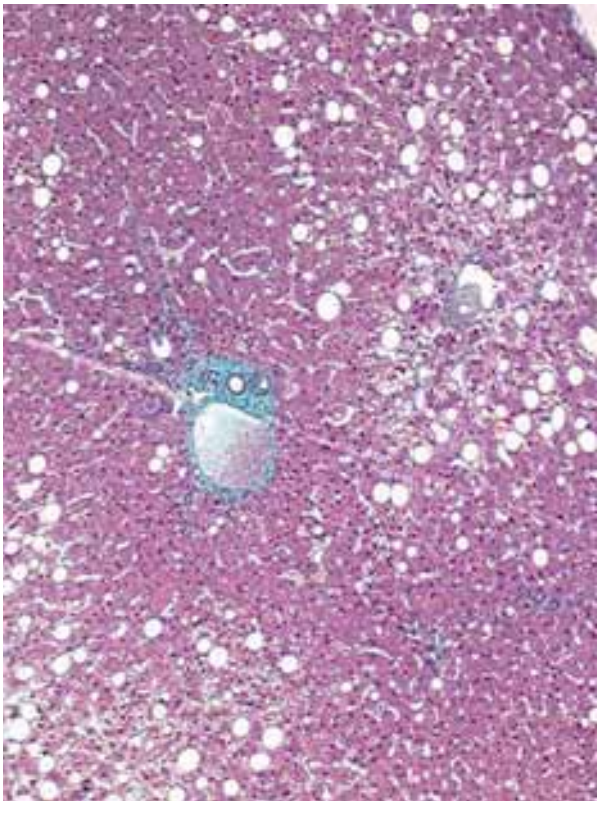

Fig. 1. Presence of moderate steatosis in the centrolobular area (trichrome stain).

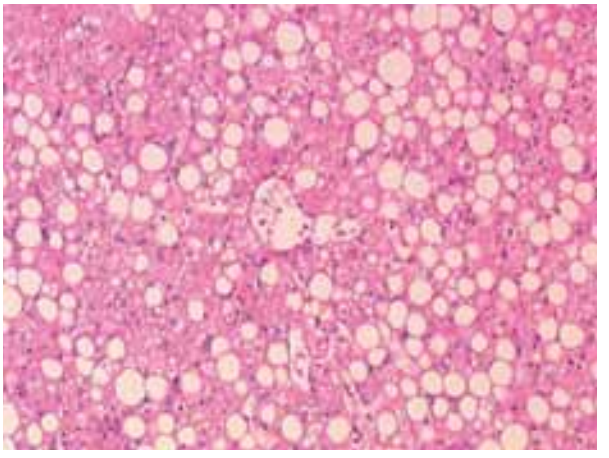

Fig. 2. Presence of macrovesicular steatosis at higher magnification (hematoxylin & eosin stain).

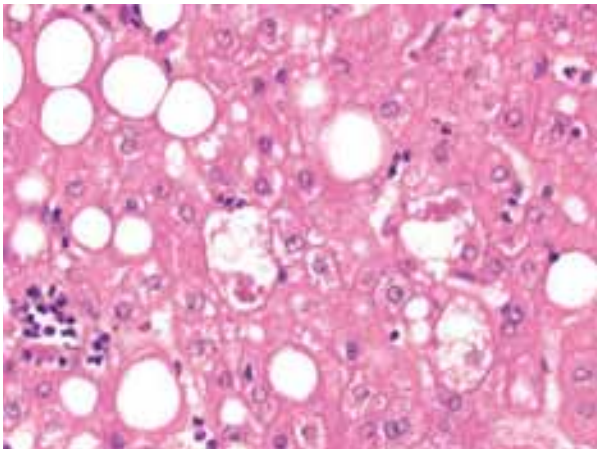

Fig. 3. Presence of balloon cells with intracytoplasmic Mallory's hyaline. Few inflammatory infiltrates are present (hematoxylin & eosin stain).

2.2. Histological scoring system of NAFLD

One major goal of the pathological analysis of patients with NAFLD is accurate evaluation of the extent of liver damage. To address this issue, histological scores of grading and staging have been developed. A system for a semi-

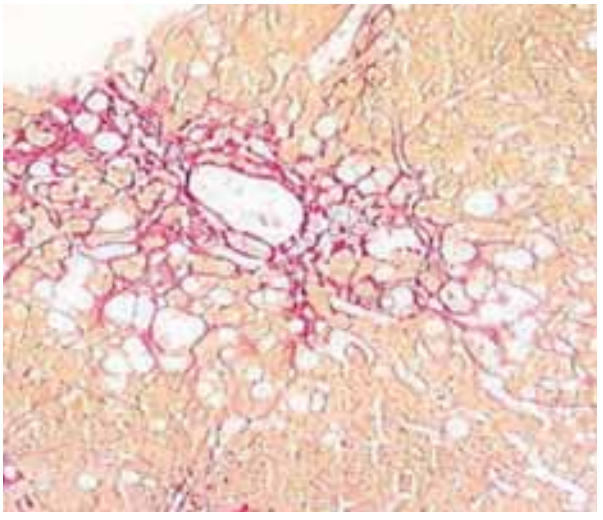

Fig. 4. Presence of moderate perisinusoidal fibrosis in the centrolobular area (sirius red stain).

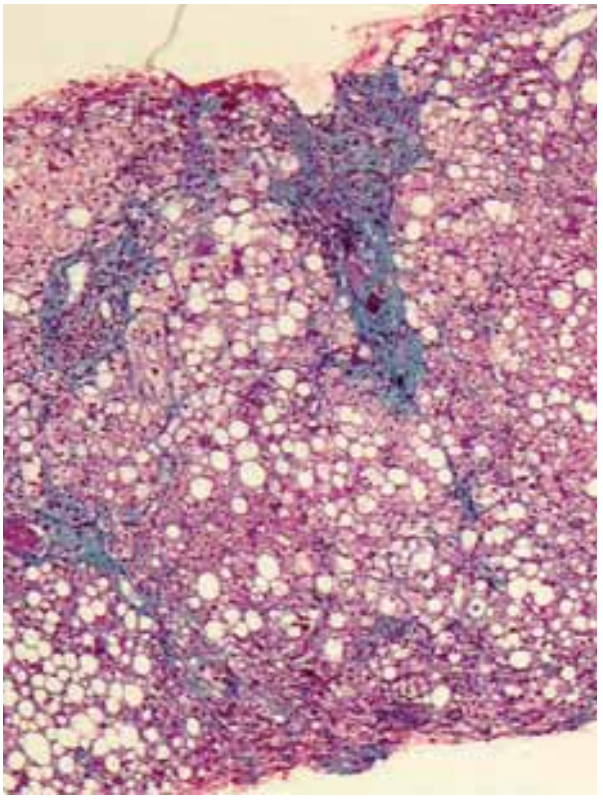

Fig. 5. Presence of portal fibrosis with few septa. Note the presence of marked steatosis (trichrome stain).

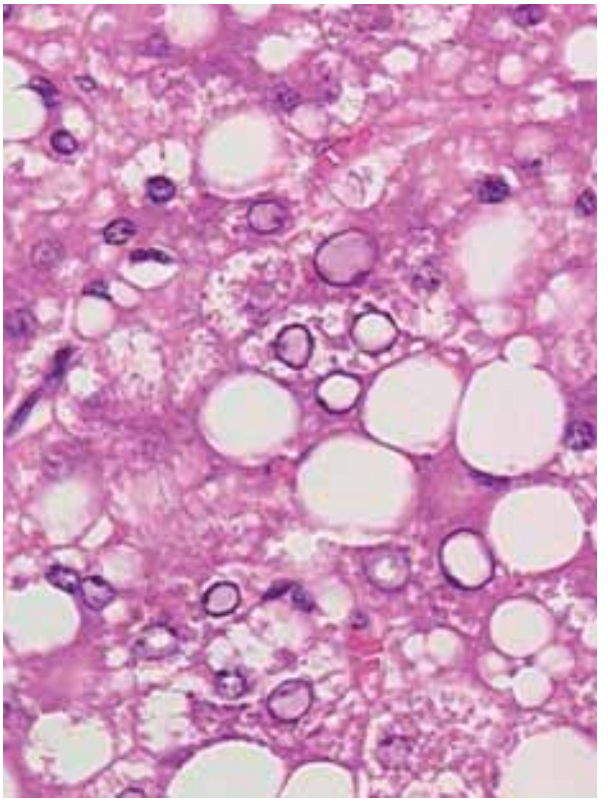

Fig. 6. Presence of glycogenated nuclei in hepatocytes. Note the presence of steatosis (hematoxylin & eosin stain).

quantitative type of evaluation, initially proposed by Brunt et al. in 1999, was based on the idea that the histological diagnosis of NASH relies on a constellation of features rather than on any one feature [10]. Such an approach was recently refined to provide a semi-quantitative feature-based scoring system for NAFLD for both pediatric and adult populations [11]. In this scoring system, histological features are grouped into five categories: steatosis; inflammation; hepatocellular injury; fibrosis; and miscellaneous features (Table 1). More

Table 1
Semi-quantitative scores for basic features according to the histological scoring system for non-alcoholic fatty liver disease by Kleiner *et al.* [11].

Basic features	Definition	Score
Steatosis	<5%	0
	5-33%	1
	>33-66%	2
	>66%	3
Lobular inflammation	No foci	0
	2 foci/200 × field	1
	2-4 foci/200 × field	2
	>4 foci/200 × field	3
Hepatocellular ballooning	None	0
	Few balloon cells	1
	Many cells/prominent ballooning	2

important, it has been demonstrated that agreement between pathologists in adult cases show reasonable concordance with the main categories of pathological features, including steatosis, fibrosis and ballooning injury, with weighted kappa values over 0.5.

In addition, a NAFLD activity score (NAS), which includes features of active injury, has been defined as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3) and ballooning (0-2). According to this scale, cases with scores ≥5 are diagnosed as NASH, and scores <3 are diagnosed as not NASH. It has been clearly emphasized that the NAS is not intended to be used as a diagnostic tool, but rather to provide a uniform tool for assessing disease severity and, ideally, in clinical trials [11].

As with viral chronic hepatitis, fibrosis is separately assessed by a 5-stage scale—ranging from no fibrosis to cirrhosis—that pays particular attention to the evaluation of the intensity of perisinusoidal fibrosis [11-13]. A description of fibrosis stages according to Kleiner *et al.* is presented in Table 2.

Table 2
Definition of fibrosis stages according to the histological scoring system for non-alcoholic fatty liver disease by Kleiner *et al.* [11].

Fibrosis	Definition	Score
Stage	None	0
	Perisinusoidal or periportal	1
	Mild, zone 3, perisinusoidal	1A
	Moderate, zone 3, perisinusoidal	1B
	Portal/periportal	1C
	Perisinusoidal, portal/periportal	2
	Bridging fibrosis	3
	Cirrhosis	4

2.3. Specific pathological aspects in diabetes

Many of the most severe complications of diabetes are the result of diabetic microangiopathy, defined as thickening of the capillary basement membranes of various tissues and organs. Hepatic abnormalities associated with diabetes have long been recognized, including NAFLD. More recently, additional histological findings have been described in patients with diabetes. Among them, hepatic hepatosclerosis, characterized by dense perisinusoidal fibrosis, has been reported in liver biopsies performed in diabetic patients for evaluation of abnormal liver test results [14]. Interestingly, perisinusoidal fibrosis was not associated with steatosis or necroinflammatory activity, but was associated with hyaline thickening of the small hepatic artery branches (Fig. 3). Glycogenic hepatopathy, characterized by marked glycogen accumulation leading to pale, swollen hepatocytes, was initially described in the context of Mauriac syndrome [15]. A pathological review of 14 liver biopsies from patients with poorly controlled type 1 diabetes demonstrated abundant cytoplasmic glycogen deposits in hepatocytes, no or mild fatty change and no or minimal necroinflammation. Such a morphological pattern clearly differs from steatohepatitis and may be reversed following adequate control of hyperglycemia [16].

3. Liver biopsy: the gold standard so far

NAFLD is defined as a clinicopathological entity that requires liver biopsy for diagnostic confirmation and estimation of disease severity. Indeed, no diagnostic laboratory test or imaging study has yet performed well enough to replace biopsy. Imaging procedures fail to detect either mild steatosis (<33%) or necroinflammation as well as biopsy does [17]. However, in addition to the potential variability in observer reproducibility and sampling errors, pathologists recognize that sample size, technique for obtaining the biopsy and the method of processing are all important considerations in liver biopsies [18,19]. Regarding sampling variability between the left and right lobes of the liver, except for necroinflammation, minimal variability was found for steatosis, NAS or fibrosis in a series of morbidly obese patients [20].

4. Pathogenesis of NAFLD

It is clear that, in patients with metabolic syndrome and diabetes, several molecular mechanisms and inflammatory mediators are involved in the development of steatosis, steatohepatitis and fibrosis. Among them, insulin resistance may play a major role in the blockade of hepatic insulin-receptor signaling through activation of different molecules, such as protein kinase C, an inhibitor of kappa B kinase. Superimposed necroinflammatory injury involves additional mechanisms, including oxidative stress, release of endotoxins, and other cytokines and chemokines. Finally, as with other forms of chronic liver disease, the production and accumulation of extracellular matrix by fibrocompetent cells, including portal fibroblasts and hepatic stellate cells, require mobilization of profibrogenic molecules such as connective tissue growth factor and TGF-β [18].

5. Conclusion

NAFLD, which represents the manifestation of metabolic syndrome in the liver, covers a wide spectrum of morphological changes, from steatosis to fibrosis and cirrhosis. Although liver biopsy comes with several drawbacks, it remains the best tool for evaluating, grading and staging the disease so far. In addition to features associated with metabolic syndrome, specific changes related to diabetes have also been described.

Conflicts of interest: The authors have none to declare.

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The role of the lipogenic pathway in the development of hepatic steatosis

C. Postic[a,b*], J. Girard[a,b]

[a]*Institut Cochin, Département d'Endocrinologie, Métabolisme et Cancer, Université Paris-Descartes, CNRS (UMR 8104), Paris, France.*
[b]*Inserm, U567, Paris, France.*

Abstract

Non-alcoholic fatty liver disease (NAFLD) represents a wide spectrum of diseases, ranging from simple fatty liver (hepatic steatosis) through steatosis with inflammation and necrosis to cirrhosis. NAFLD, which is strongly associated with obesity, insulin resistance and type 2 diabetes, is now well recognized as being part of the metabolic syndrome. The metabolic pathways leading to the development of hepatic steatosis are multiple, including enhanced non-esterified fatty acid release from adipose tissue (lipolysis), increased *de novo* fatty acids (lipogenesis) and decreased β-oxidation. Recently, several mouse models have helped to clarify the molecular mechanisms leading to the development of hepatic steatosis in the pathogenesis of NAFLD. This review describes the models that have provided evidence implicating lipogenesis in the development and/or prevention of hepatic steatosis.
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Résumé

Rôle de la lipogenèse dans le développement de la stéatose hépatique

Les maladies métaboliques du foie représentent plusieurs syndromes qui vont de la simple stéatose hépatique à la stéatose hépatique inflammatoire (stéatohépatite) pouvant évoluer vers la nécrose et même la cirrhose. La stéatose hépatique est très fortement associée à l'obésité, la résistance à l'insuline et le diabète de type 2. Les voies métaboliques, qui peuvent conduire au stockage excessif de lipides dans le foie (principalement des triglycérides), sont multiples et peuvent être liées à une augmentation exacerbée de la lipolyse adipocytaire, une synthèse accrue de la synthèse *de novo* des acides gras par la voie de la lipogenèse ainsi qu'à une réduction conjointe de la β-oxydation des acides gras. Au cours des dernières années, des modèles animaux ont permis une meilleure compréhension des mécanismes moléculaires impliqués dans le développement de la stéatose hépatique. Cette revue présente et discute certains des modèles qui ont permis de révéler l'importance de la voie de la lipogenèse dans l'apparition et/ou la prévention de la stéatose hépatique.
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Keywords: NAFLD; Hepatic steatosis; Lipogenesis; Insulin resistance; Review.

Mots clés : Maladies métaboliques du foie ; Lipogenèse ; Stéatose hépatique ; Résistance à l'insuline ; Revue.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is an increasingly common health concern that is considered to be a component of the metabolic syndrome. Excessive accumulation of triglycerides (TG) in hepatocytes is the hallmark of NAFLD. The spectrum of NAFLD can range from simple fatty liver (hepatic steatosis), with a benign prognosis, to the potentially progressive form of non-alcoholic steatohepatitis (NASH), which can lead to fibrosis and cirrhosis, resulting in increased morbidity and mortality. All features of the metabolic syndrome, including obesity, type 2 diabetes, arterial hypertension and hyperlipidemia (elevated TG levels), are associated with NAFLD/NASH [1,2]. The diagnosis of NAFLD is based clinically on high transaminase levels, a high body mass index (BMI), and ultrasound evidence of fat and features of the metabolic syndrome. Liver biopsies are, however, necessary to determine the presence of NASH and to assess the degree of fibrosis [3]. There is currently no generally accepted treatment

*Corresponding author.
E-mail Address: catherine.postic@inserm.fr

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Abbreviations:
ACC: acetyl-CoA carboxylase;
ChREBP: carbohydrate responsive element-binding protein;
CPT-1: carnitine palmitoyltransferase 1;
DAG: diacylglycerol;
DGAT: diacylglycerol acyltransferase;
FAS: fatty acid synthase;
GK: glucokinase;
GPAT: glyceraldehyde-3-phosphate acyltransferase;
HSL: hormone-sensitive lipase;
L-PK: liver pyruvate kinase;
LXR: liver X receptor;
NAFLD: non-alcoholic fatty liver disease;
NEFA: non-esterified fatty acids;
SCD1: stearoyl-CoA desaturase 1;
SREBP-1c: sterol regulatory element-binding protein-1c;
TG: triglycerides;
VLDL: very low-density lipoproteins.

for NAFLD. To date, the only effective treatments of NAFLD are lifestyle changes (diet, weight reduction and exercise). As NAFLD seems to be caused and worsened by insulin resistance, the most promising agents are drugs that restore insulin sensitivity such as thiazolidinediones (TZDs), a class of oral antidiabetic drugs that improves insulin sensitivity by acting as a selective agonist of the nuclear peroxisome proliferator-activated receptor PPAR-γ. They can reduce hepatic and peripheral insulin resistance, decrease hepatic steatosis and attenuate the inflammatory response [4-6]. TZDs exert insulin-sensitizing actions directly on adipocytes (increase number and differentiation, stimulate glucose uptake) and indirectly *via* decreased lipolysis and altered release of adipokines. TZDs decrease the secretion of anti-insulin adipokines (TNF-α and resistin), and increase the secretion of insulin-like adipokine (adiponectin) by adipocytes [7]. However, although effective in the treatment of hepatic steatosis, the limitations of TZDs in NAFLD patients are weight gain and increased body adiposity.

2. Metabolic pathways leading to the development of hepatic steatosis

Excessive accumulation of fat in hepatocytes is the earliest response to and the most common feature of NAFLD. However, the origin of the fat (mainly TG) that accumulates is complex and only partially understood. The potential sources of fat contributing to hepatic steatosis include: (i) dietary fatty acids [mainly through the uptake of intestine-derived chylomicron (CM) remnants]; (ii) increased lipolysis of peripheral fats stored in white adipose tissue that flow to the liver as plasma non-esterified fatty acids (NEFA); and (iii) fatty acids newly made within the liver through *de novo* lipogenesis. After the esterification step (converting fatty acids into TG), TG can then be stored as lipid droplets within hepatocytes or secreted into the blood as very low-density lipoproteins (VLDL), but they can also be hydrolyzed and the fatty acids channeled towards the β-oxidation pathway. Therefore, excessive fat accumulation in the liver can occur as a result of increased fat delivery, increased fat synthesis, reduced fat oxidation and/or reduced fat export in the form of VLDL.

Strong evidence demonstrates that, in NAFLD patients, insulin does not suppress lipolysis to the same extent that it does in healthy individuals [8]. Because insulin has a potent suppressive effect on hormone-sensitive lipase (HSL) [8], studies have examined whether resistance of HSL to insulin in insulin-resistant states is the predominant defect accounting for the increased flux of NEFA from adipose tissue. Studies have revealed that HSL-knockout mice show increased hepatic sensitivity due to reduced plasma NEFA and hepatic TG concentrations [9,10]. Thus, these studies suggest that restricted lipolysis could help to prevent a 'spillover' of fat from adipose tissue to the liver and so prevent hepatic steatosis and/or insulin resistance. Using a multiple stable isotope approach, Donnelly *et al.* [11] estimated that, while 60% of TG accumulated in the liver of NAFLD patients originates from NEFA, a little over 10% comes from the diet and almost 30% from *de novo* lipogenesis. This study underscores the contribution of *de novo* fat synthesis to the pathology of NAFLD.

3. Targeting the lipogenic pathway to prevent hepatic steatosis in mice

De novo fat synthesis (lipogenesis) is the metabolic pathway leading to the conversion of an excess of carbohydrates into fatty acids, which are ultimately esterified with glycerol-3-phosphate to form TG. The activity of the lipogenic pathway is strongly dependent upon nutritional conditions, and it is now clearly established that lipogenic enzyme transcription requires both insulin and glucose to be fully induced [12]. Conditions associated with high rates of lipogenesis, such as a low-fat/high-carbohydrate (LF/HC) diet, hyperglycemia and hyperinsulinemia, are associated with a shift in cellular metabolism from lipid oxidation to TG esterification, thereby increasing the availability of liver TG. The enzymes involved in the synthesis of TG in liver include: (i) glucokinase (GK) [13] and L-pyruvate kinase (L-PK) [14] for glycolysis; (ii) ATP citrate lyase [15], acetyl-CoA carboxylase (ACC) [16] and fatty acid synthase (FAS) [17] for lipogenesis, and long-chain elongase (Elovl6; LCE) [18] and stearoyl-CoA desaturase 1 (SCD1) [19], catalyzing fatty acid elongation and desaturation steps; and (iii) mitochondrial glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT) for TG synthesis [20] (Fig. 1).

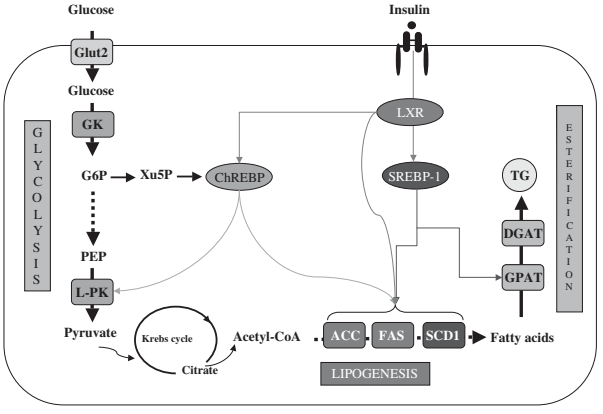

Fig. 1. Transcriptional control of glycolysis and lipogenesis.
The conversion of glucose into fatty acids through de novo lipogenesis is nutritionally regulated, and both glucose and insulin signaling pathways are elicited in response to dietary carbohydrates to synergistically induce glycolytic and lipogenic gene expression. The nature of the glucose-signaling compound was recently identified as the transcription factor ChREBP (carbohydrate responsive element-binding protein). Glucose activates ChREBP by stimulating its gene expression and mediating its post-translational modification(s). ChREBP is required for the induction of L-PK, which is exclusively dependent on glucose. Induction of lipogenic genes, such as ACC, FAS, SCD-1, is under the combined actions of ChREBP and SREBP-1c. Transcription factor SREBP-1c also mediates the effect of insulin on GPAT, although the direct action of ChREBP on GPAT gene expression has not been established. As the nuclear receptor LXR is required for insulin action on SREBP-1c expression, insulin must, in some manner, stimulate the production of an endogenous sterol ligand of LXR (oxysterols). ChREBP is also a direct target of LXR when activated by pharmacological agonists such as T0-901317, but LXR is unable to activate ChREBP expression in response to glucose (Adapted from Robichon *et al.* [54]).

Although rodent models of hepatic steatosis and/or insulin resistance do not always perfectly reproduce the human pathology of NAFDL, the use of transgenic, knockout and knockdown mouse models has helped, over the years, to achieve a better understanding of the molecular determinants of NAFDL [21]. Key enzymes of fatty acid synthesis/desaturation/elongation/esterification such as ACC, SCD1, Elovl6, GPAT and DGAT [22-28] have been shown, when knocked down, to reverse many of the metabolic defects associated with hepatic steatosis and/or insulin resistance, indicating that decreased TG synthesis in liver is a potential and interesting target for the treatment of NAFDL. Among them, SCD1 has emerged as a particularly interesting target for the reversal of hepatic steatosis and insulin resistance [29]. SCD1 is a delta-9 fatty acid desaturase that converts saturated fatty acids (SFA) into monounsaturated fatty acids (MUFA), particularly oleate (C18: 1n-9) and palmitoleate (C16: 1n-7). MUFA are major components of membrane phospholipids, TG and cholesterol esters. SCD1-deficient mice [23] or mice treated with SCD1 antisense nucleotides [24] are protected against diet-induced obesity and insulin resistance when fed a high-carbohydrate/high-fat (HC/HF) diet. The protective effect of SCD1 deficiency is attributed in these mice to a combined decrease in lipogenic rates and activation of the β-oxidation pathway, underlying the metabolic link between these two pathways. Indeed, elevated malonyl-CoA concentrations, the metabolic product of lipogenic ACC, inhibit carnitine palmitoyltransferase 1 (CPT-1), the rate-limiting enzyme of β-oxidation, and regulate the transfer of long-chain acyl-CoAs (LCCoAs) from the cytosol into the mitochondria, thereby resulting in a shift from an oxidative to a reesterification pathway [30]. However, it is not clear how SCD1 deficiency affects and/or regulates lipogenic rates in liver. Liver-specific knockout of SCD1 (LKO mice) also protects against diet-induced obesity and hepatic steatosis [31]. Under both short- and long-term conditions, LKO mice exhibit reduced rates of fatty acid synthesis in liver and decreased expression of key genes of the lipogenic pathway (namely, ACC and FAS). Interestingly, hepatic SCD1 deficiency reduces the nuclear content of two key factors—carbohydrate responsive element-binding protein (ChREBP) and sterol regulatory element-binding protein (SREBP-1c) [31]—involved in the transcriptional control of lipogenic gene expression in response to glucose and insulin, respectively, as discussed below (Fig. 1). However, once again, the mechanism by which SCD1 affects the maturation and/or translocation of these two transcription factors is not clear, but could be linked to MUFA concentrations in hepatocytes. Clearly, a better knowledge of the function and/or regulation of the transcription factors involved in the activity of lipogenic enzymes may, in the future, help in the development of potential therapeutic approaches.

4. Transcriptional control of fat synthesis *via* SREBP-1c, LXR and ChREBP

Lipogenic gene expression is coordinately controlled by key transcriptional regulators: SREBP-1c in response to insulin; and ChREBP in response to glucose [12,32]. Liver X receptors (LXRs) are ligand-activated transcription factors that belong to the nuclear hormone-receptor superfamily [33]. LXRs play a key role in cholesterol and bile acid metabolism, but are also important regulators of the lipogenic pathway, as LXRs are essential for transcriptional control of SREBP-1c by insulin [34-36]. Direct targets of LXR include FAS and SCD1 [27,37]. ChREBP is regulated by glucose at the transcriptional level [38] and was also recently identified as a direct target of LXRs [39,40]. ChREBP is particularly important for the induction of liver pyruvate kinase (L-PK), which is exclusively dependent on glucose [41]. Induction of lipogenic genes (ACC, FAS, SCD1) is under the concerted action of ChREBP, SREBP-1c and LXRs in response to nutritional signals [12,21,36] (Fig. 1).

So far, the relative importance of these transcriptional factors in controlling the synthesis of fat in response to glucose and insulin signals has been difficult to ascertain because they act either independently and/or synergistically to regulate their target genes. We have recently demonstrated that liver-specific inhibition of ChREBP by decreasing the rate of hepatic lipogenesis improved hepatic steatosis and insulin resistance in obese *ob/ob* mice [42]. These results suggest that ChREBP is a potential therapeutic target and, therefore, accurate knowledge of the mechanisms involved in regulating its expression and activation is crucial for the development of pharmacological approaches in the treatment of metabolic diseases. The mechanism responsible for ChREBP activation at the post-translational level involves an increase in intracellular glucose metabolism [43]. At low glucose concentrations, ChREBP is an inactive phosphorylated cytosolic protein whereas, at high glucose concentrations, ChREBP undergoes dephosphorylation (on Ser-196), and is translocated into the nucleus to activate its target genes [44]. Because this mechanism has only recently been demonstrated with the endogenous protein, the regulation of ChREBP by phosphorylation/dephosphorylation was controversial [45,46]. However, the use of a phospho-specific antibody that we developed provided, for the first time, a direct correlation between the modulation of Ser-196 phosphorylation and intracellular localization of the endogenous ChREBP protein in liver [40].

5. Is hepatic steatosis always associated with insulin resistance?

As already mentioned in the introduction, the excess accumulation of TG in hepatocytes is the hallmark of NAFLD, which is strongly associated with insulin resistance [2,47]. However, despite the correlation between fatty liver and insulin resistance, it remains unclear whether or not insulin resistance causes the excess accumulation of TG in liver, or whether or not the increase in TG itself or of metabolite intermediates plays a causal role in the development of hepatic or systemic insulin resistance. Recent studies have favored the hypothesis that the accumulation of intrahepatic lipids precedes the state of insulin resistance, although others have shown that hepatic TG *per se* are not toxic and may, in fact, protect the liver from lipotoxicity by buffering the accumulation of fatty acids [48,49]; this suggests that hepatic steatosis is not necessarily associated with insulin resistance. Indeed, the overexpression of key enzymes of the esterification pathway (such as DGAT2) [50] or blockade of VLDL secretion [51] show a clear dissociation between marked hepatic steatosis and insulin resistance. Recent studies have also shown that the lipid species (length of the carbon chain and/or the degree of saturation) that accumulate in the steatotic liver may not be equally deleterious for hepatic insulin sensitivity [28,31]. Further experiments are needed to better understand how fatty acid composition influences hepatic insulin sensitivity.

6. Conclusion

NAFLD appears to be one of the most frequent causes of liver dysfunction, and its incidence has increased markedly over the years. While the mechanisms involved in the pathogenesis of NAFLD in humans have not been thoroughly investigated, a recent study has reevaluated the contribution of lipogenesis to the development of hepatic steatosis and revealed that the expression of fatty acid metabolism-related genes, such as ACC and FAS, are indeed increased in NAFLD [52] (Fig. 2). Analyses of the expression of lipogenic transcription factors—namely, ChREBP, SREBP-1c and LXR—have revealed that expression levels of LXR are four times greater in the liver of NAFLD patients than in that of controls and was significantly correlated with SREBP-1c, but not ChREBP, levels [53]. In our opinion, more information on the ChREBP contribution to NAFLD in needed, and additional studies of ChREBP activity (nuclear protein content/phosphorylation levels) are also required.

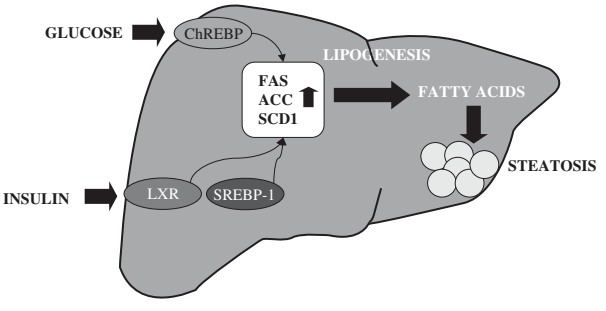

Fig. 2. Role of the lipogenic pathway in the development of hepatic steatosis. Non-alcoholic fatty liver disease (NAFLD) is one of the most frequent causes of liver dysfunction, and its incidence has increased markedly over the years. While the mechanisms involved in the pathogenesis of NAFLD in humans have not been thoroughly investigated, enhanced activity of the lipogenic pathway very likely contributes to the development of hepatic steatosis in NAFLD. In response to insulin and glucose, sterol regulatory element-binding protein (SREBP)-1c and carbohydrate responsive element-binding protein (ChREBP) are activated, respectively, and induce the expression of lipogenic genes, including ACC, FAS and SCD1. SREBP-1c and ChREBP are also transactivated by the nuclear receptor LXR that regulates the metabolism of cholesterol and fatty acids. More knowledge of the respective roles of these transcription factors in the pathogenesis of NAFLD is now needed.

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Insulin resistance and steatosis in humans

J. Capeau

Univ-Paris 6, Inserm UMR_S893 Équipe 9, Faculté de Médecine Pierre et Marie Curie, Site Saint-Antoine 27, rue Chaligny, 75571 Paris cedex 12, France. APHP, Hôpital Tenon, 75020 Paris, France.

Abstract

Insulin resistance is commonly found in a large number of adults—in particular, those with android obesity, the metabolic syndrome or type 2 diabetes. Strong adverse relationships between adipose tissue, liver and muscles in these patients result in lipotoxicity, with deposition of triglycerides (TG) within the liver and muscles together with insulin resistance. Such a situation is also seen in lipodystrophic patients with fat loss. Insulin signals in the liver through its tyrosine-kinase receptors to negatively control hepatic glucose production (HGP), replenish glycogen stores and synthesize fatty acids (FA), leading to TG exported as VLDL. In liver insulin resistance, HGP is increased mainly by activation of the gluconeogenic pathway, resulting in increased fasting glycemia. Lipogenesis is also increased possibly due to direct activation of the SREBP-1 transcription factor and together with increased FA availability results in an increased production of VLDL-TG. An imbalance between the pathways of TG synthesis and oxidation or export results in 'metabolic' steatosis. Increased cellular FA derivatives activate stress kinases, leading to phosphorylation of serine in insulin receptor substrate (IRS) proteins and, hence, insulin resistance. A number of studies in normal subjects and patients have revealed a strong association between insulin resistance and metabolic steatosis. Moreover, when insulin resistance is decreased by weight loss in obese subjects or by treatment with insulin sensitizers such as thiazolidinediones, the levels of liver fat and insulin resistance vary accordingly. An important question that remains unanswered concerns the relationship between steatosis and non-alcoholic steatohepatitis (NASH), and the potential roles of insulin resistance together with inflammation and oxidative stress in such a setting.
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Résumé

Insulinorésistance et stéatose hépatique chez l'homme

La résistance à l'insuline est une situation fréquente en clinique, en particulier chez les sujets qui présentent une obésité androïde, un syndrome métabolique ou un diabète de type 2. Les interactions délétères entre tissu adipeux, foie et muscle chez ces patients induisent un état dit « lipotoxicité » avec dépôt intrahépatique et musculaire de triglycérides et résistance à l'insuline. Une situation semblable est observée chez les patients lipodystrophiques qui présentent un défaut de tissu adipeux. L'insuline agit en activant son récepteur membranaire à activité tyrosine-kinase qui réprime la production hépatique de glucose (PHG), remplit les stocks de glycogène et active la synthèse des acides gras et aboutit à la production de triglycérides exportés sur les VLDL. En cas de résistance à l'insuline, la PHG est élevée, du fait de l'activation de la voie de la gluconéogenèse aboutissant à une hyperglycémie à jeun. La lipogenèse reste également élevée, du fait sans doute d'une activation directe du facteur de transcription SREBP-1, et avec la disponibilité accrue en acides gras libres, induit une augmentation de la production de VLDL riches en TG. Un déséquilibre entre la voie de synthèse et les voies d'oxydation ou d'export des TG aboutit à une stéatose métabolique. Dans ce cas, les dérivés d'acides gras présents dans la cellule activent des kinases de stress qui vont phosphoryler les protéines substrats du récepteur, IRS, sur des résidus sérine, inhibant la transmission du signal insuline. Plusieurs études réalisées chez des sujets en bonne santé et des patients ont mis en évidence une association étroite entre le degré de résistance à l'insuline et de stéatose métabolique. De plus, lorsque l'insulinorésistance est améliorée par une perte de poids chez le patient obèse ou par des traitements insulinosensibilisateurs comme les thiazolidinediones, la quantité de lipides hépatiques et la résistance à l'insuline varient en parallèle. Une question importante, et qui reste non résolue, concerne la relation entre la stéatose simple et la stéatohépatite non alcoolique et le rôle potentiel qu'y jouent le degré de résistance à l'insuline ainsi que l'état inflammatoire et le stress oxydant.
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Keywords: Insulin; Steatosis; Adipose tissue; Liver; Triglycerides; Lipogenesis; Hepatic glucose production; Free fatty acids; Lipotoxicity; Review.

Mots clés : Insuline ; Stéatose ; Tissu adipeux ; Foie ; Triglycérides ; Lipogenèse ; Production hépatique de glucose ; Acides gras libres ; Lipotoxicité ; Revue.

*Corresponding author.
E-mail Address: jacqueline.capeau@inserm.fr

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1. Introduction

Liver steatosis indicates the presence of triglycerides (TG) as lipid droplets within hepatocytes. In the case of metabolic disorders, metabolic steatosis has a macrovesicular pattern. The presence of steatosis, while normal in the liver of migrating birds, such as ducks and geese, that require energy stores to overcome prolonged fasting periods when flying south in autumn, is not physiologically normal in humans. Steatosis is generally associated with increased adipose-tissue stores—in particular, in the abdominal visceral and subcutaneous depots, such as found in android obesity, the metabolic syndrome and type 2 diabetes—and is also characterized by insulin resistance. This means that the association between insulin resistance and steatosis is clear. Indeed, the condition is very common and probably, when mild, requires lifestyle changes, but not aggressive pharmacological interventions. The severity of steatosis is driven by its possible evolution towards steatohepatitis (NASH) and the long-term consequences, and the roles played by insulin resistance and inflammation need to be determined, as discussed in the report by K. Clément and colleagues (also in this issue).

Insulin resistance is a common feature present in a number of physiological and pathological conditions in humans. It plays a leading role in diseases related to adipose-tissue dysfunction such as abdominal obesity and the metabolic syndrome, which are characterized by increased amounts of abdominal fat that lead to insulin resistance, and have repercussions on metabolic parameters such as altered glycemia, dyslipidemia with decreased circulating HDL and increased LDL, and raised blood pressure. Insulin resistance is also central to diseases that associate adipose-tissue dysfunction and endocrine pancreas deficiency such as type 2 diabetes. It is now thought that non-alcoholic fatty liver disease (NAFLD) is a component of the metabolic syndrome and type 2 diabetes that may progress to NASH in the long-term, along with complications of fibrosis and cirrhosis. Severe insulin resistance is observed in patients with less common diseases such as lipodystrophy, with decreases in some fat depots such as subcutaneous adipose tissue and, sometimes, with increases in other depots such as visceral fat. This abnormal fat repartitioning results in severe metabolic alterations with dyslipidemia and insulin-resistant diabetes together with NAFLD and a frequent evolution towards NASH. The origin of human lipodystrophy may be genetic—in particular, disorders leading to complete lipodystrophy such as Berardinelli– Seip congenital lipodystrophy (BSCL), or partial forms such as familial partial lipodystrophy (FPLD), that are linked to mutations of the gene encoding lamin A/C or PPARγ. Lipodystrophy may also be acquired such as observed in HIV-infected patients receiving antiretroviral drugs or in patients treated with corticoids [1]. Insulin resistance is also present in diseases that primarily affect the liver such as chronic hepatitis C.

In the liver, insulin is involved in a number of actions responsible for glucose control and lipid metabolism. In case of insulin resistance, insulin levels are raised to overcome this resistance. The resulting effects depend on the metabolic pathway: a deficient insulin response for glucose metabolism leads to increased glucose production in the fasting state, while elevated insulin leads to activation of the lipid biosynthetic pathway, resulting in increased VLDL production and dyslipidemia. Given the central role played by the liver in lipid metabolism, any imbalance between the entry and export of lipid derivatives results in steatosis.

Steatosis and insulin resistance have a number of reciprocal relationships and can enhance each other. Increased oxidative stress and stress of the endoplasmic reticulum are probably some of the altered mechanisms in this setting. Insulin resistance at the adipose-tissue level plays an important role in hepatic insulin resistance: increased free fatty acid (FFA) production favors lipid deposition in the liver. The inflammatory signals released in adipose-tissue diseases also play a leading role, with increased proinflammatory and decreased adiponectin signalling in the liver.

Inflammatory signals within the liver have also to be considered: activation of the immune system and Kupffer cells results in the local release of proinflammatory cytokines.

In addition, a role for mitochondria has recently emerged, and the close link between mitochondrial dysfunction and insulin resistance has been clearly outlined. The most likely mechanism to explain such a connection is increased oxidative stress. Furthermore, increased stress of the endoplasmic reticulum due to lipid overload is involved in hepatic dysfunction, thus linking steatosis and insulin resistance.

A number of pathways have been explored in animal models, and a number of studies have outlined the potential mechanisms resulting in liver insulin resistance in murine models of obesity (see the report by Postic and Girard in this issue). However, while steatosis is easily produced in animal models, NASH is not, thereby limiting analysis of the pathophysiological mechanisms responsible for the transition between the two stages. Nevertheless, transversal clinical studies can reveal the presence of an association between several dysfunctions, although a causal link is more difficult to demonstrate. More important, longitudinal studies in patients who are losing weight or being treated with insulin sensitizers have revealed a correlation between various stages of steatosis and insulin resistance, thereby clarifying the mechanisms acting at that level. However, the factors involved in the evolution of steatosis to NASH have yet to be confirmed in patients.

2. Insulin signalling in the liver

Hepatocytes are one of three types of insulin target cells (along with myocytes and adipocytes) that carry a large number of insulin receptors on their cell surfaces. Insulin signalling can only take place through insulin receptors [2,3].

2.1. Insulin signalling pathways

One molecule of insulin is able to bind and activate one insulin receptor, a transmembranous protein comprising four subunits—two α and two β. The β subunits possess tyrosine-kinase activity in their intracytoplasmic domain, which is activated after linkage with insulin and phosphorylated on specific tyrosine residues of the receptor itself. This phosphotyrosine signal is recognized by protein substrates of the receptor that become activated and transmit the insulin signal in the cell. The main family of substrates is the 'insulin receptor substrate' (IRS) family, which has four members (IRS1-4). In addition, phosphotyrosines of the insulin receptor β subunit can also be recognized by substrates from the SHC and CAP/cbl families. Two main pathways diverge from the receptor. One leads to activation of phosphatidylinositol 3-kinase (PI3-kinase), then PKB/Akt or atypical PKC for insulin metabolic activities such as increased glycogen synthesis, lipogenesis and inhibition of gluconeogenesis. The other—the 'canonical MAP kinase pathway'—leads to activation of cell proliferation and differentiation.

The main IRS isoforms involved in hepatocytes are IRS1 and IRS2. Recent studies have clarified their respective roles in these cells. IRS1 is always present, and has a major role after feeding in controlling glycogen synthesis and lipogenesis where there is excess glucose in the circulation that needs to be stored in hepatocytes as glycogen or exported from hepatocytes as TG on VLDL. IRS2 is involved in control during the fasting state, when its level is markedly upregulated to allow insulin to limit hepatic glucose production (HGP) by controlling the expression of two gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase [4-6].

2.2. Insulin controls hepatic glucose and lipid metabolism

Insulin controls glucose metabolism in the liver. After meals, glucose and other sugars from nutriments, released as monosaccharides in the intestine, enter hepatocytes from the portal blood through glucose transporter GLUT2, always present in the hepatocyte plasma membrane; this allows the entry of glucose when circulating levels are increased and its export during fasting periods, when glucose levels are lowered. Within hepatocytes, glucose is directed towards glycolysis and ATP production, as required for energy by the cell and to replenish glycogen stores. When glycogen stores and energy requirements are fulfilled, glucose is diverted towards lipid synthesis, first by glycolysis, then by the initiation of the mitochondrial tricarboxylic cycle and, finally, by lipogenesis in the cytosol to produce fatty acids (FA). Insulin is a strong activator of these pathways and especially of the lipogenesis pathway through activation of the expression and proteolytic maturation of the transcription factor SREBP-1, acting in conjunction with the glucose-responsive transcription factor CHREB, thereby leading to the increased expression of the enzyme glucokinase in glucose metabolism, acetyl-CoA carboxylase and FA synthase in the lipogenesis pathway. FAs are esterified as TG associated with apolipoprotein B (apoB) and exported as VLDL lipoproteins. This means that lipid metabolism is strongly related to glucose metabolism in hepatocyte and biosynthetic pathways leading to glycogen and TG synthesis activated by insulin. Circulating TG-rich lipoproteins, VLDL from the liver, and chylomicrons produced by the intestines and bearing lipids directly from the diet are all deprived of their TG by the activity of adipocyte lipoprotein lipase, activated by insulin. FAs released from TG enter adipocytes and are stored in lipid droplets as TG.

Insulin is a pleiotropic hormone that also activates protein synthesis and cell proliferation, and inhibits cell apoptosis. In the postabsorptive and fasting states, insulin levels are low and liver energy metabolism is regulated by glucagon in priority. In adipose tissue, the lipolysis of TG, which is strongly inhibited by insulin, is activated by epinephrine and allows the release of FFA. These substrates are used by the liver and muscles to produce energy as ATP, after being oxidized, which occurs mainly inside the mitochondria through the β-oxidation pathway, the tricarboxylic cycle and the respiratory chain. In the postabsorptive state, the liver produces glucose to feed the brain and glucose-dependent tissues. Glycogen breakage is activated to replenish circulating glucose, then HGP uses the glyconeogenic pathway. The key enzyme, PEPCK, is regulated at the transcriptional level and activated by transcription factor FOXO1, present in the nucleus. Glucagon activates FOXO1 and the gluconeogenic pathway. Conversely, insulin signalling leads to FOXO1 phosphorylation by the kinase Akt/PKB, which induces its cytosolic retention and degradation by the proteasome, thereby impeding PEPCK activation and HGP. Insulin inhibits FA oxidation at the level of its entry into mitochondria by its action of increasing malonyl-CoA, an inhibitor of enzyme CPT1 (carnitine palmitoyltransferase type 1) in charge of FA mitochondrial import. Glucagon decreases malonyl-CoA levels and, thus, allows the entry of FFA from adipose tissue into mitochondria to be oxidized.

3. Insulin resistance in the liver

3.1. Consequences for glucose metabolism

Insulin resistance in hepatocytes results in less storage of glycogen in the postprandial state and more HGP in the fasting state. Indeed, glucose production by glycogen degradation is normally inhibited by insulin but, when glycogen stores are reduced, the increased expression of PEPCK allows an increase of HGP by the gluconeogenic pathway [7]. Interestingly, a recent study measured the expression of FOXO1 in NASH. The authors found evidence that, in humans, fatty liver and NASH are associated with a progressive increase in the expression of PEPCK and glucose-6-phosphatase. The expression of PEPCK correlates with insulin resistance as measured by the HOMA index. Interestingly, FOXO1 expression and activity are also increased in patients with NASH, and mRNA levels correlate with hepatic insulin resistance. In the presence of oxidative stress, FOXO1 is hypophosphorylated by the insulin-signalling pathway and is localized in the nucleus. Moreover, the expression of FOXO1 is correlated with the severity of steatosis and necroinflammation [8].

3.2. Lipid metabolism in hyperinsulinaemia

A number of studies have reported that, with insulin resistance as seen in visceral obesity, the metabolic syndrome and type 2 diabetes, lipogenesis—an insulin-responsive pathway—is enhanced and not decreased as expected [9]. Although difficult to understand, one explanation could be that the different insulin-signalling pathways in the liver are differentially sensitive to insulin: for example, the pathway leading to SREBP-1 activation remains sensitive while that suppressing HGP is non-responsive. Another element to consider is the activation of lipogenesis by CHREB and SREBP-1 transcription factors, which are responsive to high glucose levels, as seen in diabetes, as well as to insulin. In addition, recent studies suggest that SREPB-1 could be activated independently of insulin due to the presence of increased stress of the endoplasmic reticulum, as observed in steatotic liver in response to fat overload and, in particular, with saturated FAs, leading to increased lipogenesis [10,11]. Finally, TNF-α, a proinflammatory cytokine that is increased with hepatic inflammation, can also activate SREBP-1, resulting in increased lipid synthesis within the liver [12].

Increased deposition of TG occurs in several tissues outside of adipose tissue in insulin resistance and contributes to lipotoxicity [7].

Given the increased production of FFA by adipose tissue together with decreased adiponectin secretion, FA derivatives such as acyl-CoA and diacylglycerol accumulate in the cytosol in muscle, liver, pancreas and cardiac cells. Indeed, most FFA are driven towards mitochondria where they are oxidized to provide energy but, with high levels, mitochondrial dysfunction can occur in some subjects (depending on, for example, age, a family history of diabetes or a high-fat diet); if adiponectin, which controls mitochondrial entry and oxidation, is deficient, then FA derivatives can accumulate in the cytosol, although most will enter the mitochondria. Increased oxidation could result in increased generation of reactive oxygen species (ROS), leading to an oxidative stress response and activation of Jun kinase. Also, in the cytosol, they may activate atypical PKC, which can phosphorylate IRS1 on serine residues, leading to insulin resistance. This pathway was first shown by Petersen and Shulman in human muscles [13], but it is also present in human liver and other tissues. The fact that TG is stored in these tissues probably represents a protective mechanism that buffers toxic FA derivatives by storing less toxic TG within lipid droplets.

Thus, insulin resistance can result from a number of adverse situations, such as hyperinsulinemia, and lead to the production of molecules able to decrease insulin action such as proinflammatory cytokines like TNF-α or IL-6, or increased FFA.

3.3. Molecular mechanisms of insulin resistance

The molecular mechanisms underlying insulin resistance have been partially described. The number of insulin receptors present on the surface of hepatocytes is regulated by the kinetics of insulin receptor biosynthesis and degradation that are normally equivalent, leading to a consistent number of receptors on the cell surface. The degradation pathway involves receptor internalization into clathrin-coated vesicles, then endocytosis and either recycling to plasma membrane or degradation. This pathway allows insulin linked to the receptor to be degraded in late endosomes or lysosomes. When insulin levels are increased, as found in insulin resistance, the insulin– insulin receptor complexes are internalized at an increased rate, thus allowing insulin degradation. But even if most receptors are recycled back to the membrane, some will be degraded together with insulin, leading to decreased receptor numbers, the so-called ‘downregulation phenomenon’, resulting in fewer surface receptors as long as insulin levels are high and, therefore, in insulin resistance. Deactivation of insulin receptors and IRS proteins requires the action of tyrosine phosphatases, and several phosphatases that decrease the insulin signal have been proposed, including PTP1B and LAR. Otherwise, increased insulin levels characteristic of insulin resistance will further decrease insulin signalling by acting on IRS protein serine phosphorylation through the activation of stress kinases such as Jun kinase or IKKβ kinase in the NFκB pathway. A number of studies have shown that serine phosphorylation of critical residues

on IRS1—in particular, serine 307 or 312—blocks transmission of the insulin signal [3,14,15]. Thus, instead of being a natural enhancer of insulin action through tyrosine phosphorylation, IRS when phosphorylated on serine becomes an important step in impeding the action of insulin and leading, in turn, to further desensitization to insulin.

The proinflammatory cytokines are also able to induce insulin resistance [14,16,17]. TNF-α, produced either locally by activated Kupffer cells or at a distance in adipose tissue, is able, through binding to surface receptors, to activate stress kinases such as Jun kinase and IKKβ, leading again to serine phosphorylation of IRS proteins and the blocking of insulin signal transduction. IL-6 can induce insulin resistance by acting on the suppressor of cytokine signalling (SOCS) protein family and, in particular, by inducing SOCS3, a protein that inhibits insulin signalling through insulin receptors and IRS proteins driven towards degradation by the proteasome pathway.

The ability of FFA to induce insulin resistance has been explained by the ability of their intracellular derivatives, acyl-CoA and diacylglycerol, to activate isoforms of atypical PKC that are able to phosphorylate IRS proteins on serine residues, thereby leading to insulin resistance. In addition, recent studies have revealed that FFA can also activate toll-like receptor 4 (TLR4), present on Kupffer cells [18]. These receptors, activated also by bacterial lipopolysaccharides, are connected to the proinflammatory pathways in macrophages and, through activation of the NFκB pathway, lead to increased production of the proinflammatory cytokines TNF-α, IL-6 and IL-1β.

3.4. The role of adipokines

Adiponectin and leptin produced by adipose tissue may also play a role in the liver. Adiponectin is the most abundant adipokine, and its production by adipose tissue is strongly related to insulin sensitivity. It acts on signalling pathways within a number of cell types by binding to two surface receptors—AdipoR1 and AdipoR2—which are involved in the activation of PPARα in hepatocytes, leading to the expression of genes involved in FA oxidation and activation of AMP kinase, which also increases FA oxidation, in mitochondria. In addition, adiponectin inhibits the expression of the gluconeogenic enzymes PEPCK and glucose-6-phosphatase, thus decreasing HGP. In general, adiponectin favors insulin action, is protective against FA-derivative accumulation in the cytosol and prevents stress-kinase activation. In NAFLD patients, circulating adiponectin levels were reported to be negatively related to hepatic insulin resistance and the amount of fat [19]. Accordingly, in human studies of lipoatrophic and obese individuals, adiponectin concentration was inversely related to liver fat content and insulin resistance [20-22].

The role of leptin in the liver is complex. This adipokine is secreted by adipose tissue, and its levels are elevated in obesity. Leptin also activates AMP kinase, allowing increased FA oxidation. However, obese subjects are resistant to leptin.

Adiponectin and leptin are produced in stellate cells, the former when cells are not activated and exert antifibrogenic properties, the latter in myofibroblasts which participate in liver fibrosis [12].

4. Cellular stress

In addition to these molecules acting at the extracellular level and inducing a decreased response to insulin, other intracellular mechanisms are also probably involved. Mitochondrial dysfunction has been shown to induce insulin resistance. Mitochondria play a central role in glucose and FA oxidation to synthesize energy. During the process of respiratory chain function, some ROS are generated, which is useful for the cell. However, in situations of defective mitochondrial function in terms of required FA oxidation, the level of ROS is increased within mitochondria and leaks out of the mitochondria into the cytosol, where it activates stress kinases and leads to insulin resistance. Mitochondrial dysfunction is present in tissues during the aging process, which leads to age-related insulin resistance. It has also been observed in relatives of type 2 diabetic patients even before the occurrence of diabetes, suggesting a role for genetic factors in mitochondrial defects [13]. It may even be induced by a number of drugs acting on the liver. In addition, it may result from the increased FA pool leading to steatosis in humans. An important role has been established for the transcription factor PGC1α, activated by PPAR receptors and involved in mitochondrial biogenesis in the face of increased oxidative requirements [23].

The importance of oxidative stress and mitochondrial dysfunction in NASH has been outlined by Sanyal *et al.* [24], who studied liver samples from patients with steatosis compared with NASH. Both NASH and steatosis were associated with insulin resistance in muscle and adipocytes, as indicated by decreased glucose control during a euglycemic– hyperinsulinemic clamp and abnormal glycerol production by adipose tissue. However, it is worth noting that only NASH samples showed mitochondrial alterations with loss of mitochondrial cristae and inclusions. This was associated with markers of increased oxidative stress in the liver. Therefore, mitochondrial dysfunction was present in NASH samples. Increased FA influx to the liver was associated with increased β-oxidation, which suggests that it could be responsible for increased ROS production.

Another stress pathway that has been more recently identified in the liver is endoplasmic reticulum stress. Increased fat and FAs in the liver could activate this pathway, which is ini-

tiated at the level of the endoplasmic reticulum. The synthesis of proteins is normally controlled by chaperone proteins such as BIP. In cases of increased saturated lipid contents in the endoplasmic reticulum, its integrity and morphology are compromised. BIP dissociates from neosynthesized proteins and stops protein synthesis, then initiates a stress pathway that could activate stress kinases and, thus, lead to insulin resistance. ER stress can also activate SREBP-1, leading to increased lipogenesis and steatosis [11].

5. What is the origin of the agents acting on hepatocytes to control insulin sensitivity?

Both extrahepatically and intrahepatically produced agents are involved in hepatic insulin resistance, and adipose tissue is among the main contributors. In the presence of increased visceral as well as upper-body subcutaneous fat, it has been observed that adipose tissue presents a low-grade state of inflammation that results in an increased production of FFA and proinflammatory cytokines, and a decreased production of adiponectin. These alterations result in increased insulin resistance in the liver (see the report by K. Clément and colleagues in this issue).

Diet is also important. The level of saturated FAs from diet can increase insulin resistance and liver fat content. Studies in humans have revealed that about 15% of dietary FAs are incorporated in hepatic fat and secreted as VLDL-TG after a meal [25]. This suggests that the liver takes up a significant fraction of dietary lipids in the postprandial period. A study by Westerbacka *et al.* evaluated liver fat by proton spectroscopy and markers of insulin sensitivity in 10 non-diabetic obese women at baseline and after 2 weeks of isocaloric periods containing either 16% or 56% of total energy intake as fat. Interestingly, liver fat content decreased in the low-fat period together with insulin levels, and increased in the high-fat period in parallel with insulinemia. The other fat depots and metabolic parameters were not altered. This means that dietary fat influences liver fat content and possibly also hepatic insulin sensitivity [26].

The production of proinflammatory cytokines by activated Kuppfer cells are also involved.

6. Pathophysiology of steatosis in humans

The occurrence of fat as TG in liver cells results from an imbalance between TG synthesis and degradation. The synthesis pathway results from the *de novo* synthesis of FAs, then TG from glucose, and is activated by hyperinsulinemia, as seen in patients with the metabolic syndrome, type 2 diabetes or lipodystrophy syndromes. Increased TG also results from increased FFA in the liver due to increased lipolysis by insulin-resistant adipose tissue in cases of increased visceral and subcutaneous abdominal fat depots. The sources of FAs stored in the liver and secreted via lipoproteins in patients with NAFLD have been identified: 60% comes from FFA; 26% from *de novo* lipogenesis; and 15% from diet [25]. It has been shown that the contribution of splanchnic lipolysis to hepatic FFA delivery ranged from < 10% in lean subjects to almost 50% in obese subjects, and increased as a function of visceral fat amounts in both women and men. Leg and splanchnic tissues contributed to a greater release of systemic FFA in obese *vs* lean men and women. Nevertheless, visceral fat does not account for the majority of portal FFA, and subcutaneous fat from the upper body plays a leading role [27]. In addition, as indicated above, about 15% of dietary lipids are recovered in VLDL. Indeed, increased TG synthesis also results from the increase in TG-rich lipoproteins due to an increased production of chylomicrons with a fat-rich diet and an increased production of VLDL by the liver. Insulin resistance in adipose tissue leads to less exportation of lipoprotein lipase of the vessel surface and less lipoprotein hydrolysis, thereby increasing levels of remnant TG-rich lipoproteins going back to the liver. In addition to increased FA flux, another factor that controls the fat pool is the efficiency of FA oxidation in the mitochondria. In studies of patients with steatosis or NASH, FA oxidation was not impaired [24].

Finally, TG equilibrium requires the efficient export of TG on VLDL, which is controlled by levels of apoB, the protein required to recover the VLDL particle. An increased degradation of apoB has been reported in response to insulin relative to the level required to sort VLDL, therefore leading to increased TG storage within hepatocytes i.e. in steatosis [9]. This impairment of apoB is relative as, in cases of massive FA availability due to increases in the different biosynthetic pathways, a relative deficiency of apoB leads to steatosis together with an increased release of VLDL and increased circulating TG levels.

For these reasons, steatosis appears to be a consequence of insulin resistance both at the level of adipose tissue and in the liver.

7. Relationship between steatosis and insulin resistance in humans

The link between steatosis and insulin resistance has been reported in several studies in patients with the metabolic syndrome or diabetes as a reciprocal, positive relationship. Interestingly, such a link has also been observed in young, lean, healthy subjects of different ethnic origins: Asian-Indian men present an increased prevalence of insulin resistance

and NAFLD compared with other ethnic groups, and this is associated with an increased circulating level of IL-6 [28]. Also, insulin-resistant fatty liver overproduces a number of molecules that are deleterious at the vascular level and generate a state of low-grade inflammation that is prothrombotic, increasing cardiovascular risk and including high glucose levels with the production of advanced glycation end-products (AGE) involved in diabetic complications [12], VLDL-TG, plasminogen activator inhibitor-1 (PAI-1), coagulation factors, C-reactive protein (CRP) and fibrinogen [17,29].

Several studies have revealed an association between levels of liver fat and insulin resistance in patients with obesity, the metabolic syndrome or type 2 diabetes [29,30]. The presence of NAFLD predicts type 2 diabetes: in the NHANES-III survey, adults with NAFLD were twice as likely to have type 2 diabetes than those without NAFLD, after adjustment for age, gender, race and BMI [29].

Yki-Jarvinen evaluated whether the amount of liver fat was related to components of the metabolic syndrome and to insulin sensitivity in 271 non-diabetic subjects. Liver fat was fourfold higher in those with *vs* without the metabolic syndrome, and this association was independent of age, gender and BMI. Liver fat was associated with levels of transaminases ASAT and ALAT as surrogate markers of NAFLD. The amount of liver fat has been related to the amount of subcutaneous fat in both men and women [31], although the relationship was more striking with visceral fat. In fact, the best correlate of liver fat was fasting plasma insulin and C-peptide in both men and women, indicating that it is not the visible fat, but rather the fat hidden in the liver that is the most accurate indicator of insulin resistance [29].

The possible involvement of dietary fat in liver fat content and insulin resistance has already been mentioned. Bugianesi *et al.* [19] investigated insulin sensitivity in non-obese, non-diabetic patients with NAFLD, but without dyslipidemia, increased visceral fat or hypertension, who were matched with controls by age, BMI and body composition. Insulin resistance was present in patients with NAFLD in a pattern consistent with accelerated lipolysis, the result of resistance in adipose tissue leading to an increased FFA supply and oxidative use of lipid at the whole-body level. In the liver, which normally extracts FFA with high efficiency, higher rates of lipid oxidation and impaired suppression of hepatic lipid oxidation by insulin indicated the presence of insulin resistance. This study suggested that, in such patients, most of the hepatic insulin resistance results from the increased fatty substrate delivery by adipose tissue.

As previously described, insulin resistance was present in patients with NAFLD in the study of Sanyal *et al.* [24], who compared them with patients without steatosis, but with increased oxidative stress and FA oxidation. Indeed, only patients with NASH presented altered mitochondrial function.

8. Steatosis and insulin resistance after weight loss or treatment with insulin sensitizers

Weight loss is effective in reducing liver fat. Eight obese patients with type 2 diabetes were studied before and after weight stabilization using a moderately hypocaloric very low-fat diet. Weight losses of about 8 kg resulted in normalization of fasting plasma glucose and HGP together with an 81% reduction in intrahepatic lipid. However, there were no significant changes in either insulin-stimulated peripheral glucose uptake or intramyocellular lipid levels. Therefore, moderate weight loss was able to reverse liver fat and liver insulin resistance independently of any change in peripheral glucose metabolism [32]. Similar data have been reported in other studies [22,30], indicating that steatosis and insulin resistance evolve in parallel.

Moreover, longitudinal studies of patients with NASH have evaluated the ability of PPARγ agonists, such as pioglitazone or rosiglitazone, and metformin to improve steatosis and insulin resistance. Metformin is used in the treatment of type 2 diabetes, and its positive action on liver fat and insulin sensitivity has been revealed in several studies, but was not confirmed in a double-blind, randomized study of type 2 diabetics, where metformin treatment improved basal hepatic insulin sensitivity, but did not change the amount of liver fat [22].

Thiazolidinediones (TZD) lower levels of FFA by reducing adipose-tissue lipolysis while dramatically increasing circulating levels of adiponectin, which is correlated with changes in liver fat independent of BMI and increased insulin sensitivity [20,29,30].

A recent randomized, double-blind study compared the effects of rosiglitazone and placebo on steatosis and insulin sensitivity. In this so-called FLIRT trial, 63 NASH patients received either rosiglitazone 8 mg/d or placebo for 1 year. Rosiglitazone improved steatosis and transaminase levels despite weight gains, an effect related to improvement in insulin sensitivity. However, other parameters of liver injury were not modified [33].

In rare situations of lipodystrophy with massive steatosis and severe insulin resistance, treatment with recombinant human leptin in such patients devoid of leptin resulted in a spectacular decrease in liver fat quantities and steatosis together with major improvements in insulin resistance [34].

These studies reveal that, in most cases, the quantity of liver fat is closely related to the degree of insulin resistance. Also, that they vary in parallel suggests a strong link between these two parameters.

9. Conclusion

A number of arguments suggest that insulin resistance leads to steatosis and that steatosis, or rather the presence of

FA derivatives used for TG synthesis, enhances insulin resistance. In humans, steatosis and insulin resistance are clearly associated, and lead to an increased prevalence of diabetes and cardiovascular risk. A number of mechanisms might explain this association, some of which are extrahepatic, such as adipose tissue or diet, while some are intrahepatic.

An important question is to ask why, in some patients, does steatosis evolve towards NASH while insulin resistance is present in both entities. The degree of insulin resistance is greater in patients with NASH than with steatosis [35,36], and its involvement in the transition has been reported in an animal model [37]. It is evident that the presence of abnormal mitochondria and increased oxidative stress in NASH compared with steatosis [24] favors an important role for mitochondrial dysfunction. However, adipokines and cytokines may also play major roles. Adiponectin is thought to be more decreased in NASH than in steatosis, and an adiponectin gene polymorphism can predict the severity of disease in NASH [35,36,38]. In addition, TNF-α is overexpressed at the mRNA level both in adipose tissue and the liver, suggesting an important role for TNF-α in the pathogenesis of NASH [39]. The roles of inflammation (see the report by K. Clément and colleagues in this issue) and immunity also need to be considered in such a setting [40].

Conflicts of interest: The author has none to declare.

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Adipose tissue inflammation and liver pathology in human obesity

J. Tordjman[a], M. Guerre-Millo[a], K. Clément[a, b*]

[a]*Inserm, U872, 15, rue de l'École de Médecine, 75007 Paris, F-75006 France; Université Pierre et Marie Curie-Paris 6, Centre de Recherche des Cordeliers, UMRS 872, Paris, F-75006 France; Université Paris Descartes-Paris 5, UMRS 872, Paris, F-75006 France.*
[a, b]*APHP, Pitié-Salpêtrière Hospital, Nutrition and Endocrinology Department, Paris, F-75013 France; CRNH-Ile-de-France, Paris, F-75013 France.*

Abstract

The increase in circulating inflammatory factors found in obese subjects and the recent discovery of macrophage infiltration in white adipose tissue (WAT) have opened up new fields of investigation, allowing a reevaluation of the pathophysiology of human obesity. The so-called 'low-grade' inflammatory state, which characterizes this complex disease, is revealed by the moderate, but chronic, systemic rise of a growing panel of molecules with proinflammatory functions. The qualitative and quantitative alterations in the production of these molecules (free fatty acids, cytokines) by the different WAT cell types, particularly in the omental fat depot, are considered new factors with the potential to modify local WAT biology and to contribute, *via* the portal system, to liver alteration. The aim of this review is to present the most up-to-date knowledge regarding the relationships between inflammatory processes in WAT and non-alcoholic liver disease in human obesity.
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Résumé

Inflammation du tissu adipeux et pathologie hépatique chez l'obèse

L'augmentation des facteurs inflammatoires circulants chez les sujets obèses et la découverte récente d'une infiltration macrophagique du tissu adipeux blanc sont des avancées majeures qui ont conduit à une réévaluation de la physiopathologie de l'obésité humaine. L'état inflammatoire « à bas bruit » qui caractérise cette maladie complexe se traduit par une augmentation modérée, mais chronique, d'une série de molécules qui possèdent des propriétés pro-inflammatoires. Les modifications qualitatives et quantitatives de la production de ces facteurs (acides gras libres, cytokines) par les différents types cellulaires présents dans le tissu adipeux blanc, plus particulièrement dans le tissu adipeux blanc omental, sont considérées comme de nouveaux facteurs pouvant modifier localement la biologie du tissu adipeux blanc et contribuer, *via* le système porte, aux altérations hépatiques. Dans cette revue, nous présentons l'état des connaissances actuelles concernant les liens entre processus inflammatoires dans le tissu adipeux blanc omental et pathologie hépatique non alcoolique dans l'obésité humaine.
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Keywords: Obesity; Liver; Inflammation; NASH; NAFLD; Review.

Mots clés : Obésité ; Inflammation ; Foie ; Stéatohépatite non alcoolique ; Revue.

Obesity is characterized by an increase in adipose tissue mass. The number of obese subjects is growing worldwide, reaching epidemic proportions in adults and children in some countries [1]. The progression of obesity-linked pathologies, including type 2 diabetes, increased cardiovascular risk and liver diseases, is to be expected and necessitates the identification of the underlying mechanisms. An emerging theory suggests that inflammation is a major contributing factor to obesity co-morbidity. Animal models and human studies have identified white adipose tissue (WAT) as a major site of inflammatory damage in obesity, which is revealed by macrophage infiltration [2-5]. Although the subject of intensive investigations, the consequences of adipose tissue inflammation and macrophage accumulation remain elusive in the human diseases related to obesity. Non-alcoholic fatty liver disease (NAFLD) is a frequent complication of human obesity [6,7] and has been linked to the amount of visceral fat [8,9]. Here, we review the recent hypothesis linking adipose tissue inflammation with liver histopathology in human obesity.

*Corresponding author.
E-mail Address: karine.clement@psl.aphp.fr

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1. Inflammation in obesity

The increase in fat mass—particularly in the splanchnic region (visceral fat) of the body—is associated with chronic elevation of circulating levels of inflammatory mediators. This includes non-specific markers such as C-reactive protein, acute-phase inflammatory proteins and proinflammatory cytokines [10,11]. Adhesion and remodelling molecules of the extracellular matrix are part of these systemic changes [11-13]. The liver and lymphoid organs are the usual production sites of inflammatory factors but, in obesity, WAT is converted into a major producer of these molecules, leading to a chronic and constant local and systemic inflammatory milieu [14]. The role of WAT as a major site of production of proinflammatory molecules was first suggested about 15 years ago by Hotamisligil *et al.* [15], who showed that WAT synthesizes tumor necrosis factor-α (TNF-α) and that the expression of this proinflammatory cytokine was elevated in adipocytes of obese mice. Moreover, insulin sensitivity could be improved by the action of TNF-α in neutralizing antibodies administered to obese insulin-resistant rats. These pioneer observations underscored the link between a proinflammatory cytokine produced and secreted by WAT, and the development of insulin resistance in rodents. More recently, the expression and secretion of a myriad of factors linked to inflammation have been identified in WAT. They include members of the cytokine family [interleukins (IL)-1, IL-1Ra, IL-8, IL-18 and IL-10], growth factors such as transforming growth factor-β (TGF-β), proteins secreted in the acute phase of inflammation (IL-6, plasminogen activator inhibitor-1), haptoglobin, serum amyloid A (SAA), chemokines (monocyte chemoattractant proteins; MCP-1, -3, -4), angiopoietins, metallothioneins, macrophage inflammatory protein-1α (MIP-1α), complement factors and retinol-binding protein-4 (RBP4) [16-19]. Even though the expression of a large number of cytokines is found in WAT, some of which may influence local biological processes, these molecules are not necessarily significantly secreted into the circulation to exert a major systemic role. Within WAT itself, there are factors that are produced specifically by adipocytes (such as leptin, adiponectin and SAA) and those that are produced by other, non-adipocyte cell types (described below).

2. Adipose tissue infiltration with inflammatory and immune cells

Cell types that make up WAT include mature adipocytes, specialized metabolic cells and a variety of other cells lumped together in the so-called 'stromal vascular fraction' (SVF), which are yet to be precisely characterized in humans. In WAT, the presence of macrophages, except in specific experimental conditions leading to adipose cell death in mice, has gone virtually unnoticed until recently [20]. It is now established that, in fact, macrophages are scarce in the WAT of normal-weight individuals, but increase markedly in animal models of obesity and in obese humans [2-5]. Transplant studies in mice suggest that these macrophages derive mostly from bone marrow [2] rather than from preadipocyte differentiation in the macrophage lineage [21]. Substantial infiltration of inflammatory cells occurs around necrotic-like adipocytes in experimental models of adipose cell death [20,22]. Interestingly, in 2000, Bornstein *et al.* noted the presence of CD68+ cells in direct contact with mature adipocytes in normal-weight individuals, but this was initially considered an experimental artefact [23]. More recently, a specific crown-like disposition of macrophages around single adipocytes exhibiting features of necrosis has been reported in obese subjects [5,24] (Fig. 1). In addition, weight loss-induced improvements in systemic inflammation has been associated with a reduction in macrophage infiltration and improved inflammatory profile in subcutaneous WAT [5,25].

The possible infiltration of WAT in obesity by other inflammatory cells is also suggested by recent analyses in mice showing the modulation of T- and NK-cell subtypes in animals fed a high-fat diet [26]. In a mouse model of high-fat-diet-induced insulin resistance, a recent study has shown that infiltration of T lymphocytes into visceral WAT precedes the recruitment of macrophages. The authors hypothesized that proinflammatory T lymphocytes may contribute to local inflammatory cell activation, and play an important role in the initiation and perpetuation of WAT inflammation and the subsequent development of insulin resistance [27]. In humans, our team has recently shown that other lymphoid cells are present within the WAT of obese subjects (Fig. 1). We observed the presence of NK and T lymphocytes in obese WAT, although they appeared to be less abundant than macrophage cells [28]. Only a few comparative studies have described lymphoid cell accumulation in WAT in obese subjects [29]. In type 2 diabetes patients with moderate-to-morbid obesity, a correlation between WAT lymphocyte number and waist circumference has been reported [27].

WAT is composed of distinct, non-contiguous depots with different characteristics [18,19]. We have recently shown that macrophage accumulation in WAT is dependent on anatomical location. Indeed, there are twice as many macrophages in omental as in subcutaneous WAT on comparing these depots in the same obese subject (Fig. 1) [30]. Other studies have shown that CD68+ cells (activated macrophages and lymphocytes) are more frequently seen in visceral than in subcutaneous WAT in lean, overweight and obese individuals [23,31,32]. The degree of macrophage infiltration might represent a new WAT site-related difference in addition to distinct metabolic capacities, gene expression, secretory function and hormonal responsiveness [18,33].

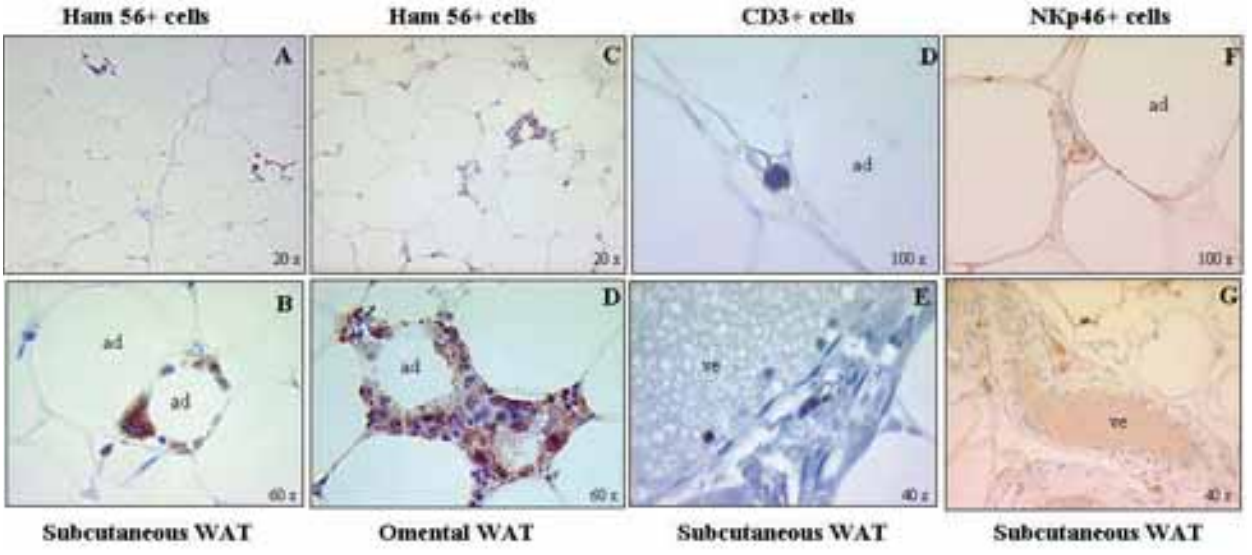

Fig. 1. Presence of macrophages in subcutaneous (A-B) and omental WAT (C-D), T lymphocytes (D-E) and NK cells (F-G) in subcutaneous WAT of morbidly obese subjects. Immunohistochemical detection of HAM 56+ macrophages in subcutaneous WAT (A-B) and omental WAT (C-D) of one representative obese woman shows that macrophages are both dispersed into WAT parenchyma and in crown arrangement. Immunohistochemistry on paraffin-embedded adipose tissue and nuclei staining with haematoxylin (blue) shows CD3+ cells between adipocytes (D) and in vessel walls (E), as well as NK cells (anti NKp46) (F-G). **Ad refers to adipocytes and ve as vessels.**

3. Role of adipose tissue macrophages in liver pathology

As a source of proinflammatory factors, WAT macrophages are thought to contribute to various co-morbidities related to obesity. In animal models, a role for WAT macrophages in inducing systemic insulin resistance has been demonstrated through diet-induced, genetic or pharmacological manipulations of macrophage numbers in adipose tissue [3,34-36]. However, in humans, the pathological consequences of macrophage infiltration in WAT remain largely hypothetical. We have recently addressed this point by focusing on non-alcoholic liver pathology, a frequent complication of human obesity [6,7]. In a population of morbidly obese subjects (BMI > 35 kg/m^2) undergoing gastric surgery, we obtained paired biopsies of subcutaneous WAT, omental WAT and liver [37]. The number of HAM56+ macrophages in WAT was quantified microscopically, and correlations with clinical and biological parameters, and histological liver lesions, were investigated. Liver histopathology was precisely evaluated by experts of liver anatomopathology. In this population, metabolic risk factors and significant liver histopathology of steatosis, NASH or fibroinflammation were present in roughly half the participants. Only a minority of subjects (9%) showed no detectable histological liver damage, and no severe damage, such as cirrhosis, was found. The proportion of participants with significant liver histopathology was greater in men than in women. We found no evidence that the duration of obesity aggravates liver histopathology. On the contrary, the proportion of subjects with significant liver damage was greater among individuals with late-onset obesity *vs* those with early-onset obesity. One important finding in the present study was that omental WAT macrophage accumulation was significantly associated with significant hepatic fibroinflammatory lesions (including fibrosis, and portal and lobular inflammation (Fig. 2). To our knowledge, this is the first identified association between macrophage infiltration in WAT and co-morbidity in human obesity. Interestingly, no association was found with the number of macrophages in subcutaneous WAT, thus suggesting a specific link between omental macrophages and liver damage [37].

4. Potential links between WAT macrophages and liver pathology

The mechanisms underlying the deleterious association between accumulation of macrophages in omental WAT and liver pathology could involve increased free fatty acid

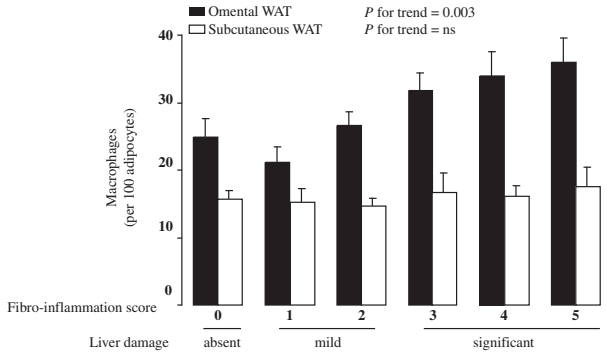

Fig. 2. WAT macrophages in morbidly obese subjects classified according to severity of hepatic fibroinflammation. The number of HAM56+ macrophages was counted on slides of omental (black bars) and subcutaneous (open bars) WAT obtained at the time of gastric surgery in morbidly obese subjects. Patients were scored for severity of fibroinflammation, based on histological evaluation of liver biopsy obtained during gastric surgery. Data are shown as means ± SEM for the number of patients indicated below each bar. Comparison between means was performed by ANOVA, followed by *P* for linear trend post-test.

(FFA) flux and/or delivery of proinflammatory factors to the liver through the portal circulation. Increased IL-6 concentrations measured in the portal vein of obese subjects suggests a role for this proinflammatory cytokine in promoting liver damage in human obesity [38]. Through their capacity to secrete a myriad of inflammatory molecules, macrophages could profoundly influence WAT biology *via* paracrine interaction with preadipocytes or adipocytes. *In vitro* experiments using cell lines or human primary cells have shown that

Fig. 3. Schematic representation of the potentially deleterious relationship between accumulation of macrophages in omental WAT and increased liver histopathology in human obesity. Local alteration of obese WAT biology by macrophage-secreted factors results in an increased delivery of free fatty acids (FFA) and/or proinflammatory cytokines to the liver *via* the portal system.

macrophage-secreted products enhance preadipocyte and adipocyte proinflammatory states and adipocyte lipolytic capacity [39-41]. In turn, increased FA and inflammatory molecules released by visceral WAT into the portal system could impact liver function [19]. The relationship between WAT-secreted products (leptin, adiponectin, TNF-α) and hepatic damage has been recently evaluated in humans [42-44]. Interestingly, in our population of severely obese patients, neither leptin nor TNF-α circulating levels were significantly associated with the severity of hepatic lesions. However, patients with significant hepatic fibroinflammation had reduced adiponectin levels. A similar association of low serum adiponectin with worsening grades of hepatic necroinflammation has recently been reported in different populations, including non-obese and non-diabetic subjects with simple steatosis or NASH [42-44].

The exact phenotype of infiltrating macrophages in WAT is still a matter of debate. The classical M1 macrophages initiate the inflammatory reaction, while the alternative M2 macrophages terminate the inflammatory process [45]. Several markers, including cell-surface receptors, chemokines, cytokines, free-radical-producing enzymes and matrix-degrading enzymes, have all been described as hallmarks of macrophage phenotype. This includes TNF-α, IL-6, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX_2) expressed in M1 macrophages, and IL-10 and transforming growth factor-β (TGF-β) expressed in M2 macrophages. In mice, a shift in the activation state of WAT macrophages from an M2 'alternatively activated' state to an M1 'proinflammatory state' has been recently described in response to diet-induced obesity [46]. In response to a high-fat diet, M1 macrophages are recruited from the circulation and accumulate in WAT in addition to resident M2 macrophages [47,48]. However, the macrophage populations found in WAT in humans are not fully defined [49]. Cell-surface markers characterizing the M2 phenotype (CD206 and CD163) have been identified on the basis of gene-expression analyses in subcutaneous WAT in non-obese subjects. Nevertheless, investigation of the secretome of these macrophages revealed the production of proinflammatory cytokines, suggesting a 'mixed' phenotype [50]. Similarly, macrophages that are immunoisolated from subcutaneous WAT in normal-to-overweight subjects express both markers of M1 and M2 polarization [51]. In the morbidly obese, our observations suggest that the phenotype of WAT macrophages might be influenced by changes in fat mass. Indeed, the M2 marker, IL-10, while not detectable in the subcutaneous WAT of morbidly obese subjects, was readily immunodetected in WAT after drastic weight loss induced by bariatric surgery [5]. More recently, a preliminary study of a limited number of morbidly obese subjects showed a higher proportion of macrophages expressing proinflammatory (M1) markers in omental WAT than in subcutaneous WAT (Wis-

newsky J, unpublished data). The relationship between the phenotype of macrophages infiltrating omental WAT and liver pathology remains to be explored. Indeed, depending on their phenotype, the *in vivo* paracrine dialogues between inflammatory and adipose cells could be modified.

In conclusion, the discovery of low-grade inflammation in human obesity has provided new concepts in the pathophysiology of this complex disease. From a temporal perspective, human obesity can be considered a set of phenotypes of variable severity that develop successively over time. Progressive biological alterations of WAT probably contribute to the development of obesity-linked metabolic, hepatic and cardiovascular complications. As suggested by studies in mice and, to a lesser degree, in humans, inflammation characterized by the infiltration of various types of circulating immune-system cells appears to follow the different phases of fat-mass accumulation. However, the mechanisms and roles of these inflammatory phenomena in the different stages of human obesity remain to be established. In particular, more information is needed of the dynamics of inflammatory processes (types and phenotypes of cells) and their local roles in the perturbation of preadipocyte and adipocyte biology, and of the development of the complications associated to obesity. Defining precisely these pathophysiological processes in human conditions is mandatory for paving the way towards a greater understanding, and eventually the discovery, of new candidate molecules for therapeutic uses.

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How to measure hepatic insulin resistance?

S.-P. Choukem[a], J.-F. Gautier[a*]

[a]*Service de Diabétologie et d'Endocrinologie, Inserm-CIC9504 Centre d'Investigations Cliniques, Hôpital Saint-Louis, Université Paris-Diderot Paris-7, 1, avenue Claude Vellefaux, 75475 Paris Cedex 10, France;*
Inserm UMRS 872, Centre de Recherche des Cordeliers, 15, rue de l'École de Médecine, 75270 Paris cedex 06, France.

Abstract

The liver plays a pivotal role in energy metabolism. Under the control of hormones, especially insulin, the liver stores or releases glucose as needed by the body's systems. It is also responsible for an important part of non-esterified fatty-acid and aminoacid metabolism. Assessing hepatic insulin resistance is almost always synonymous with measuring hepatic glucose production (HGP) and calculating indices of hepatic insulin resistance. The most frequently used method to this end is the isotope dilution technique using a tracer. Among tracers, stable isotope-labelled glucose molecules are particularly advantageous over radioactive isotope-labelled glucose and are, therefore, the tracers of choice. The tracer is infused either on its own after an overnight fast to evaluate fasting HGP, or with some among the usual insulin-sensitivity tests to assess HGP suppression by insulin and/or glucose. In a fasting state, HGP is easily calculated whereas, during insulin or glucose infusion, some formula are needed to correct for the non-steady-state condition. The hepatic insulin-resistance index is the product of HGP and the corresponding plasma insulin concentration. Although subject to error, the isotope dilution method nevertheless remains an irreplaceable tool for assessing hepatic insulin resistance in clinical research. From a practical point of view, some easily obtainable indices and clinical or biochemical parameters can serve as surrogates or markers of hepatic insulin resistance in clinical practice. Finally, drugs such as metformin or glitazones can improve hepatic insulin resistance, hence their use in hepatic insulin-resistant states such as type 2 diabetes and non-alcoholic fatty liver disease.
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Résumé

Comment mesurer la résistance hépatique à l'insuline ?

Le foie joue un rôle central dans le métabolisme énergétique. Sous le contrôle des hormones, notamment l'insuline, il met en réserve ou produit du glucose en fonction des besoins de l'organisme. Il est aussi responsable d'une part importante du métabolisme des acides gras libres et des acides aminés. Évaluer l'insulinorésistance hépatique est presque toujours synonyme de mesurer la production hépatique de glucose (PHG) suivie du calcul des indices d'insulinorésistance hépatique. La méthode la plus fréquemment utilisée à cette fin est la technique de dilution isotopique utilisant un traceur. Le glucose marqué par un isotope stable est plus avantageux que celui marqué à l'isotope radioactif, et constitue le traceur de choix. Le traceur est perfusé soit isolément et à jeun pour évaluer la PHG de base, soit au cours de certains tests usuels de mesure de la sensibilité à l'insuline pour mesurer l'effet suppressif de l'insuline et/ou du glucose sur la PHG. À jeun, la PHG est facilement calculée, mais lorsque du glucose ou de l'insuline est perfusé, des équations sont nécessaires pour tenir compte de l'état de non-équilibre créé. L'index de résistance hépatique à l'insuline est le produit de la PHG et de l'insulinémie correspondante. Malgré les possibilités d'erreurs, la méthode de dilution isotopique reste un outil irremplaçable pour évaluer l'insulinorésistance hépatique en recherche clinique. D'un point de vue pratique, certains indices ou paramètres cliniques ou biochimiques facilement mesurables peuvent servir de substituts ou de marqueurs de l'insulinorésistance hépatique en pratique clinique. Enfin, certains médicaments comme la metformine et les glitazones améliorent l'insulinorésistance hépatique, d'où leur utilisation dans le diabète de type 2 ou la stéatose hépatique non alcoolique.
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Keywords: Hepatic insulin resistance; Hepatic glucose production; Tracer; Isotope; Review.

Mots clés : Insulinorésistance hépatique ; Production hépatique de glucose ; Traceur ; Isotope ; Revue.

*Corresponding author.
E-mail Address: jean-francois.gautier@sls.aphp.fr

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1. Introduction

Insulin resistance is considered the primary defect underlying the development of type 2 diabetes [1,2] and associated diabetes subtypes [3]. It is a multisite dysfunction that involves the liver, skeletal muscle and adipose tissue, which are the body's three main insulin-sensitive tissues [3-5]. Hepatic insulin resistance is of particular interest because it is a major determinant of fasting hyperglycemia and is consequently the major dysfunction in impaired fasting glucose, a prediabetic state [6].

The liver is the first organ to pick up nutrients that enter the body from the intestines after a meal and, therefore, plays a pivotal role in energy storage. Its major metabolic function is to maintain plasma glucose levels by storing exogenous carbohydrates after a meal and, later, by releasing glucose [7]. It also has an important role in protein metabolism as amino-acid catabolism occurs mainly in the liver. Indeed, it is the only organ capable of synthesizing urea to eliminate amino-acid nitrogen. Although lipids from a meal bypass the liver as they enter the circulation, the liver takes up non-esterified fatty acids from plasma, which are oxidized or esterified to form triacylglycerol. Glucose is, however, considered to be more important than fats as an energy substrate because it is the only energy source for the cells of tissues found in the brain, retina, blood and germinal epithelium of the gonads [8].

Discussions of hepatic insulin sensitivity are usually restricted to carbohydrate metabolism whereas insulin is also involved in fat and amino-acid metabolism. This should be borne in mind when considering that, in some insulin-resistant states such as non-alcoholic fatty liver disease (NAFLD), the liver may be resistant to insulin with respect to glucose production, but very insulin-sensitive for the synthesis of fatty acids and triacylglycerol from glucose [9].

This report reviews the hepatic metabolic pathways while paying special attention to those that depend on insulin control. Also, among the *in vivo* tests that are used to assess insulin sensitivity, we discuss those that allow hepatic insulin sensitivity to be explored while addressing some practical aspects. Finally, we briefly discuss the involvement of hepatic insulin in various diseases and its ensuing practical implications.

2. Role of the liver in energy metabolism

Carbohydrates and lipids are the major substrates of energy production. Hepatocytes constitute a large chemically reactant pool with a high rate of metabolism, sharing substrates and energy from one metabolic system to another [8]. Thus, the liver is a central organ for carbohydrate, lipid and amino-acid metabolism. As for carbohydrate metabolism, the liver plays the role of a 'glucose-buffering system' [8] in that it takes up glucose and stores it in the form of glycogen when blood glucose concentration rises, and releases it back into the blood when blood glucose concentration falls. Non-esterified fatty acids (NEFA) are the main form of lipids taken up by the liver and oxidized or esterified into triglycerides which, in turn, are either used to synthesize very low-density lipoproteins (VLDL) or are transiently stored within hepatocytes [7,10]. The liver is also the main site of protein synthesis and amino-acid catabolism. The role of insulin in the regulation of different steps of carbohydrate and NEFA metabolism in the liver is far more important and better understood than it is in protein metabolism [10].

2.1. Carbohydrate metabolism in the liver and its control by insulin

The liver performs two major functions that are reciprocally regulated by insulin and glucagon: (1) glucose storage into glycogen; and (2) glucose production by glycogenolysis and gluconeogenesis, essential processes for maintaining plasma glucose during fasting. Numerous other functions, such as the conversion of galactose and fructose to glucose, and the conversion of excess glucose into fatty acids when glycogen-storing capacity is overtaken, also take place in the liver [8]. In addition, as with many other cell types in the body, glycolysis takes place in hepatocytes to provide energy.

2.1.1. Glucose storage

When glucose concentration outside the liver rises—for instance, during or after a meal—glucose is rapidly taken up into hepatocytes, especially the periportal cells, *via* GLUT2 [10,11]. GLUT2 transporters have a high K_m and are not sensitive to insulin. In the hepatocyte, glucose is temporarily 'trapped' by phosphorylation to glucose-6-phosphate, a reaction catalyzed by hepatic glucokinase, which also has a high K_m (around 12 mmol/L) and a high capacity, and is unaffected by insulin—at least in the short term [10]. Thus, the process of glucose uptake and phosphorylation by the hepatocyte depends on the glucose concentration outside of the cell. The role of insulin is crucial in the subsequent steps of the storage process. It stimulates glycogen synthase, the rate-controlling enzyme responsible for polymerization of glucose to glycogen [12]. Insulin also inhibits glycogen phosphorylase, which catalyzes glycogen breakdown, although glucose is the most potent inhibitor of glycogen phosphorylase [12,13]. The liver can then store up to the equivalent of 5-6% of its weight in glycogen, which is about 100 g [8]. When the quantity of glucose entering the liver exceeds the hepatocyte glycogen-storing capacity, insulin promotes the conversion of all the excess glucose into fatty acids that are subsequently packaged as triglycerides [8,14]. This process, known as *de*

novo lipogenesis, is stimulated by insulin *via* the transcription factor sterol regulatory element-binding protein-1c (SREBP-1c) [15] and by glucose *via* the transcription factor carbohydrate response element-binding protein (ChREBP) [14]. Both transcription factors are inducers of lipogenic enzyme genes, especially fatty acid synthase, and are upregulated by a high-carbohydrate diet [7,14,15]. However, contrary to what is observed in rodents, *de novo* lipogenesis is limited in humans under normal conditions (responsible for < 5% of the circulating triacylglycerol pool) [16]. By contrast, in patients with NAFLD, this increases to up to 25% of the triacylglycerol pool [17].

2.1.2. Hepatic glucose production

In the postabsorptive state, the liver is responsible for at least 75% of the total endogenous glucose production. In humans, the hepatic glucose production (HGP) rate is around 2 mg/kg body weight/min [11]. HGP originates from two mechanisms: glycogenolysis and gluconeogenesis. Glycogenolysis—glycogen breakdown to release glucose—depends on two key enzymes: glycogen phosphorylase and glucose-6-phosphatase. Glycogen phosphorylase breaks down glycogen into glucose-1-phosphate which, in turn, is converted in a reversible reaction to glucose-6-phosphate (G6P) by phosphoglucomutase, and glucose-6-phosphatase then dephosphorylates to produce glucose, which diffuses out of hepatocytes [8,10]. Gluconeogenesis is glucose synthesis *de novo* from non-carbohydrate precursors—namely, lactate, amino acids (especially alanine) and glycerol. If fasting is prolonged, the relative contribution of gluconeogenesis in relation to total HGP increases. When labelled nuclear magnetic resonance (MR) spectroscopy was used to measure glycogenolysis rate, the average relative contribution of gluconeogenesis rate (obtained by subtraction of glycogenolysis from HGP) was 64% during the first 22 hours of fasting, 82% after 22-46 hours and 96% after 46-64 hours of fasting [18]. The key enzyme of gluconeogenesis is phosphoenolpyruvate carboxykinase (PEPCK). Glycogen phosphorylase, glucose-6-phosphatase and PEPCK are inhibited by insulin and activated by glucagon.

2.1.3 Effects of insulin on hepatic glucose production

Insulin inhibits HGP through direct and indirect effects. Insulin binds to receptors on the hepatocyte membrane and, in the course of its signalling pathway, it also inhibits the enzymes involved in gluconeogenesis and glycogenolysis, the two mechanisms of HGP [19,20]. These direct effects have been well demonstrated in dogs and have been recently shown to dominate the indirect effects [21]. SREBP-1c mediates the inhibitory effect of insulin on the PEPCK gene, the central enzyme of gluconeogenesis [22]. Indirect inhibitory effects of insulin on HGP include: (1) inhibition of glucagon secretion by acting on islet alpha cells; (2) inhibition of lipolysis and proteolysis in muscle, and of lipolysis in adipose tissue, thereby decreasing the availability of gluconeogenic precursors; and (3) central action through the hypothalamus [19].

2.2. Fatty acid metabolism

NEFA taken up by the liver have two major fates: β-oxidation or esterification [10]. β-oxidation splits NEFA into acetyl coenzyme A (acetyl-CoA). Acetyl-CoA either enters the tricarboxylic acid cycle in hepatocyte mitochondria to produce energy—used, in particular, for energy-demanding pathways such as gluconeogenesis—or is condensed into ketone bodies (acetoacetic and hydroxybutyric acids) and exported in the circulation to other cells [8,10]. In these cells, ketone bodies are converted back to acetyl-CoA and used in the tricarboxylic acid cycle to provide energy. Esterification of NEFA forms triglycerides, which are stored in the hepatocyte and used for VLDL formation. The balance between β-oxidation and esterification is controlled by insulin and glucagon [10]. Insulin promotes storage either directly, by activating triglyceride synthesis, or indirectly by its 'fat-sparing' effects (insulin does not favor the use of NEFA as energy substrates), whereas glucagon promotes oxidation.

2.3. Amino-acid metabolism

Amino acids are not direct energy substrates *per se*. The major pathways of amino-acid metabolism in the liver (such as protein synthesis, deamination, interconversion of amino acids and formation of urea for removal of ammonia) are not intended to produce energy [8]. However, some amino acids, such as alanine, are substrates for gluconeogenesis and, therefore, contribute indirectly to energy production. Insulin promotes protein synthesis, and inhibits amino-acid uptake and use as gluconeogenic substrates.

3. Overview of methods used to assess insulin sensitivity in humans

Liver, skeletal muscle and adipose tissue are the three major insulin-sensitive organs involved in glucose homeostasis. Some common methods and indices used to evaluate insulin sensitivity in terms of glucose metabolism are:

– clamps (euglycemic-hyperinsulinemic, and hyperglycemic);

– frequently sampled intravenous glucose tolerance test with minimal modelling;

– indices calculated from oral glucose tolerance test;

– indices computed from fasting plasma insulin and glucose, such as HOMA;

– insulin-sensitivity test;

– short insulin tolerance test;

– continuous infusion of glucose with model assessment (CIGMA).

These have been extensively reviewed [23-26], and readers are referred to these published articles for more details. Insulin sensitivity to lipid metabolism can be assessed by many methods; the most commonly used is the measurement of NEFA suppression in response to insulin infusion [3-5].

4. Methods to measure hepatic insulin resistance

Before choosing which test to use for hepatic insulin-resistance assessment, the first question should be: Which pathway of insulin-sensitive hepatic metabolism (glucose or fatty-acid metabolism) is being explored? When it comes to glucose metabolism itself, another question raised is: Which fate of glucose (glycogen synthesis or fatty-acid synthesis) is being studied? Most techniques focus on carbohydrate metabolism by measuring HGP rate. Although HGP is clinically the most useful indicator of hepatic insulin resistance because of the spectrum of diseases in which its study can be applied, it should be remembered that, in some 'insulin-resistant' states such as NAFLD, the resistance of insulin to suppress HGP is reflected by the sensitivity of insulin to stimulate NEFA synthesis from glucose. Apart of direct methods that can be used to measure HGP or NEFA uptake and reesterification by the liver, metabolic indices and liver imaging features may be used as surrogates or correlates, and clinical and biological parameters may be used as markers of hepatic insulin resistance.

4.1. Direct measurement of HGP, and NEFA uptake and reesterification

Measurement of HGP is by far the most commonly used method of assessing hepatic insulin resistance. HGP itself is measured as part of glucose turnover. Three direct techniques can be used: (1) the arteriovenous-difference technique; (2) the isotope dilution technique; and (3) labelled nuclear MR spectroscopy.

4.1.1. The arteriovenous-difference technique

Also known as Fick's principle, this consists of the simultaneous measurement of liver blood flow, and the difference between arterial and venous glucose concentrations [27]. HGP is then calculated as the product of the two parameters [28]. Although it measures the net hepatic glucose output, Fick's principle is invasive, as it requires venous and arterial catheterization. Moreover, the liver incoming blood flow originates from two sources, the portal vein and the hepatic artery. These reasons preclude its practical application.

4.1.2. The isotope dilution technique

This is the most widely used technique. Depending on the tracer used, it can study hepatic insulin sensitivity through glucose metabolism (measurement or HGP) or NEFA metabolism (measurement of NEFA uptake and reesterification by the liver).

For glucose metabolism, one or many tracers are infused either alone or during glucose or insulin administration to estimate basal or suppressed HGP. Various 'indices of hepatic insulin resistance' are then calculated. Among the methods for assessing insulin sensitivity, the labelled euglycemic– hyperinsulinemic clamp is the most frequently used for measuring hepatic insulin sensitivity in response to glucose and insulin infusions. However, minimal modelling during the labelled intravenous glucose tolerance test can be used as well [29,30]. Multiple (dual- or triple-) tracer approaches can also provide more precise measurements of glucose metabolic pathways. For instance, using labelled gluconeogenic precursors (^{13}C-lactate or ^{13}C-glycerol) or measuring the incorporation of $^{2}H_2O$ (deuterated water) allows estimation of gluconeogenesis and, therefore, its relative contribution to HGP [31]. In addition, labelled mixed meal or oral glucose can also be used in a dual- or triple-tracer approach to evaluate the postprandial or post-load outcome of ingested glucose [32,33].

• *4.1.2.1. Tracers used to study HGP*

Definition: A tracer is a labelled form of a substance [34]. To label a substance, atoms in the unlabelled form of that substance are replaced by their rare isotopes. Metabolically speaking, a tracer is used to study another substance, usually a naturally available molecule in the organism where the tracer is being introduced. The label makes the tracer detectable by the observer. Its structure and metabolic outcome should be identical to that of the molecule studied and, ideally, it should not interfere with the normal behavior of the system being studied [28,34]. These conditions are achieved by using the tracer in very small quantities. In glucose metabolism, glucose itself is usually labelled and used as a tracer. In labelled glucose, the isotope may be an atom of hydrogen or carbon, it may be stable or radioactive and it may be located at any of the six carbon atoms of the glucose molecule.

Choice of tracer: *Hydrogen-* vs *carbon-labelled glucose:* To label the glucose molecule, either hydrogen is replaced by ^{2}H (deuterium) or ^{3}H (tritium), or carbon is replaced by ^{13}C or ^{14}C [28]. In hydrogen-labelled glucose, the major labelled degradation product is deuterated or tritiated water. The reincorporation of labelled water in glucose is unlikely—in other words, when a hydrogen-labelled tracer enters a metabolic pathway, it is totally cleared from the body. Thus, it is called an 'irreversible' (non-recycling) tracer, as opposed to a 'reversible' (recycling) tracer such

as carbon-labelled glucose [28,31]. When glucose is carbon-labelled, lactate, the major metabolic product of glucose degradation, is labelled and may be reincorporated in glucose through gluconeogenesis. This leads to an underestimation of glucose turnover, and specific methods are needed to take this recycling into account.

Position of the isotope in the glucose molecule: The position of the isotope is a matter only for hydrogen-labelled glucose. In the liver, chemical reactions may occur concomitantly with their opposites, but catalyzed by different enzymes: for example, the first steps of glycolysis occur simultaneously with their opposites, the last steps of gluconeogenesis [28]. In this case, a molecule of glucose may undergo one or two steps of glycolysis and lose the label, then enter the reverse reaction of gluconeogenesis and be released from the liver as a neosynthesized glucose molecule. As the marker has been lost, this will be counted as used glucose molecule whereas no molecule has been used until the energy producting step, thus leading to overestimation of glucose use. Labelled hydrogen atoms can be located on any carbon, but the most common sites are positions 2 (C-2) ([2-^{2}H]glucose or [2-^{3}H]glucose), 3 ([3-^{2}H]glucose or [3-^{3}H]glucose) or 6 ([6,6-$^{2}H_{2}$]glucose or [6,6-$^{3}H_{2}$]glucose). The Figure 1 is based on the assumption that ^{2}H is the isotope and C-2, C-3 and C-6 are all labelled [31]. 2-^{2}H is released precociously in the second step of glycolysis, when glucose-6-phosphate is converted to fructose-1,6-biphosphate; 3-^{2}H is released later, in the fourth step; 6,6-$^{2}H_{2}$ is particularly advantageous because C-6 can handle two isotopes of ^{2}H, and the ^{2}H on C-6 is released later in the course of glycolysis (after the sixth step) thereby avoiding the overestimation of glucose turn over [28,31].

Stable- vs *radioactive-isotope tracers:* Among the isotopes used in glucose-labelling, ^{3}H and ^{14}C are radioactive whereas ^{2}H and ^{13}C are stable. Radioactive-isotope tracers have been used in humans since the early 1950s [36]. Since the first use of stable-isotope tracers to quantify glucose metabolism in humans in the late 1970s, they have progressively replaced radioactive-isotope tracers as the tracers of choice to study *in vivo* metabolic dynamics in humans, especially because of the advantages they present. Table 1 compares and contrasts the two types of tracers [37]. Although stable-isotope tracers can yield equally accurate results, their main limitation is their cost compared with radioactive-isotope tracers. On the other hand, the health and environmental concerns of radioactive tracers is a major inconvenience, as they cannot be used in high-risk population groups such as infants, children and pregnant or lactating women. Furthermore, with stable-isotope tracers, the two variables (tracer and tracee) used in the calculations are both measured by mass spectrometry whereas, with radioactive tracers, they are measured by two different techniques [scintillation counting for the tracer, and a chemical method for the tracee (glucose)], leading to an increased variability of results [31]. Also, the high selectivity provided by mass spectrometry allows the simultaneous use of multiple stable tracers during the same experiment, which cannot be done when a radioactive tracer is used [31].

Finally, among stable-isotope glucose tracers, [6,6-$^{2}H_{2}$]glucose appears to be the most suitable because, apart from being safe and non-recycling, it is also considered to give the best estimate of true endogenous glucose production [31].

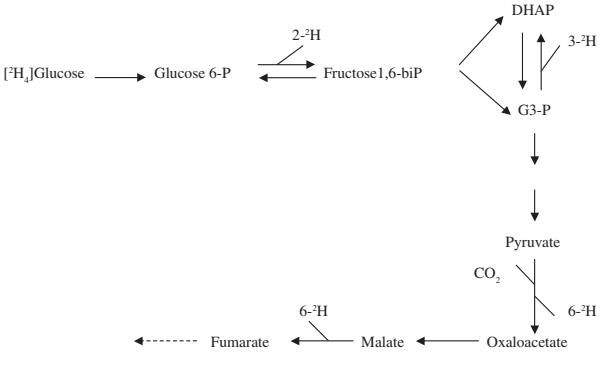

Fig. 1. Level of release of ^{2}H during glycolysis depends on the carbon location in the glucose molecule (adapted from Coggan [31]). [$^{2}H_{4}$]Glucose, ^{2}H-labelled glucose on C-2, C-3, C-6. Glucose-6-P: glucose– phosphate; Fructose 1,6-biP: fructose 1,6-biphosphate; DHAP: dihydroxyacetone phosphate; G3-P: glyceraldehyde-3-phosphate; 2-^{2}H, 3-^{2}H, 6-^{2}H: ^{2}H loss from C-2, C-3, C-6, respectively.

Table 1
Comparison of stable- and radioactive-isotope tracers for metabolic purposes (adapted from Solomon [37]).

	Radioisotopes	Stable isotopes
Safety	Some risk, especially for pregnant or lactating women, and children	No significant risk
As tracers	True tracers, as they are not naturally present	Naturally present, sufficient amounts must be given to be detectable
Study time	Half-life of the radioisotope can affect duration of study	Tracer may be followed for extended periods of time
Combination of tracers in one study	Generally only one radioisotope is given	Multiple isotopes of an element and/or isotopes of different elements can be given simultaneously
Analysis	Sample analysis must be timely, based on the half-life of the isotope; sample preparation minimal; tracer and tracee are measured by different techniques	Samples can be stored without loss of tracer; may require extensive sample preparation; tracer and tracee are measured by the same technique

• *4.1.2.2. Basic principles and technical procedures*

Concepts and terminology: *Assumptions:* For mathematical purposes, the constituents of a living system can be represented as being located in distinguishable volumes called 'pools' or 'compartments' [34]. Some basic assumptions govern tracer experiments, but they are not always valid in all circumstances and, thus, there may be a need for appropriate corrections. The first basic assumption is that the tracer element follows its unlabelled isotope faithfully in all biological reactions [38]-in other words, the metabolic behavior of the tracer is the same as that of its unlabelled counterpart [34]. However, an isotope effect is to be expected and has been demonstrated in the rates of certain reactions, particularly those involving ^{14}C and isotopes of hydrogen. However, for hydrogen-labelled tracers, the isotope effect is usually negligible. The second assumption is that, within a given compartment, the substance being studied is uniformly distributed at all times. This assumption implies instantaneous and homogeneous mixing within the compartment, and is invalid for many physiological conditions [38].

Terminology: "Steady-state": This term applies to compartments where the rates of removal of the substances under study are equal to the rates of replacement, so that the concentrations and amounts of the substances are constant during the period of observation. Such a situation is obtained after an overnight fast, when a primed constant infusion of tracer is performed for at least 60 min—the amount of time it takes the tracer to become completely mixed with the body glucose pool. Blood glucose is very constant, and the glucose produced by the liver is assumed to equal glucose uptake by peripheral tissues, especially the brain. "Non-steady-state": When exogenous insulin or non-labelled glucose is introduced into the system either orally or intravenously, this creates a disequilibrium within the system that is called a 'non-steady-state'. To account for this, formulas such as Steele's equation [39] are used to calculate HGP. The non-steady-state is of major importance as it reflects what actually occurs in the physiological state most of the time.

Technical procedures: The model described here includes the use of a stable-isotope tracer. To measure basal (fasting) HGP, the tracer is infused alone after an overnight fast either as a single injection, or as a priming bolus followed immediately by a continuous infusion (primed constant infusion), which is the most suitable method for administering the tracer [40] because it allows a simpler estimation of HGP [41]. With the single bolus injection commonly used in the past, estimation of HGP was more complicated, and tended to produce too-high values for the body's glucose pool [40]. Blood samples for measuring fasting HGP should be collected after at least 60 min of constant tracer infusion, the time needed for the priming dose to completely mix with the glucose pool. This was described in dogs using ^{14}C, where the plasma glucose-specific activity reached a plateau and remained stable after 60 min of constant tracer infusion [40]. Sample analysis is performed by mass spectrometry that allows measurement of both the tracer and tracee, and the tracer enrichment is calculated as the plasma tracer concentration divided by the plasma tracee concentration.

Measurement of HGP under suppression (glucose and/or insulin administration) can be performed during a labelled euglycemic– hyperinsulinemic clamp or a labelled frequently sampled intravenous glucose tolerance test (FSIVGTT). Measuring HGP under such conditions gives an idea of the dynamics of liver response to the suppressive effects of glucose and/or insulin. During a euglycemic clamp, for instance, the constant tracer infusion is used at the same rate as during fasting, and is maintained until the end of the test. Blood samples collected during the last 20 or 30 min of the clamp, or from each clamp step in the case of a multistep clamp, are used to measure plasma tracer enrichment by mass spectrometry under non-steady-state conditions.

• *4.1.2.3. Calculation of HGP rate and indices of hepatic insulin resistance*

Calculation of HGP rate: Basal HGP rate corresponds to the hepatic response to physiological plasma insulin. To facilitate estimations, a one-compartment model with constant volume is used (Fig. 2). Based on the above-mentioned assumptions, under steady-state conditions, the glucose rate of appearance (Ra) equals its rate of disappearance (Rd); and the ratio of plasma tracer/tracee (C*/C), which corresponds to tracer enrichment (ε), is equal to the ratio of tracer infusion rate/glucose rate of appearance (Ra*/Ra). Thus, $\varepsilon = Ra^*/Ra$ ($Ra = Ra^*/\varepsilon$). As Ra corresponds to the HGP rate in steady-state, $HGP = Ra^*/\varepsilon$.

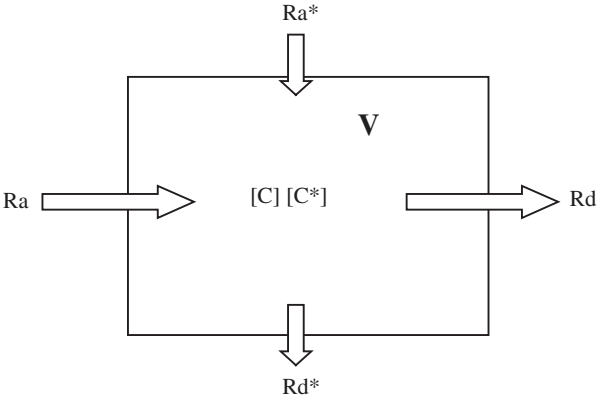

Fig. 2. Monocompartment model (modified from Steele [38]).
Ra: hepatic glucose production rate; Ra*: tracer infusion rate;
C: plasma glucose concentration; C*: plasma tracer concentration;
Rd: rate of glucose disappearance; Rd*: rate of tracer disappearance;
V: volume of compartment.

HGP measured during the clamp is an estimate of hepatic response to supraphysiological insulin concentrations (residual HGP). Under non-steady-state conditions created by glucose and insulin infusion, special models are needed to calculate the residual HGP. The most widely used of these models is Steele's equation [39], which proposes a monocompartmental model with constant volume. Computation is based on the derivation of tracer and tracee measurements performed in plasma. For more details, readers are referred to Steele *et al.* [39]. Another, more complex, model was proposed by Radziuk *et al.* [42], and a more recent model that accounts for the error in Steele's equation has also been proposed to estimate HGP in non-steady-state conditions [43].

When HGP is measured during labelled FSIGTT, the HGP rate is obtained by deconvolution using minimal modelling [29,44].

Indices of hepatic insulin resistance: The HGP rate offers an idea of hepatic resistance to the suppressive action of insulin on glucose production: the higher the HGP rate, the higher the hepatic insulin resistance. To provide a more metabolically significant estimate of hepatic insulin resistance, HGP should be related to plasma insulin concentration. Hepatic insulin resistance indices are calculated as the product of HGP rate and the corresponding plasma insulin concentration [6,45] either at baseline (basal HGP × fasting plasma insulin) or during the plateau phase of the clamp.

• *4.1.2.5. Limitations of HGP measurement*

It should be noted that, throughout the above-described procedures for measuring HGP, a number of inconsistencies make the isotope dilution method less than perfect, although it nevertheless remains one of the best available tools for studying hepatic glucose metabolism *in vivo*. Indeed, what we measure using the tracer is, in reality, endogenous glucose production, and it should be borne in mind that the liver is not the only glucose-producing organ during fasting conditions. The kidney cortex produces glucose by gluconeogenesis, and its relative contribution to endogenous glucose production in the postabsorptive state is estimated to range from 5% to 28% [19, 46-48]. Also, as already stated above, the monocompartmental model and the assumptions that constitute the basic principles of tracer methodology may be subject to error. Another inconsistency is the computation of HGP rate using Steele's equation, which often generates negative values of HGP during the euglycemic clamp perhaps due to an error in the equation itself. These negative values are assumed to correspond to zero—that is, complete suppression of HGP. For instance, in our team's study of insulin sensitivity in Africans with ketosis-prone diabetes *vs* controls [3], 22 out of 32 participants had negative HGP values during the high-dose (80 $mU.m^{-2}.min^{-1}$) insulin-infusion clamp step.

• *4.1.2.6. Isotope dilution technique to study NEFA metabolism*

The principles and procedures for studying NEFA turnover are, in general, the same as for the study of glucose metabolism. To measure hepatic insulin sensitivity to plasma NEFA (released from adipose tissue triglycerides) uptake and reesterification, a labelled fatty acid is infused, and the relative and absolute contributions of labelled triglyceride secreted from the liver measured. The test can also be performed at baseline and in response to insulin. Labelled palmitate ([1-^{13}C]palmitate or [1,2,3,4-$^{13}C_4$]potassium palmitate) has been used to this end to show that, in patients with NAFLD compared with control subjects, the relative contribution of triglycerides produced by *de novo* lipogenesis to the total secreted triglycerides released by the liver was increased at the expense of triglyceride synthesized from NEFA reesterification [17,49].

4.1.3. Labelled nuclear magnetic resonance spectroscopy

^{13}C-labelled nuclear MR spectroscopy has been used to measure net hepatic glycogen synthesis and glycogenolysis in humans [13,18]. Net hepatic glycogenolysis is calculated as the slope of glycogen concentration decrements over a period of time, multiplied by liver volume (measured by MR imaging) [18]. When combined with the isotope dilution technique, it allows calculation of the relative contribution of glycogenolysis and gluconeogenesis (obtained by subtraction of glycogenolysis rate from total HGP rate) either during fasting, or in response to insulin or glucagon [50].

MR spectroscopy is also the technique used to measure hepatic triglyceride content (HTGC). An increased HTGC is the hallmark of NAFLD as well as a good correlate of hepatic insulin resistance.

4.2. Surrogates, correlates and clinical markers of hepatic insulin resistance

Many metabolic indices and features of liver imaging may correlate well with the index of hepatic insulin resistance obtained by the isotope dilution method, while some biochemical parameters may be associated with the increased needs of exogenous basal insulin doses in type 2 diabetic patients (supposedly a suppressor of HGP). In addition, anthropometric measurements may be associated with hepatic insulin-resistant states. These indices, features and parameters may therefore, be considered either surrogates, correlates, or clinical markers of hepatic insulin resistance. Their importance in clinical practice is that, as direct methods are reserved for clinical research and cannot be used in routine practice, these markers may therefore serve as screening or diagnostic tools for hepatic insulin-resistant conditions.

HGP is almost the only source of plasma glucose in the fasting state. As HGP is normally inhibited by insulin, fasting plasma insulin or indices of basal (fasting) insulin resistance are good correlates of hepatic insulin resistance and may be used as surrogates. For instance, the HOMA-IR has been shown to correlate well ($r = 0.64$) with the basal hepatic insulin-resistance index [51].

A good correlation ($r = 0.64$) with the basal hepatic insulin-resistance index was also reported recently for an index obtained with the oral glucose tolerance test (OGTT). This index was computed as the product of the total area under the curve (AUC) for glucose and insulin during the first 30 min of the OGTT ($glucose_{0\text{-}30}$[AUC] × $insulin_{0\text{-}30}$[AUC]) [51].

Hepatic triglyceride content measured by MR spectroscopy to ascertain NAFLD is also well correlated with the hepatic insulin-resistance index, and was recently reported to be a strong predictor of insulin action in liver, skeletal muscle and adipose tissue [52,53].

Alanine aminotransferase (ALT) is an enzyme secreted by hepatocytes. In the LANMET study, comparing insulin glargine with NPH insulin as basal insulin treatment in metformin-treated type 2 diabetics, serum ALT levels were among the positive predictors of basal insulin dose in these patients [54]. For this reason, serum ALT levels may be a rough clinical marker of hepatic insulin resistance.

Furthermore, features of the metabolic syndrome have been shown to be highly prevalent in patients with NAFLD [55], including central obesity (waist circumference > 102 cm in men and > 88 cm in women) (47%), hypertriglyceridemia > 2 mmol/L (47%) and levels of HDL cholesterol < 1 mmol/L (43%). These parameters may also be useful markers or predictors of hepatic insulin resistance in clinical practice.

5. Hepatic insulin resistance in diseases

Hepatic insulin resistance is an underlying factor or feature of many non-communicable diseases or syndromes that, in turn, may be related between each other. Non-alcoholic fatty liver disease (NAFLD) is an increasing health problem that is associated with insulin resistance, the metabolic syndrome, type 2 diabetes and other conditions [55,56] (this topic is discussed in depth in the other articles in this issue). In patients with NAFLD, basal HGP rates may be similar [55,57] or higher [58] compared with those of control subjects, but the hepatic insulin-sensitivity index or insulin-mediated HGP suppression is concordantly impaired. More important, intrahepatic triglyceride (IHTG) content, measured by MR spectroscopy to ascertain NAFLD, correlates well with the hepatic insulin-resistance index, and was recently reported to be a strong predictor, independent of body mass index (BMI), of insulin action in liver, skeletal muscle and adipose tissues [52,53]. In patients with NAFLD, pioglitazone, an antidiabetic drug, was shown to decrease hepatic fat content by 54%, and to improve hepatic insulin sensitivity by 47% as well as biochemical markers of hepatic insulin resistance [33]. This study was of special interest as they used a double-tracing technique (intravenous and orally labelled glucose).

In type 2 diabetes, HGP is often increased, and contributes to fasting and postprandial hyperglycemia. The increased HGP is attributed to an increased rate of gluconeogenesis [59,60] and impaired glycogen metabolism [61]. The oral antidiabetic agent metformin decreases HGP through inhibition of hepatic gluconeogenesis [60].

In liver cirrhosis, glucose metabolism is impaired, yet HGP rates are similar to those in normal subjects [62]. Therefore, there is no hepatic insulin resistance *per se* in cirrhosis; instead, there is a disequilibrium in the glucose-producing mechanisms, with gluconeogenesis being increased at the expense of glycogenolysis [62].

6. Conclusion

Hepatic glucose production and the ensuing index are the best clinically accessible indicators of hepatic insulin resistance which, in turn, is an important pathogenic factor or feature of type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) and the metabolic syndrome. Measuring HGP requires methods that use isotope-labelled glucose, but caution should be used in light of the dangers of radioactive isotopes to specific patient populations and to the environment. Although subject to error, the method, in use for more than half a century, nevertheless remains an irreplaceable tool for assessing hepatic insulin resistance in clinical research when used rigorously. From a practical point of view, there are easily obtainable indices and clinical or biochemical parameters that can serve as surrogates or markers of hepatic insulin resistance in clinical practice. Finally, drugs such as metformin or glitazones can improve hepatic insulin resistance and are, therefore, of value in hepatic insulin-resistant conditions such as NAFLD.

Conflicts of interest: The authors have none to declare.

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Non-invasive diagnosis of steatosis and fibrosis

L. Castera

Département d'Hépatologie, Hôpital Saint-André & Haut Lévêque, CHU Bordeaux, Bordeaux, France.

Abstract

The prognosis and management of liver disease greatly depends on the amount of liver fibrosis. Non-alcoholic fatty liver disease (NAFLD), ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), is emerging as a major cause of liver disease in Western countries because of the increasing prevalence of obesity and type 2 diabetes. A key issue in patients with NAFLD is the differentiation of NASH from simple steatosis. It is particularly important to identify NASH patients as they are at greatest risk of developing complications such as cirrhosis, liver failure and hepatocellular carcinoma. The limitations of liver biopsy (invasive procedure, sampling errors, interobserver variability and non-dynamic fibrosis evaluation) have stimulated the search for non-invasive approaches for the assessment of steatosis and liver fibrosis in patients with NAFLD. A variety of methods, including serum markers, imaging techniques such as ultrasound, CT, MRI and measurement of liver stiffness by transient elastography, have been proposed for the non-invasive assessment of steatosis and hepatic fibrosis. This review discusses the advantages and limitations of these different methods in clinical practice.
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Résumé

Diagnostic non invasif de la stéatose et de la fibrose hépatiques

L'importance et la progression de la fibrose hépatique conditionnent à la fois le pronostic et la prise en charge des maladies chroniques du foie. La stéatose hépatique non alcoolique (NAFLD), qui va de la stéatose simple jusqu'à la stéatohépatite non alcoolique (NASH), est une cause émergente de maladie du foie dans les pays occidentaux, en raison de la prévalence croissante de l'obésité et du diabète de type 2. Différencier la NASH de la stéatose simple est d'une importance fondamentale chez les patients atteints de NAFLD. En effet, les patients atteints de NASH sont les plus à risque de développer d'une part, une fibrose hépatique progressive et, d'autre part, des complications telles qu'une cirrhose, une insuffisance hépatique ou un carcinome hépatocellulaire. Les limites de la biopsie hépatique (examen invasif avec biais d'échantillonnage et variabilité interobservateur qui ne permettent pas une évaluation dynamique de la fibrose) ont stimulé la recherche d'approches non invasives pour évaluer la stéatose et la fibrose hépatiques chez les patients atteints de NAFLD. Plusieurs méthodes comprenant des marqueurs sériques, des techniques d'imagerie comme l'échographie, le scanner ou l'IRM, et plus récemment la mesure de l'élasticité hépatique par élastométrie impulsionnelle (FibroScan), ont ainsi été proposées. Cette revue a pour but de discuter les avantages et les limites respectives de ces méthodes en pratique clinique.
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Keywords: Type 2 diabetes; Obesity; Non-alcoholic steatohepatitis; Liver fibrosis; Steatosis; Non-invasive procedure; Transient elastography (FibroScan); Serum markers; Liver biopsy, Review.

Mots clés : Diabète de type 2 ; Obésité ; Stéatohépatite non alcoolique ; Fibrose hépatique ; Stéatose ; Méthode non invasive ; Élastométrie (FibroScan) ; Marqueurs sériques ; Biopsie hépatique ; Revue.

Non-alcoholic fatty liver disease (NAFLD) is emerging as a major cause of liver disease in Western countries because of the increasing prevalence of obesity and type 2 diabetes. NAFLD encompasses a spectrum of diseases, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), a more severe entity [1]. It is estimated that 30% of the adult population in the US now have NAFLD and that 3-6% have NASH [2]. A key issue in patients with NAFLD is the differentiation of NASH from simple steatosis. It is particularly important to identify NASH patients who are at greatest risk of developing complications of chronic liver

*Corresponding author.
E-mail Address: laurent.castera@chu-bordeaux.fr

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disease, such as cirrhosis, liver failure and hepatocellular carcinoma [3,4]. The diagnosis of NASH, which includes necroinflammation, ballooning degeneration and fibrosis, is essentially based on histological examination of a liver specimen obtained by liver biopsy. However, liver biopsy is a painful and invasive procedure [5]—with rare, but potentially life-threatening, complications [6,7]—that is prone to sampling error [8,9]. In addition, given the numbers of patients with NAFLD, the use of liver biopsy is clinically and financially impractical.

These limitations have stimulated the search for new non-invasive approaches. Ideally, a non-invasive marker of liver fibrosis should be liver-specific, easy to perform, reliable and inexpensive. It should, in addition, be accurate not only for the staging of fibrosis, but also for monitoring disease progression. A variety of methods, including serum markers, imaging techniques such as ultrasound, CT, MRI and measurement of liver stiffness by transient elastography, have been proposed for the non-invasive assessment of steatosis and hepatic fibrosis. Although most of these methods have been mainly validated in the context of hepatitis C, there has been considerable interest in extending this work into the field of NAFLD because of its increasing prevalence. This review is aimed at discussing the advantages and limitations of these different methods in clinical practice.

1. Non-invasive diagnosis of steatosis

1.1. Imaging techniques

Non-invasive techniques such as ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (1H-MRS) can detect hepatic steatosis, but currently cannot distinguish between simple steatosis and NASH.

1.1.1. Ultrasound

Hepatic ultrasound is a simple, non-invasive technique that is widely used in clinical practice to detect fatty infiltration of liver. Hepatic steatosis causes increased echogenicity on ultrasound, making the liver appear brighter than the cortex of the right kidney. Several studies have shown that ultrasound for detecting hepatic steatosis has a sensitivity of 60% to 94%, and a specificity of 84% to 95% [10]. The sensitivity of ultrasound increases with increasing degrees of fatty infiltration. However, ultrasound is unable to provide a precise grading of hepatic fat content. Also, its sensitivity is reduced in the morbidly obese, and its performance is highly operator-dependent.

1.1.2. Computed tomography

Non-contrast-enhanced CT is the most accurate CT technique to detect and characterize hepatic steatosis [11]. The CT diagnosis of hepatic steatosis is made by measuring the difference in liver and spleen attenuation values in Hounsfield units. In subjects with steatosis, as the mean attenuation value of the liver is lower that of the spleen, the liver appears darker than the spleen. Although non-contrast-enhanced CT is useful for the qualitative diagnosis of macrovesicular steatosis of 30% or greater, there is conflicting evidence as to whether or not it can accurately quantify hepatic fat content. In addition, it exposes subjects to ionizing radiation.

1.1.3. MRI and proton MR spectroscopy

Chemical-shift MRI uses the difference in resonance frequency of water and lipid to differentiate tissue containing only water from those containing water and lipid, known as the Dixon method. Several studies have recently demonstrated a good correlation between the severity of hepatic steatosis on MRI and liver biopsy [12,13]. Multiecho imaging may also be a promising method [14]. Similarly, *in vivo* ^{1}H-MRS is a fast and safe technique for the quantitative assessment of hepatic steatosis. Several studies have shown a good correlation between quantification of hepatic fat content by H-MRS and liver biopsy [13,15]. Both techniques will be useful tools in the future.

1.2. Serum markers

So far, the only serum test that has been proposed to detect steatosis is the SteatoTest [16]. This test includes the 6 parameters of FibroTest– ActiTest plus BMI, serum cholesterol, triglycerides and glucose adjusted for age and gender. It has been constructed from a training group of 310 patients with various chronic liver diseases, using the presence of steatosis (> 5%) on liver biopsy as the reference, and validated in three different groups of patients with hepatitis C and alcoholic liver disease (n = 434). At a cutoff of 0.3, the sensitivity of SteatoTest ranged from 85% to 100% whereas, at a cutoff of 0.7, the specificity ranged from 83% to 100%. Validation of this test in other groups of patients (including NAFLD) by independent studies is awaited.

More interest has been focused on whether or not non-invasive serum tests can differentiate NASH from simple steatosis among patients with NAFLD. Several groups have proposed tests, including the NashTest [17], and scores combining age, gender, AST, BMI, AST/ALT ratio and hyaluronic acid [18] or adiponectin, HOMA-IR, and serum type IV collagen [19] (Table 1).

Table 1
Diagnostic performance of currently available non-invasive indices of NASH in NAFLD.

Markers	*N*	Parameters	Endpoint	Cut-offs	AUROC	Se (%)	Sp (%)	PPV (%)	NPV (%)
NashTest [17]	257 97* 383**	Age, gender, BMI, triglycerides, cholesterol, α-2-macroglobulin, γGT, AST, ALT, haptoglobin apolipoprotein A1, total bilirubin	NAS ≥ 5 (Kleiner)	ND	0.79*	29	98	91	71
Palekar index [18]	80	Age ≥ 50 yrs; female gender; AST ≥ 45 UI/L; AST/ALT ratio ≥ 0.8; BMI ≥ 30 kg/m²; hyaluronate ≥ 55 mcg/l	NASH (Brunt)	≥ 3	0.76	74	66	68	71
Shimada index [19]	85	Serum adiponectin level; HOMA-IR; serum type IV collagen 7s level	NAS ≥ 5 (Kleiner)	ND	ND	94	74	94	74

NAS: NAFLD activity score.
AUROC: area under ROC curve; Se sensitivity; Sp specificity; PPV and NPV: positive and negative predictive values.
*Validation group: performances correspond to validation group.
**Control group.

2. Non-invasive diagnosis of fibrosis in NAFLD

Scoring of liver fibrosis by histology is used in a variety of scoring systems, including some that have been specifically designed for NAFLD, such as the Brunt [20] and Kleiner scores [21], and others, such as METAVIR [22] and Scheuer [23], designed for the scoring of fibrosis in the context of viral hepatitis. The most attention has been focused on whether or not non-invasive tests can detect advanced fibrosis (F3-F4) or cirrhosis (F4). Such an approach is clinically relevant because the presence of advanced fibrosis or cirrhosis is an indication for specific monitoring of complications related to portal hypertension and to the increased risk of developing hepatocellular carcinoma.

The clinical and biological variables most commonly associated with advanced fibrosis in patients with NAFLD are: increasing age; elevated BMI; presence of diabetes; presence of the metabolic syndrome; increased homeostatic insulin resistance (HOMA-IR); increased aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio; decreased platelet count; and hyaluronic acid [24].

2.1. Serum markers

Compared with hepatitis C [25], only a limited number of serum markers have been evaluated for their ability to assess liver fibrosis in patients with NAFLD. They include the BAAT score [26], NAFLD score [27], ELF score [28, 29], FibroMeters [30], FibroTest [31], hyaluronic acid [32] and NS score [33] (Table 2). Markers have been validated against the current clinical gold-standard liver biopsy using, as an expression of effectiveness, the area under the receiver operating characteristic (AUROC) curve, which plots the sensitivity over 1 – specificity, with optimal values being as close to 1.0 as possible. The diagnostic performances of most of the proposed indices are summarized in Table 3. Importantly, the results of the training set were confirmed in an independent validation set in only a few studies [27-31]. In addition, most of these studies included small numbers of patients with heterogeneous scoring systems and endpoints for fibrosis assessment. Some indices such as the FibroTest have also been proposed for the screening of fibrosis in large populations at risk of developing fibrosis such as diabetics [34] or hyperlipidemic patients [35].

Table 2
Proposed serum indices for non-invasive evaluation of fibrosis in NAFLD.

– BAAT score (BMI, age, ALT, triglycerides)
– NAFLD Fibrosis Score (NFS) (age, hyperglycemia, BMI, platelet count, albumin, AST/ALT ratio)
– European Liver Fibrosis score (ELF) (age, hyaluronate, MMP-3, TIMP-1)
– FibroMeter NAFLD (age, weight, platelet count, ferrtin, glucose, AST, ALT)
– FibroTest (α-2-macroglobulin, ψGT, apolipoprotein A1, haptoglobin, total bilirubin, age, gender)
NS score (type IV collagen 7s, hyaluronate)

Three indices are protected by patents and are currently commercially available: the FibroTest® in Europe (BioPredictive, Paris, France) or FibroSURE® in the USA (LabCorp, Burlington, NC, USA); FibroMeters® (BioLiveScale, Angers, France); and ELF® (Enhanced Liver Fibrosis Test, iQur Ltd, Southampton, UK).

Table 3
Diagnostic performance of currently available non-invasive indices of liver fibrosis in NAFLD.

Markers	*N*	Score	Fibrosis stage	Cut-offs	AUROC	Se (%)	Sp (%)	PPV (%)	NPV (%)
BAAT [26]	93	METAVIR	F ≥ 2	2	0.84	71	80	61	86
NFS [27]	733 253*	Brunt	F ≥ 3	≤ 1.455 > 0.676	0.82*	77	71	52	88
ELF [28,29]	61	Scheuer	F ≥ 3	0.375 0.462	0.87	89 78	96 98	80 87	98 96
	192	Kleiner	F ≥ 1 F ≥ 2 F ≥ 3	-0.207 -0.1068 0.3576	0.76 0.82 0.90	61 70 80	80 80 90	81 70 71	79 80 94
FibroMeters [30]	235 114*	METAVIR	F ≥ 2	ND	0.94	78.5	95.9	87.9	92.1
FibroTest [31]	170 97	Brunt/Kleiner	F ≥ 2	0.3 0.7	0.81	77	77	54	90
			F ≥ 3	0.3 0.7	0.88	15 92 25	98 71 97	73 33 60	76 98 89
Hyaluronate [32]	79	Brunt	F ≥ 3	46.1	0.92	85	80	51	96
NS [33]	112	Brunt	F ≥ 3	Coll ≥ 5 or HA ≥ 50 Coll ≥ 5 and HA ≥ 50	ND	96 54	63 92	66 84	95 73

AUROC: area under ROC curve; Se sensitivity; Sp specificity; PPV and NPV: positive and negative predictive values
* Validation group: performances correspond to the validation group.

2.2. Transient elastography

Transient elastography (TE) (FibroScan®, Echosens, Paris, France) has recently been proposed for measuring liver stiffness [36]. Briefly, an ultrasound transducer probe is mounted on the axis of a vibrator. Vibrations of mild amplitude and low frequency are transmitted by the transducer, inducing an elastic shear wave that propagates through the underlying tissues. Pulse-echo ultrasound acquisitions are used to follow the propagation of the shear wave and to measure its velocity, which is directly related to tissue stiffness, expressed as the elastic modulus: the stiffer the tissue, the faster the shear wave propagates.

TE is painless, rapid (less than 5 min) and easy to perform at the bedside or in the outpatients clinic. The results are immediately available and expressed in kilopascals (kPa), corresponding to the median value of 10 validated measurements and ranging from 2.5 to 75 kPa, with normal values being around 5.5 kPa [37]. The main limitation of TE in clinical practice is the impossibility of obtaining any liver stiffness measurements in around 5% of cases, mainly obese patients, which may represent a concern for its use in NAFLD patients.

TE has been shown to be reliable in the assessment of liver fibrosis initially in patients with chronic hepatitis C [38,39], with a strong correlation of liver stiffness values with METAVIR fibrosis stages, and with AUROCs ranging from 0.79 to 0.83 for the diagnosis of significant fibrosis and from 0.95 to 0.97 for cirrhosis. So far, only two studies have investigated TE in patients with NAFLD [40,41] (Table 4). However, these TE results should be interpreted with caution as these studies were conducted in a Japanese [40] and a pediatric population [41], with low mean BMIs (26.6 ± 4.2 and 26 ± 4, respectively) and small sample sizes (97 and 52, respectively). This may be an explanation for the rather low LSM failure rate in these two studies (5% and 4%, respectively), similar to that reported in patients without NAFLD.

Liver stiffness values may be influenced by the metabolic syndrome even in the absence of biological features of NAFLD. Indeed, in a recent study conducted in 429 healthy Western subjects without overt causes of liver disease and normal liver enzymes, liver stiffness values were significantly higher in subjects with the metabolic syndrome (n= 59; 13.7%) than in those without (6.5 ± 1.6 *vs* 5.3 ± 1.5 kPa, respectively; $P< 0.0001$) [42]. Interestingly, in four of the seven subjects with the metabolic syndrome who had liver stiffness values above 8 kPa and who underwent liver biopsy, all had NASH lesions with portal fibrosis, but mild or absent steatosis, suggesting that TE may be a sensitive tool for the detection of fibrosis. More data are awaited regarding the use of TE in NAFLD patients.

Table 4
Diagnostic performance of transient elastography (TE) in NAFLD.

Authors	*N*	Score	TE cut-offs (kPa)				AUROC	Se (%)	Sp (%)	PPV (%)	NPV (%)
			F ≥ 1	F ≥ 2	F ≥ 3	F = 4					
Yoneda *et al.* [40]	97	Brunt									
	79		5.9				0.93	86	89	97	59
	51			6.6			0.86	88	74	79	85
	27				9.8		0.90	85	81	64	93
	9					17.5	0.99	100	97	75	100
Nobili *et al.* [41]	50	Brunt									
	39		5.1				0.97	97	91	97	91
	12			7.4			0.99	100	92	80	100
	3				10.2		1.00	100	100	100	100
	2					–	–	–	–	–	–

AUROC: area under ROC curve; Se sensitivity; Sp specificity; PPV and NPV: positive and negative predictive values.

2.3 Other imaging techniques

Conventional imaging techniques such as ultrasound coupled with Doppler, CT and MRI can be used for the diagnosis of cirrhosis. However, the ability to detect early and intermediate stages of fibrosis with these techniques remains limited. Novel techniques, including magnetic resonance (MR) spectroscopy, diffusion-weighted MR and MR elastography, have also emerged for detecting hepatic fibrosis [43]. The theoretical advantages of these methods include the ability to analyze nearly the entire liver and their applicability in obese patients. MR elastography has recently been suggested to have better diagnostic accuracy than TE for the diagnosis of significant fibrosis (AUROC: 0.99 *vs* 0.84, respectively; $P< 0.05$) in a series of 96 patients with liver disease (eight with NASH) [44]. Although such results are encouraging, so far, these techniques remain too expensive and time-consuming for implementation in clinical practice for screening hepatic fibrosis.

Conflicts of interest: The author has none to declare.

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Cannabinoid receptors as novel therapeutic targets for the management of non-alcoholic steatohepatitis

A. Mallat*, S. Lotersztajn

Inserm, U841, Créteil, F-94010 France; Université Paris XII-Val-de-Marne, Créteil, 94000 France;
Groupe hospitalier Henri Mondor-Albert Chenevier, Service d'Hépatologie et de Gastroentérologie, AP-HP, Créteil, 94000 France.

Abstract

Prevalence of non-alcoholic steatohepatitis (NASH) rises steadily in Western countries with the obesity epidemic. NASH is associated with activation of liver fibrogenesis and predisposes to cirrhosis and associated morbi-mortality. The cannabinoid system is increasingly emerging as a crucial mediator of acute and chronic liver injury. Recent experimental and clinical data indicate that peripheral activation of cannabinoid CB1 receptors promotes insulin resistance and liver steatogenesis, two key steps in the pathogenesis of non-alcoholic fatty liver disease. Moreover, CB1 receptors enhance progression of liver fibrogenesis. These findings provide a strong rationale for the use of CB1 antagonists in the management of NASH.
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Résumé

Les récepteurs des cannabinoïdes: de nouvelles cibles thérapeutiques dans la prise en charge de la stéatohépatite non alcoolique

La prévalence de la stéatohépatite non alcoolique est en progression dans les pays occidentaux, parallèlement à celle de l'obésité. La stéatohépatite non alcoolique est associée à une activation des mécanismes de fibrogenèse avec un risque d'évolution cirrhogène et de morbidité significative. Le système cannabinoïde est un médiateur important la physiopathologie des hépatopathies aiguës et chroniques. Des données expérimentales et cliniques récentes indiquent que l'activation des récepteurs CB1 des cannabinoïdes dans les tissus périphériques joue un rôle déterminant dans l'insulinorésistance et la stéatogenèse hépatique, deux étapes clés dans le développement de la stéatopathie métabolique. Les récepteurs CB1 sont également impliqués dans la progression de la fibrose associée aux hépatopathies chroniques. L'ensemble de ces données suggère que les antagonistes du récepteur CB1 des cannabinoïdes pourraient offrir une nouvelle approche thérapeutique au cours de la stéatohépatite non alcoolique.
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Keywords: Fatty liver; Non-alcoholic steatohepatitis; Endocannabinoids; Cannabinoid receptors; Liver fibrosis; Review.

Mots clés : Stéatose ; Stéatohépatite non alcoolique ; Endocannabinoïdes ; Récepteurs des cannabinoïdes ; Fibrose hépatique ; Revue générale.

Preparations of the hemp plant *Cannabis sativa* have been used for medicinal purposes over centuries. THC was identified in 1964 as the predominant cannabinoid compound responsible for psychoactive effects of marijuana. Thereafter, cloning of cannabinoid receptors CB1 and CB2 in the early 1990s constituted a determinant milestone in the characterization of a novel biological system with a wide array of biological functions. Moreover, improvement in the understanding of the signaling mechanism responsible for cannabinoid actions has fostered research efforts in the development of therapeutic applications. Consequently, capsules of THC and its synthetic analog nabilone are approved in several countries for the management of chemotherapy-induced nausea and vomiting [1], and rimonabant, a selective CB1 receptor antagonist, has been available for 2 years in Europe as an adjunctive treatment of obesity or overweight with associated type 2 diabetes or dyslipidemia [2-4].

*Corresponding author.
E-mail Address: ariane.mallat@hmn.aphp.fr

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In this context, accumulating experimental and clinical data have stressed the crucial role of the cannabinoid system in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). NAFLD is closely linked to the metabolic syndrome and the obesity epidemic [5], and is currently a rising cause of liver injury, with a 20-30% prevalence in Western countries. The spectrum of the disease ranges from simple steatosis, a condition generally associated with a benign liver outcome, to steatohepatitis, an entity that comprises steatosis, liver inflammation and hepatocellular injury. The latter stage is associated with activation of fibrogenic pathways and carries a 10-20% risk of cirrhosis after 10 or 20 years. As shown in several recent studies, non-alcoholic steatohepatitis (NASH) leads to increased liver-related mortality due to end-stage liver disease or development of hepatocellular carcinoma [6]. The present review summarizes evidence that cannabinoid receptor antagonism may offer novel therapeutic approaches for the management of NAFLD.

1. The endocannabinoid system

The endocannabinoid system comprises endogenous lipid ligands, specific G-protein-coupled receptors (CB1 and CB2), and proteins that are responsible for their biosynthesis, cellular uptake and degradation [7-9].

The CB1 receptor was originally cloned from a rat brain library due to its high level of expression in the central nervous system [10], and subsequent studies have shown its presence at lower levels in many peripheral tissues. Expression of CB2 receptors predominates in the immune system and, although more restricted, is increasingly demonstrated in several cells [8,11,12]. Recent reports also suggest the existence of additional cannabinoid receptors.

Endocannabinoids are hydrophobic fatty-acid-derived compounds with predominantly autocrine/paracrine effects, among which anandamide (arachidonoyl ethanolamide) and 2-arachidonoyl glycerol (2-AG) are the best known. Both compounds are synthesized on demand and are rapidly degraded by fatty-acid amide hydrolase (FAAH) or monoacylglycerol lipase, following ligand binding and cellular reuptake [8,9,11,12]. Anandamide shows a higher affinity for CB1 than CB2 receptors and is therefore considered a major endogenous CB1 ligand, whereas 2-arachidonoyl glycerol binds both receptors with similar affinity [13]. In addition, both compounds also induce CB1- and CB2-independent effects. Lipid mediators other than anandamide and 2-AG have been reported to bind CB receptors, but their biological significance remains undetermined.

2. Modulators of cannabinoid receptors as therapeutic agents

Rimonabant has been the first CB1 antagonist to reach the market in Europe [2-4]. The drug was initially developed for the treatment of obesity in light of the positive impact of phyto- and endocannabinoids on central appetite-regulating pathways. It soon became clear that CB1 antagonism produces metabolic effects beyond those expected from weight loss alone, including improvements in dyslipidemia, insulin resistance and diabetes [14]. In keeping with clinical data, experimental studies have established that multiple peripheral mechanisms contribute to the beneficial effects of CB1 antagonism by enhancing energy expenditure, peripheral lipolysis and insulin sensitivity, among others [15,16]. Accordingly, trials are underway to further define the impact of CB1 antagonism on dyslipidemia, type 2 diabetes and cardiovascular morbidity. Other therapeutic applications under evaluation also include management of alcohol- and nicotine-dependence or neurodegenerative disorders [9]. The safety of CB1 antagonists in obesity has been questioned, given the occurrence of modest rates of anxiety and depression in susceptible individuals [14]. As a result, the FDA denied approval of rimonabant pending additional data, whereas Merck recently suspended the development of taranabant for obesity due to safety concerns. In this context, the development of peripherally restricted CB1 antagonists could prove of interest by avoiding central adverse effects.

Although selective agonists and antagonists of CB2 receptors have not yet reached a clinical stage, preclinical studies nevertheless suggest meaningful therapeutic applications as anti-inflammatory, analgesic or anti-allergenic compounds [9,17]. Of particular interest, such compounds should be devoid of central adverse effects.

Identification of cannabinoid receptors as potential therapeutic targets for the management of liver diseases [7] has emerged recently with the demonstration that CB1 receptors contribute to the pathogenesis of cirrhotic portal hypertension [18,19]. Soon after, additional studies uncovered a key role of cannabinoids in metabolic and ethanol-induced fatty liver, ischemia reperfusion, and in the scarring process associated with chronic liver disease [20-25].

3. Pathogenesis of NAFLD

It is now admitted that metabolic steatosis and insulin resistance are in tight relationship [26]. Thus, rodent models have shown that resistance to insulin promotes lipolysis in adipose tissue, thereby increasing delivery of free fatty acids to the liver [26]. Moreover, in the liver, hyperinsulinemia triggers *de novo* fatty acid, and impairs β-oxidation and lipid dis-

posal. Conversely, however, steatosis may also contribute to hepatic insulin resistance [26]. The transition from steatosis to NASH is poorly understood and appears to be multifactorial. Excess accumulation of free fatty acids leads to increased oxidative stress and lipid peroxidation, thereby resulting in cellular injury. Moreover, enhanced cytokine production by infiltrating macrophages in adipose tissue and the liver are also incriminated in the progression of injury [5].

4. Cannabinoid receptor antagonism reduces development of NAFLD

4.1. CB1 receptors promote metabolic steatosis and insulin resistance

Recent findings have shown that the hepatic cannabinoid system is activated in NAFLD. Thus, in the experimental model of diet-induced obesity, hepatic anandamide levels are increased following inhibition of its degradation by FAAH, and CB1 receptor expression is strongly induced in hepatocytes [23].

Accumulating experimental evidence indicates that CB1 receptors contribute to metabolic steatosis and the related insulin resistance [23,24,27]. CB1 receptor knockout mice are resistant to high-fat diet (HFD)-induced obesity and steatosis, and to the associated increase in hepatic lipogenesis; moreover, HFD-fed CB1-ablated mice display reduced insulin resistance [23,28]. In keeping, genetically obese fa/fa rats treated with rimonabant show reversal of hepatic steatosis and improved insulin sensitivity [27]. Interestingly, mice bearing a selective deletion of CB1 receptors in hepatocytes become obese under a HFD, but are protected from hepatic steatosis and impaired glucose tolerance [24]. Finally, characterization of functioning of upregulated hepatic CB1 receptors during steatogenesis suggests combined enhancement of lipogenesis and inhibition of fatty acid β-oxidation [23,24]. Collectively, these data indicate that peripheral overactivation of the cannabinoid system promotes obesity-associated fatty liver and insulin resistance. Beyond its contribution to steatogenesis, CB1-dependent endogenous cannabinoid tone may also favor the inflammatory response associated with NASH. Thus, it has been shown that endogenous CB1 activation reduces secretion of adiponectin [29], an adipocytokine with potent anti-inflammatory effects in the liver [30]. In keeping with these observations, administration of rimonabant to genetically obese rats induces a significant improvement in the hepatic inflammatory response [27].

Clinical studies also indirectly support the potential role of endocannabinoids and their receptors in the pathogenesis NAFLD. Analysis of pooled 1-year data from four pivotal trials in overweight patients indicates that rimonabant reduces alanine aminotransferase levels, a marker of NAFLD [14]. In addition, we recently investigated the impact of cannabis use on steatosis grade in 307 patients with chronic hepatitis C and found that daily cannabis consumption is an independent predictor of severe steatosis [31]. Overall, these results provide strong evidence for a steatogenic role of cannabinoids in humans.

4.2. CB receptors regulate liver fibrogenesis

As stated previously, transition from steatosis to NASH is associated with activation of fibrogenic pathways and predisposes to the development of liver fibrosis [32]. We recently found that expression of CB1 and CB2 receptors is markedly upregulated in cirrhotic liver samples, predominantly in liver fibrogenic cells, and demonstrated that CB1 and CB2 receptors display potent pro- and antifibrogenic properties, respectively [22,25]. Antifibrogenic properties of CB2 receptors were established in CB2 knockout mice repeatedly exposed to carbon tetrachloride, based on findings that these mice show enhanced liver fibrosis and increased accumulation of liver fibrogenic cells compared with wild-type animals [22]. The function of CB1 receptors in liver fibrogenesis was assessed in three different experimental models (chronic carbon tetrachloride or thiacetamide administration and bile duct ligation). Administration of rimonabant or genetic inactivation of CB1 receptors significantly reduced progression of fibrosis [25]. Profibrogenic properties of CB1 receptors were ascribed to the overactivation of CB1 receptors expressed by liver fibrogenic cells, leading to a combined enhancement of cell proliferation and decrease in apoptosis rate.

The clinical relevance of these experimental findings was confirmed in an epidemiological study of the impact of cannabis use on fibrosis severity in HCV-infected individuals. Daily cannabis use was documented as an independent predictor of fibrosis severity, suggesting that CB1 signaling dominates over CB2 during chronic hepatitis C [33]. A subsequent independent study in a Canadian cohort reported similar findings [34].

5. Emerging role of CB2 receptors in the pathogenesis of NAFLD

Several studies have shown that obesity generates a low-grade inflammatory state that contributes to the development of insulin resistance and NAFLD [35-37]. CB2 receptors are potent regulators of innate immunity [38] and we recently investigated their potential role in the pathogenesis of NAFLD. Compared with wild-type counterparts, mice invalidated for CB2 receptors are less prone to HFD-induced obesity [39].

Moreover, CB2 knockout mice are resistant to steatosis and display improved glucose tolerance. The mechanism underlying steatogenic effects of CB2 receptors appears to involve proinflammatory effects of upregulated CB2 receptors in adipose tissue.

6. Conclusion

Accumulating data indicate that the endocannabinoid system is upregulated in NAFLD and plays an important role in the pathogenesis of steatosis and insulin resistance *via* peripheral pathways. CB1 antagonism has proven efficient in the control of experimental NAFLD and liver fibrogenesis. Recent clinical trials have also established that inactivation of CB1 receptors not only reduces overweight, but also improves several parameters of the metabolic syndrome, including insulin resistance and dyslipidemia [14]. These observations undoubtedly provide a strong rationale for the evaluation of CB1 antagonists in the management of NASH, as currently underway in phase III clinical trials. Concern over potential adverse central effects of CB1 antagonists should stimulate ongoing efforts to develop peripherally restricted molecules.

Conflicts of interest: A. Mallat: Occasional involvements: advisory services (Sanofi-Aventis); Close relatives employed by Sanofi-Aventis.

S. Lotersztajn: none.

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The farnesoid X receptor (FXR) as a new target in non-alcoholic steatohepatitis

B. Cariou

Inserm, U915; Université de Nantes, Faculté de Médecine; CHU Nantes
Clinique d'Endocrinologie, Institut du Thorax, CHU Hôtel-Dieu, 1, place Alexis Ricordeau, 44093 Nantes cedex, France.

Abstract

The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily that is mainly expressed in liver, intestine, kidney and adipose tissue. On activation by bile acids, FXR regulates a wide variety of target genes that are critically involved in the control of bile acid, lipid and glucose homeostasis. Thus, FXR appears to be a promising target for the treatment of non-alcoholic steatohepatitis (NASH). Notably, FXR activation inhibits hepatic *de novo* lipogenesis, increases insulin sensitivity and protects hepatocytes against bile acid-induced cytotoxicity. More recent data also indicate a critical role of FXR in liver regeneration and hepatocarcinogenesis. For this reason, the development of FXR agonists and/or modulators (SBARMs) may prove to be clinically useful for treating NASH. While preclinical studies in rodents support this hypothesis, clinical studies are still warranted in humans.
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Résumé

Le récepteur nucléaire FXR *(farnesoid X receptor)* : une nouvelle cible moléculaire pour le traitement de la NASH

FXR *(farnesoid X receptor)* fait partie de la superfamille des récepteurs nucléaires. Il est exprimé principalement au niveau du foie, de l'intestin, des reins et du tissu adipeux. Une fois activé par ses ligands endogènes, les acides biliaires, FXR module l'expression d'un grand nombre de gènes cibles impliqués dans le contrôle du métabolisme des acides biliaires, des lipides et du glucose. De par cette action au carrefour de plusieurs voies métaboliques, FXR apparaît comme une cible prometteuse pour le traitement de la NASH. L'activation de FXR diminue la lipogenèse *de novo* au niveau du foie, augmente la sensibilité à l'insuline et protège l'hépatocyte contre l'action cytotoxique des acides biliaires. Plus récemment, des travaux ont démontré que FXR intervient également dans le contrôle de la régénération et de la carcinogenèse hépatiques. Le développement d'activateurs ou de modulateurs sélectifs (SBARMs) de FXR pourrait être efficace pour le traitement de la NASH. Si des études précliniques chez les rongeurs semblent confirmer cette hypothèse, les études chez l'homme font encore défaut à l'heure actuelle.
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Keywords: FXR; Bile acids; Liver steatosis; Liver regeneration; Lipogenesis; Review.

Mots clés : FXR ; Acides biliaires ; Stéatose hépatique ; Régénération hépatique ; Lipogenèse ; Revue.

1. Introduction

The farnesoid X receptor (FXR, NR1H4) is an adopted member of the nuclear-receptor superfamily that is predominantly expressed in the liver, gut, kidneys and adrenals, with much lower levels in white adipose tissue. FXR is expressed from a single gene locus in humans (chromosome 12q23.1); (for reviews, see Cariou and Staels, and Lee *et al.* [1,2]). Two alternative promoters, in the presence of an internal cryptic splicing site, lead to the expression of four isoforms—FXRα1/FXRα2 and FXRα3/FXRα4—which are not equivalent in terms of gene transactivation [3]. FXR was originally named for its weak activation at supraphysiological concentrations by farnesol, an intermediary in the mevalonate biosynthetic pathway [4,5]. In 1999, three independent teams demonstrated

*Corresponding author.
E-mail Address: bertrand.cariou@univ-nantes.fr

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that bile acids (BAs) bind to and activate this nuclear receptor [6-8]. The hydrophobic BA chenodeoxycholic acid (CDCA) is the most effective activator of FXR, whereas hydrophilic ursodeoxycholic (UDCA) and muricholic acids are inactive. Several synthetic FXR ligands have been generated, especially the non-steroidal GW4064 compound, and have been extensively used both *in vitro* and *in vivo* in rodents [9]. Ligand-activated FXR binds to DNA elements called 'FXR response elements' (FXREs). It is worth noting that FXR can bind to and activate or repress a large variety of FXREs either as a classical FXR/RXR heterodimer or as a monomer [10]. Although BAs can also influence gene expression *via* FXR-independent pathways, it is now well established that FXR activation by BAs results in the regulation of several genes controlling BA metabolism, thereby acting as the 'master intracellular BA sensor'. In this review, we specifically focus on the role of FXR in liver steatosis and fibrosis. The potential therapeutic value of pharmacological modulation of FXR in non-alcoholic fatty liver diseases (NAFLD) is also discussed.

2. FXR and bile acid metabolism

The main physiological role of FXR is to act as a BA sensor in enterohepatic tissues. FXR activation regulates the expression of various transport proteins and biosynthetic enzymes crucial to the physiological maintenance of BA and lipid homeostasis. BAs are actively secreted by the liver into bile and discharged into the intestinal lumen upon ingestion of a meal. BAs exhibit detergent-like properties that are crucial for their physiological functions in hepatic bile formation, and absorption of dietary lipids and fat-soluble vitamins from the small intestine. Efficient reabsorption of BAs in the terminal ileum results in the accumulation of a certain mass of BAs within the body, referred to as the 'BA pool', which cycles ≈ 12 times between intestine and liver in the enterohepatic circulation. Only ~5% of the pool escapes reabsorption per cycle and is lost *via* the large intestine in the feces [11]. This fecal loss of BAs, which is compensated for by *de novo* BA biosynthesis in the liver to maintain the pool size, represents a major route for cholesterol removal in humans. On the other hand, the physical characteristics of BAs, which allow them to form micelles, also impose a certain risk to cells that are exposed to high concentrations of these natural detergents. When present at high concentrations, BAs can become cytotoxic. In particular, hepatocytes and bile duct cells are at risk, for instance, in conditions of cholestasis, and protective mechanisms appear to become active when intracellular BA concentrations are elevated. BAs themselves are directly involved in the regulation of gene expression in liver and intestine *via* interaction with FXR, which provides a sensory function (Fig. 1).

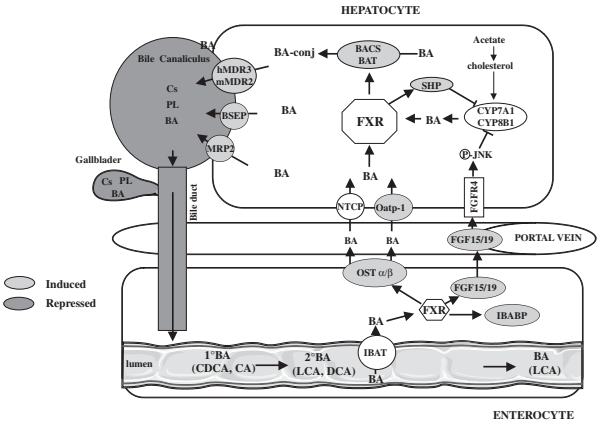

Fig. 1. Overview of FXR action in bile-acid metabolism. Bile acids (BAs) directly bind and activate FXR in both hepatocytes and enterocytes, resulting in transcription of target genes. Genes that are induced and repressed after FXR activation are shown in light grey and dark drey, respectively. BAs are synthesized in hepatocytes conjugated with taurine or glycine *via* BACS or BAT, and secreted across the bile canalicular membrane by transporters (BSEP, MRP2 and MDR2/3). BAs are then stored in the gallbladder before being excreted into the intestinal lumen. In the enterocyte, FXR represses the expression of the ileal BA transporter (IBAT) while inducing the expression of IBABP and two transporters—OSTα and OSTβ—which facilitate BA transport into the portal vein. Intestinal FXR also increases expression of FGF15/19, which circulates in the portal vein and subsequently binds to the hepatic cell surface receptor FGFR4. Activation of FGFR4 leads to the repression of *Cyp7a1* and *Cyp8b1* through the JNK pathway. Repression of *Cyp7a1* and *Cyp8b1* is also mediated by induction of SHP, a direct FXR target gene. BAs are taken up in the liver by NTCP and organic anion transport protein-1 (OATP-1) at the basolateral membrane of hepatocytes. In summary, FXR acts as the 'master BA sensor' by decreasing BA synthesis and liver uptake while simultaneously increasing BA conjugation, detoxification and excretion in bile canaliculi.

Recent extensive reviews have focused on the role of FXR in BA metabolism [10,12]. Briefly, FXR induces the small heterodimer partner (SHP) in liver that, in turn, down-regulates the expression of both *Cyp7a1* and *Cyp8b1* genes, encoding enzymes that synthesize BAs from cholesterol, and represses the Na+ -taurocholate pump (NTCP) that transports BAs from serum to the liver. In addition, FXR also induces the expression of transporters, such as the bile salt export pump (BSEP), to transport BAs from the liver into the bile canaliculi. In the intestine, FXR represses the expression of the ileal apical sodium-dependent BA transporter (ABST; also called 'intestinal BA transporter' or IBAT), and induces both ileal bile-acid-binding protein (IBABP), and the organic solute and steroid transporters (OST) α/β that serve to transport BAs from the gut to the circulation, where they are then transported back to the liver. In addition, in response to BA flux in the intestine, FXR activates fibroblast growth-factor 15/19 (FGF15/19) gene expression in the enterocyte. Once secreted, FGF15/19 is transported to the liver where, through the FGF receptor-4 (FGFR4) signal-transduction pathway, it

downregulates *Cyp7a1* and *Cyp8b1* expression [13]. In summary, FXR activation suppresses *de novo* BA synthesis, and accelerates hepatic biliary excretion and detoxification, while simultaneously reducing their importation from the portal vein in a tightly coordinated fashion [2,10]. Thus, FXR protects liver cells from the deleterious consequences of cellular BA overload.

3. FXR and liver steatosis

Besides its classical role in BA and lipid homeostasis, recent data have underlined an unexpected function of FXR in glucose metabolism (for review, see Cariou and Staels [1]). The first indication came from the observation that hepatic FXR expression is reduced in several rodent models of diabetes [14] and varies during nutritional changes: it is increased during fasting and decreased on refeeding [15,16]. Interestingly, FXR can impact several steps of the pathophysiological process of liver steatosis by modulating insulin sensitivity and the rate of *de novo* lipogenesis.

3.1. FXR and insulin sensitivity

Three independent teams simultaneously identified a role for FXR in regulating insulin sensitivity [17-19]. FXR deficiency leads to impaired glucose tolerance and insulin resistance. While hyperinsulinemic– euglycaemic clamp studies clearly concluded that $FXR^{-/-}$ mice display peripheral insulin resistance, reflected by reduced peripheral glucose disposal [17,18], there are discordant data concerning the level of hepatic insulin sensitivity in $FXR^{-/-}$ mice. While some studies found a reduced inhibition of hepatic glucose output during a low-dose insulin clamp [18], FXR deficiency was also shown to be associated with normal hepatic insulin sensitivity [15,17]. The reason for this discrepancy is unclear, but may be linked to differences in the genetic backgrounds of the mice and/or the insulin dose used during the clamp. If FXR acts as an insulin sensitizer, then FXR activation would be expected to promote insulin sensitivity. In support of this hypothesis, treatment with GW4064 improved insulin sensitivity *in vivo* in both *db/db*, KK-A(y) [19] and *ob/ob* [17] diabetic mice.

Nevertheless, the molecular mechanisms behind the insulin-sensitizing effects of FXR remain poorly defined. Insulin signalling was found to be impaired in peripheral insulin-sensitive tissues such as skeletal muscle and white adipose tissue, whereas liver data remain conflicting [17,18]. As FXR is not expressed in skeletal muscle, it is conceivable that FXR deficiency indirectly alters insulin signalling in this tissue. One hypothesis is that FXR deficiency promotes ectopic lipid deposition in insulin target tissues, a phenomenon usually referred to as 'lipotoxicity' [20]. Indeed, $FXR^{-/-}$ mice have elevated circulating FFA levels [17,18], and increased intramuscular triglyceride and FFA contents [18]. A similar mechanism could also operate in liver as hepatic triglyceride content is increased in $FXR^{-/-}$ mice [15,18].

An interesting alternative pathway to explain the insulin-sensitizing effects of FXR is its role in white adipose tissue. FXR expression increases progressively during adipocyte differentiation *in vitro*, both in 3T3-L1 cells and mouse embryonic fibroblast (MEFs) cells [17,21]. Using MEFs as a model system, it has been shown that FXR deficiency leads to impaired adipogenic processing with defective triglyceride accumulation [17]. Conversely, the synthetic FXR ligand 6α-ECDCA/INT-747 promotes adipocyte differentiation and lipid storage in 3T3-L1 adipocytes [21]. Consistent with these *in vitro* data, $FXR^{-/-}$ mice exhibit a moderate lipoatrophic phenotype that may contribute to their impaired insulin sensitivity. Moreover, GW4064 treatment improves insulin signalling and insulin-induced glucose uptake in 3T3-L1 differentiated adipocytes [17,21]. Recently, FXR has been shown to directly stimulate the expression of the insulin-responsive glucose transporter GLUT4 [22].

Although beyond the scope of this review, it should be noted that BAs can also modulate metabolic homeostasis in a FXR-independent manner. The addition of cholic acid (CA) to the diet increases energy expenditure in brown fat in mice, and prevents the development of high-fat diet-induced obesity and insulin resistance. This metabolic effect of BAs was found to be critically dependent on induction of type 2 iodothyronine deiodinase (DIO2), and was suggested to be mediated by cAMP production induced by BAs binding to the G-protein-coupled receptor TGR5 (or Gpbar1) [23]. In addition, a recent study indicates that taurine-conjugated UDCA (tUDCA) can act as a molecular chaperone, thereby protecting hepatocytes against endoplasmic reticulum (ER) stress. As a consequence, *in vivo* treatment with tUDCA protects mice against diet-induced obesity and insulin resistance as well as fatty liver disease [24]. The physiological relevance of these signalling pathways, however, remains to be established in humans.

3.2. FXR and lipogenesis

FXR is involved in the control of hepatic *de novo* lipogenesis, one source of the fatty acids used for the assembly of very low-density lipoproteins (VLDL) (Fig. 2). FXR activation by BAs or synthetic agonists represses the expression of the transcription factor SREBP-1c and its lipogenic target genes in mouse primary hepatocytes and in liver, at least in part, in an SHP-dependent manner [16,25]. In addition, FXR modulates the kinetics of the response to dietary carbohydrate intake, as the maximum induction of glyco-

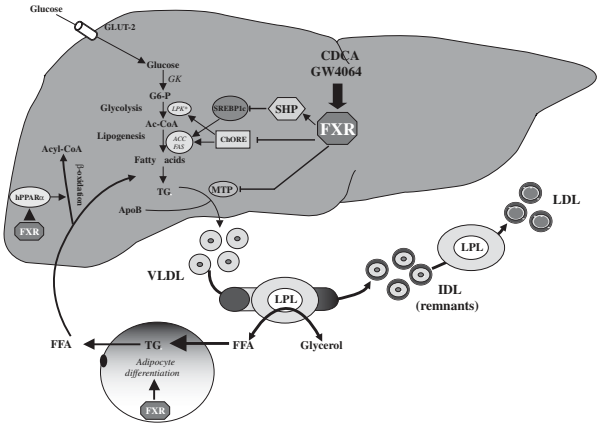

Fig. 2. Role of FXR in hepatic lipogenesis. Bile acids directly bind and activate hepatic FXR, leading to a variety of responses modulating triglyceride (TG) metabolism. FXR inhibits hepatic lipogenesis by both interfering with promoters (ChOREs) of glucose-regulated genes and decreasing the expression of SREBP-1c in an SHP-dependent manner. Conversely, FXR can promote FFA catabolism by increasing PPARα expression. FXR also controls the assembly of VLDL by repressing the expression of MTP. Finally, FXR can promote adipose TG storage by stimulating adipocyte differentiation (see text for details and references). CDCA, chenodeoxycholic acid; SHP, small heterodimer partner; SREBP-1c, sterol regulatory element-binding protein-1c; ChORE, carbohydrate response element; GK, glucokinase; G6-P, glucose-6 phosphate; LPK, L-pyruvate kinase; FAS, fatty-acid synthase; ACC, acetyl-CoA carboxylase-1; MTP, microsomal triglyceride transfer protein; LPL, lipoprotein lipase; VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; FFA, free fatty acid.

lytic and lipogenic genes occurs earlier during the refeeding phase in $FXR^{-/-}$ than in wild-type mice. Lack of FXR therefore leads to enhanced glycolytic flux, which provides substrates for lipogenesis [15]. At the molecular level, FXR activation by GW4064 attenuated glucose-induced mRNA expression as well as promoter activity of several glucose-regulated genes, such as L-pyruvate kinase and acetylCoA carboxylase 1, in rodent primary hepatocytes [15]. Triglycerides derived from *de novo* lipogenesis efficiently mobilize apolipoprotein B and induce VLDL assembly [26]. Consistent with this observation, hepatic VLDL production is significantly increased upon refeeding $FXR^{-/-}$ mice with a carbohydrate-rich diet [15]. Finally, FXR ligands induce the expression of PPARα and its target gene pyruvate dehydrogenase kinase-4 (PDK-4), both of which are known to promote fatty-acid oxidation [27,28].

Very recently, it has been demonstrated that FXR-deficiency induces non-alcoholic steatohepatitis (NASH) in LDL receptor knockout mice, a mouse model of hypercholesterolemia, feeding a high-fat diet. In addition to liver macrosteatosis, FXR-deficiency was specifically associated with inflammatory infiltrates [29,30]. Based on these results, it would be expected that FXR activation by its ligands would reduce hepatic steatosis. Data from rodent models appear to confirm this suggestion. CA lowers hepatic triglyceride accumulation, VLDL secretion and elevated serum triglycerides in KK-A(y) mice, a mouse model of hypertriglyceridemia [25]. Furthermore, GW4064 treatment reduces neutral lipid accumulation in the liver of *db/db* mice [19].

Altogether, these results suggest that FXR activation may have a beneficial role in NAFLD by decreasing hepatic *de novo* lipogenesis that constitutes the 'first hit' of the disease.

4. FXR and liver fibrosis

Inflammatory processes are the 'second hit' in the course of NAFLD, and lead to the development of hepatitis and subsequent liver fibrosis [29]. Hepatic FXR appears to be downregulated during the acute-phase response in rodents in a similar manner as seen in other nuclear receptors such as PPARα and LXR [31,32]. This indirectly suggests that FXR may also modulate the expression of genes participating in the inflammatory response. Treatment of mice with CA induces the expression of ICAM-1, VCAM-1, serum amyloid A2 and TNF-α. Moreover, *in vitro* experiments in human hepatocytes demonstrate that FXR increases the transcriptional activity of the human ICAM-1 promoter [33]. Based on these results, FXR activation in liver could be associated with a deleterious proinflammatory profile.

However, a recent study indicates that FXR activation inhibits the expression of inflammatory mediators in response to NF-κB activation *in vitro* in hepatoma cell line and in primary hepatocytes. Interestingly, $FXR^{-/-}$ mice are more prone to develop necrosis and severe inflammation after treatment with lipopolysaccharide (LPS) than wild-type mice [35,36]. In addition, FXR has been shown to be expressed in both rat and human stellate cells (HSCs) [34,35]. Activated HSCs are responsible for the deposition and accumulation of extracellular matrix in fibrotic liver. In chronic liver disease, HSCs undergo a progressive process of transdifferentiation from a resting, fat-storing phenotype, toward a myofibroblast-like phenotype characterized by increased expression of fibroblast cell markers such as α-smooth muscle actin [37]. Activation of FXR with its synthetic agonist 6E-CDCA reduces HSC transdifferentiation *in vitro*, thereby protecting the liver against fibrosis in rodent models of liver injury [34,38]. This effect is thought to be mediated by the induction of hepatic PPARγ [39]—and potentially PXR [40]—expression following FXR activation.

5. FXR and other liver diseases

5.1. Cholestasis

Due to its hepatoprotective action, FXR has been proposed as an attractive target for treatment of cholestatic liver diseases. $FXR^{-/-}$ mice were found to be less sensitive to bile duct-ligated (BDL)-induced liver damage, a model for obstructive extrahepatic cholestasis [41,42]. This is due, at least partly, to the fact that these mice, in contrast to wild-type mice, do not maintain expression of the transporter BSEP. In rat models of chemically induced intrahepatic cholestasis, activation of FXR with GW4064 resulted in significant reductions in serum alanine and aspartate aminotransferases as well as other markers of liver damage [43]. GW4064 also decreased the incidence and extent of necrosis, decreased inflammatory cell infiltration and bile duct proliferation. Based on analyses of gene expression profiles, the beneficial effects of FXR activation have been ascribed to the reduction of BA synthesis genes such as *Cyp7a1*, and the induction of genes involved in biliary transport such as the phospholipid transporter Mdr2/Abcb4 [43]. FXR also induces UGT2B4 expression and activity in human hepatocytes, indicating a feed-forward reduction of BA toxicity in humans by glucuronidation [44]. In addition, upregulation of Mrp2/ABCC2, Mrp4/ABCC4 and Ostα/Ostβ on the basolateral surface of renal tubular cells in the kidney will increase the overall elimination capacity for such hydrophilic BA metabolites from the body [45].

5.2. Liver regeneration and hepatocarcinogenesis

During the past few years, accumulating data have indicated that FXR is involved in carcinogenesis (for review see Wang *et al.* [46]). The incidence of hepatocellular carcinoma (HCC) has doubled over the last two decades in the United States often as a complication of NAFLD. Partial hepatectomy experiments in mouse models have revealed that FXR is crucial for the control of liver regeneration [47], a critical process for restoring liver mass following liver injury. However, uncontrolled regeneration of hepatocytes, which occurs after repeated cycles of necrosis and regeneration in chronic hepatitis, appears to be an important factor in hepatocarcinogenesis. While elevated BAs stimulate liver growth after partial hepatectomy, BA sequestrants strongly decrease the rate of liver regeneration. As these effects are lost in $FXR^{-/-}$mice, FXR appears to be the molecular link of the effect of BAs on liver regeneration [47]. FXR activation contributes to cell cycle entry of hepatocytes by inducing the expression of transcription factors that regulate cell cycling such as FoxM1b [48,49]. Recently, it was also demonstrated that FXR protects liver cells from apoptosis induced by serum deprivation *in vitro* and starvation *in vivo* [50]. The protective role of FXR is strongly underlined by the increased prevalence of liver tumors in old (12-15 months) male and female $FXR^{-/-}$ mice, a tumorigenic response characterized by general liver injury, irregular regeneration and severe inflammation [48,49]. These tumors include hepatocellular adenoma, carcinoma and hepatocholangiocellular carcinoma. As $FXR^{-/-}$ mice display elevated BA pool size, the tumorigenic process is probably related to the cytotoxic effects of BAs. In accordance with this hypothesis, a CA-enriched diet favors chemically induced liver tumor progression. In contrast, BA sequestrants decrease tumors in treated mice [48,49].

In summary, FXR exerts its hepatoprotective effect by tightly controlling the BA level in liver and promoting liver repair though controlled regeneration (Fig. 3). This dual effect of FXR helps the liver to protect against hepatocarcinogenesis.

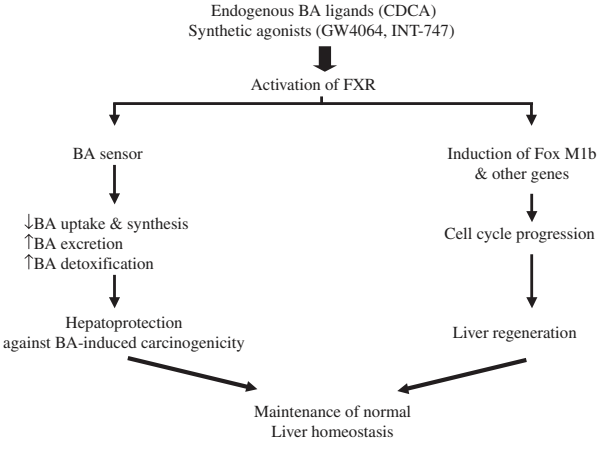

Fig. 3. A model of the dual action of FXR in the control of hepatic carcinogenesis (adapted from Wang *et al.* [44]).

6. Human studies

To date, there is no identified mutation in the FXR gene related to human diseases. However, based on quantitative trait locus analyses, it has been proposed that polymorphisms of FXR are likely to be primary genetic determinants of cholesterol gallstone susceptibility [51]. Interestingly, we found recently that plasma BA concentrations are negatively correlated with insulin sensitivity—but not glycemic status—in a wide variety of subjects, including healthy volunteers, abdominally obese and type 2 diabetes patients [52]. While reinforcing the hypothesis of a link between BA metabolism and

insulin sensitivity, these data remain correlative. Therefore, whether or not elevated circulating BAs are agents or markers of insulin resistance is still an unresolved issue. More direct evidence of a metabolic role of BAs is found in the results of human studies employing BA sequestrants, which disrupt the enterohepatic circulation of BA [53]. Indeed, in lipid-lowering trials, BA sequestrants have been shown to lower plasma glucose and HbA_{1c} [54,55]. Future interventional clinical studies with BAs, especially CDCA, or FXR agonists [56] are clearly needed to ascertain their functional relevance in the treatment of obesity, type 2 diabetes and NAFLD.

7. Conclusion

FXR is a multipurpose nuclear receptor that interferes with BA and metabolic homeostasis. As a BA sensor, it exerts a liver-protective role, and is an interesting target in both liver inflammation and carcinogenesis. Accumulating data, at least in rodents, suggest that FXR acts as an insulin-sensitizer. Given this dual effect, it is tempting to speculate that FXR activation by natural or synthetic agonists, or FXR modulation with selective bile-acid receptor modulators (SBARMs), may have a beneficial action in the pathogenesis of NAFLD. The findings of ongoing studies in humans will help to definitively resolve this issue.

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Conflict of interest: The author has none to declare.

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Hepatitis C virus and glucose and lipid metabolism

M. Alaei[a], F. Negro[a,b*]

Divisions of [a]Gastroenterology and hepatology and of [b]Clinical pathology, University Hospitals, Geneva, Switzerland.

Abstract

The hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. Its spectrum of severity, however, varies widely, as does its rate of progression towards cirrhosis. This depends on several host-related cofactors, such as age, gender, alcohol consumption, overweight and co-infections. The objective of this review is to discuss two of these cofactors: steatosis and insulin resistance. Although both may occur independently of HCV, a direct role of HCV infection in their pathogenesis has been reported. Whereas the virus-induced steatosis does not seem to have major clinical consequences, the so-called 'metabolic' steatosis and underlying insulin resistance may not only modify the clinical and histological course of chronic hepatitis C, but may also influence the response to interferon alpha-based therapy.
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Résumé

Virus de l'hépatite C et métabolisme du glucose et des lipides

Le virus de l'hépatite C (VHC) est une importante cause de maladies chroniques du foie dans le monde entier. Sa gravité est pourtant très variable, tout comme son taux de progression vers la cirrhose. Cela dépend de la présence de plusieurs cofacteurs liés à l'hôte, tels que l'âge, le sexe, la consmmation d'alcool, le surpoids et certaines coinfections. Dans cette revue, nous allons discuter de deux de ces facteurs, la stéatose et l'insulinorésistance. Bien que ces deux facteurs puissent être présents indépendammant du VHC, un rôle direct de l'infection par VHC dans leur pathogenèse a été rapporté. La stéatose virale ne paraît pas avoir des conséquences majeures sur le plan clinique. Au contraire, la stéatose métabolique (ainsi que la sous-jacente insulinorésistance) est capable de modifier l'histoire clinique ainsi que la progression histologique de l'hépatite C chronique, et aussi influencer la réponse au traitement par interféron alpha.
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Keywords: Hepatitis C virus; Diabetes; Steatosis; Cirrhosis; Interferon alpha; Review.

Mots clés : FXR ; Virus de l'hépatite C ; Diabète ; Stéatose ; Cirrhose ; Interféron alpha ; Revue.

1. Introduction

The hepatitis C virus (HCV) is a major cause of chronic liver disease, affecting an estimated 170 million people worldwide. The spectrum of severity of the liver disease associated with HCV—from minimal liver damage to full-blown hepatitis, both acute and chronic—varies widely as does its rate of progression towards cirrhosis. This rate seems to depend on many host-related cofactors such as age, gender, alcohol consumption, overweight and co-infections [1,2]. The objective of this review is to discuss two such cofactors-steatosis and insulin resistance. Although both may occur independently of HCV, a direct role of HCV infection is now accepted in their pathogenesis. Steatosis and insulin resistance may not only modify the course of chronic hepatitis C, but may also influence the response to interferon alpha-based therapy.

2. HCV and steatosis

Liver steatosis is the accumulation of triglycerides in hepatocytes, and is a common finding in any chronic liver disease. Liver steatosis and steatohepatitis, once thought to arise only

*Corresponding author.
E-mail Address: Francesco.Negro@hcuge.ch

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as a result of alcohol consumption, are now most often seen in association with the metabolic syndrome. The concomitance of increased fatty acid synthesis and the free fatty acid overflow to hepatocytes that accompanies the metabolic syndrome are the major pathogenic mechanisms leading to fatty liver in these patients [3]. Given the current pandemics of overweight, it is not surprising that steatosis is so frequent in chronic hepatitis C. In fact, the prevalence of steatosis in patients with chronic hepatitis C varies between 50% and 80%, depending on the prevalence of alcohol consumption, overweight, diabetes and other risk factors of fatty liver [2,4]. This prevalence is higher than in the general population. In comparison, 30-35% of potential liver donors in the United States have steatosis at liver biopsy [5]. When all major factors of fatty liver are excluded, the prevalence of steatosis in chronic hepatitis C is still about 40%, which is twofold higher compared with the average prevalence of steatosis in chronic hepatitis B [2,6]. This observation alone suggests that HCV may be directly affecting intrahepatic lipid metabolism, resulting in a fatty liver; indeed, in the pre-serology era, the presence of fatty liver was widely considered diagnostic of non-A, non-B hepatitis.

The association between HCV and fatty liver is, in part, genotype-specific. Among patients with chronic hepatitis C, those with genotype-3 infections have more frequent and more severe steatosis than those with non-genotype-3 infections [7-10], hinting at the presence of *steatogenic* sequences within the genome of genotype 3: in patients with genotype-3 infection, fatty liver can occur in the complete absence of obesity and insulin resistance [11,12]. In addition, the severity of steatosis in patients with genotype 3 correlates with the level of HCV replication, both in liver [8] and in serum [9]. Steatosis is reduced or disappears when patients are successfully treated with antivirals, particularly if infected with genotype 3, while those with non-3 genotypes may retain a fatty liver even when cured of the virus [13,14]. A relapse after the end of therapy may cause the reappearance of steatosis in patients in whom it had disappeared during therapy [15]. These observations suggest a viral etiology of fatty liver, at least in patients with genotype-3 infection. In non-genotype-3 infections, steatosis is most common in patients who are obese and insulin-resistant, and insulin resistance seems to be central in accounting for the pathogenesis of fatty liver in such cases [3,9,12].

The mechanism of triglyceride accumulation by HCV is multifactorial [16]. HCV can interfere with lipid metabolism at three levels: impaired lipoprotein secretion; increased lipogenesis; and impaired fatty acid degradation. Impaired secretion of lipoproteins from infected hepatocytes was the first mechanism proposed to explain HCV-induced steatosis. Serum levels of apolipoprotein B (apoB) and cholesterol are diminished in chronic hepatitis C [10,14,17], but return to normal after successful antiviral therapy, suggesting that HCV may interfere with very low-density lipoprotein (VLDL) assembly and/or secretion. The potential relevance of this viral effect on virion assembly and release is discussed below.

Experimental models have shown that the HCV core protein is sufficient to induce triglyceride accumulation in hepatocytes [18-20]. The accumulation seems to occur to a slight extent in most viral genotypes, but genotype 3a is the most efficient [20]. In the transgenic mouse, the HCV core protein inhibits microsomal triglyceride transfer protein (MTP) activity [19]. Since this enzyme plays a key, rate-limiting role in VLDL assembly, the consequence of its inhibition is the accumulation of triglycerides. Recent data in human liver are in agreement with this mechanism, since the MTP mRNA levels are reduced in the liver of chronic hepatitis C patients, especially those with steatosis [21].

As a second mechanism, HCV may induce steatosis *via* increased synthesis of fatty acids by upregulating the sterol response element binding protein 1c (SREBP-1c) [22,23]. Yang *et al.* [24] confirmed these data indirectly by providing evidence of a causal relationship between HCV infection and the level of fatty acid synthase (FAS). They hypothesized that upregulation of FAS results in increased lipogenesis. If so, HCV infection could directly increase lipogenesis, contributing to the formation of steatosis. The HCV core protein may also bind to and activate the DNA-binding domain of retinoid receptor α (RxRα), a transcription factor that controls, among other functions, lipid synthesis [25].

Finally, HCV may impair fatty acid oxidation. Transfection of hepatoma cells with the HCV core protein leads to reduced expression of peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor regulating several genes involved in fatty acid degradation [26]. PPARα mRNA is significantly reduced in the liver of patients with chronic hepatitis C [27,28].

A phenylalanine residue at position 164 of the core encoding sequence—present in genotype 3a, but replaced by tyrosine in all other genotypes—seems to be associated with activation of fatty acid synthetase and accumulation of big lipid droplets in hepatocytes [29,30]. However, in a recent study by Piodi *et al.* [31], although cells transfected with genotype 3a contained larger lipid droplets than cell transfected with genotype-1b sequences, there were no genetic differences between genotype-3a core proteins in patients with and without HCV-induced steatosis. The authors suggested that other viral proteins—or even host factors—could modulate the development of hepatocellular steatosis in patients infected by HCV genotype 3a.

The recurring question is: If HCV induces steatosis, why does it do so? Is HCV benefiting from the accumulation of triglycerides? Is steatosis increasing viral fitness or its rate of replication? Clinical trials of the treatment of chronic hepa-

titis C patients with steatosis [13,14] have shown that HCV replication *precedes* steatosis—not the other way around—and, therefore, it is unlikely that steatosis *per se* is a necessary factor for the HCV life cycle to proceed. However, some comments are needed in view of the known activation of HCV replication—at least *in vitro*—by fatty acids, especially saturated and monounsaturated, and by the observation that, in the same *in vitro* model, inhibition of fatty acid synthesis blocked HCV replication [32]. HCV replicates in association with cell membranes [33]: fatty acids are likely to be required to maintain a proper membrane structure. The HCV core protein has a strong affinity for intracytoplasmic lipid droplets (LD) and accumulates on their surfaces [34], a process mediated by its middle domain [35]. The transfer of core protein from the endoplasmic reticulum, where it is synthesized, to the surface of LD requires proteolytic processing [36]. Once localized to the LD, the core protein recruits the HCV replication complex, an event that requires interaction with non-structural protein 5A (NS5A) [37]. It must be mentioned that LD are fat-storing organelles physiologically found in hepatocytes and that, therefore, they are preexisting HCV infection. HCV core binds to LD independently of viral genotype and the presence or absence of steatosis in the liver of patients from which the isolate has been derived [31]. Thus, co-localization of the HCV core with LD and accumulation of fatty acids within hepatocytes are two events that should be considered as independent of each other. While it is clear that HCV replication and virion assembly requires fatty acids and LD, there is no evidence that steatosis—the excess accumulation of fat in cytoplasm—is indeed increasing viral replication. On the contrary, coalescence of LD into big steatosis droplets would reduce the effective surface area needed by the virus to correctly assemble mature virions.

Other evidence points towards the hypothesis that virus-induced steatosis is unfavorable to HCV. We noted that HCV decreases MTP activity, which results in the blockade of VLDL assembly and steatosis formation [19,21]. On the other hand, it has been shown that HCV virions are secreted *via* the intact VLDL pathway [38], and that MTP activity is necessary for HCV to be secreted, since silencing apoB or inhibiting MTP activity with the grapefruit flavonoid naringenin would block HCV secretion by about 80% [39]. Thus, HCV, while inducing steatosis, seems to block a pathway that is necessary for its mature virion secretion: for this reason, virus-induced steatosis is as favorable to HCV as would be its attempted suicide. We can only deduce that proper secretion of genotype 3 virions relies on any residual activity of MTP and that steatosis is, in fact, reducing virion secretion. Whether this results in a benefit for the virus in terms of reduced viral spread, replication and viral protein expression, a device frequently used by viruses to favor its persistence of infection, remains purely speculative at present. In conclusion, the significance (if any) of the occurrence of steatosis in some patients infected with HCV, especially genotype 3, remains unclear.

3. Clinical impact of HCV-induced steatosis

Steatosis has been reported to contribute to disease progression in chronic hepatitis C [2,3,9]. Steatosis on the initial biopsy has been associated with a more rapid development of fibrosis [40-45], higher risk of hepatocellular carcinoma (HCC) [46] and decreased response to antiviral therapy [43]. In view of the discordant data reported in the literature, especially concerning the relative contribution of the different genotypes to liver fibrosis [41-45], we carried out a vast meta-analysis on individual patients' data (the HCV MAID Study), collecting information on 3,068 patients from 10 centers around the world [47]. The results of this analysis suggest that the relationship between steatosis and fibrosis holds true essentially for patients with non-genotype-3 infections, notably those with genotype 1. In other words, the steatosis observed in patients with genotype-3 infection, despite its frequent occurrence and severity, does not appear to lead to an accelerated course towards cirrhosis [47]. Previous reports suggesting otherwise may have artefactually emphasized a spurious association between HCV-induced steatosis and fibrosis due to center-specific features of the study population such as overrepresentation of cirrhosis patients [44].

The association between steatosis and response to interferon alpha-based therapy is similarly true only for patients with non-virus-induced steatosis. An extensive study has confirmed this view [14] and, in another report [48], patients with genotype 3 and the most severe steatosis had very high rates of sustained virological response. Conversely, steatosis of other origin, essentially insulin resistance, is certainly associated with a poor response to therapy [14]: the mechanisms underlying this relationship are discussed below.

Finally, steatosis is often considered as a pathological condition that worsens an insulin-resistant state. However, in the case of HCV-induced steatosis, this does not seem to be true. In fact, not only do patients with HCV genotype 3 seem to have the lowest levels of insulin resistance [49], but they are also comparable to patients with and without steatosis [45]. Experimental models have also elegantly shown how hepatocellular steatosis and insulin resistance are not necessarily linked to each other [50].

In conclusion, steatosis can be induced directly by HCV, especially in genotype-3 infected patients. In some, fatty liver, mostly macrovesicular, can be severe, and as much as 80-90% of hepatocytes may contain big lipid droplets. Despite this, the clinical impact seems overstated, and virus-induced fatty liver does not accelerate liver disease progression, reduce the rate

of response to interferon alpha or worsen insulin resistance. Further studies may clarify the significance of viral steatosis in the HCV life cycle and its interaction with the host.

As far as the clinical management of virus-induced fatty liver is concerned, no therapeutic measures are indicated in addition to the standard management of chronic hepatitis C. Extensive diagnostic workups aimed at the identification of rare forms of fatty liver should be limited to special cases where the pathogenesis of steatosis is unclear. In most patients, severe steatosis, with genotype-3 infection, accompanied by high levels of HCV RNA and, if available, low levels of apoB in serum [17], do not warrant additional diagnostic procedures.

4. HCV and insulin resistance

Insulin resistance is defined as a condition in which higher-than-normal insulin concentrations are needed to achieve normal metabolic responses or, alternatively, normal insulin concentrations are unable to achieve normal metabolic responses [51]. Even before we started measuring the level of insulin resistance in chronic hepatitis C patients, most often by measuring the homeostasis assessment score of insulin resistance, known as HOMA score, several reports suggested an association between HCV infection and diabetes.

Diabetes is a common complication of all liver diseases, independent of the etiology, and especially in the advanced stages. However, clinical and experimental data suggest a direct role of HCV in the perturbation of glucose metabolism. Historically, the first observation that cirrhotic patients infected with HCV may have type 2 diabetes more frequently than do patients with cirrhosis of other origin was published by Allison *et al.* in 1994 [52]. A subsequent retrospective analysis of 1,117 patients with chronic viral hepatitis [53] reported type 2 diabetes among 21% of HCV-infected patients, but only in 12% of HBV-infected persons. Multivariable analyses showed that HCV infection and age were independent factors predicting diabetes. In a further case-control study conducted by the same authors in a cohort of 594 diabetics and 377 patients evaluated for thyroid disorders, 4.2% of diabetic patients were infected with HCV, compared with only 1.6% of controls [53]. Another study conducted within the Third National Health and Nutrition Examination Survey (NANHES-III) suggested a significant association between HCV infection and diabetes among persons 40 years of age or older, with a risk increased by a factor of about 3 [54]. This raised the suspicion that diabetes may be due to the stage of advancement of liver disease rather than the viral infection. However, when the insulin-resistance score, a more sensitive and earlier marker of glucose metabolism derangement, was measured in a population of 121 chronic hepatitis C patients with portal or no fibrosis—in other words, at the early stages of disease—this was higher compared with the average HOMA score found among 137 healthy volunteers matched by gender, body mass index (BMI) and waist-to-hip ratio [49]. This work suggested that HCV may disturb glucose metabolism at a very early stage and, thus, independently of the degree of fibrosis.

All of the above studies failed to completely rule out the possibility that the higher prevalence among HCV-infected persons may partly depend on the higher risk of exposure to HCV through invasive medical procedures undergone by diabetic patients. If this were true, then HCV could merely be considered an iatrogenic infection of patients with diabetes repeatedly exposed to blood-contaminated tools, hence *following* the diagnosis of diabetes. To dispel this potential bias, compelling evidence comes from longitudinal studies. In a community-based cohort of 1,084 persons, aged between 44 and 65, enrolled in the Atherosclerosis Risk in Communities (ARIC) study and free of diabetes at baseline, 548 developed diabetes during a follow-up of 9 years [55]. After categorization of participants as low-risk or high-risk for diabetes, based on their age and BMI, and considering only those at high risk of diabetes, persons with HCV infection at the start were more than 11 times as likely as those without HCV infection to develop diabetes during follow-up. Among those at low risk, no increased incidence of diabetes was detected among HCV-infected persons. The authors concluded that a preexisting HCV infection could increase the risk of type 2 diabetes in those with recognized diabetes risk factors. A similar synergistic effect of HCV with other risk factors was observed in a more recent study from Taiwan. Wang *et al.* [56] analyzed a community characterized by a high prevalence of HBV and HCV infections to assess the temporal relation between these infections and the occurrence of diabetes. This study demonstrated that HCV infection—including HBV/HCV co-infection, but not HBV infection—could increase the risk of incident diabetes. The risk of diabetes for HCV-infected persons increased among younger persons. Again, a synergistic effect on the risk of diabetes was found in overweight and obese patients infected with HCV. The authors went as far as to recommend regular diabetes screening among anti-HCV-positive people, starting at a young age, especially for those at high risk. Finally, additional epidemiological evidence comes from longitudinal studies carried out in transplant patients. In the liver-transplant setting, HCV infection is a risk factor for development of type 2 diabetes after transplantation [57-60], and a recent meta-analysis has shown that anti-HCV-positive renal-transplant recipients are also characterized by a marked increase of the risk of post-transplant diabetes [61]. This risk is clinically meaningful because the excess risk of death in HCV-positive renal-transplant recipients may be at least partially attributed to post-transplant diabetes and its complications [61].

Ascertaining glucose metabolism disturbances among HCV-infected population clearly depends not only on the sensitivity of the diagnostic tool, but also on the baseline epidemiology of the population under study. Recent reports show how conflicting the data may be. In Sweden, the prevalence of HCV is lower than elsewhere—estimated to be around 0.33% [62]. Sjöberg *et al.* [63] determined the HCV prevalence in a large cohort of patients with diabetes to assess if such an association could be found in a region with a low prevalence of HCV. In this cohort of diabetic patients (including both type 1 and type 2), the prevalence of HCV was comparable to that found in Swedish healthcare workers (0.68%). They concluded that, in a region with low HCV prevalence, hepatitis C has no etiological role in the development of diabetes, suggesting the involvement of other pathophysiological mechanisms. Another recent study from Japan, based on a different design, came to a similar conclusion. Imazeki *et al.* [64], in a cross-sectional study, investigated the prevalence of diabetes and insulin resistance in patients with chronic hepatitis C, and compared it with that in patients infected with HBV and those who cleared HCV after interferon treatment as controls. They found the prevalence of insulin resistance to be higher in patients infected with HCV than in those whose HCV had been cleared, but multivariable logistic-regression analysis did not identify HCV infection as an independent risk factor for insulin resistance after adjusting for age, BMI and transaminase levels. There were no differences in the prevalence of diabetes or insulin resistance between patients with genotypes 1 and 2 (genotype 3 is uncommon in Japan). They concluded that, in Japan, factors other than HCV, such as older age, male gender, increased BMI and presence of cirrhosis, might be important risk factors for the development of glucose abnormalities in chronic hepatitis C.

In an attempt to definitively clarify this issue, an important meta-analysis was performed and recently published [65], the first such study to specifically address the association between HCV infection and risk of diabetes in the general population. The significant excess risk observed in the meta-analysis of prospective studies (adjusted hazard ratio = 1.67) was highly consistent with the significant excess risk observed in the meta-analysis of retrospective studies (adjusted odds ratio = 1.70), adding further support to the retrospective data. Similarly, the overall unadjusted pooled estimator demonstrated a significant twofold excess risk. Taken together, the findings of this meta-analysis clearly indicate that chronic hepatitis C is associated with a modest, but significant, increase in the risk of developing type 2 diabetes in comparison to uninfected controls [65].

Data suggesting a relationship between the severity of insulin resistance and HCV replicative levels are inconclusive. Recent work seems to suggest that this is the case [66,67], but it is still not clear whether HCV replication in these patients directly increases insulin resistance or whether hyperinsulinemia stimulates viral replication, as suggested by previous *in vitro* data [68]. The poor correlation may be due to the fact that the overall score of insulin resistance largely depends on contributions from adipose tissue and muscle, two extrahepatic compartments not affected by HCV.

If HCV is increasing the level of insulin resistance or predisposes to the development of type 2 diabetes in high-risk individuals, then curing hepatitis C should result in an improvement in HOMA score and a lower incidence of glucose metabolism dysfunction in the post-treatment follow-up. Romero-Gómez *et al.* [69] assessed the effects of sustained virological response, together with host and viral factors, on the incidence of impaired fasting glucose and/or type 2 diabetes in 1,059 patients with chronic hepatitis C treated with a combination of pegylated interferon alpha plus ribavirin. Their results showed that the eradication of HCV reduced by half the incidence of type 2 diabetes and/or impaired fasting glucose in the course of post-treatment follow-up. Abnormal glucose values were detected more often in chronic hepatitis C, in older patients, those with steatosis and those who were overweight. Similarly, Kawaguchi *et al.* [70], in their study on 89 patients who underwent repeated liver biopsy before and after therapy, demonstrated that clearance of HCV improved the HOMA score and the intrahepatic expression of insulin receptor substrates (IRS) 1 and 2, two hepatocellular transducers of the insulin signal. Both studies seemed to indicate that HCV itself is involved in the development of insulin resistance. Conversely, in another study, Giordanino *et al.* [71] evaluated and followed-up 202 patients with chronic hepatitis C treated with antiviral therapy. They concluded that the cumulative incidence of both impaired glucose tolerance and diabetes in chronic hepatitis C patients who maintain a long-term clearance of the virus is better predicted by baseline-recognized risk factors of diabetes than by HCV eradication. In fact, there was no significant difference between non-responders and long-term responders regarding the incidence of diabetes. The baseline features predicting diabetes, such as older age, BMI and family history of diabetes, maintain their critical role even in sustained virological responders. It is possible that HCV eradication is beneficial in the short term and that, as follow-up proceeds, major risk factors of diabetes take over.

5. HCV interference with insulin signaling

Experimental data suggest a direct interference of HCV with the insulin cascade. This was first shown by a study where liver specimens obtained from 42 non-obese and non-diabetic HCV-infected subjects and 10 non-HCV-infected subjects matched for age and BMI were exposed *ex vivo* to insulin, and examined for the contents and phosphorylation/activation status of insulin-signaling molecules [72]. Insulin-stimu-

lated IRS-1 tyrosine phosphorylation was decreased twofold in HCV-infected patients compared with non-HCV-infected subjects, and this was accompanied by significant reductions in IRS-1/p85 phosphatidylinositol-3-kinase association, IRS-1-associated PI3-kinase enzymatic activity and insulin-stimulated Akt phosphorylation [72]. Thus, in patients with chronic hepatitis C, direct interactions between viral products and insulin-signaling components occur that may contribute to insulin resistance, thereby leading to the development of type 2 diabetes in high-risk individuals, as already stated above. However, the nature of such molecular interaction(s) is still under debate. In the transgenic mouse model [73], the core-encoding region of HCV is sufficient to induce insulin resistance. This effect is annulled by treatment with anti-TNF-α antibodies, suggesting an increased level of serine phosphorylation of IRS-1 induced by TNF-α. The effect of the HCV core protein has been also tested *in vitro*, where an increased proteasomal degradation of IRS-1 and -2 *via* activation of the suppressor of cytokine signaling (SOCS)-3 was observed [74]. However, *in vitro* data are diverse, and the mechanisms may be variable, depending on the system and/or the viral genotype tested. Increased endoplasmic reticulum (ER) stress has been reported that may render the cell insulin-resistant [23]. Work from Pazienza *et al.* [75] showed downregulation of peroxisome proliferator-activated receptor γ (PPARγ) and upregulation of SOCS-7 in cells transfected with the core protein of genotype 3, whereas the core protein of genotype 1b activated the mammalian target of rapamycin (mTOR), findings that were confirmed using agonists for PPARγ (rosiglitazone) or short interfering RNA for SOCS-7 [75]. Another study has identified the overexpression of PP2A in cells expressing HCV and in the liver of chronic hepatitis C patients as a factor contributing to the pathogenesis of insulin resistance associated with HCV [76]. Recently, the role of the HCV-induced activation of the c-Jun N-terminal kinase (JNK) has been emphasized: the HCV core protein-mediated Ser(312) phosphorylation of IRS-1 was inhibited by a JNK inhibitor in an *in vitro* infection assay using cell-culture-grown HCV genotypes 1 and 2 [77].

The role of oxidative stress is suggested by results obtained in chronic hepatitis C patients. Mitsuyoshi *et al.* [78] evaluated 203 histologically confirmed chronic hepatitis C patients with HCV genotype 1 or 2 infection. HOMA and serum levels of thioredoxin (Trx), a marker of oxidative stress, were found to be significantly correlated with each other, even after adjustment for BMI. Further studies are, however, necessary to clarify the role of oxidative stress in the pathogenesis of insulin resistance in the liver of chronic hepatitis C patients. An additional indirect viral effect, mediated by increased levels of TNF-α as suggested by the transgenic mouse model, has been corroborated by some human studies in which an exaggerated intrahepatic TNF-α response, resulting in insulin resistance and a higher risk of developing diabetes, has been reported [79,80].

6. Clinical consequences of insulin resistance in hepatitis C

There are two major clinical consequences of the insulin-resistant state associated with hepatitis C, independent of its pathogenesis: the accelerated progression of liver fibrosis; and the reduced response to therapy. We have already mentioned the role of non-virus-induced steatosis as an independent predictor of fibrosis [47]. The mechanisms by which non-viral steatosis can promote liver fibrosis range from oxidative stress to proinflammatory cytokines, insulin resistance and increased susceptibility to apoptosis.

The association between HCV and oxidative stress has been reported in transgenic mice [81,82]. In the presence of steatosis, oxidative stress is increased in HCV infection and may promote fibrogenesis, similar to the so-called 'second strike' proposed in the pathogenesis of non-alcoholic steatohepatitis. Proinflammatory cytokines may also mediate fibrogenesis in patients with steatosis, although it is unclear how steatosis can promote and/or amplify this process. Our multicenter meta-analysis [48] using individual data from 3,068 patients with chronic hepatitis C showed that steatosis is associated with both increased liver inflammatory activity and fibrosis.

Insulin resistance is associated with liver fibrosis and, when the multivariable model includes both steatosis and insulin resistance, only the latter is found to be an independent predictor of fibrosis stage [49]. These observations have been largely confirmed [83-85], although the molecular mechanisms leading from the insulin-resistant state to accelerated fibrogenesis are unclear. In non-alcoholic steatohepatitis, hyperglycemia/hyperinsulinemia may be directly stimulating hepatic stellate cells to produce connective tissue growth factor leading, in turn, to increased collagen fiber deposition [86]. Interestingly, weight reduction and increased physical activity in patients with chronic hepatitis C and steatosis were sufficient, in the short term, to reduce both liver fibrosis score and hepatic stellate cell activation [87], although these data await independent confirmation. Finally, increased liver cell apoptosis has been reported to correlate with steatosis [88], as reflected by the elevated caspase activity in serum [89]. In the presence of steatosis, increased apoptosis was associated with activation of stellate cells and a higher stage of fibrosis, which is in agreement with the hypothesis that a steatotic liver is more vulnerable to liver injury and suggesting another mechanism of accelerated liver disease progression in the presence of steatosis [88].

Steatosis decreases the response to interferon alpha-based therapy in chronic hepatitis C [14,90,91]. As in the case of accelerated fibrogenesis, this association seems to be limited

to patients with metabolic steatosis, suggesting that the mechanism of reduced response to treatment may be again mediated by insulin resistance. This was confirmed by studies in patients with genotype 1 [92] or genotypes 2 and 3 [93], where the sustained virological response rate was inversely correlated with the HOMA score before therapy. Indirect evidence in favor of this negative association comes also from the reduced response to treatment reported among African Americans, very likely due to a high rate of visceral obesity and insulin resistance [94], and the correlation between high levels of circulating TNF-α, typically observed in insulin resistance, and reduced response to interferon alpha therapy [95]. The molecular link between insulin resistance and resistance to interferon alpha seems to be represented by the increased levels of SOCS-3 in liver [96]. SOCS-3 is not only promoting the proteasomal degradation of IRS-1, leading to impaired insulin signaling [74] but is also, together with other members of the SOCS family, a negative regulator in the transduction of the interferon alpha signaling [97]. Thus, HCV may activate some members of the SOCS family as a mechanism to inhibit interferon alpha signaling while simultaneously impairing insulin signaling. Whether this mechanism can be exploited pharmacologically, with drugs aimed at reducing insulin resistance while improving the responsiveness to interferon alpha, remains to be fully explored. Although preliminary data from a pilot study [98] have been disappointing, further schedules should be evaluated. For the time being, the only clinical-management measure that can be reasonably proposed for patients with chronic hepatitis C and an insulin-resistant state associated with the metabolic syndrome involves the lifestyle changes that are commonly recommended for all patients with an increased cardiovascular risk. Given the impact that diabetes has not only on liver fibrosis progression, but also on the development of hepatocellular carcinoma [99], more targeted and effective drugs are eagerly awaited.

Conflicts of interest: The authors have none to declare.

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