Marqueurs diagnostiques et pronostiques de la stéatohépatite métabolique

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Listes des abréviations

AUDC	Acide Ursodésoxycholique
CHC	Carcinome Hépatocellulaire
F	Fibrose
FTIR	Microspectroscopie à Transformée de Fourier en Infrarouge
GGT	Gamma Glutamyl Transférase
HR	Hazard Ratio
IC	Intervalle de Confiance
IMC	Indice de Masse Corporelle
IR	Insulino-Résistance
LCFA	Acide Gras à Longue Chaine
LSN	Limite Supérieure à la Normale
NAFL	Non Alcoholic Fatty Liver
NAFLD	Non Alcoholic Fatty Liver Disease
NAS	NAFLD Activity Score
NASH	Non Alcoholic Steato-Hepatitis
OR	Odd Ratio
PPAR	Récepteur Activé par les Proliférateurs des Peroxysomes
SAF	Score de Stéatose, d'Activité et de Fibrose
TH	Transplantation Hépatique
VLCFA	Acides Gras à Très Longue Chaîne

Liste de publications en relation avec la thèse

Articles principaux de la thèse

Article # 1. Article original, publié en Avril 2017

Metabolism dysregulation induces a specific lipid signature of nonalcoholic steatohepatitis in patients

Chiappini F, Coilly A, Kadar H, Gual P, Tran A, Desterke C, Samuel D, Duclos-Vallée JC, Touboul D, Bertrand-Michel J, Brunelle A, Guettier C, Le Naour F. Sci Rep. 2017 Apr 24;7:46658.

Article # 2. Article original, publié en décembre 2019

FABP4 and MMP9 identified as predictive factors of poor prognosis in patients with nonalcoholic fatty liver using data mining approaches and gene expression analysis

Coilly A, Desterke C, Guettier C, Samuel D, Chiappini F. Sci Rep. 2019 Dec 24;9(1):19785.

Article # 3. Article original, soumis

Quantitative assessment of triglycerides by Fourier Transform InfraRed (FTIR) spectroscopy of donor liver helps predicting outcome after liver transplantation

Coilly A, Desterke C, Kaščáková C, Chiappini F, Bertrand Michel J, Peng C, Samuel D, Vibert E, Le Naour F.

Article en annexe

Article # 4. Revue, publié en Mars 2016

Recent Insights into Treatment of Non-Alcoholic Steatohepatitis

Coilly A, Chiappini F. Journal of Endocrinology and Diabetes. 3. 01-11. 10.15226/2374-6890/3/1/00142.

Article # 5. Consensus international, publié en Janvier 2019

International Liver Transplantation Consensus Statement on End-stage Liver Disease Due to Nonalcoholic Steatohepatitis and Liver Transplantation.

Tsochatzis E, Coilly A, Nadalin S, Levistky J, Tokat Y, Ghobrial M, Klinck J, Berenguer M. Transplantation. 2019 Jan;103(1):45-56.

1. Introduction

Au cours des deux dernières décennies, la stéatose hépatique non alcoolique (ou NAFLD pour non alcoholic fatty liver disease, en anglais) est passée d'une maladie relativement méconnue à une des causes les plus fréquentes de maladie hépatique dans le monde. En fait, on estime que 25% de la population mondiale est touchée actuellement par la NAFLD. La stéatohépatite non alcoolique ou métabolique (dite NASH en anglais pour non alcoholic steato-hepatitis) est un des sous-groupes de la NAFLD qui peut évoluer vers la cirrhose, le carcinome hépatocellulaire (CHC) et le décès. La NAFLD et la NASH se retrouvent non seulement chez les adultes, mais également chez les enfants et les adolescents. En raison de l'association étroite entre la NAFLD, le diabète de type 2 et l'obésité, les derniers modèles prévoient une augmentation de la prévalence de la NAFLD et de la NASH, ce qui entraînera une charge clinique et économique considérable dans les prochaines décennies.

1.1 Définitions et données épidémiologiques

1.1.1 Définitions

Décrite pour la première en 1980, la stéatose non alcoolique du foie est une entité reproduisant les lésions histologiques de l'hépatite alcoolique, pouvant évoluer vers la cirrhose mais sans consommation d'alcool excessive (1). Dans la publication princeps, les auteurs observaient que cette maladie concernait majoritairement les femmes et s'associait souvent avec un surpoids et un diabète de type 2.

Depuis, la définition de la NAFLD a évolué et regroupe plusieurs entités dont la stéatose simple (dite NAFL en anglais pour non alcoholic fatty liver) et la stéato-hépatite métabolique ou NASH.

La stéatose simple est classiquement définie par la présence de vésicules chargées en triglycérides dans le cytoplasme des hépatocytes et affectant plus de 5% d'entre eux. Là encore, il existe 2 types de stéatose selon la taille des vésicules ou vacuoles lipidiques:

- la stéatose macrovacuolaire lorsque les vacuoles ont une taille supérieure à celle du noyau cellulaire et il est déplacé vers la périphérie cellulaire ;
- la stéatose microvésiculaire : les vacuoles ont une taille inférieure à celle du noyau cellulaire et ne le déplacent pas en périphérie.

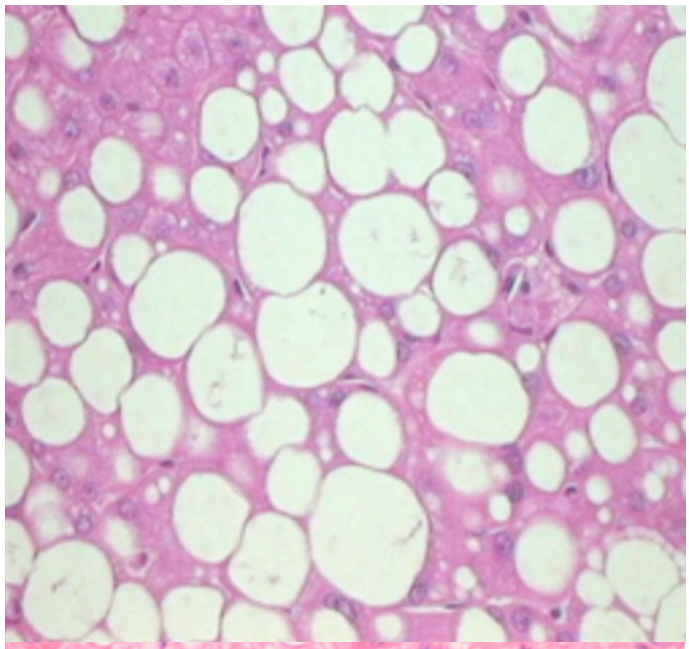
La définition historique de la NASH est histologique et comprend une stéatose touchant plus de 5% des hépatocytes associée à des lésions d'inflammation lobulaire ou de ballonisation hépatocytaire avec ou sans fibrose (Figure 1) (2). Plusieurs scores ont été développés, évalués puis publiés pour tenter de standardiser la définition qui repose sur la lecture de l'anatomopathologiste. Quelques décennies plus tard, la définition fait encore débat bien que le score NAS soit retenu par les recommandations actuelles comme ayant les performances les meilleures (3-5). Le score NAS attribue des notes à chaque lésion: la stéatose (de 0 à 3), l'inflammation lobulaire (de 0 à 3), et la ballonisation (de 0 à 2). La note globale trouvée en additionnant ces trois notes peut donc aller d'un minimum de 0 à un maximum de 8. La NASH est définie selon un score NAS ≥ 5.

Les sociétés savantes européenne et américaine ont complété la définition de la NAFLD comme diagnostic d'exclusion (5, 6). En effet, il faut en premier lieu exclure tout autre cause de maladie chronique du foie, au premier rang desquelles la maladie alcoolique. La consommation excessive d'alcool est définie par un seuil de 20 grammes par jour pour les femmes et 30 grammes par jour pour les hommes car au-delà de ces seuil, la présence d'une stéatose est jugée significative (7). Cependant, il n'est pas exceptionnel en pratique médicale courante, d'observer plusieurs facteurs étiologiques concomitants qui favorisent l'évolution de la maladie hépatique. Quelques publications vont dans ce sens et suggèrent un rôle synergique de la NAFLD associée à une autre maladie hépatique comme les hépatites virales, par exemple (8-10).

Figure 1

Principales lésions histologiques permettant de définir la NAFLD. Stéatose simple sans lésions de nécroinflammation (A, Hematoxyline-eosine).
Steatohépatite métabolique (NASH) : stéatose >5% avec lésions d'inflammation lobulaire et de ballonisation hepatocytaire (B, Hematoxyline-eosine)

A



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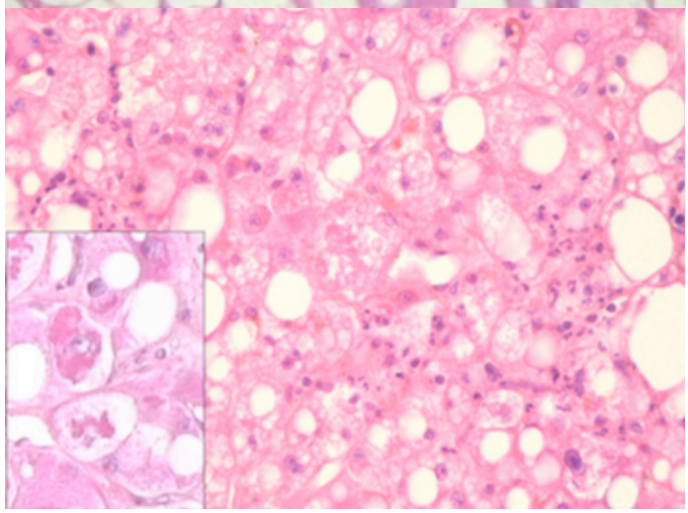


1.1.2 Données épidémiologiques

La prévalence de la NAFLD peut beaucoup varier en fonction des régions du monde, mais aussi de la façon dont les études sont menées (population générale ou population cible, méthodes diagnostiques utilisées, etc…). La prévalence de la NAFLD, par exemple,

semble augmenter avec l'âge. Chez les septuagénaires, la prévalence est d'environ 40% (11).

En Europe, la prévalence de la NAFLD est estimée à 20-30% dont 3% de NASH (5, 12). Les taux de prévalence de la NAFLD varient selon les pays européens. En Allemagne, une étude de population a montré que la prévalence de la stéatose, découverte à l'échographie, était de 29,9%, mais seuls 15,9% de ces patients avaient de façon concomitante des anomalies du bilan biologique hépatique (13). Des taux similaires ont été décrits dans la population générale du nord de l'Italie avec 25% en Espagne (25,8%) et au Royaume-Uni (26,4%) (4). En France, il n'existe pas de données actuellement publiées mais une étude dite « constance » menée chez 118 664 sujets à visée de dépistage. Les premiers résultats communiqués semblent indiquer une prévalence de la NAFLD moindre en France qu'escomptée, de 16% dont 2,6% de NASH ayant une fibrose extensive sur les données de marqueurs non-invasifs (Serfaty, et al. CO, AFEF 2018).

En Amérique du Nord, la prévalence de la NAFLD dans la population générale est estimée à environ 24% (14). En revanche, en Amérique du Sud, la prévalence est plus importante de 32% mais une grande variabilité est observée entre les pays, la prévalence la plus élevée étant à Belize où 35% de la population est obèse (12).

Des résultats similaires sont observés dans toutes les régions du monde, y compris les pays émergents d'Asie et d'Afrique.

Si les études de prévalence convergent pour qu'un quart de la planète soit atteint de NAFLD, très peu d'études ont été rapportées concernant l'incidence de la NAFLD en Europe et aux Etats-Unis. Il semblerait qu'elle varie de 28,01 pour 1000 personnes-année à 52,34 pour 1000 personnes-année (4). Cependant, les facteurs associés à la NAFLD, obésité et diabète, eux augmentent au cours du temps. En outre, un nombre croissant de composants du syndrome métabolique semble augmenter le risque de progression vers la NASH (15, 16). On peut alors s'attendre à une augmentation du nombre d'individus ayant une NAFLD durant la prochaine décennie. C'est également ce que montre une étude récente de modélisation selon un modèle de Markov (17). Le modèle a été utilisé pour estimer la progression de la NAFLD et de la NASH dans huit pays (Chine, France, Allemagne, Italie, Japon, Espagne, Royaume-Uni et Etats-Unis) sur la base de données sur la prévalence de l'obésité chez les adultes et du diabète de type 2. Les auteurs montrent que même si l'obésité et la prévalence du diabète de type 2 diminuent à l'avenir, on devrait observer une croissance modeste du nombre total de

NAFLD (0-30%) entre 2016 et 2030, avec la plus forte croissance en Chine due à l'urbanisation et la plus faible croissance au Japon, résultat d'une diminution de la population. Cependant, dans le même temps, la prévalence de la NASH augmentera de 15 à 56%, tandis que la mortalité hépatique et l'hépatopathie avancée feront plus que doubler en raison du vieillissement de la population.

1.1.3 Cadre nosologique

Il est devenu de plus en plus clair que la NAFLD est la manifestation hépatique du syndrome métabolique et est très répandue chez les sujets obèses, diabétiques et/ou dyslipidémiques. Dans une étude de registre américaine, la prévalence du syndrome métabolique parmi les patients ayant une NAFLD était de 79% (16).

Le syndrome dit métabolique englobe plusieurs pathologies, corrélées les unes aux autres et appartiennent au cadre nosologique de l'insulinorésistance (IR).

1.1.3.1 NAFLD et obésité

Les études longitudinales de chirurgie bariatrique montre que la très grande majorité des obèses ont une NAFLD. Si la prévalence de la NAFLD est de 90% parmi les obèses, en revanche, un peu moins d'un sujet sur deux aurait une NASH (18). Dans une récente étude française ayant inclus 1489 patients ayant eu une biopsie hépatique lors de la chirurgie bariatrique, la prévalence de la NASH semble plus faible de 11,9% seulement (19). Tous les sujets obèses n'ont donc pas de NAFLD, encore moins de NASH. Ils sont fréquemment dénommés « healthy obese » en langue anglaise. Il semble qu'ils soient également moins prédisposés à développer d'autres complications du syndrome métabolique comme le diabète, les maladies cardiovasculaires, replaçant l'IR au cœur de la pathogénèse de la NAFLD (20).

A contrario, dans une méta-analyse récente, les estimations de la prévalence globale de l'obésité chez les patients ayant une NAFLD et chez les patients ayant une NASH étaient respectivement de 51,34% (IC 95%: 41,38-61,20) et 81,83% (IC 95%: 55,16-94,28) (12).

1.1.3.2 NAFLD et diabète de type 2

De nombreuses cohortes de patients diabétiques de type 2 sont disponibles. Dans ces études, la prévalence de la NAFLD varie de 40 à 70% (21). L'ancienneté du diabète et

l'importance de l'IR semblent être deux facteurs corrélés à la présence d'une NAFLD. Dans une étude de cohorte ayant inclus 270 patients ayant une NAFLD sans fibrose avancée, suivis pendant une durée moyenne de 4,4 ans, les facteurs associés à la progression de la fibrose de 16% étaient le diabète de type 2, le syndrome métabolique et l'index HOMA qui évalue l'IR (22).

De nouveau, *a contrario*, les estimations globales de la prévalence du diabète chez les patients ayant une NAFLD et chez les patients ayant une NASH étaient respectivement de 22,51% (IC à 95%: 17,92 à 27,89) et de 43,63% (IC à 95%: 30,28 à 57,98, dans la méta-analyse de Younossi *et al*. (12).

1.1.3.3 NAFLD et autres entités du syndrome métabolique

La présence d'une NAFLD est retrouvée chez 50% des sujets dyslipidémiques, 45% des hypertendus et 42% des femmes souffrant du syndrome des ovaires polykystiques. Cependant, les études sont réalisées à partir de données échographiques, montrant une stéatose et ne reposent pas sur des données histologiques.

Dans la méta-analyse de Younossi *et al*., les estimations de la prévalence globale de la dyslipidémie et de l'hypertension artérielle chez les patients ayant une NAFLD et chez les patients ayant une NASH étaient respectivement de 69,16% (IC 95%: 49,91-83,46) et 72,13% (IC 95%: 54,59-84,78) et 39,34% (IC 95%: 33,15-45,88) et 67,97% (IC 95%: 56,31-77,74) (12).

Enfin, le syndrome d'apnée de sommeil émerge également comme facteur de risque associé avec la sévérité des lésions hépatiques dans la NAFLD (23-25).

1.1.4 Morbi-mortalité

Comme pour toutes maladies hépatiques, le pronostic de la NAFLD, et plus particulièrement de la NASH repose sur la présence d'une fibrose hépatique, sa progression vers la cirrhose et ses complications, mais aussi de l'apparition d'un CHC. Il est souvent difficile d'apprécier le pronostic de la maladie en tant que telle car les patients ayant une NAFLD ont d'autres comorbidités (Cf. 1.1.3) qui les prédisposent à des maladies extra-hépatiques, pouvant être mortelles comme les cancers ou les maladies cardio-vasculaires. Dans une étude longitudinale suédoise de 256 patients, le pourcentage de décès de maladie hépatique était de 14,5 à 10 ans et de 44% à 21 ans (26). Cependant, la mortalité globale à 10 ans est augmentée de 55% chez les patients

ayant une NALFD par rapport à la population générale après appariement selon l'âge et le sexe (27).

1.1.4.1 NAFLD et progression vers la cirrhose

L'histoire naturelle de la NAFLD a été décrite à partir d'études transversales menées en population générale reposant le plus souvent sur des marqueurs non invasifs de fibrose ou d'études avec biopsies hépatiques séquentielles.

Dans les séries rétrospectives issues de centres spécialisés, une fibrose hépatique extensive est retrouvée dans environ un tiers des cas au moment du diagnostic dont 10 à 20% de cirrhoses constituées (28).

Il est en revanche, difficile d'estimer réellement la prévalence de la cirrhose NASH. En effet, au stade de cirrhose, les lésions permettant d'établir le diagnostic de NASH sont souvent absentes du fait du remaniement architectural. Aussi, beaucoup de cirrhose NASH sont classées comme cryptogéniques ou indéterminées. La présence d'un syndrome métabolique permet parfois de rectifier le diagnostic (29, 30). En effet, la stéatose diminue avec la progression de la fibrose.

La NAFLD est une cause fréquente de cirrhose cryptogénétique retrouvée dans 30 à 75% des cas (30, 31).

Plusieurs études ont mis en évidence des facteurs associés à la progression de la fibrose et à l'apparition de la cirrhose. L'âge et un diabète de type 2 sont les plus fréquents facteurs associés à la progression de la fibrose (32). Le diabète de type 2 est un facteur de risque indépendant associé avec la sévérité de la fibrose (33). D'autre part, la cirrhose est diagnostiquée à un âge plus tardif, peut–être dû au retard diagnostic (34). L'obésité est un facteur de risque indépendant connu pour la progression de la fibrose et le développement de la cirrhose. Elle est présente chez 55% des patients présentant une cirrhose cryptogénétique. Le risque relatif de développer une cirrhose augmente de 28% pour une augmentation de 5 unités de l'indice de masse corporelle (35).

D'autres facteurs sont associés à des formes plus sévères de NAFLD comme des facteurs génétiques, la composition du microbiote ou le syndrome d'apnée de sommeil. Certaines prédispositions génétiques sont maintenant établies, bien que non utilisées en pratique clinique. Les variants génétiques de la PNPLA3 (pour patatin-like phospholipase domain-containing 3 protein), également appelée adiponutrine, peuvent aider à prédire le risque de progression de la maladie. Une méta-analyse récente a confirmé le lien du variant I148M pour la progression de fibrose et développement du CHC (36). D'autres

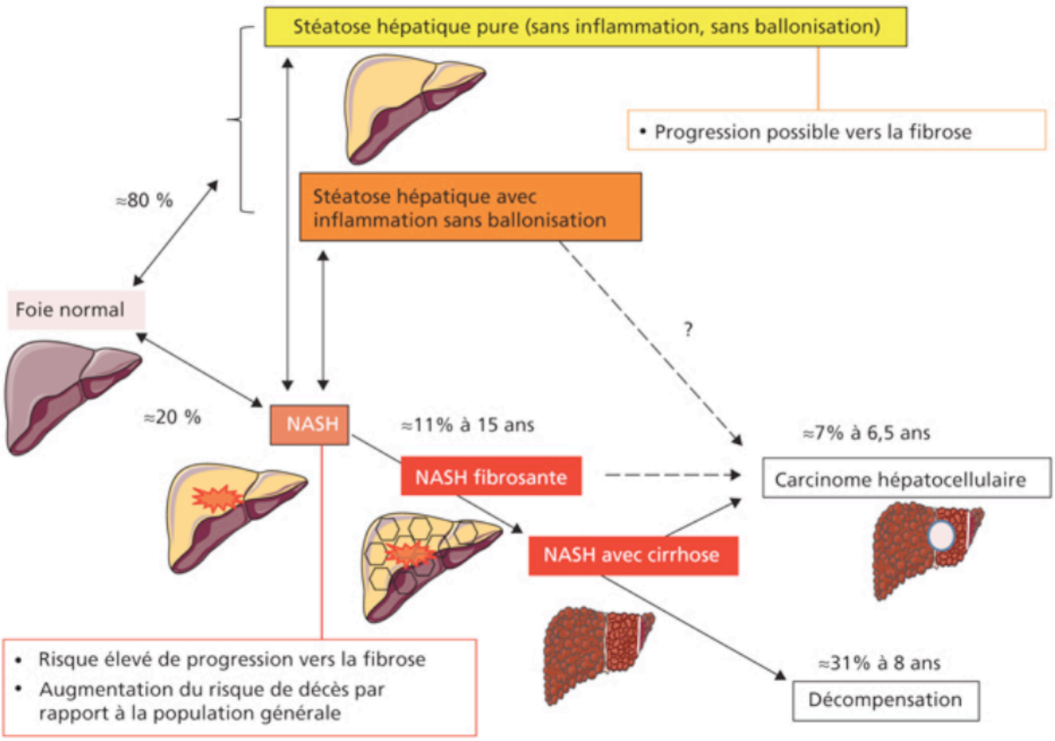
mutations sont identifiées et impliquées dans la pathogénèse comme TM6SF2, MBOAT7 et GCKR, régulant le remodelage et la sécrétion de triglycérides et de phospholipides dans les gouttelettes lipidiques intracellulaires et la lipogenèse *de novo*.

Plus surprenant, plusieurs études ont montré que la stéatose simple n'était pas une maladie bénigne et qu'il était possible d'évoluer vers une fibrose extensive voire une cirrhose (37). Une analyse systématique de la littérature composée de 11 études de cohorte incluant 411 patients avec NAFLD prouvée par biopsie (150 avec stéatose simple et 261 avec NASH) a contribué à contredire ce dogme. Le taux de progression annuel de la fibrose chez les patients atteints de NAFL qui présentaient au début une fibrose au stade 0 était de 0,07 stades (IC à 95%, 0,02 à 0,11 stades), par rapport à 0,14 chez les patients atteints de NASH (IC à 95%, 0,07 à 0,21 stades). Ces résultats correspondent à un stade de progression sur 14,3 ans chez les patients atteints de stéatose simple (IC à 95%, 9,1 à 50,0 ans) et à 7,1 ans chez les patients atteints de NASH (IC à 95%, 4,8 à 14,3 ans) (38).

1.1.4.2 NAFLD et cirrhose constituée

La cirrhose décompensée est une cause importante de décès des patients ayant une NAFLD (Figure 2). Entre 8% et 25% des patients ayant une NASH évoluent vers la cirrhose (39). L'obésité est associée à un risque 4 fois plus élevé de décompensation de cirrhose et d'hospitalisation (22). Le risque de décompensation de la cirrhose est de 15% chez les sujets avec un poids normal contre 30% chez les patients en surpoids et 43% chez les patients obèses (40).

Figure 2 : Histoire naturelle de la NAFLD et risque de morbi-mortalité



En cas de cirrhose compensée (Child-Pugh A), le pronostic des patients ayant une NAFLD isolée semble meilleur en termes de survie ou de risque de décompensation, en comparaison aux autres causes connues de cirrhose (41). En revanche, en cas de cirrhose décompensée, le risque de rupture de varices œsophagiennes, d'ascite, d'encéphalopathie hépatique et de décès, est similaire en comparaison aux patients ayant une cirrhose virale C dans une étude américaine (41). La première cause de décès dans les deux groupes était le risque hépatique mais le décès de causes extra-hépatiques, de maladies cardiovasculaires était significativement supérieur dans le groupe NAFLD.

Ces résultats n'ont cependant pas été reproduits dans une étude internationale multicentrique, de 247 patients avec NAFLD ayant une fibrose avancée (F3 ou cirrhose compensée) comparés à 264 patients ayant une cirrhose virale C. Alors que le risque de décompensation était plus élevé chez les patients ayant une hépatite C, le risque de mortalité globale était similaire dans les deux groupes (42).

En effet, les patients ayant une NAFLD parvenue au stade de fibrose sont plus à risque de décéder d'une complication ou hépatique mais également cardiovasculaire en comparaison à la population générale. Dans une étude américaine de 2015, 229 patients

ont été comparés à la population générale sur une durée moyenne de 26 ans (43). Les patients ayant une NAFLD avaient une mortalité accrue par rapport à la population de référence (Hazard Ratio (HR) 1,29, *p*=0,020), avec un risque accru de maladie cardiovasculaire, CHC, maladie infectieuse et cirrhose. La mortalité globale n'augmentait pas chez les patients ayant un score NAS entre 5 et 8 et un stade de fibrose 0-2, alors que les patients présentant une fibrose de stade 3-4, indépendamment du NAS, avaient une mortalité accrue (HR 3,3, IC 2,27-4,76, p <0,001).

Une autre étude a des résultats similaires, montrant une association entre fibrose et mortalité globale et spécifique chez 619 patients suivis pendant 12 ans (44).

1.1.4.3 NAFLD et CHC

Le cancer primitif du foie est l'autre grande cause de décès spécifique hépatique (Figure 3). Si les données épidémiologiques en termes de prévalence et de risque de CHC sont robustes concernant les hépatites virales ou même en cas de maladie alcoolique du foie, les études robustes dans la NAFLD manquent. Quelques études longitudinales ont exploré la prévalence du CHC dans la NALFD, rapportant une prévalence variant de 0% à 3% sur une période de suivi comprise entre 5,6 et 21 ans (45). Lorsqu'une cirrhose NASH est diagnostiquée, 10% à 25% des patients sont à risque de développer CHC. Compte tenu de la prévalence actuelle de la NAFLD dans la population générale, il résulte qu'environ 200 000 à 500 000 individus sont potentiellement à risque de développer un CHC dû à la NASH (46).

Ce risque augmente si on s'intéresse à la population présentant un excès pondéral voire une obésité. Il est maintenant établi que l'obésité augmente le risque de développer un cancer, quelque soit le site mais le CHC est le cancer le plus corrélé à l'obésité. Dans une large étude prospective américaine conduite chez plus de 900 000 participants suivis durant 16 ans, le risque relatif de décès lié au CHC chez les personnes obèses (IMC > 35 kg/m^2) était de 4,52 chez les hommes et 1.68 chez les femmes (47). Dans une étude européenne, le risque de CHC était 3,5 fois plus élevé chez les sujets obèses avec une augmentation supplémentaire du risque chez les sujets obèses et diabétiques (Odd Ratio (OR) = 11,8) (48). Ces résultats sont similaires à ceux rapportés de plusieurs cohortes longitudinales, coréenne, nordique, italienne, ayant inclus plusieurs dizaines de milliers de sujets (49-53).

Dans une analyse de la base de données MEDICARE aux Etats Unis, le risque relatif de CHC dû à l'obésité et au diabète de type 2 était de 2,5 alors que ce risque était de 39,9

pour l'hépatite C (54). Cependant dans cette même étude, le diabète de type 2 et l'obésité étaient responsables de plus de cas de CHC que l'hépatite C.

Dans deux méta-analyses, le risque de CHC était augmenté de 17% chez les patients en surpoids, de 90% chez les obèses et 28% des CHC étaient attribuables à un excès pondéral (55, 56). La présence d'une autre maladie hépatique qu'elle soit alcoolique ou virale synergise le risque de développer une CHC. Dans une étude taiwanaise, les patients ayant une hépatite virale B ou C et diabétiques ou obèses avaient un risque 100 fois plus élevé de développer un CHC (57). Une analyse rétrospective du registre des anciens combattants américains a révélé que le diabète augmentait le risque de cancer primitif du foie uniquement en présence d'autres facteurs de risque tels que l'hépatite C ou B ou la cirrhose alcoolique (58).

Parmi les facteurs de risque de développer un CHC, plusieurs études soulignent le risque ajouté d'une consommation d'alcool excessive (59-61).

Egalement, le temps d'exposition aux facteurs de risque métabolique semble avoir une influence sur le risque de développer un CHC. Dans une étude cas-témoin, le risque de développer un CHC à l'âge adulte était 2 fois plus élevé chez les patients qui avaient des antécédents d'obésité à 20 ans (62).

Comme pour l'obésité, le diabète de type 2 est associé avec une augmentation du risque de cancer en général et de CHC (53, 63). Une étude de population publiée en 1996 a suivi 153 852 sujets et a révélé sur-risque de développer un CHC chez les patients diabétiques de 4,1 (64). Le risque de CHC semble être corrélé avec la durée et le contrôle du diabète.

Dans une étude américaine, le risque relatif de CHC était significativement plus élevé, multiplié par 2, chez les diabétiques dont la durée de la maladie était de plus de 10 ans (65). Plusieurs études ont montré une association entre la glycémie à jeun ou l'insulinémie à jeun et le risque de CHC (66, 67). Dans une étude autrichienne, le risque était d'autant plus élevé que la glycémie à jeun l'était (67). Enfin, le risque de décès dû au CHC est 2 fois plus élevé chez les patients ayant une NASH et diabétiques (68).

De façon récente, plusieurs études rapportent un risque de développer un CHC sur foie non cirrhotique, voire ayant seulement une stéatose simple. La prévalence du CHC varie entre 0% et 0.5% chez les patients ayant une stéatose et 12.8% chez les patients ayant une NASH (69-71). Mais la proportion des CHC développés en l'absence de la cirrhose varie entre 23% et 55% en Europe, aux Etats Unis et au Japon (72, 73). Une analyse anatomopathologique des tumeurs développées sur un foie non-cirrhotique révèle que

ces tumeurs sont de plus grande taille, sont mieux différenciées et moins souvent encapsulées que les tumeurs survenant sur un foie de cirrhose. Une hypothèse est la transformation d'adénomes, dont certains sont associés au syndrome métabolique, en CHC (74).

1.1.4.4 NAFLD et décès de causes non-hépatiques

Les maladies cardiovasculaires et le cancer extra-hépatique sont les premières causes de décès des patients ayant une NAFLD, plaçant finalement la mortalité hépatique au 3e rang (27). En effet, le syndrome métabolique est le facteur de risque conjoint de la NAFLD et des maladies cardiovasculaires.

Plusieurs études, majoritairement rétrospectives, retrouvent une mortalité de l'ordre de 20 à 30% de maladie cardiovasculaire dans la NAFLD (75-80). Certaines suggèrent même un sur-risque de mortalité par maladie cardiovasculaire en cas de NAFLD après ajustement sur les facteurs de risque cardiovasculaires dont le syndrome métabolique (76). Une méta-analyse incluant 10 études réalisées en population générale montre que l'augmentation de la GGT (pour Gamma Glutamyl Transférase) est significativement associée à la survenue de décès par maladie cardiovasculaire, 20% évènements coronariens et 54% d'accidents vasculaires cérébraux (81).

En résumé, la NAFLD est une maladie fréquente, qui concerne un quart des patients dans le monde. Sa prévalence va encore augmenter car elle est liée à notre mode de vie sédentaire et corrélée au syndrome métabolique, particulièrement obésité et diabète de type 2. Les risques pour la santé d'avoir une NAFLD sont hépatiques (cancer du foie, cirrhose décompensée) mais aussi extra-hépatiques avec un haut risque de développer et décéder d'une maladie cardiovasculaire. Il est donc capital de s'intéresser à la NAFLD, en diagnostiquant mieux les sujets à risques.

1.2 Enjeux de la prise en charge des patients

Compte tenu du problème majeur de santé publique que représente la NAFLD, dépister, diagnostiquer les patients à risque de développer une fibrose avancée ou un CHC est un enjeu majeur. Les outils de dépistage et diagnostic disponibles aujourd'hui ne permettent pas de répondre de façon simple à cette question si bien que le diagnostic est souvent tardif, lorsque les patients sont symptomatiques. De plus, la grande majorité des patients n'ont pas connaissance de leur maladie et ne sont pas adressés en hépatologie, même si aujourd'hui le nombre exact est difficile à évaluer. Son évolution lente et longtemps asymptomatique explique en partie cela. En revanche, compte tenu de leurs comorbidités, ces patients sont souvent pris en charge en médecine générale, endocrinologie ou cardiologie.

1.2.1 Etablir le diagnostic

Les recommandations américaines et européennes publiées montrent quelques différences dans le dépistage et la gestion des patients ayant une NAFLD (5, 82, 83). Alors que le dépistage des formes sévères est recommandé en Europe, ce n'est pas le cas aux Etats-Unis car il n'existe aucun traitement approuvé sauf les modifications de style de vie. De plus, aucune cohorte de vie réelle n'est disponible à ce jour pour le dépistage et diagnostic de la NAFLD. TARGET-NASH est une étude de cohorte observationnelle longitudinale en cours mais les résultats ne seront publiés que dans plusieurs années (84).

1.2.1.1 Stéatose simple

Le diagnostic de la stéatose simple peut être posé soit de façon fortuite, lorsque les patients font un examen d'imagerie pour une autre raison que le dépistage d'une maladie du foie soit de façon ciblée devant le terrain ou la présence d'anomalie des tests hépatiques. Une étude récente a montré que 11% des patients présentant une stéatose simple découverte fortuitement pourraient en réalité avoir une fibrose hépatique avancée (85).

Le diagnostic de stéatose simple repose sur :

1. La découverte d'une stéatose sur un examen d'imagerie ou en anatomopathologie
2. L'absence de consommation d'alcool significative

3. L'absence de cause connue de stéatose (Table 1)

Table 1 : Principaux diagnostics différentiels de la stéatose simple

Stéatose macrovacuolaire	Stéatose microvésiculaire
Consommation excessive d'alcool	Syndrome de Reye
Hépatite C (génotype 3)	Médicaments (valproate de sodium, antirétroviraux)
Maladie de Wilson	Stéatose aigue gravidique
Lipodystrophie	Syndrome de HELLP
Malnutrition	Maladies orphelines touchant le métabolisme des lipides
Nutrition parentérale	
Abétalipoprotéinémie	
Médicaments dont l'amiodarone, méthotrexate, tamoxifène, corticostéroïdes	

Même si elles peuvent coexister, il convient également de rechercher d'autres causes de maladie chronique du foie, notamment l'hémochromatose, les maladies hépatiques auto-immunes, l'hépatite virale chronique, le déficit en alpha-1 antitrypsine. La ferritine sérique peut être cependant légèrement augmentée dans la NAFLD et n'indique pas nécessairement une surcharge en fer hépatique, bien qu'elle puisse avoir un impact sur la progression de la maladie. Bien que les données soient quelque peu contradictoires, une ferritine sérique > 1,5 limite supérieure de la normale (LSN) est associée à une fibrose plus avancée (86). De la même façon, de faibles titres d'auto-anticorps sériques, en particulier les anticorps anti-muscles lisses et antinucléaires, sont retrouvés chez 21% des patients atteints de NAFLD et sont généralement considérés comme un épiphénomène sans conséquence clinique, bien qu'ils nécessitent souvent une biopsie du foie pour exclure les maladies auto-immunes (87).

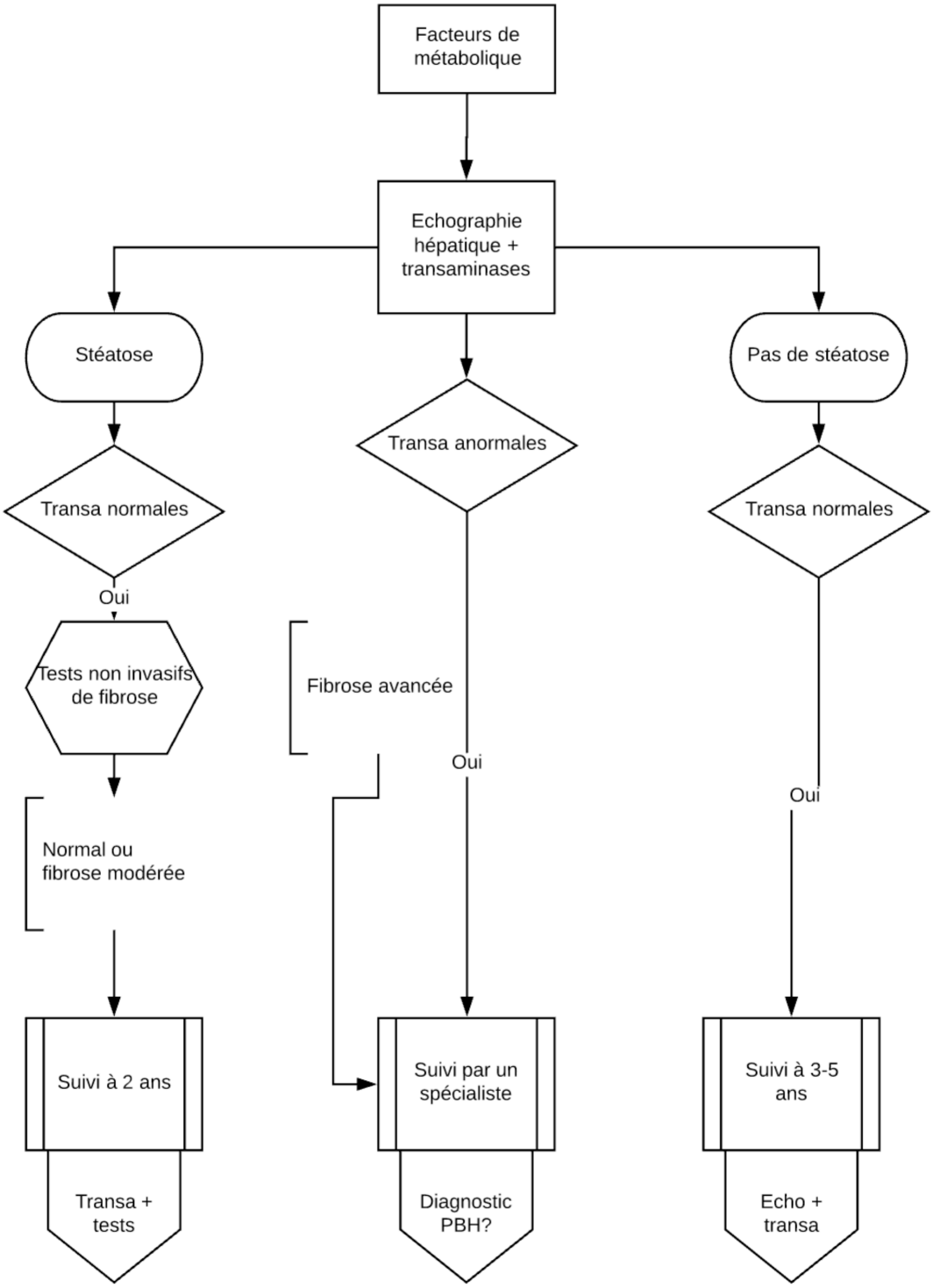
L'échographie est la méthode d'imagerie la plus largement utilisée car disponible et moins chère que l'IRM, dont les performances sont meilleures. L'échographie a en effet une sensibilité limitée et ne détecte pas de manière fiable la stéatose lorsqu'elle est inférieure à 20% ou chez les individus ayant un IMC élevé (> 40 kg/m2) (88). Pour les études de dépistage à grande échelle, les biomarqueurs sériques sont préférés, car la disponibilité et le coût de l'imagerie ont un impact substantiel sur la faisabilité. Les scores de stéatose les mieux validés sont l'indice de stéatose hépatique (FLI), le SteatoTest® et le score de graisse du foie de la NAFLD (NAFLD liver fat score) (89). Ils ont tous été validés dans la population générale ou chez des obèses et permettent de

prédire de manière variable la morbi-mortalité métabolique, hépatique et cardiovasculaire. En revanche, ils ne permettent pas de prédire de manière fiable la sévérité de la stéatose.

Enfin, une autre technique d'imagerie, le paramètre d'atténuation contrôlée (CAP) peut diagnostiquer la stéatose, mais a une capacité limitée à discriminer les grades histologiques et n'a jamais été comparé à la stéatose mesurée par ^{1}H-MRS (spectroscopie par résonnance magnétique) (90). Dans une analyse prospective de 450 patients atteints de stéatose hépatique, le CAP et l'élastométrie évaluaient respectivement la stéatose hépatique et la fibrose, avec des valeurs d'AUROC comprises entre 0,70 et 0,89 (91).

Une fois la NAFLD diagnostiquée, et en l'absence de recommandation, le suivi n'est pas clairement validé. Cependant, les recommandations européennes proposent un organigramme pour la surveillance de ces patients représenté dans la Figure 3 (5).

Figure 3 : Algorithme décisionnel de prise en charge et surveillance de la stéatose hépatique



1.2.1.2 NASH

Le diagnostic de NASH fournit des informations pronostiques importantes et indique un risque accru de progression de la fibrose, de la cirrhose et éventuellement de comorbidités hépatiques (CHC). Cela peut également nécessiter un suivi plus étroit et modifier la prise en charge thérapeutique. Malheureusement, les tests cliniques, biochimiques ou d'imagerie ne permettent pas de distinguer la NASH de la stéatose.

Aussi, la prise en compte du terrain est importante. Bien que la NAFLD soit fortement associée aux composants du syndrome métabolique, la présence d'un nombre croissant de maladies métaboliques, telles que le diabète de type 2, l'hypertension, la dyslipidémie et l'obésité viscérale semblent augmenter le risque de maladie hépatique progressive. Par conséquent, les patients atteints de NAFLD et de multiples facteurs de risque sont les plus exposés (88).

Les taux circulants de fragments de cytokératine-18 ont été étudiés en tant que biomarqueurs de la présence de NASH chez des patients atteints de NAFLD, avec des résultats controversés (92, 93). Les taux diminuent en parallèle à une amélioration histologique parallèle mais ne font pas mieux que les taux de transaminases pour identifier les répondeurs histologiques (94). C'est pourquoi, ce biomarqueur n'est pas utilisé en routine et il n'existe pas à l'heure actuelle de marqueurs non-invasifs de la NASH.

L'analyse histologique demeure donc le gold-standard pour le diagnostic de NASH. Mais en raison du caractère invasif d'une biopsie hépatique, elle ne peut être pratiquée chez tous les patients ayant une NAFLD, surtout si la prise en charge thérapeutique ne s'en trouve pas modifié.

Le diagnostic de stéatose simple ou NAFL comprend: a) la stéatose seule, b) la stéatose avec atteinte lobulaire ou inflammation portale, sans ballonisation hépatocytaire, ou c) la stéatose avec ballonisation mais sans inflammation. Le diagnostic de NASH nécessite la présence conjointe de stéatose, de ballonisation et inflammation lobulaire (3, 95). La NASH présente d'autres caractéristiques histologiques, qui ne sont cependant pas nécessaires pour établir le diagnostic comme l'inflammation portale, l'infiltrat polymorphe, les présences de corps de Mallory, de corps apoptotiques, de noyaux vacuolés clairs, de stéatose microvacuolaire et de mégamitochondries. La fibrose périsinusoïdale est également fréquente mais ne fait pas partie des critères de diagnostiques. L'algorithme FLIP consortium, conçu de manière prospective, augmente

les performances inter-observateur. Il existe deux systèmes de notification semi-quantitative des lésions nécrotico-inflammatoires le score NAS et le score de stéatose, d'activité et de fibrose (SAF). Ils ne devraient être utilisées qu'après le diagnostic histologique établi.

1.2.2 Etablir le pronostic de l'atteinte hépatique

1.2.2.1 Evaluation de la fibrose

En l'absence de marqueurs non-invasifs de la NASH, il est important de rechercher la présence d'une fibrose, principal élément pronostic.

Les outils non-invasifs couramment étudiés sont des aides cliniques simples (par exemple, le score NFS pour NALFD fibrosis score, le score FIB-4, le score APRI) ou des biomarqueurs sériques plus complexes faisant souvent l'objet d'un brevet et plus coûteux (ELF pour Enhanced Liver Fibrosis, Fibromètre®, FibroTest® et Hepascore). Associée à ces biomarqueurs, l'élastographie (impulsionnelle ou par résonance magnétique complète le panel de marqueurs non-invasifs (96, 97).

Le score NFS est basé sur six variables facilement disponibles (âge, IMC, hyperglycémie, nombre de plaquettes, albumine et ratio ASAT/ALAT). Dans une méta-analyse de 13 études portant sur 3 064 patients, ce score obtenait une AUROC de 0,85 pour prédire la fibrose avancée (98). Le score FIB-4 est un algorithme basé sur la numération plaquettaire, l'âge, l'AST et l'ALT, offrant deux valeurs seuil (les patients avec un score <1,45 sont peu probables, alors que les patients avec un score> 3,25 sont susceptibles d'avoir une fibrose avancée) (96). Ils ont, tous deux, été validés dans des populations de NAFLD, ethniquement différentes, avec des résultats robustes.

Le score ELF comprend les taux plasmatiques de 3 protéines (acide hyaluronique, inhibiteur tissulaire de la métalloprotéinase 1 et peptide procollagène III N-terminal) présentant un AUROC de 0,90 avec une sensibilité de 80% et une spécificité de 90% pour la détection de la fibrose avancée (99). Une étude récente comparant ces scores couplés à l'élastographie à l'histologie hépatique a montré que les scores NFS et FIB-4 étaient les plus performants et qu'ils faisaient aussi bien pour prédire la fibrose avancée chez les patients avec NAFLD que l'élastographie (100).

Plus récemment encore, le score MACK-3 combinant HoMa, ASAT et cytokératine 18, a permis d'obetenir des AUROC significativement plus élevées (0,847 ± 0,030, P ≤ 0,002) que les tests de fibrose sérique. MACK-3 était précis pour le diagnostic de la NASH avec

93,3% de patients bien classés (sensibilité: 90,0%, spécificité: 94,2%. , valeur prédictive positive: 81,8% et valeur prédictive négative: 97,0%) (101).

Tous ces scores prédisent la mortalité globale, la mortalité cardiovasculaire et la mortalité hépatique. Tous ces tests permettent de distinguer une fibrose avancée (≥F3) d'une fibrose modérée mais distinguent mal une fibrose modérée (F2) versus une fibrose minime voie absente (97). De plus, les valeurs prédictives négatives pour exclure la fibrose avancée sont supérieures aux valeurs prédictives positives correspondantes.

L'élastographie ultrasonore (FibroScan®) donne de meilleurs résultats pour prédire la cirrhose (F4) que pour la fibrose avancée (F3). L'élastographie a un taux plus élevé de faux positifs que de faux négatifs (102, 103). Le principal défaut de l'élastographie est l'absence de fiabilité des résultats en présence d'un IMC élevé et/ou d'une épaisseur de pli cutané importante. Dans une grande série européenne, jusqu'à 20% des examens avaient des résultats peu fiables, principalement chez les patients obèses (104).

Aussi, les recommandations américaine et européenne préconisent de combiner ces tests non-invasifs entre eux, sans pour autant privilégier une stratégie.

1.2.2.2 Dépister et diagnostiquer le cancer

Bien qu'il s'agisse d'une complication redoutée, le diagnostic de CHC en cas de NAFLD est souvent tardif (105). Cet état de fait traduit probablement un manque de standardisation et d'application des stratégies de dépistage. Aux Etats Unis comme en Europe, seuls 20 et 23% des patients chez qui l'on diagnostique un CHC dans un contexte de NAFLD ont été en réalité dépistés (72, 106).

Le taux d'alphafœtoprotéine est souvent normal, en tout cas plus bas que dans d'autres étiologies comme les hépatopathies virales (107). L'échographie de dépistage peut être également difficile en cas d'IMC élevée. Une particularité importante du CHC chez les patients ayant une NAFLD est sa survenue en l'absence de la cirrhose, parfois secondaire à la transformation maligne d'adénomes hépatiques (Cf 1.1.4.3). Autant il est clair qu'il faut dépister les patients ayant une cirrhose, autant il n'existe pas de recommandation de dépistage clair pour les patients qui ont une fibrose de moindre importance. Le grand nombre de cas de NAFLD présentant un risque de CHC rend la surveillance systématique pratiquement irréalisable. Ni les sociétés européennes ni américaines ne proposent un algorithme décisionnel de surveillance du CHC en dehors de la cirrhose. Le polymorphisme du gène rs738409 C> G de PNPLA3 a été associé à un risque accru de CHC et pourrait fournir une stratification du risque du patient pour une surveillance

personnalisée du CHC dans les NAFLD, mais il n'est pas encore considéré comme rentable (108).

1.2.3 Prise en charge thérapeutique

La prise en charge thérapeutique des patients ayant une NAFLD a fait l'objet d'une publication « Coilly A, Chiappini F (2016) Recent Insights into Treatment of Non-Alcoholic Steatohepatitis. J Endocrinol Diab 3(1): 1-11» correspondant à l'Article 4 de cette thèse, en annexe.

Brièvement, la prise en charge thérapeutique des patients ayant une NAFLD repose principalement sur des modifications du style de vie. Les données épidémiologiques suggèrent une relation étroite entre le mode de vie sédentaire et la NAFLD. Une consommation quotidienne d'alcool allant jusqu'à 30 g pour les hommes ou 20 g pour les femmes est insuffisante pour induire une stéatose alcoolique.
La perte de poids améliore la stéatose et l'insulino-résistance. Dans une étude non contrôlée de 12 mois comportant 261 biopsies par paires, une perte de poids modérée induite par le mode de vie était associée à une régression de la NASH (25% du total des cas) sans aggravation de la fibrose (109). Des approches pragmatiques associant restriction alimentaire et augmentation progressive de l'exercice physique sont préférables et doivent être adaptées à chaque cas (110). Aucune donnée n'est disponible sur leurs effets à long terme sur l'histoire naturelle de la NAFLD.
Si plusieurs traitements sont utilisés en pratiques courantes, aucun médicament n'a reçu d'autorisation de mise sur le marché ce jour dans cette indication. Mais plusieurs traitements sont en cours de développement et parvenus aux phases III de développement. Aussi, peut-on espérer une commercialisation prochaine d'un ou plusieurs d'entre eux. L'objectif d'un traitement serait de réduire la mortalité liée à la NASH, réduire la progression vers la cirrhose ou le CHC. La résolution des lésions histologiques définissant la NASH est maintenant acceptée comme critère de substitution, en particulier dans les essais cliniques.
Il existe peu de preuves d'une efficacité histologique de la metformine dans la NASH (111). L'effet de la metformine sur la stéatose est faible en raison de son incapacité à rétablir les taux sériques d'adiponectine à court terme (112). Certaines données précliniques soutiennent une activité anti-tumorigène de la metformine sur le cancer du

foie, alors que la démonstration de la réduction des taux de CHC chez l'homme se limite à des études rétrospectives (113, 114).

Les thiazolidinediones sont des agonistes γ du récepteur activé par les prolifférateurs des peroxysomes (PPAR) ayant des effets sensibilisants à l'insuline. L'étude PIVENS a comparé la pioglitazone à faible dose, la vitamine E et le placebo pendant 2 ans chez des patients sans diabète déclaré. La pioglitazone améliorait toutes les caractéristiques histologiques (sauf la fibrose) (115). Les effets secondaires des glitazones sont préoccupants: prise de poids, fractures osseuses chez la femme et, rarement, insuffisance cardiaque congestive.

Les incrétino-mimétiques, agissant sur l'interaction glucose-insuline, ont donné des résultats satisfaisants en améliorant les tests biologiques hépatiques (116). Un essai pilote d'injections quotidiennes de liraglutide a permis une rémission des signes histologiques de la NASH sans aggravation de la fibrose (117).

Dans l'étude PIVENS, la vitamine E améliorait la stéatose, l'inflammation et améliorait les signes histologiques de la NASH chez 36% des patients (115). Cependant, cet essai positif n'a pas été reproduit. De plus, des inquiétudes concernant l'innocuité à long terme de la vitamine E existent, principalement une augmentation de la mortalité globale, des accidents vasculaires cérébraux hémorragiques et du cancer de la prostate chez les hommes de plus de 50 ans.

L'acide ursodésoxycholique (AUDC) a été étudié dans plusieurs essais randomisés, à des doses différentes et jusqu'à deux ans, mais n'a montré que quelques améliorations biochimiques mais aucune amélioration histologique (118).

Un agoniste synthétique du récepteur X du farnésoïde, l'acide obéticholique, est actuellement étudié. Dans l'étude de phase IIb FLINT, un traitement de 72 semaines par acide obéticholique chez des patients non cirrhotiques atteints de NASH a permis d'améliorer toutes les lésions de la NASH tout en améliorant la fibrose. La phase III est actuellement en cours (119).

De nouveaux agents prometteurs aux propriétés anti-inflammatoires, sensibilisantes à l'insuline (doubles agonistes de PPARα/δ, antagonistes du récepteur des chémokines [CCR]2/CCR5 et des acides conjugués d'acides gras/biliaires) et antifibrotiques (anticorps monoclonaux anti-lysyl oxydase-like [anti -LOXL2]) sont également testés dans des essais cliniques randomisés en phase terminale dans NASH.

En résumé, établir le diagnostic et le pronostic de la NAFLD est un enjeu majeur en médecine aujourd'hui. On pourrait attendre des tests qu'ils prédisent ou diagnostiquent les patients à risque d'avoir une fibrose avancée ou un CHC. Seule l'histologie permet de poser formellement le diagnostic même si plusieurs combinaisons de marqueurs non-invasifs permettent une sélection de patients à risque. De nouveaux outils sont nécessaires pour améliorer la prédiction et le diagnostic de NAFLD.

1.3 Mécanismes physiopathologiques de la NAFLD

L'histoire naturelle de la NAFLD est encore mal élucidée. La NAFLD est une maladie complexe résultant de l'interaction de multiples facteurs et mécanismes pathologiques dont le résultat conduit à une grande diversité de phénotypes.

Particulièrement le profil de malades à risque de développer une maladie hépatique sévère ainsi que les facteurs responsables de l'aggravation des lésions histologiques sont mal définis. Une des questions majeures concernent les patients ayant une stéatose simple. Certains ne vont jamais progresser alors que d'autres au contraire vont développer une NASH voire une fibrose sévère ou un CHC.

1.3.1 Stéatose simple et NASH : continuum ou entité distincte ?

Il est dogmatique de dire que la stéatose simple est une maladie bénigne. Toutefois, certaines études montrent une évolution de la stéatose simple vers la NASH. Plusieurs cas ont d'abord été rapportés. Les études ayant inclus un nombre plus important de patients sont restreintes. Parmi 13 et 36 malades ayant une stéatose simple, 3 et 17 ont développé une NASH et une fibrose pendant une période de suivi de 14 ans dans deux études longitudinales (120, 121). Plus récemment, McPherson et *al*. Ont décrit l'évolution de 27 patients ayant une stéatose simple pendant une période de suivi moyen de 6,6 ans. Parmi ces patients, 12 (44%) ont développé une NASH (122).

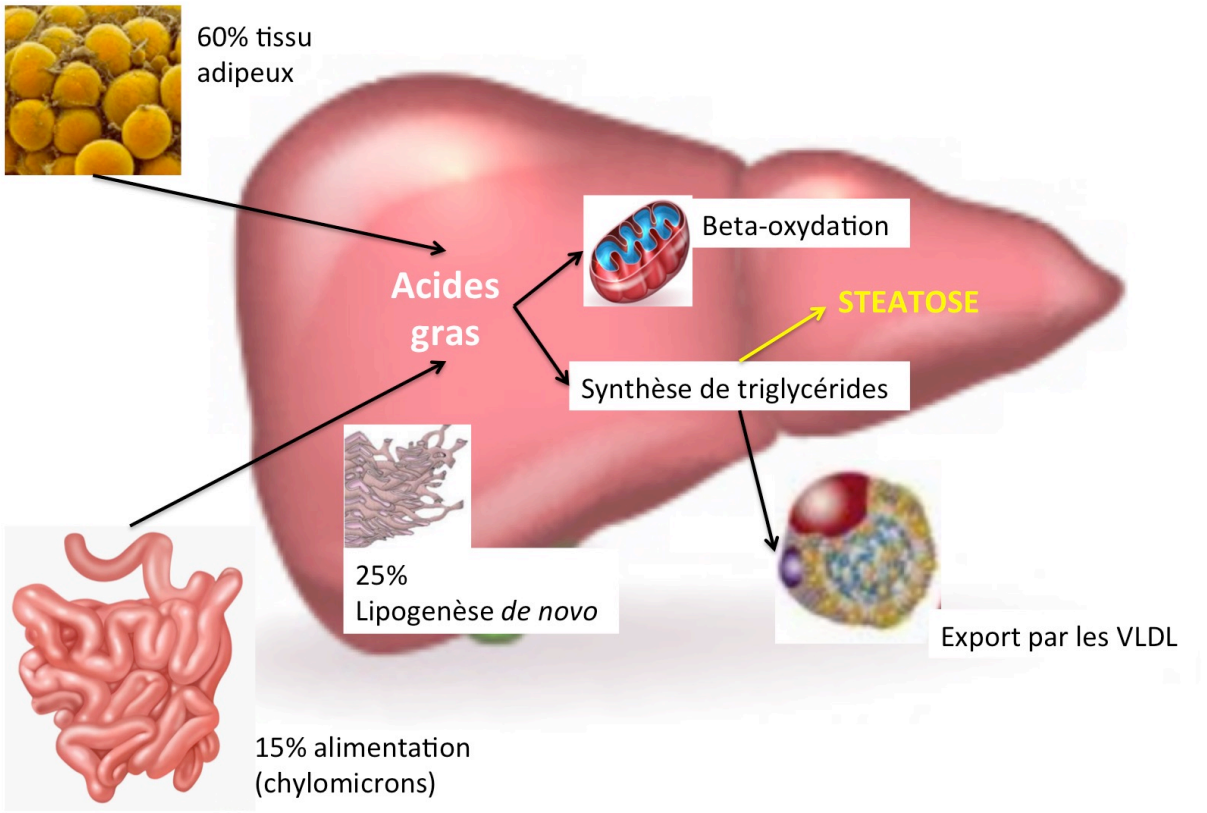
Ces résultats suggèrent qu'il pourrait exister plusieurs catégories de stéatose, pouvant résulter des mécanismes physiopathologiques qui la sous-tende et qui dans certains cas sont voisins des mécanismes impliqués dans la NASH et dans certains cas différents (Figure 4).

En effet, la stéatose est présente dans la NAFL et la NASH (3). La stéatose se produit chaque fois que l'importation ou la synthèse de graisse dépasse l'exportation ou la dégradation de graisse dans le foie. Les triglycérides sont les lipides prédominants et visibles en histologie (123). Ainsi, ils sont la base de la classification de la stéatose. Mais ils ne sont pas à proprement parler hépatotoxiques, de sorte que le degré ou la sévérité de la stéatose ne prédisposent ni à l'inflammation ni à la fibrose (124-126). D'autre part, les acides gras, le diacylglycérol, le cholestérol et les phospholipides peuvent endommager les hépatocytes. La prise de conscience du fait que la lipotoxicité est

provoquée par des lipides autres que les triglycérides a incité à développer des stratégies de prévention ou de traitement de la NASH en bloquant l'accumulation hépatique de lipides lipotoxiques. La lipotoxicité initie donc le développement de la NASH et constitue une cible thérapeutique. En revanche, la composition lipidique hépatique n'est pas utilisée pour prédire ou diagnostiquer la NASH.

Figure 4 : Mécanismes de la stéatose hépatique

La stéatose hépatique résulte d'un afflux accru de lipides dans le foie ou d'une diminution de l'élimination des lipides. Les principales sources sont les acides gras plamatiques (provenant principalement du tissu adipeux), la lipogenèse d*e novo* et les acides gras alimentaires. Le foie les élimine par oxydation ou en les exportant sous forme de VLDL. Les hépatocytes peuvent également utiliser les lipides en excès pour synthétiser les triglycérides et les stocker dans des gouttelettes lipidiques. *Figure adaptée de Machado, MV. Gastroenterology. 2016.*



1.3.2 Lipotoxicité : la pierre angulaire

Le tissu adipeux en condition d'excès produit des adipokines qui empêchent les adipocytes d'assimiler les acides gras et favorisent leur libération. Cela se traduit par une libération accrue d'acides gras dans le foie et alimente la synthèse des triglycérides

par les hépatocytes (127). La capacité de la synthèse des triglycérides à compenser une exposition accrue aux acides gras hépatiques semble déterminée par la lipotoxicité. En effet, certaines données provenant d'études sur des modèles murins de NASH ont montré que l'inhibition de la synthèse des triglycérides hépatiques augmentait l'accumulation hépatique d'acides gras libres et la gravité des lésions hépatiques et de la fibrose, malgré la réduction de la stéatose (124). Plus récemment, des études sur des souris ayant inhibé simultanément l'oxydation des acides gras libres et de la lipogenèse *de novo* ont montré que l'accumulation de lipides intermédiaires et de faibles niveaux de triglycérides génèrent un stress oxydatif, une inflammation et des dommages cellulaires (128). La quantité de triglycérides stockée est également régulée par des protéines qui lient les gouttelettes lipidiques, comme la périlipine-5. Chez les souris présentant un défaut pour cette protéine, une réduction de la taille des gouttelettes lipidiques était associée à une lipolyse et une lipotoxicité accrues (129). L'ensemble de ces données indique que la capacité de stocker les lipides sous une forme plus inerte, sous forme de triglycérides, aux dépens d'autres espèces, protège les individus de la progression vers NASH.

1.3.2.1 Les lipides toxiques

La lipotoxicité dépend aussi du type d'acide gras qui s'accumulent. Li et *al*. ont montré que l'inhibition de la stéaroyl-CoA désaturase (qui convertit les acides gras saturés en acides gras monoinsaturés) exacerbait les lésions hépatiques dans des modèles murins de NASH (128). Les acides gras sont classés en fonction de la longueur de la chaîne carbonée et du nombre de doubles liaisons. Les acides gras saturés comprennent le palmitate (C16:0) et le stéarate (C18:0), qui sont des composants majeurs du régime alimentaire ou peuvent être synthétisés *de novo* à partir de glucides. Plusieurs études ont mis en évidence les effets toxiques puissants des acides gras libres insaturés, en particulier le palmitate et le stéarate, qui induisent l'apoptose et l'inflammation via plusieurs mécanismes (130). Les acides gras monoinsaturés tel que l'oléate (C18:1) sont moins toxiques bien qu'ils contribuent à la stéatose, renforçant ainsi l'idée que l'accumulation de graisse et la lipotoxicité ne sont pas synonymes. De plus, ces lipides peuvent même protéger de la mort cellulaire, réduisant les niveaux de protéines pro-apoptotiques et favorisant la séquestration du palmitate dans les triglycérides (131).
Certaines classes de lipides protègent même des lésions cellulaires. Les acides gras polyinsaturés contribuent à l'élimination des graisses des hépatocytes (132). De même,

il a été démontré que l'acide α-linolénique protège les hépatocytes de l'apoptose et réduit de JNK entraînant l'expression de médiateurs proinflammatoires (133).

D'autres lipides jouent un rôle incertain dans les mécanismes induisant la NASH comme les lipides dérivés de la phosphatidylcholine ou les céramides. La lysophosphatidylcholine est augmentée dans les modèles animaux et dans la NASH humaine et plusieurs sources de données indiquent que la LPC pourrait être l'un des effecteurs en aval de la toxicité de l'acide palmitique (134). Également, des taux élevés de céramides ont été rapportés dans les modèles murins et chez les patients atteints de NASH (135). Enfin, des données récentes ont mis en évidence le caractère toxique du cholestérol libre qui jouerait un rôle dans les lésions hépatiques, l'inflammation et la fibrose dans le contexte de la NASH (136). Une expression accrue de la protéine de liaison aux éléments régulateurs des stérols (SREBP) entraîne l'activation de la HMGCoA réductase, étape limitante de la synthèse du cholestérol, entraînant une accumulation de cholestérol libre, en particulier dans la mitochondrie (137). En dépit de ces données, aucune preuve appuyant l'utilisation de statines dans le traitement de la NASH n'a encore été fournie, bien que cette classe de médicaments puisse être indiquée chez les patients atteints de NASH afin de réduire le risque cardiovasculaire.

1.3.2.2 Mécanisme de la lipotoxicité

Les lipides peuvent causer une toxicité par divers mécanismes. L'oxydation des acides gras mitochondriaux et peroxisomaux génère des espèces réactives de l'oxygène qui peuvent être immédiatement toxiques ou qui peuvent éventuellement épuiser les réserves d'antioxydants, ce qui rend les hépatocytes plus vulnérables à d'autres facteurs générant un stress oxydatif (138). Cela conduirait à de graves dysfonctions de la chaîne respiratoire mitochondriale qui elle-même conduirait à l'apoptose (139, 140). L'accumulation d'acides gras serait également responsable de l'IR et l'hyperinsulinémie, ce qui entraîne une accumulation supplémentaire des lipides hépatiques et favorise les réponses inflammatoires et fibrogéniques, ainsi que les oncogéniques (141).

Un autre mécanisme de lipotoxicité implique des modifications de la signalisation cellulaire. Les acides gras sont capables d'interagir et modifier d'autres molécules, comme certains facteurs de transcription ou récepteurs comme les Toll-like récepteurs, entraînant des modifications profondes des voies de signalisation qui régulent le métabolisme et les réponses au stress (142). La lipotoxicité induit plusieurs types de

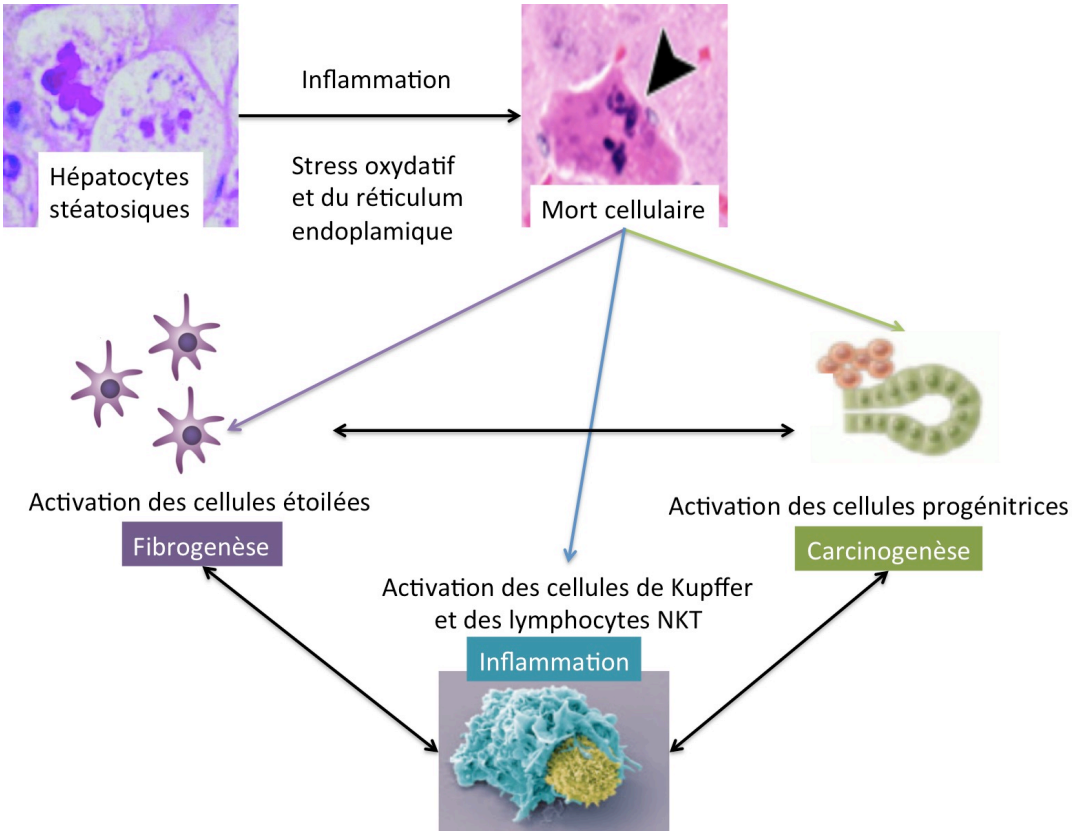
stress cellulaire, y compris le stress du réticulum endoplasmique et l'autophagie (143). Malgré les preuves croissantes du rôle important de la lipotoxicité dans la physiopathologie de la NASH, il n'existe pas de méthode simple pour identifier et quantifier les types de lipides qui s'accumulent dans le foie.

1.3.2.3 Vers la NASH

La NASH est due au fait que les hépatocytes lipotoxiques libèrent des facteurs qui initient des réponses cicatrisantes pour remplacer les hépatocytes altérés. La cicatrisation est un processus complexe qui englobe l'activation des cellules immunitaires résidentes et le recrutement de cellules inflammatoires dérivées de la moelle osseuse (inflammation), le remodelage de la matrice (fibrogenèse et fibrinolyse), l'angiogenèse et la mobilisation des populations de progéniteurs du foie (Figure 6).
Cependant, les aberrations dans les réponses de cicatrisation peuvent être dangereuses. Celles-ci peuvent conduire à une réparation défectueuse du parenchyme hépatique lésé et favoriser le développement d'un cancer du foie et/ou le remplacement progressif du parenchyme hépatique par un tissu cicatriciel conduisant à la cirrhose.

Figure 6 : Réponse hépatique à la lipotoxicité

La lipotoxicité provoque un stress cellulaire et la mort des hépatocytes. Pour favoriser la régénération ou la réparation, le foie recrute et active les cellules étoilées hépatiques afin d'éliminer les cellules nécrosées et de maintenir les épithéliums restants, ainsi que des progéniteurs pour les remplacer. Si la réponse n'est pas adaptée, elle favorise l'inflammation, la fibrogenèse et l'hépatocarcinogenèse. *Figure adaptée de Machado, MV. Gastroenterology. 2016.*



En résumé, les lipides et leur toxicité propre joue un rôle central dans la physiopathologie de la NAFLD. Leur concentration mais également leur composition dans le foie stéatosique peuvent induire des lésions inflammatoires, un remodelage de la matrice extracellulaire. Si ces aspects physiopathologiques sont bien validés, aucune signature lipidique de la NASH n'a été identifiée à ce jour.

1.4 NAFLD et Transplantation hépatique

Comme nous venons de le décrire, l'augmentation de la prévalence et de l'incidence de la NAFLD est bien documentée. L'enjeu de cette tendance épidémiologique est double. En premier lieu, parmi les patients atteints de NASH, un quart vont évoluer vers une cirrhose décompensée ou développer un CHC. La transplantation hépatique (TH) demeure le seul traitement curatif des patients dont le pronostic vital est engagé et non candidat ou non répondeur à un autre traitement. Ainsi, l'augmentation des patients candidats à une TH pour NASH est déjà observée dans de nombreux pays, positionnant la NASH comme une indication majeure de TH. Le deuxième enjeu concerne les foies transplantés. L'augmentation de l'incidence de la NAFLD contraint les équipes de greffe à choisir des foies stéatosiques, foies qui une fois transplantés obtiennent de moins bons résultats en termes de fonction immédiate et influencent péjorativement les résultats de la TH.

1.4.1 TH pour complications hépatiques de la NASH

1.4.1.1 Période d'attente et activité de TH

Certaines études ont mis en évidence une augmentation disproportionnée de la cirrhose liée à la NASH en tant qu'indication de la LT par rapport à d'autres étiologies (144, 145). En 2017, Cholankeril *et al*. a rapporté une augmentation de 162% de l'activité de TH pour NASH entre 2003 et 2014 selon les données de l'UNOS, 54% et 33% pour la cirrhose alcoolique ou virale C compliquée, respectivement.

Le nombre de patients inscrits sur liste d'attente TH pour NASH augmente également. Entre 2004 et 2013, le nombre de nouvelles inscriptions pour NASH sur liste d'attente a augmenté de 170% aux Etats-Unis (146). Cette augmentation est liée à l'augmentation de l'incidence de la NAFLD bien sûr mais également à la diminution de certaines indications comme celle liée au virus de l'hépatite C grâce à l'avènement des antiviraux directs (147).

L'une des préoccupations importantes est que la mortalité sur liste d'attente à 90 jours semble plus importante que pour les autres indications, en particulier alcool et virus de l'hépatite C (146). Mais cette surmortalité disparaissait après ajustement en fonction du sexe, de l'âge, de la présence d'un diabète de type 2, d'un CHC ou de la gravité de la cirrhose. Ce constat montre bien le poids des comorbidités dans le pronostic d'un

patient ayant une maladie hépatique terminale. Une étude récente a montré que l'obésité morbide et le diabète de type 2 étaient associés à des taux plus élevés de sortie de liste ou décès sur liste d'attente de TH (148). Mais ces patients sont peut-être plus exposés aux complications de la cirrhose comme la thrombose portale (149).

Enfin, la probabilité pour un patient inscrit pour un NASH et candidat à la TH de recevoir un greffon semble inférieure à celle d'autres indications, en particulier alcool et hépatite C (146).

Les données actuelles sont principalement américaines. Malheureusement, en France, l'item NASH n'a été implémenté dans le thésaurus de l'Agence de Biomédecine que cette année. Il est cependant quasi-certain que la NASH « pure » représente une indication minoritaire de TH en France (150).

1.4.1.2 Résultats de la greffe pour NAFLD

Les résultats de mortalité à long terme semblent similaires que les receveurs soient transplantés pour une NASH ou pour une autre indication (151, 152). La survie rapportée fluctue de 79–90%, 82–83% et 72–78% à 1, 3 et 5 ans, respectivement (153). Cependant, un sous-groupe de patients à haut risque ayant un âge > 60 ans, un diabète, une hypertension artérielle et un IMC >30 Kg/m^2 ont une mortalité à 30 jours et à un an étaient de 31% et 50%, respectivement (153). Une méta-analyse réalisée en 2014 a également révélé que les patients transplantés pour NASH avaient un risque plus élevé de décès par complications cardiovasculaires (OR=1,65, IC95%: 1,01–2,70, p=0,05) (154). Les mêmes facteurs de risque du syndrome métabolique qui prédisposent les patients à la NAFLD, notamment l'obésité, le diabète, l'hyperlipidémie et l'hypertension artérielle prédisposaient à des évènements cardiovasculaires. Malgré ces facteurs de risque connus, des études récentes ont montré que les taux de survie et les résultats à long terme sont similaires pour les cirrhoses liées à l'alcool au virus C ou à la NASH (154).

Ces études montrent que chez des patients bien sélectionnés, la NASH est une indication acceptable de TH, en augmentation. L'influence des comorbidités est importante pour la mortalité pré comme post-TH. Cependant, aucune étude à ce jour ne permet de contre-indiquer la TH de façon formelle, en fonction des comorbidités du patient. Un consensus international récent de l'ILTS a permis d'établir les examens devant être réalisés avant d'indiquer une TH pour NAFLD (Article 5 de cette thèse).

1.4.2 NASH chez le patient transplanté hépatique

La NAFLD et la NASH peuvent être diagnostiquées sur un greffon hépatique après TH, soit sous forme d'une maladie récurrente pour les patients greffés pour cette indication, soit *de novo*. Le taux de récidive dépend du fait que le diagnostic repose sur une évaluation parfois différente en fonction des pratiques et des centres allant de simples tests hépatiques aux techniques d'imagerie ou au gold-standard anatomopathologique. De plus, la plupart des données disponibles proviennent d'études portant sur un petit nombre de patients, majoritairement sans biopsie post-greffe et avec un suivi court. Seules quelques études ont examiné la fréquence et les facteurs de risque de la NAFLD *de novo*, compris entre 18% et 33%. NASH après TH. Une étude récente suggère que la récidive de NASH après TH est plus fréquente et plus sévère que la NASH *de novo*, retrouvée chez 78% des patients transplantés pour NASH et chez 44% des patients transplantés pour une autre indication (155, 156).

Comme pour d'autres maladies récidivantes sur le greffon comme l'hépatite C, l'histoire naturelle de la maladie semble accélérée après la TH avec une fibrose extensive chez 30% des patients à 5 ans (157). Une étude monocentrique a montré que tous les patients ayant été transplantés entre 1995 et 2013 pour NASH ou cirrhose cryptogénique pour laquelle une NASH était suspectée, 88,2% des 34 patients présentaient une récidive de la NAFLD, et 41,2% présentaient une récidive de la NASH, histologiquement prouvée, et avec un recul médian de 47 mois (158).

Généralement après la TH, les patients présentent une propension connue à la prise de poids et à l'obésité. Dans une étude sur l'IMC après la TH, parmi les 320 patients non obèses avant la TH, 21,6% sont devenus obèses dans les 2 ans suivant la TH (159). De nombreux facteurs ont été rapportés comme associés à une récidive de la NASH, notamment l'obésité avec un IMC>30 Kg/m^2, l'utilisation de corticostéroïdes comme agents immunosuppresseurs, le diabète de type 2, l'hypertension artérielle et l'hypertriglycéridémie (160).

Des études ont également décrit l'évolution de la NAFLD *de novo* après la TH (161). Une étude rétrospective monocentrique a révélé que, parmi 170 patients, 32,9% avaient une NAFLD et la dose de corticoïdes après TH (5,2±2,4 mg/jour versus 7,1±4,7 mg/jour; p=0,014) était associée au syndrome métabolique *de novo* (162).

En cas de récidive grave ou de NASH de novo évoluant au stade terminal de la maladie, la reTH peut être un recours. Mais, malgré des taux relativement élevés de récidive de NAFLD, les études de suivi après 5–10 ans n'ont pas suggéré d'augmentation du risque de reTH dans cette indication. Dans une étude monocentrique, 6 patients représentant seulement 30% de la cohorte de patients NASH ont été retransplantés, dont 3 pour la NASH décompensée à proprement parler.

Ces études suggèrent que la récidive ou la survenue de la NAFLD voire de la NASH est fréquente et donc une préoccupation quotidienne du clinicien. Lutter contre les éléments du syndrome métabolique mais aussi adapter la stratégie immunosuppressive semble être les éléments clés même si les données de la littérature restent conflictuelles et minces.

1.4.3 Stéatose du greffon

Une stéatose hépatique est de façon récente, fréquemment retrouvée chez les donneurs potentiels de greffons hépatiques, probablement due à une augmentation de l'obésité dans le monde entier. La biopsie met en évidence une stéatose hépatique chez 76% des donneurs potentiels de foie présentant un IMC supérieur à 28 Kg/m^2 (163).

De plus, la stéatose du greffon est associée à des résultats globaux de la TH moins bons. Après la reperfusion, la stéatose induit des modifications de la microcirculation et des cellules du greffon hépatique, pouvant entraîner une nécrose des hépatocytes, aggravant ainsi les lésions d'ischémie perfusion. En outre, le potentiel de régénération des greffons stéatosiques est altéré (164, 165).

La stéatose hépatique est un facteur de risque indépendant du succès d'une TH, entraînant une augmentation de la morbidité et de la mortalité. Cela inclut une durée plus longue de séjour en réanimation, ou en salle conventionnelle, un risque accru de non-fonctionnement primaire et un retard de reprise de fonction (166, 167). En plus de l'influence à court terme, la stéatose du greffon peut avoir une influence à plus long terme, comme cela a été montré chez les receveurs ayant une récidive de l'hépatite C sur le greffon, récidive plus sévère s'ils recevaient des greffons stéatosiques (168).

Cependant, le seuil de non transplantabilité repose sur des études anciennes. La limite supérieure à ne pas franchir a été fixée à 60% de stéatose macrovacuolaire. Pourtant, bien qu'en général récusés pour la greffe par les chirurgiens, ces greffons ont montré des résultats similaires à court et long terme que les greffons sans stéatose lorsqu'ils sont

utilisés chez des receveurs dont le score MELD est faible (169). En revanche, la plupart des études publiées indiquent un taux relativement faible inférieur à 5% d'échec de la TH pour les greffons ayant une stéatose macrovacuolaire modérée de 30 à 60% et aucun effet majeur sur les résultats à long terme. Accepter ces greffons semble possible. Cependant, une publication récente a montré que la stéatose, même modérée était responsable de plus de 70% des dysfonctions primaires du greffon (170). De plus, dans cette étude, la stéatose hépatique était le seul facteur prédictif associé à une moins bonne survie en analyse multivariée. Les données de la littérature sont donc conflictuelles. En attendant de nouvelles données, il est prudent de réserver les greffons stéatosiques aux receveurs à faible risque (score de MELD bas, receveur jeune, temps d'ischémie froide plus courte).

L'influence du type de stéatose, macrovacuolaire ou microvésiculaire, sur le résultat de la TH est encore incertaine. La stéatose macrovacuolaire semble être une affection bénigne potentiellement réversible. Elle est principalement associée à l'obésité et à la consommation d'alcool. En revanche, la stéatose microvésiculaire est considérée comme une affection plus grave souvent associée à une altération de la bêta-oxydation mitochondriale et donc à un pronostic moins favorable (171). L'impact de la stéatose microvésiculaire sur le fonctionnement du greffon demeure très controversée (172, 173).

Il est important de souligner que l'évaluation de la stéatose du greffon est biaisée dans la plupart des études mentionnées en raison de l'absence d'une méthode fiable, facile à réaliser, objective et reproductible. Dans de nombreuses études, le degré de stéatose est évalué par une évaluation visuelle et une palpation au moment du prélèvement par l'équipe chirurgicale. Cette modalité d'évaluation subjective repose malheureusement sur l'expérience des chirurgiens.

Le gold-standard pour évaluer la stéatose hépatique est une analyse histologique réalisée par un anatomopathologiste expérimenté. Même dans ce cas, l'évaluation peut également être sujette à plusieurs biais, notamment l'hétérogénéité de la taille de l'échantillon ou les techniques de coloration pouvant affecter le degré de stéatose. Un autre biais est la variabilité inter-observateurs, même entre anatomopathologistes expérimentés (174). Tous ces facteurs peuvent contribuer aux divergences observées entre les études.

En résumé, l'augmentation de l'incidence de la NAFLD est responsable d'une augmentation de TH dans cette indication mais aussi d'une augmentation du nombre de greffons proposés ayant une stéatose. Seule l'histologie permet d'évaluer la stéatose au moment du prélèvement et avant la TH. Mais cette évaluation fait l'objet de nombreux biais. De nouveaux outils sont nécessaires pour améliorer l'évaluation de la stéatose du greffon, rapide et reproductible afin de prédire l'évolution de la TH.

2 Objectifs de la thèse

La NAFLD est une maladie fréquente, qui concerne un quart des patients dans le monde et sa prévalence devrait encore augmenter. Etablir le diagnostic et le pronostic de la NAFLD est un enjeu majeur en médecine aujourd'hui. Non diagnostiquée à un stade précoce, la maladie peut être découverte au stage de fibrose avancée, de cirrhose décompensée voire de CHC qui menace le pronostic vital des patients. A ce jour, seule l'histologie permet de poser formellement le diagnostic même si plusieurs combinaisons de marqueurs non-invasifs permettent une sélection de patients à risque. De nouveaux outils sont nécessaires pour améliorer la prédiction et le diagnostic de NAFLD. Les lipides et leur toxicité propre joue un rôle central dans la physiopathologie de la NAFLD. Leur concentration mais également leur composition dans le foie stéatosique peuvent induire des lésions inflammatoires, un remodelage de la matrice extracellulaire. Si ces aspects physiopathologiques sont bien validés, aucune signature lipidique de la NASH n'a été identifiée à comme marqueur diagnostic ou pronostic de la NAFLD.

Enfin, l'augmentation de l'incidence de la NAFLD est responsable d'une augmentation de TH dans cette indication mais aussi d'une augmentation du nombre de greffons proposés ayant une stéatose. Seule l'histologie permet d'évaluer la stéatose au moment du prélèvement et avant la TH. Mais cette évaluation fait l'objet de nombreux biais. De nouveaux outils sont donc nécessaires pour améliorer l'évaluation de la stéatose du greffon, de façon rapide et reproductible afin de prédire l'évolution de la TH.

L'objectif général de cette thèse était d'identifier de nouveaux outils diagnostiques ou pronostiques de la NAFLD, dans le contexte général, comme celui de la TH : (1) une signature lipidique diagnostique de la NASH permettant de distinguer stéatose et NASH à partir de la composition lipidique du foie (2) une signature génétique pronostique des différents stades de NAFLD, obtenue à partir d'une analyse in silico (3) une analyse spectrométrique de la teneur en triglycérides des greffons hépatiques pour diagnostiquer la stéatose et prédire le devenir de la TH.

3 Article 1

Metabolism dysregulation induces a specific lipid signature of nonalcoholic steatohepatitis in patients

La stéatopathie non alcoolique ou métabolique (NAFLD) comprend un éventail large de lésions hépatiques allant de la simple stéatose (NAFL) à la stéato-hépatite non alcoolique (NASH) pouvant évoluer vers une fibrose hépatique, une cirrhose et un CHC (175). La lésion primaire de la stéatose hépatique est l'accumulation intracellulaire de lipides, en particulier de triglycérides, qui se traduit par la formation de gouttelettes lipidiques dans les hépatocytes. Cette accumulation résulte d'un déséquilibre entre absorption, synthèse, exportation et oxydation des acides gras. La stéatose simple est une lésion réversible et asymptomatique longtemps considérée comme bénigne. Cependant, il est maintenant admis qu'elle peut être précurseur de la NASH (3).

Nous avons déjà démontré à l'aide d'une analyse transcriptomique que les gènes impliqués dans les processus inflammatoires étaient déjà surexprimés chez les patients ayant une stéatose dite simple (176). Ce résultat était également concordant avec le rôle toxique de certains lipides (177). Plus précisément, il a été rapporté que les acides gras saturés, les phospholipides présentaient des propriétés pro-inflammatoires et pro-apoptotiques (178).

Ainsi, nous avons formulé l'hypothèse que la progression de la NAFLD peut être liée à la composition même des lipides. Quelques études ont porté sur une analyse lipidomique du foie humain visant à identifier les lipides de la NAFLD (123, 179, 180). Ces études ont révélé des altérations de l'homéostasie des triglycérides, du cholestérol, des phospholipides et des acides gras à longue chaîne (LCFA) dans la NAFLD. Cependant, aucune d'entre elle n'a été en mesure de caractériser une signature lipidique spécifique de la NASH. Récemment, nous avons également utilisé une approche mathématique originale pour définir une signature lipidique de la NASH dans un modèle murin (181).

L'objectif principal de notre étude était d'établir une signature lipidique de la NASH. Les objectifs secondaires étaient d'évaluer le rôle toxique des lipides retrouvés dans cette signature.

3.1 L’article

SCIENTIFIC REPORTS

OPEN

Metabolism dysregulation induces a specific lipid signature of nonalcoholic steatohepatitis in patients

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Nonalcoholic steatohepatitis (NASH) is a condition which can progress to cirrhosis and hepatocellular carcinoma. Markers for NASH diagnosis are still lacking. We performed a comprehensive lipidomic analysis on human liver biopsies including normal liver, nonalcoholic fatty liver and NASH. Random forests-based machine learning approach allowed characterizing a signature of 32 lipids discriminating NASH with 100% sensitivity and specificity. Furthermore, we validated this signature in an independent group of NASH patients. Then, metabolism dysregulations were investigated in both patients and murine models. Alterations of elongase and desaturase activities were observed along the fatty acid synthesis pathway. The decreased activity of the desaturase FADS1 appeared as a bottleneck, leading upstream to an accumulation of fatty acids and downstream to a deficiency of long-chain fatty acids resulting to impaired phospholipid synthesis. In NASH, mass spectrometry imaging on tissue section revealed the spreading into the hepatic parenchyma of selectively accumulated fatty acids. Such lipids constituted a highly toxic mixture to human hepatocytes. In conclusion, this study characterized a specific and sensitive lipid signature of NASH and positioned FADS1 as a significant player in accumulating toxic lipids during NASH progression.

Nonalcoholic fatty liver disease (NAFLD) is a pathological condition involving a broad spectrum of lesions ranging from nonalcoholic fatty liver (NAFL) so-called steatosis to nonalcoholic steatohepatitis (NASH). It has been established that NASH may progress to hepatic fibrosis, cirrhosis and hepatocellular carcinoma[1,2]. NAFLD is a systemic disease associated with obesity, insulin resistance, type 2 diabetes mellitus and metabolic syndrome[3]. In addition, NAFLD can also be found in non-obese individuals and its prevalence can range from 3.5% to 27% in lean individuals[4]. Therefore, the dramatic increased incidence of NAFLD makes it the most common cause of chronic liver diseases and a major public health problem worldwide[5,6].

The hallmark of fatty liver disease is an intracellular accumulation of lipids, and particularly triglycerides, which causes the formation of lipid droplets in hepatocytes. This accumulation results from an imbalance between the uptake, synthesis, export and oxidation of fatty acids[7]. Fatty liver is a reversible and asymptomatic lesion that has long been considered as benign. However, it is now admitted that fatty liver is a precursor for steatohepatitis

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	Control n = 7	NAFL1 n = 9	NAFL2 n = 12	NAFL3 n = 18	NASH_Lds n = 15	NASH_Vds n = 7
Gender F/M	5/2	6/3	6/6	4/14	10/5	7/0#
Age (years)	36.4 ± 5.2*	49.7 ± 5.7	60.1 ± 3.5	61.1 ± 3.6	54.7 ± 2.5	54.6 ± 3.9
BMI (kg/m^2)	21.0 ± 1.0	22.1 ± 1.1	26.1 ± 1.3*	27.7 ± 0.8*	31.5 ± 1.6*	42.9 ± 1.8*†
Fasting glucose (mmol/L)	6.1 ± 0.4	6.9 ± 1.3	5.1 ± 0.3	6.1 ± 0.4	7.2 ± 0.6	5.1 ± 0.2
AST (normal range 0–65 IU/L)	27.4 ± 2.5	18.5 ± 3.7	82.2 ± 52.1	29.2 ± 2.9	33.8 ± 3.1	35.4 ± 3.2
ALT (normal range 0–65 IU/L)	29.6 ± 4.2	19.0 ± 3.6	59.4 ± 24.2	34.7 ± 4.9	40.6 ± 5.1	49.9 ± 5.3
γ-GT (IU/L)	106.0 ± 41.3	50.4 ± 6.3	193.1 ± 93.6	83.5 ± 22.6	129.2 ± 29.8	36.7 ± 6.5*†
Alkaline phosphatase (IU/L)	132.9 ± 42.8	87.8 ± 7.3	182.5 ± 68.0	91.0 ± 8.3	114.3 ± 25.7	69.9 ± 6.4
Total bilirubin (mgL)	14.3 ± 2.2	12.6 ± 2.4	11.6 ± 2.4	13.9 ± 1.8	10.4 ± 1.2	8.2 ± 1.7
Albumin (g/L)	37.4 ± 1.1	39.4 ± 0.9	38.5 ± 0.8	35.7 ± 0.8	37.8 ± 0.7	43.6 ± 1.4
Platelets (giga/L)	271.9 ± 40.5	252.6 ± 21.8	267.5 ± 32.6	224.2 ± 16.7	230.8 ± 26.3	279.0 ± 11.3
Steatosis grade (%)	0	15 ± 3	32 ± 8	53 ± 5	58 ± 6	81 ± 3
NAFLD activity score (NAS)	0.6 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	2.5 ± 0.2	5.9 ± 0.2*	5.0 ± 0*
Fibrosis stage, 0/1a/1b/1c/2/3						
n patients	5/2/0/0/0/0	6/2/1/0/0/0	7/3/1/0/1/0	5/6/5/0/2/0	0/6/3/1/3/2	0/5/1/1/0/0
(%),	(71/19/0/0/0/0)	(67/22/11/0/0/0)	(59/25/8/0/8/0)	(28/33/28/0/11/0)	(0/40/20/7/20/13)	(0/72/14/14/0/0)

Table 1. Characteristics of the study population. All patients are Caucasian. Data are expressed as mean ± SEM. The different groups were compared using ANOVA-test. *$p < 0.05$ *versus* Control (unpaired *t*-test); †$p < 0.01$ *versus* NASH_Lds (unpaired *t*-test). Genders repartition between the 5 groups of patients with $p = 0.05317$ and # between 6 groups of patients $p = 0.008243$ by Kruskal-Wallis rank sum test. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; F: female; γ-GT, gamma-glutamyl transferase; m: male; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis. Control, NAFL1, NAFL2, NAFL3 and NASH_Lds groups of patients selected at Paul Brousse Hospital (Villejuif, France). NASH_Vds are patients from L'Archet Hospital (Nice, France). NASH_Lds: learning dataset cohort; NASH_Vds: validation dataset cohort.

defined by the presence of steatosis, ballooning of hepatocytes, Mallory's bodies and lobular inflammation with infiltrated macrophages and leukocytes on liver histology[8,9]. In line with this assumption, we have demonstrated using transcriptomic analysis that genes involved in inflammatory processes were significantly up-regulated in patients with bland steatosis[10] leading to the main idea that NAFL is not so benign[11], and suggesting that lipids themselves may trigger inflammation. More precisely, it has been reported that some lipid species such as saturated fatty acids, phospholipids and disturbances in ceramide-signaling or cholesterol content exhibit pro-inflammatory and pro-apoptotic properties[7,12–14]. Therefore the progression of fatty liver to NASH may be related to the lipid composition. A few studies in human have focused on comprehensive hepatic lipidomic analysis in order to identify the lipids involved in NAFLD[15–17]. These studies revealed changes to the homeostasis of triglycerides, cholesterol, phospholipids and long-chain fatty acids (LCFA) in that context. However, none of these studies was able to characterize a specific lipid signature and mechanism for NASH. Therefore, identifying lipids with potential toxicity related to the progression of NASH is still an unmet need.

In this study, we have performed a comparative lipidomic analysis on human liver biopsies from patients with NAFL or NASH. We employed the unbiased mathematical approach that we recently implemented on animal models. Indeed, we have demonstrated that a random forests-based mathematical approach was enabled to characterize hepatic lipid signature specific to NASH in murine models[18]. In patients with NASH, we showed here the universal hallmark of the lipid signature of NASH that was related to alterations of the metabolic pathway involved in the synthesis of LCFA and very long-chain fatty acids (VLCFA). Finally, we demonstrated that lipids selectively accumulated in the context of NASH constituted a mixture highly toxic to human hepatic cells.

Results

Lipidomic and machine learning analysis revealed a lipid signature of NAFLD. A quantitative lipidomic analysis was performed in order to identify lipids discriminating the pathological statuses of the liver. The study was realized on 61 liver biopsies including normal livers as controls (n = 7), nonalcoholic fatty livers (NAFL, n = 39) and nonalcoholic steatohepatitis (NASH, n = 15), from the same hospital (Hôpital Paul Brousse, Villejuif, France) (Table 1). Lipids were extracted from the liver tissue and further identified by gas phase or liquid phase chromatography coupled to mass spectrometry (GC/LC-MS). This led to the identification and quantification of 104 lipid species such as cholesterol, cholesteryl esters (CE, n = 3), one diacylglycerol (DG), triglycerides (TG, n = 5), fatty acids (n = 21), ceramides (Cer, n = 4), phosphatidylcholines (PC, n = 18), phosphatidylethanolamines (PE, n = 16), phosphatidylinositols (PI, n = 14), phosphatidylserines (PS, n = 11) and sphingomyelins (SM, n = 11).

First, we sought to distinguish different grades of steatosis among the 39 liver biopsies from NAFL. Thus, in order to avoid any bias related to an imbalance and to assure a homogenous repartition of patients between NAFL groups, we employed the classification and regression trees (CART) analysis. Indeed, we have previously

demonstrated by using this approach that the total amount of triglycerides (TG) allowed the unbiased discrimination of the grade of steatosis[19] much better than histological examination usually considered as the "gold standard"[20]. In the present study, CART approach led to individualize 3 groups of nonalcoholic fatty livers based on the total amount of TG, namely NAFL1 ($41.7 < TG < 220$ nmol/mg of protein; $n = 9$), NAFL2 ($220 < TG < 465.5$ nmol/mg of protein; $n = 12$) and NAFL3 ($TG > 465.5$ nmol/mg of protein; $n = 18$) (see Supplemental Fig. S1). We confirmed whether the number of patients per group was suitable for further statistical analyses. We performed a statistical parametric test by multivariate analysis of variance (MANOVA) that showed a minimum number of 54 patients with an average of 9 patients per group. Our study groups that enrolled a total of 61 patients was in the upper range compared to other studies published in the field (Table 1)[15,21–24].

Investigations were further conducted using a random forest-based machine learning approach. This unbiased statistical analysis allows comparing several groups -each exhibiting hundreds variables- thus leading to characterize the predictor variables of a given status[18,25]. Studies were performed on the whole set of data corresponding to 104 variables from the 61 liver biopsies distributed in 5 groups including normal liver ($n = 7$), NAFL1 ($n = 9$), NAFL2 ($n = 12$), NAFL3 ($n = 18$) and NASH called "learning data set" (NASH_Lds, $n = 15$). After calculating the best number of randomly preselected splitting variables ($mtry = 28$, see Supplemental Fig. S2a), random forests analysis led to the characterization of a signature constituted by 32 lipids based on mean decreased accuracy (MDA) and mean decreased Giny (MDG) scores (Fig. 1a, see Supplemental Fig. S2b). Such a signature allowed discriminating the 5 groups from each other (Fig. 1b) and was associated to out-of-bag (OOB) estimate of error rate of 14.75% (see Supplemental Fig. S2b). Indeed, normal livers were distinguished from low level of steatosis NAFL1 along the first dimension that is related to the highest variance. The three groups NAFL1, NAFL2 and NAFL3 were well discriminated (Fig. 1b). Finally, the NASH_Lds patients appeared as a compact group onto the second dimensions of the plot that was completely separated from the other groups (Fig. 1b).

The study was further focused on the validation of the lipid signature of NASH. Additional liver biopsies from 7 patients with NASH called "validation data set" (NASH_Vds) were obtained from another hospital (Hôpital L'Archet, Nice, France). The characteristics of the study groups were summarized in Table 1. Lipids were extracted from these biopsies followed by quantitative lipidomic analysis. Using the signature of the 32 lipids, the projection into the two dimensional plot of the validation group NASH_Vds was superimposed to NASH_Lds (Fig. 1c). Furthermore, receiver operating characteristic (ROC) curve demonstrated that the overall signature of 32 lipids was able to discriminate NASH_Lds (Fig. 1d, see Supplemental_Excel_File_S1) as well as NASH_Vds (Fig. 1e, see Supplemental_Excel_File_S2) from other groups of patients with 100% sensitivity and specificity (Fig. 1d and e).

Among the 32 discriminant lipids, 9 lipids were significantly decreased in NASH, mainly ceramides and phospholipids. On the other hand, 23 lipids exhibited a significant increase in NAFL and/or NASH, including 6 fatty acids, 2 cholesteryl esters, 1 diglyceride, 5 triglycerides and 9 phospholipids (Fig. 2).

Altogether, these results demonstrated that the random forests mathematical approach allowed characterizing a lipid signature of the grades of fatty liver diseases discriminating NASH patients with 100% accuracy.

Lipid signature of NASH was related to dysregulations along fatty acid synthesis pathway. The metabolic pathways deregulated in NASH were investigated. Interestingly, the 6 fatty acids increased in NASH (C14:0, C16:0, C16:1n-7, C18:1n-7, C18:1n-9 and C18:2n-6) belong to the LCFA synthesis pathway (Fig. 3). Thus, investigations were performed on the activity of the enzymes along this metabolic pathway involving elongases and desaturases. The activity of each enzyme was estimated by measuring the ratio between its product and substrate[26] based on our lipidomic analysis.

First, we focused on the two main elongases named ELOVL (elongation of very long chain fatty acids), ELOVL5 and ELOVL6. ELOVL5 activity was significantly increased (Fig. 4a, Supplemental Fig. S3a), whereas ELOVL6 activity was significantly decreased (Fig. 4b). ELOVL6 is involved in the elongation of lauric acid (C12:0) to stearic acid (C18:0) (Fig. 3). The decrease of ELOVL6 activity in NASH was consistent with the marked increase in LCFA in NASH culminating with the accumulation of myristic acid (C14:0) (Fig. 2). Investigations were further performed on the activity of the desaturases fatty acid desaturase 1 (FADS1), FADS2 and steroyl-CoA desaturase 1 (SCD1). FADS2 and SCD1 activities were increased in accordance with the increase of monounsaturated (C16:1n-7 and C18:1n-9) and polyunsaturated (C18:2n-6) fatty acids in NASH (Fig. 4c and d, Supplemental Fig. S3b and c). In contrast, a significant decrease in FADS1 activity was observed in NASH (Fig. 4e). Furthermore, the estimation of amount of substrate and product on multiple steps suggested that ELOVL6 and FADS1 were limiting enzymes along the metabolic pathway. Indeed, the decreased activity of these two enzymes was driving the global activity of the pathway significantly down in NASH patients (Supplemental Fig. S3d–h). Accordingly, this led to a significant increase in n-6 to n-3 ratio and significant decrease n-3 index in NASH patients (Fig. 3, Supplemental Fig. S3i), which are both markers of inflammatory process during NASH progression.

The decrease in FADS1 desaturase activity in NASH may constitute a bottleneck leading to the accumulation of fatty acids upstream. As a consequence, the synthesis of lipids downstream of this enzyme such as the eicosanoid precursors (arachidonic acid (C20:4n-6), eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3)) exhibited a significant decrease in livers of NASH compared to controls (Fig. 5a). It should be noted that eicosanoid precursors are involved in the synthesis of phospholipids. Therefore, the deficiency in the synthesis of polyunsaturated LCFA has to produce a global decrease in phospholipids that was observed in NASH patients (Fig. 5b).

To determine if changes in desaturase and elongase activities were related with their expression levels, liver mRNA gene expressions of *ELOVL5*, *ELOVL6*, *FADS1*, *FADS2* and *SCD1* were investigated by RT-Q-PCR on NASH compared to normal livers and NAFL matched NASH patients. To avoid any variations of mRNA gene expression levels due to the age, gender, BMI and steatosis grade (*i.e.* the amount of lipid content), patients from NAFL2 group were matched to NASH patients (Fig. 2 and see Supplemental Fig. S4). We first verified

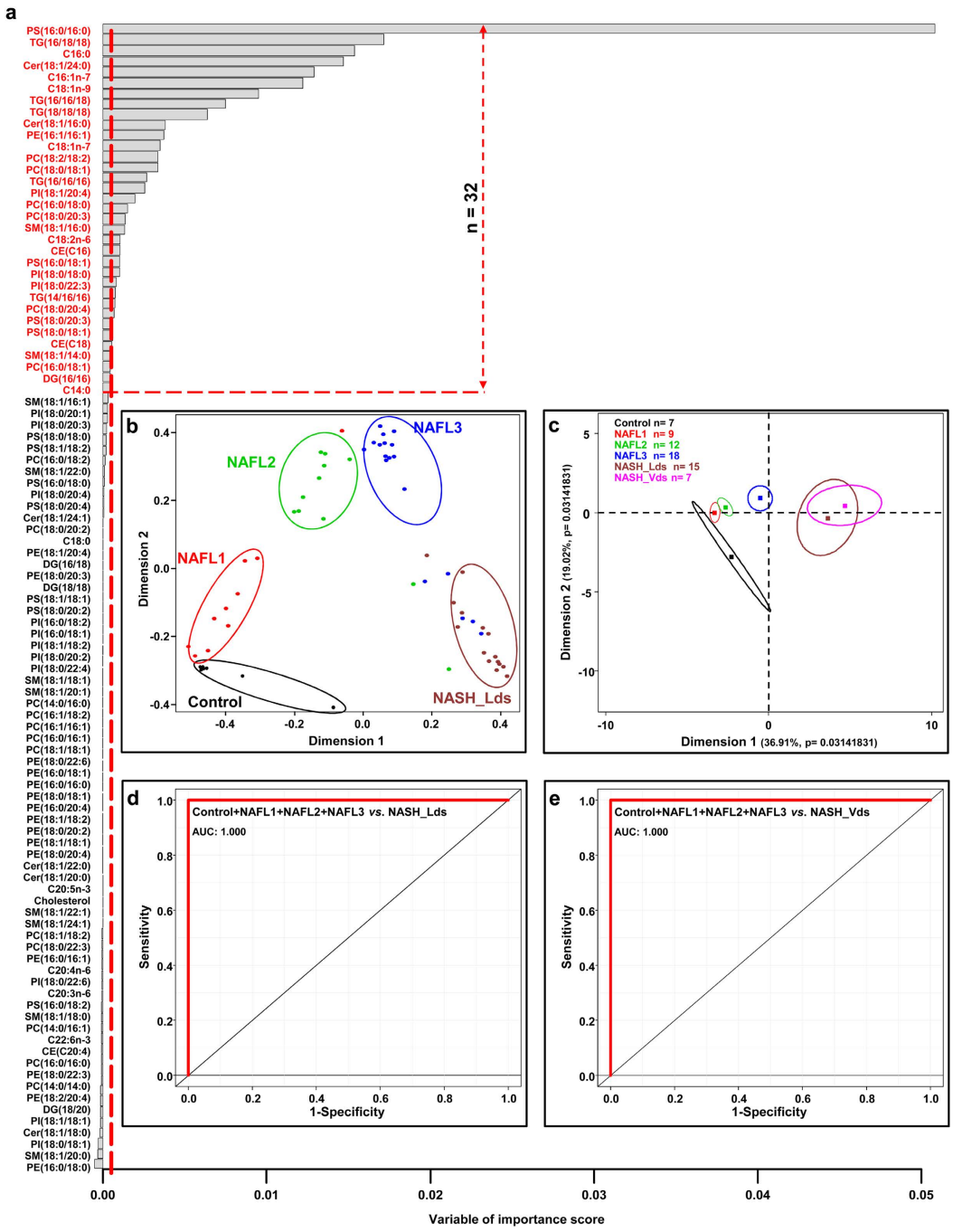

Figure 1. Selected lipids using random forests analysis discriminate NASH patients. (**a**) Data are represented as a bar plot of a matrix with 104 lipids (column) analyzed by mass spectrometry and ordered based on their variable of importance score. The threshold in the x-axis is calculated as the absolute value of the less abundant lipid represented as vertical red dot line. Discriminant lipids (n = 32) are those over the horizontal red dot line. Random forests analysis was run with the 5 groups of patients. (**b**) Multidimensional scaling plot discriminating NASH_Lds group from Control, NAFL1, NAFL2 and NAFL3 groups based on random forests results. (**c**) Principal component analysis based on the 32 lipids identified discriminating specifically NASH patients (NASH_Lds). Lines are the ellipses centered to the mean (colored squares) representing 95% interval confidence, and p the probability associated with the F- test of the analysis of variance along the axes. ROC curves based on the 32 lipids combined comparing (**d**) NAFLD groups of patients (*i.e.* Control + NAFL1 + NAFL + NAFL3) and NASH group (NASH_Lds) from Paul Brousse Hospital, and (**e**) NASH group (NASH_Vds) from L'Archet Nice Hospital and analyzed with Youden's test. • Control n = 7; • NAFL1 n = 9; • NAFL2 n = 12; • NAFL3 n = 18; • NASH_Lds n = 15, • NASH_Vds n = 7. AUC: Area under the curve; CE: cholesteryl ester; Cer: Ceramides; DG: diacylglycerols; NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis; PC: Phosphatidylcholines; PE: Phosphatidylethanolamines; PI: Phosphatidylinositols; PS: Phosphatidylserines; PV: predictive value; SM: Sphingomyelins, TG: triglycerides.

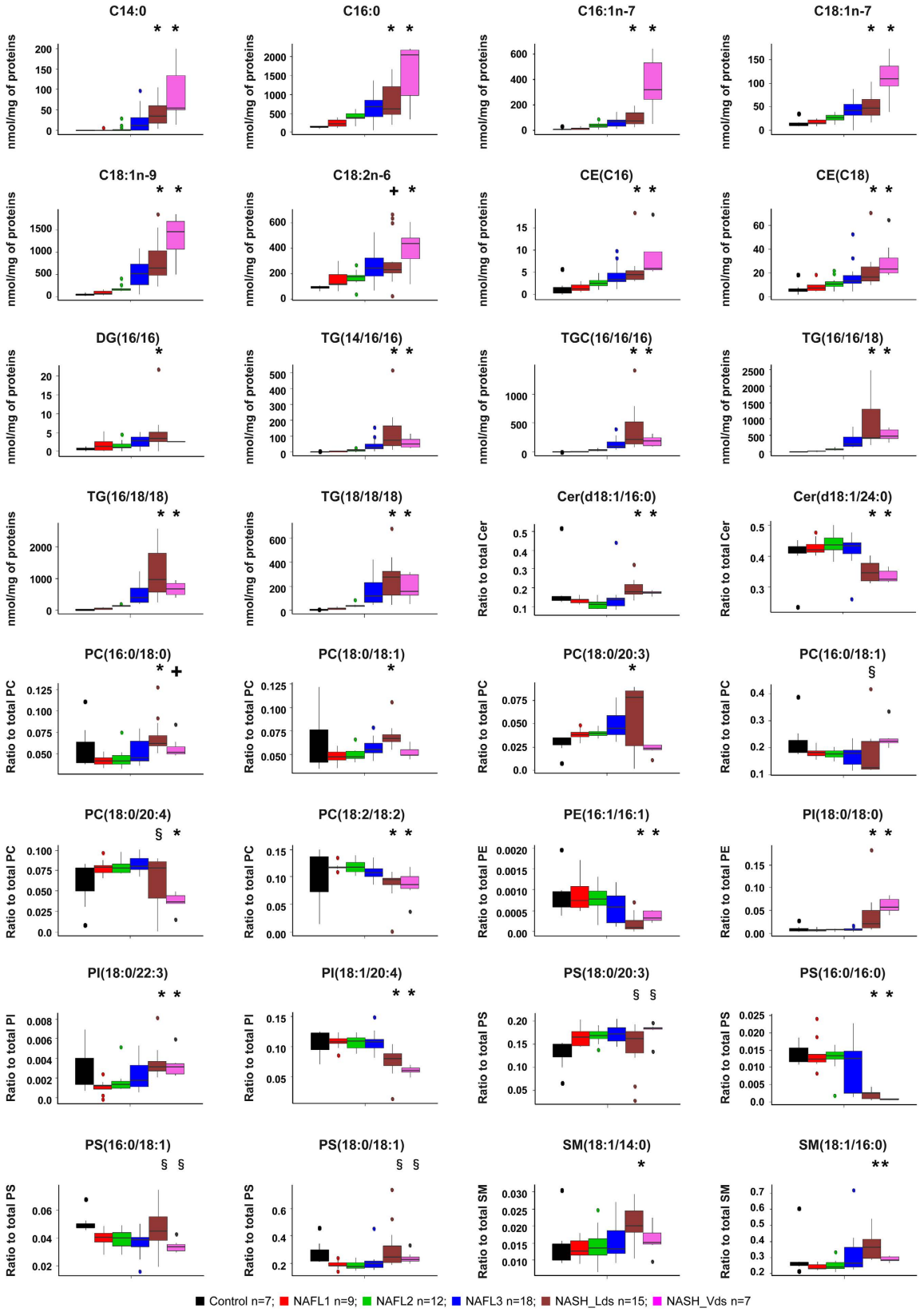

Figure 2. Hepatic levels of the 32 lipids discriminating NASH group based on random forests analysis. Data are represented as boxplot. *$p < 0.05$, by unpaired *t*-test compared to Control, NAFL1, NAFL2 and NAFL3 groups. $^{+}p < 0.05$ by unpaired *t*-test compared to Control, NAFL1 and NAFL2 groups. $^{§}p < 0.05$ by unpaired *t*-test compared to Control group. Unpaired *t*-test was done after ANOVA test. ■ Control n = 7; ■ NAFL1 n = 9; ■ NAFL2 n = 12; ■ NAFL3 n = 18; ■ NASH_Lds n = 15; ■ NASH_Vds n = 7. NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis; NASH_Lds: learning dataset; NASH_Vds: validation dataset.

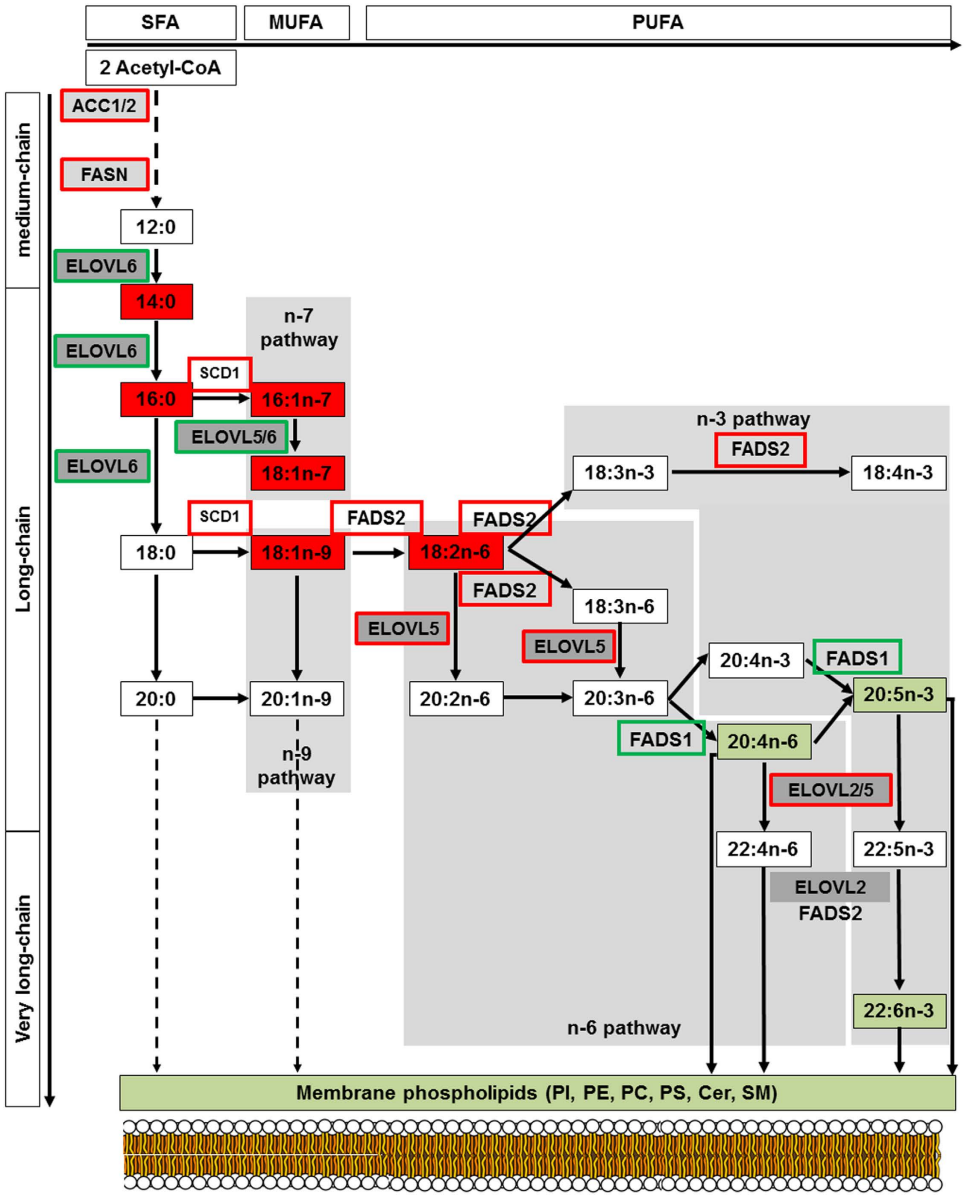

Figure 3. Scheme of short-, long- and very long-chain fatty acid biosynthesis leading to membrane phospholipids synthesis. The long chain saturated fatty acids and unsaturated fatty acids of the n-3, n-6, n-7 and n-9 series can be synthesized from myristic acid (C14:0) and palmitic acid (C16:0) produced by ACC and FASN. Long-chain fatty acids of the n-6 and n-3 series can also be synthesized from precursors obtained from dietary precursors to elongation (ELOVL) and desaturation (FADS) steps as indicated in these pathways. Lipids in red and in green are those found "up" and "down" in our analysis, respectively. Increase in enzyme activities is framed in red whereas a decrease is framed in green. ACC: acetyl-CoA carboxylase; ELOVL: elongase of very long chain fatty acid; FASN: fatty acid synthase; FADS: fatty acid desaturase; SCD: stearoyl-CoA desaturase.

that the lipid signature in these subgroups of patients was identical to the whole group of patients (Fig. 2 and Supplemental Fig. S4, respectively). *ELOVL5* mRNA expression was slightly but significantly increased in NASH compared to NAFL2 group (Supplemental Fig. S5a) whereas *ELOVL6* mRNA liver expression was significantly decreased in NASH patients (Supplemental Fig. S5b), consistent with low enzyme activity observed in NASH. The gene expression of *FADS2* and *SCD1* were significantly increased in NASH patients (Supplemental Fig. S5c and d) according to the enzyme activities observed. Regarding *FADS1*, liver mRNA gene expression level was similar in NASH compared to control group and only a slight decrease was observed in NASH compared to NAFL2 group (Supplemental Fig. S5e). Studies were further focused on genes related to *de novo* fatty acids synthesis such as sterol regulatory element-binding proteins 1c (*SREBP1c*), fatty acid synthase (*FASN*) and acetyl-CoA carboxylase 1 (*ACC1*) (Fig. 3). As previously reported in human livers[27–33], *SREBP1c* expression was significantly decreased in NAFL patients compared to control whereas it was increased in NASH patients compared to NAFL2 (Supplemental Fig. S5f). Interestingly, *FASN* and *ACC1* gene expression levels were significantly decreased in

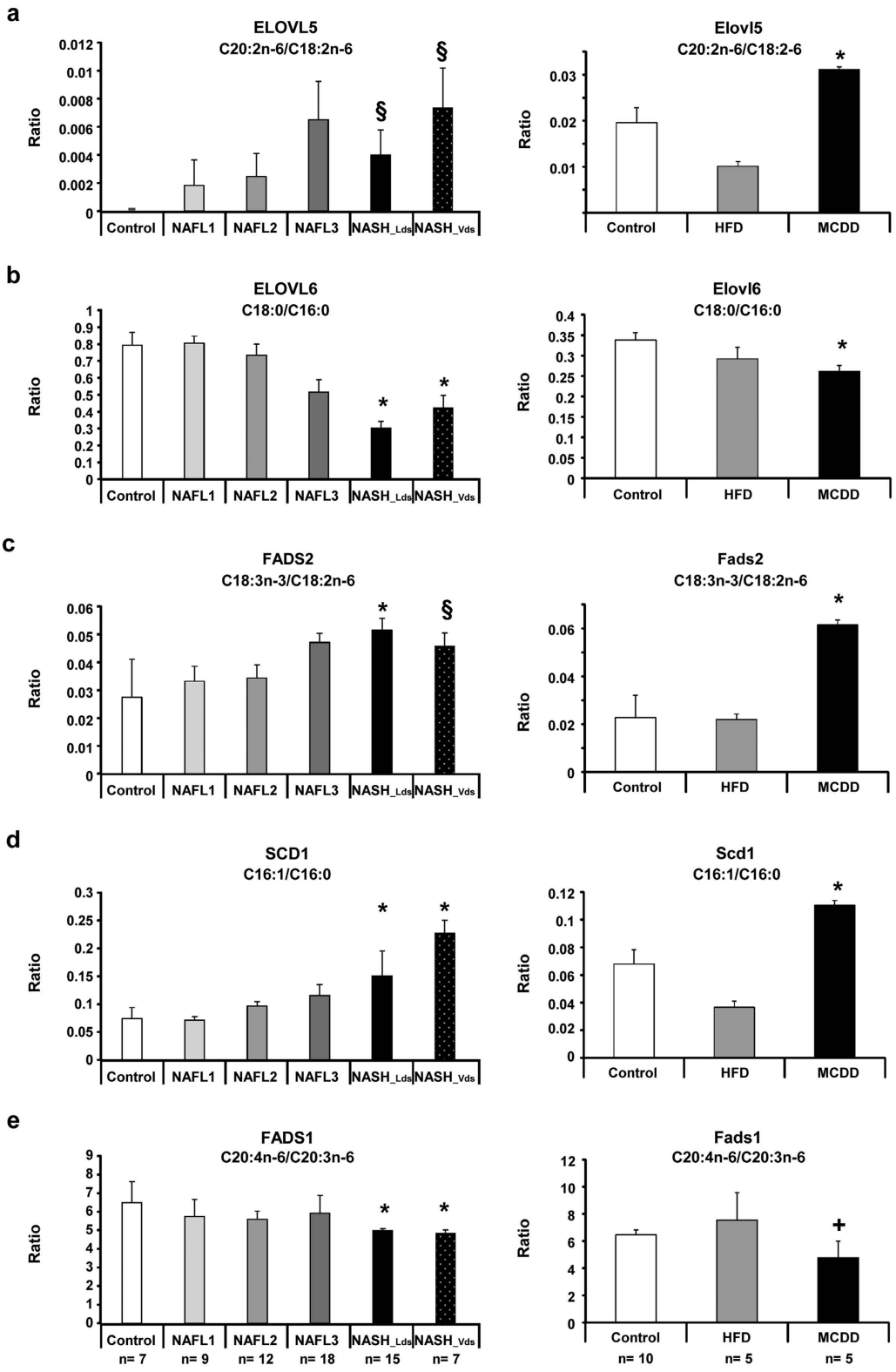

Figure 4. Decrease in ELOVL 6 and FADS1 activities in NASH patients and mouse feeding a methionine-choline deficient diet. In order to evaluate enzyme activities, ratio between product to precursor of each reaction has been used in human groups (left panel) and mouse models (right panel). (**a**) Evaluation of ELOVL5 activity using C20:2n-6 to C18:2n-6 ratio, (**b**) ELOVL6 activity using C18:0 to C16:0 ratio, (**c**) FADS2 activity using C18:3n-3 to C18:2n-6 ratio, (**d**) SCD1 activity using C16:1 to C16:0 ratio and (**e**) FADS1 activity using C20:4n-6 to C20:3n-6 ratio. Data are shown as mean ± SEM. *$p < 0.05$ by unpaired *t*-test compared to each other groups. +<0.05 by unpaired *t*-test compared to Control and $^{\S}p < 0.05$ by unpaired *t*-test compared to Control. NAFL1 and NAFL2 after ANOVA analysis. Control patients n = 7; NAFL1 n = 9; NAFL2 n = 12; NAFL3 n = 18; NASH_Lds n = 15; NASH_Vds n = 7. Control mice n = 10; HFD n = 5; MCDD n = 5.

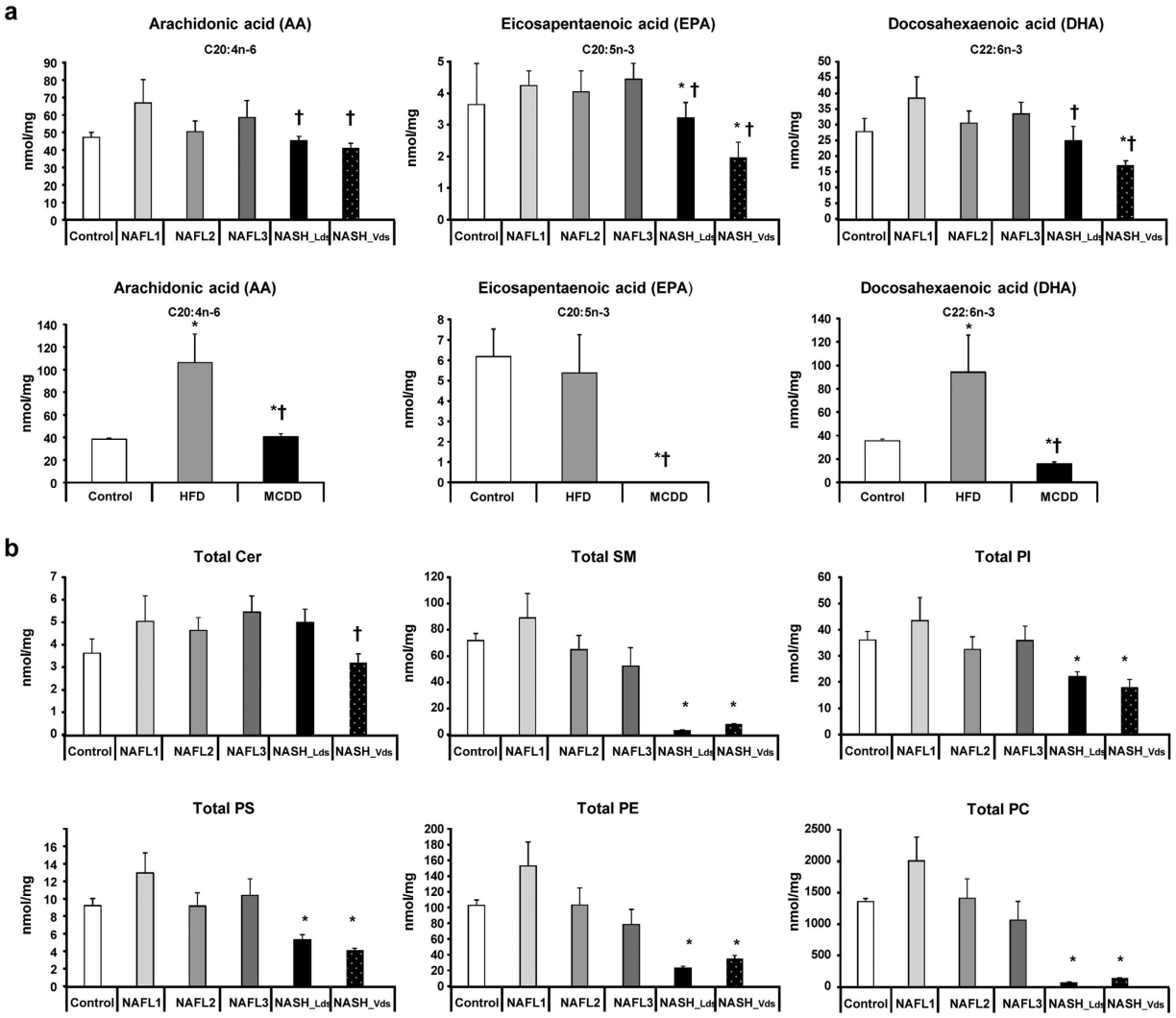

Figure 5. Decrease in eicosanoid precursors in NASH. Hepatic levels of (**a**) arachidonic acid (C20:4n-6), eicosapentanoic acid (C20:5n-3), and docosahexaenoic acid (C22:6n-3) in the patients studied (upper panel) and mouse models (lower panel). (**b**) Total phospholipids in each group of patients. Data are means ± SEM. $^{*}p < 0.05$, by unpaired *t*-test compared to Control group and $^{\dagger}p < 0.05$, by unpaired *t*-test compared to NAFL groups after ANOVA analysis. Control n = 7; NAFL1 n = 9; NAFL2 n = 12; NAFL3 n = 18; NASH_Lds n = 15; NASH_Vds n = 7 and Control n = 10; HFD n = 5; MCDD n = 5.

NAFL group but significantly increased in NASH, suggesting that *de novo* fatty acid synthesis may contribute to LCFA accumulation in NASH (Supplemental Fig. S5g and h).

Altogether, these results highlighted the major dysregulation of fatty acid synthesis pathway in NASH. Changes in lipid composition in NASH resulted of the additional effect of increase in *de novo* short-chain fatty acids synthesis and in FADS2 and SCD1 activities associated to a decrease in ELOVL6 and FADS1 activities. These results also positioned FADS1 as a bottleneck leading upstream to the accumulation of LCFA, and downstream to the deficiency in VLCFA and thus in phospholipids synthesis.

Dysregulations along fatty acid synthesis pathway were confirmed in animal models. The metabolic features observed in human were investigated using animal models. NAFL and NASH can be induced in mice by using specific high-fat diet (HFD) and methionine-choline deficient diet (MCDD), respectively[14,34–38]. We exploited the lipidomic analysis of these mouse models that we published recently[18] to confirm the data observed in patients. Interestingly, Elovl5, Fads2 and Scd1 activities were also increased in MCDD mice (Fig. 4a,c and d, respectively). In contrast, Elovl6 and Fads1 activities were decreased (Fig. 4b and e, respectively). The amount of lipids synthesized downstream Fads1 such as arachidonic acid (C20:4n-6), eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3) were dramatically decreased in livers of mice fed MCDD (Fig. 5a).

These observations demonstrated the common metabolic dysregulation along the fatty acid synthesis pathway in both human patients and animal models, leading to the development of NASH.

Mass spectrometry imaging on tissue section revealed spreading of lipids in NASH. A major feature of NASH revealed by our lipidomic analysis was the failure in total phospholipids (Fig. 5). The low amount of phospholipids may impact cellular membranes in which phospholipids are important components. Furthermore, it has been reported that the ratio between phosphatidylcholine (PC) and phosphatidylethanolamine (PE) can be used as a surrogate to assess cell membrane integrity[39]. Investigations were performed in both humans and mouse model thus demonstrating a significant decrease of the PC to PE ratio in NASH (Fig. 6a). These data suggested cell membrane impairments leading to a possible spreading of hepatocyte content into hepatic parenchyma. In order to address the distribution of lipids in the liver tissue, experiments were performed using time-of-flight-secondary ion mass spectrometry (ToF-SIMS) imaging. This approach allows investigating the lipid composition at the subcellular level. By rastering a tissue section, the distribution of lipids can be visualized. Mass spectrometry imaging using ToF-SIMS was performed on tissue sections from patients with NAFLD. The distribution of fatty acids C14:0 as well as C16:0, C18:0, C16:1, C18:1, C18:2 and C20:4 to consolidate data (data not shown) were addressed. The distribution of diacylglycerols (DAG) corresponding mostly to the fragmentation of triglycerides under mass spectrometry analysis was also addressed. In NAFL patients, lipids were accumulated into lipid droplets (Fig. 6b and c). In patients with NASH, the lipids were also accumulated into lipid droplets but an important diffusion was observed into hepatic parenchyma (Fig. 6b and c). It should be noted that the comparison in the lipid repartition between NAFL and NASH was performed from images exhibiting similar amount of the lipid species studied as attested by the total count (TC) values ($TC_{NAFL3} = 2.25 \times 10^5$ vs $TC_{NASH} = 2.37 \times 10^5$), thus strengthening a real difference in terms of distribution.

These results suggested a disruption of cell membrane integrity in NASH, most likely due to a defect in phospholipids, leading to a leak and spreading hepatocyte content out into the parenchyma.

Specific mixture of lipids accumulated in NASH exhibited higher toxicity on hepatocytes. The toxicity of the lipids identified in NASH was addressed. Studies were focused on 5 fatty acids accumulated in NASH and available for cell culture. Toxicity of myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n-7), vaccenic acid (C18:1n-7) and oleic acid (C18:1n-9) were investigated in cell culture on HepG2 cells and human primary hepatocytes (HPH). Lipotoxicity was first addressed for each individual lipid at various concentrations (50, 100, 250, 500 and 1000 μM). HepG2 cell line and HPH showed different sensitivities to lipids most likely due to differences in the lipid metabolism of such cells[40]. Moreover, the 5 lipids at the highest concentration exhibited toxicity on both HepG2 cells and HPH by triggering 25% to 90% cell death (Supplemental Fig. S7a and b). Toxicity of combined lipids was further investigated. Three mixes corresponding to the composition and proportion of the 5 fatty acids into the normal liver, NAFL2/3 and NASH were composed based on the mean concentrations obtained from our lipidomic analysis, respectively (Table 2). HepG2 cells and HPH were incubated with such mixes at the same final concentrations from 50 μM to 1000 μM. Interestingly, NASH mix was significantly more toxic on both hepatic cells. Furthermore, lipotoxicity of the NASH mix was also observed at low concentration (Fig. 6d and e).

These results demonstrated the potent toxicity of the specific mixture of lipids accumulated in NASH.

Discussion

The aim of this study was to characterize lipid markers specific to all patients with NASH independently of patient's background such as mild (between 30 and 35 kg/m^2), severe (between 35 and 40 kg/m^2) or morbid (>40 kg/m^2) obesity associated or not with type II diabetes assessed by the homeostasis model assessment of insulino-resistance ("low-HOMA-IR" or "High-HOMA-IR"), and/or a metabolic syndrome. In order to find universal lipid markers for the NASH, we intentionally not focused in patients' medications or systemic complications such as type II diabetes and insulin-resistance as well as their polymorphisms or even gender. Difference in female gender distribution between groups of patients was not linked to the progression of the steatohepatitis but due to the selection between both hospitals due to their specificities (*i.e.* liver diseases and liver transplantation *vs* bariatric surgery). Also, the role between genders and steatohepatitis progression is not elucidated yet[8,41,42].

Here, we used an innovative unbiased random forests-machine learning statistical analysis[25] that was never used before to find biomarkers in patients. This approach allowed not being dependent of the size of the patient cohort which is usually a limiting factor when analyzing multiple groups of patients involving hundreds of variables using conventional statistical methods (*e.g.* ANOVA, MANOVA).

For the first time, this study established a lipid signature of nonalcoholic steatohepatitis based on the quantification of 32 lipids. Such complex signature highlighted the major interest of combining a global approach such as lipidomic with an unbiased RF analysis. Indeed, none of the lipids identified allowed by itself the discrimination of NAFL or NASH, but in contrast the overall lipid signature allowed discriminating between control patients, NAFL groups and NASH patients. The robustness of the lipid signature of NASH was underlined by 100% specificity and 100% sensitivity on samples from two independent hospital centers. Indeed, using two independent groups of patients with NASH from these hospitals emphasizes the sturdiness and the universality of the lipid signature of NASH. Moreover, the main difficulty for a pathologist is to discriminate between steatosis and steatohepatitis, especially before the inflammation and hepatocyte degenerations appeared (*i.e.* ballooning and Mallory's hyaline bodies). Therefore, this specific lipid signature is able to discriminate between both steatosis and steatohepatitis.

Dysregulations of the metabolic pathway involved in synthesis of fatty acids were highlighted in NASH. The major impact of alterations in this metabolic pathway was reinforced by the similar biochemical features (*i.e.* dysregulation of enzyme activities) observed in both human and animal models for this pathology, strengthening the idea that these alterations are universal in NASH. Indeed, we used animal models that developed fatty liver when fed on high fat diet or NASH when fed on methionine choline deficient diet. It should be noted that MCDD mouse model is the most well-known and used model to study NASH[18,21,34–37,43]. A short period of 5 weeks is

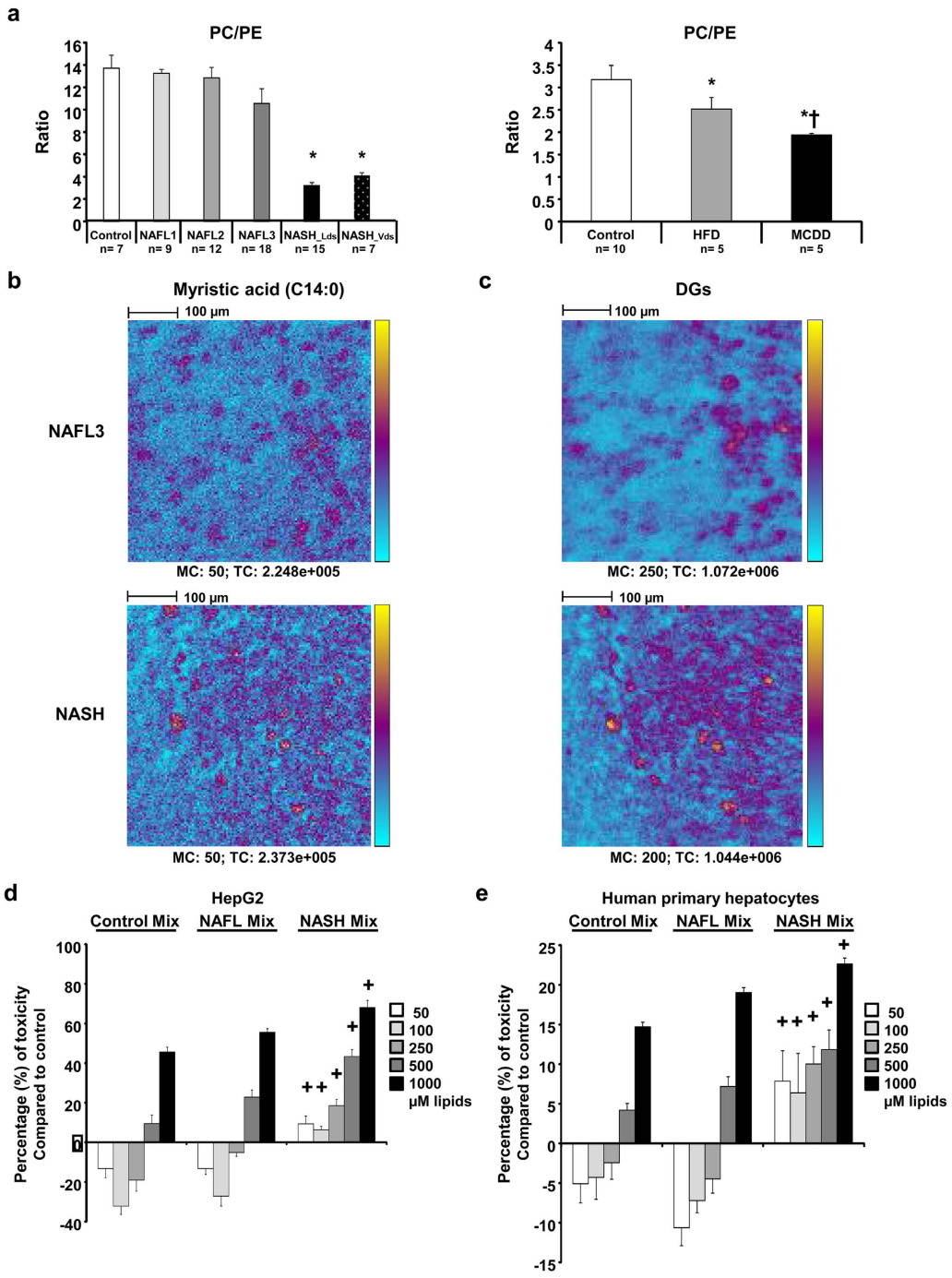

Figure 6. Membrane integrity is disrupted in NASH patients and mice leading to release of lipids in parenchyma. (**a**) Phosphatidylcholine (PC) to phosphatidylethanolamine (PE) ratio in the patients studied (left panel) and mouse models (right panel). (**b**) Myristic acid (C14:0) and (**c**) sum of all diacylglycerols (DG) liver contents in NAFL3 and NASH patients. Both selected patches regarding myristic acid were matched according to the total count (TC). Each patch is representative of the 6–12 patches recorded from central vein to portal triad. Between 2 to 3 slices per sample were processed. Color scale bar with amplitude in number of counts are indicated to the right of each image. Field of view of 500 µm × 500 µm. Scale bars: 100 µm. Data are means ± SEM. $^{*}p < 0.05$ by unpaired *t*-test compared to Control group and $^{\dagger}p < 0.05$ by unpaired *t*-test compared to NAFL groups after ANOVA analysis. Control n = 7; NAFL1 n = 9; NAFL2 n = 12; NAFL3 n = 18; NASH_Lds n = 15, NASH_Vds n = 7 and Control n = 20; HFD n = 5; MCDD n = 5 mouse males. (**d**) HepG2 cell and (**e**) human primary hepatocytes treated with lipid mixes at different concentrations. Cells are treated in triplicate during 24 h with 50. 100. 250. 500 and 1000 µM final concentration of lipids with lipid mixes (Control Mix. NAFLD Mix. NASH Mix) based on the percentage of C14:0, C16:0, C16:1n-7, C18:1n-7, and C18:1n-9 found in liver tissues of control. NAFL2/3 and NASH patients. Two independent experiments were done. Data are mean ± SEM. $^{+}p < 0.05$ by unpaired *t*-test compared to Control Mix and NAFL Mix at the same concentrations after ANOVA test.

	Patients	Myristic acid	Palmitic acid	Palmitoleic acid	Vaccenic acid	Oleic acid	Total
FA concentration* in human livers expressed in nmol/mg of proteins	Control	0	138.04	10.54	14.32	52.52	215.41
	NAFL2 + 3	14.24	558.87	56.37	35.87	393.44	1058.81
	NASH	56.95	1044.95	180.52	70.89	976.53	2329.34
Percentage of FA in liver tissues	Control	0	64	5	24	7	100
	NAFL2 + 3	1.5	53	5.5	3	37	100
	NASH	2.5	45	7.5	3	42	100

Table 2. Concentrations and proportions of the 5 discriminant fatty acids in human livers. FA: fatty acid; NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis. *Mean of each lipid assessed by lipidomic analysis found in each group of patients: Control (n = 7), NAFL 2 and NAFL3 (n = 30), NASH (n = 22).

enough to develop NASH. Thus, the murine models led to control the environment and to confirm the observations found in humans. Most of the time when NASH is diagnosed in human patients, the disease is already progressing and therefore the main underlying mechanism is blunt by subsequent modifications during the chronic phase. Finally, using mouse models permitted to control food and obesity and demonstrated that the decreased in FADS1 activity in mice fed MCDD and human patients with NASH is not dependent of diet and obesity as demonstrated also by others[44].

The concomitant increase of saturated and unsaturated LCFA with the significant decrease of polyunsaturated VLCFA resulted from the decreased activity of FADS1. Impairment of FADS1 activity has created a bottleneck leading to the accumulation upstream of fatty acids up to 20 carbons as also described before[45], but interestingly in our study we found it associated to the NASH. This phenomenon was accentuated by an increase in *de novo* fatty acids synthesis as demonstrated by the increase in mRNA expression levels of *ACC1* and *FASN*. Furthermore, decreased expression and activity of ELOVL6 contributed also to the marked increase of LCFA culminating in NASH with the accumulation of myristic acid (C14:0). Recently, it has been demonstrated that fatty acid metabolism was altered in NASH independent of obesity and diet[44]. Expression and activities of FADS1 (also called delta-5 desaturase, D5D), FADS2 (also called delta-6 desaturase, D6D) and SCD1 were dysregulated. Lower activity of FADS1/D5D was observed[44] in NASH groups and was independent of diet and obesity as we demonstrated in our study in both patients and murine models. In addition, FADS2 and SCD1 mRNA liver expression were significantly increased in NASH group according to our observations and by others[44]. On the other hand, a consequence of the impaired expression and activity of FADS1 was the extremely low amount of polyunsaturated LCFA added to the hepatic imbalance between n-6 and n-3 levels. Indeed, our results demonstrated that n-6 to n-3 ratio was significantly increased, associated with a significant decrease in n-3 index in livers of both patients with NASH and MCDD mice, according to recent studies[26,45–47]. Such metabolic alterations may generate broad effects since LCFA represent substrates for the synthesis of eicosanoids and phospholipids, thus impacting the properties of membranes. LCFA also serve as substrate precursors for the biosynthesis of lipid signaling molecules with pro-inflammatory properties[48–50]. Therefore, the current study strengthened the central role of FADS1 in lipid homeostasis and positioned this desaturase as a major player in NASH[45].

Thus, due to the impairment in FADS1 activity, the deficiency in phospholipid synthesis can damage the cellular membranes in which phospholipids are major components. Indeed, half of the phospholipids from the signature were significantly down in NASH patients Furthermore, we have shown that a surrogate of membrane integrity the PC to PE ratio was significantly decreased in patients with NASH[39] and could lead to necrosis of hepatocytes[51,52]. Next, we demonstrated that indeed membrane integrity was altered in the liver tissues with NASH. Therein, mass spectrometry imaging using ToF-SIMS imaging revealed lipid spreading in the hepatic parenchyma in NASH whereas lipids were mostly located in vesicles in NAFL. Such a spreading may result of loss of membrane integrity of hepatocytes in the context of NASH. Therefore, our study showed a potent loss of membrane integrity in NASH leading to a potent toxicity of the lipids released in the hepatic parenchyma, thus favoring the progression of the pathology.

By using an original approach combining the five fatty acids from the signature, toxicity of lipids accumulated in NASH was demonstrated on the human hepatoma cell line HepG2 and HPH. Although each of the five fatty acids studied showed toxicity individually[12,13,51], the combination corresponding to the lipid composition observed in NASH based on our lipidomic analysis exhibited a much higher toxicity compared to those combination related to the composition observed in normal liver or NAFL. Our results suggested that lipotoxicity was not only related to the amount of lipids but also to their specific composition and proportion. It should be noted that the range of concentration tested from 50 μM to 1000 μM were lower than the concentration of these lipids estimated by lipidomics in the liver. This strengthened the idea that these five lipids at least could be highly toxic when in contact with neighboring hepatocytes.

In conclusion, we clearly characterized a specific and sensitive lipid signature, universal for all patients with NASH. This study highlighted dysregulations of the metabolic pathway involved in the synthesis of fatty acids and eicosanoid precursors. In particular, our study positioned ELOVL6 and FADS1 as major players in the progression to NASH. Finally, the current study suggested also a direct role of lipids accumulated in NASH in the progression of the pathology by their toxicity. This opens new avenues for further development of early diagnosis and therapeutic approaches.

Materials and Methods

Study Cohort. A total of 68 patients were enrolled in this study selected from two hospital centers (Paul Brousse Hospital, n = 61 and L'Archet Hospital, n = 7). A pathologist expert (CG) reviewed all liver biopsies. Seven patients had a normal liver forming the control group (hepatic steatosis <5%). Fifty four patients have been recorded as affected with NAFLD. To differentiate NAFL and NASH, a histological discrimination was made based on a separate system of scoring the features of NAFLD called the NAFLD Activity Score (NAS)[8]. By definition, a NAS < 5 represents NAFL and a NAS ≥ 5 represents NASH[8,42]. Clinical and biological data on general status, metabolic syndrome and liver function were retrospectively recorded. Exclusion criteria were liver diseases such as viral hepatitis B, viral hepatitis C, primary biliary cirrhosis, sclerosing cholangitis, autoimmune hepatitis, hemochromatosis, Wilson's disease, α1-antitrypsin deficiency, drug-induced liver disease and alcohol consumption more than 20 g/day for women and 30 g/day for men. The institutional review board of each hospital (Paul Brousse Hospital through Centre des ressources biologiques Paris-Sud and L' Archet Hospital) approved the study and written informed consent was obtained from all patients. Access to this material and all experiments were performed in accordance with the relevant guidelines and regulation of the French ethical laws.

Animal Models. Male C57Bl/6J mice were fed on chow diet, HFD and MCDD. Mice fed a HFD and MCDD developed NAFL and NASH, respectively as described in our previous publication[18]. A total of 20 animals underwent chow diet (n = 10, Teklad Rodent Diet no. 5053; 5% kcal from fat; 3.1 kcal/g), HFD (n = 5; 15 weeks on diet, Research Diet D12492i; 60% kcal from fat; 5.24 kcal/g) and MCD diet (n = 5; 5 weeks on diet, TekladRef# TD.90262). Mice were housed at room temperature (22–24 °C) with a 12-hour light/12-hour dark cycle. Food and water were provided *ad libitum*. Animal protocols were accepted by the Institutional Animal Care and Use Committee in Main (Jackson Laboratories) and by the "Comité d'Ethique pour l'Expérimentation Animale" registered to the "Comité National de Réflexion Ethique sur l'Expérimentation Animale 05" (Protocol # Ce5/2012/075). All experiments were performed in accordance with the relevant guidelines and regulation of each country.

Real-time quantitative PCR of genes involved in lipid metabolism. Total RNA was extracted from frozen liver biopsies using RNA-STAT 60 reagent (AMS Biotechnology Europe LTD). Quantity and quality of RNA were assessed using NanoDrop®-ND1000 (Thermo Scientific). cDNAs were generated by using the RivertAid® First Strand cDNA Synthesis (Thermo Scientific), and Syber Green from FastStart Essential DNA Green Master mixes (Roche, Life Science) were used to quantify hepatic mRNA levels with specific primers of each gene described in Supplemental Table S1.

Q-RT-PCR was performed using LightCycler® 96 Instrument (Roche, Life Science). Gene expression levels were normalized to actin RNA levels and data analyzed with LightCycler® 96 SW 1.1 software (Roche, Life Science). For each sample, the gene to actin ratio was calculated based on an arbitrary value of copies determined by the standard curve for each gene, as previously described[53].

Activity indexes of desaturases and elongases. As standard method to evaluate fatty acid-synthetizing enzyme activities, assay measurement of the rate of radiolabeled precursor FA to their respective products is used *in vitro* and *in vivo*, but for practical and ethical reasons is not possible in human studies. Therefore product-to-precursor ratio as surrogate measure to estimate desaturase and elongase activities is assessed in this study as it has been extensively used in different other studies before[17,21,26,54,55]. Using the comprehensive lipid analysis by mass spectrometry data from human and mouse livers we assessed activity indexes of desaturases and elongases belonging to the long chain and very long chain saturated, monounsaturated and polyunsaturated of fatty acid synthesis pathway as summarized in Fig. 3.

Isolation and primary culture of human hepatocytes. Normal liver tissue was obtained from adult patients undergoing partial hepatectomy at Saint Antoine Hospital (generous gift from Dr. Filomena Conti and Pr. Yvon Calmus, Paris, France). The first donor was a 63 years old woman treated for liver metastasis for colorectal adenocarcinoma. The second donor was a 36 years old female treated for hepatocellular carcinoma developed on normal liver. The third patient is a 65 years old man treated for liver metastasis of pancreatic cancer. Experimental procedures were performed in accordance with French laws and regulations. Human primary hepatocytes isolation was made based on previous protocol[56,57]. Briefly, immediately after hepatectomy liver resection specimen was stored in Celsior solution (IMTIX-SangStat), followed by a 2-steps perfusion method, less than 3 h after resection. Visible vessels were first perfused with Liver Perfusion Medium (Invitrogen) at 37 °C to eliminate blood cells. A second perfusion then was performed with collagenase- and dispase-containing Liver Digest Medium (Invitrogen) at 37 °C, at constant flow rate until the tissue was fully digested. Liver fragments were shaken gently in Hepatocyte Wash Medium (Invitrogen) to free loose cells, and then were filtered before centrifugation. The fibroblast- and Küpffer cell–containing supernatant was discarded, and hepatocytes were washed a second time before assessing viability by trypan blue dye exclusion. Cells were re-suspended in complete hepatocyte medium and seeded at a density of 5×10^5 viable cells per well onto 96-well plates that had been pre-coated with a solution type I collagen from calf skin between 1 and 10 hours before plaiting cells. The medium was replaced 16–20 hours later with fresh complete hepatocyte medium supplemented with 1 mol/L hydrocortisone hemi-succinate [56,57] and 100 units/mL penicillin, and 100 g/mL streptomycin[56,57]. All cell cultures were maintained at 37 °C in a 5% CO_2 atmosphere.

Cell culture. HepG2 cells, derived from differentiated human hepatoblastoma[51], were obtained from ATCC (Manassas, VA). Cells were cultured in DMEM containing 10% (v/v) FBS, 100 units/mL penicillin, and 100 g/mL

streptomycin. The medium was changed 12 h before treatment. All cell cultures were maintained at 37 °C in a 5% CO_2 atmosphere.

Lipids preparation for treatments. Myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n-7), vaccenic acid (C18:1n-7) and oleic acid (C18:1n-9) were all obtained from Sigma (Ref# M3128, 43051, S4751, P9417, V0384, O1257, respectively). Fatty acids were dissolved in absolute ethanol at a concentration of 40 mM stock solutions, sonicated 15 min and then warmed at 70 °C for 15 min, for complete dissolution. FA solutions were filtered through a 0.22 μm filter before use and stored at −20 °C. Then, FA were dissolved in bovine serum albumin (10% BSA, Sigma) at the final ratio 1/10 (v/v) in William's E plus Glutamax™ medium or in OptiMEM™ (Gybco, Invitrogen) and warmed again at 55 °C for 10 min before use[58]. The final concentration of ethanol did not exceed 1%. Myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) palmitoleic acid (C16:1n-7), vaccenic acid (C18:1n-7) and oleic acid (C18:1n-9) were diluted individually or mixed in bovine serum albumin and ethanol to obtain 50, 100, 250, 500 and 1000 μM at the final concentration. Three mixtures so-called Control, NAFL2/3 and NASH were prepared based on the percentage of myristic acid, palmitic acid, palmitoleic acid, vaccenic acid and oleic acid found in liver of patients (Table 2). HepG2 cells or human primary hepatocytes (HPH) were treated with each lipid individually or with the lipid mix during 24 h. Lipotoxicity was assessed by the content of total ATP into the cells (CellTiter-Glo® Luminescent Cell Viability Assay, Promega, France).

Lipid Profiling by Mass Spectrometry Analysis. Liver biopsies (5–10 mg) were homogenized in 2 ml of methanol/EGTA (2:1 v/v) with FAST-PREP (MP Biochemicals) tissue lyser for further lipid analyses. Also, the equivalent of 0.5 mg of tissues was evaporated. The dry pellets were dissolved in 0.25 ml of NaOH (0.1 M) overnight and proteins were measured with the Bio-Rad assay. The quantification of the lipids is expressed in nmol/mg of total proteins.

Briefly, lipids were extracted from liver tissues according to Bligh and Dyer[59] in dichloromethane/methanol/water (2.5:2.5:2.1, v/v/v), in the presence of the internal standards (stigmasterol, cholesteryl heptadecanoate, glyceryl trinonadecanoate) to quantify neutral lipids. Dichloromethane phase were evaporated to dryness, and the residue dissolved in 20 μl of ethyl acetate. 1 μl of the lipid extract was analyzed by gas-liquid chromatography on a FOCUS Thermo Electron system using a Zebron-1 Phenomenex fused silica capillary columns coupled to mass spectrometry according to previous publication[60].

Phospholipids for relative quantification were extracted as neutral lipids but with 2% acetic acid in the presence of the internals standards (Cer(d18:1/15:0) 16 ng; PE(12:0/12:0) 180 ng; PC(13:0/13:0) 16 ng; SM(d18:1/12:0) 16 ng; PI(16:0/17:0) 30 ng; PS(12:0/12:0) 156.25 ng). After centrifugation the organic phase was collected and dried under azote, then dissolved in 50 μL of methanol. Lipids were separated using a Kinetex HILIC column with a mobile phase A of acetonitrile and B with 10 mM ammonium formate in water at pH 3.2 following a gradient and the injection volume was 5 μL. Sample solutions were analyzed using an Agilent 1290 UPLC system coupled to a G6460 triple quadrupole mass spectrometer (Agilent Technologies) and using MassHunter software (Agilent Technologies) for data acquisition and analysis. Data were treated using QqQ Quantitative (version B.05.00) and Qualitative analysis software (version B.04.00).

For Fatty Acid Methyl Ester (FAME) analysis, homogenate were extracted as neutral lipids in the presence of the internal standards glyceryl triheptadecanoate (2 μg) and transmethylated 1 h in boron trifluoride methanol solution 10% at 55 °C. After addition of water (1 ml) to the crude, FAMEs were extracted with hexane (3 ml), evaporated to dryness and dissolved in ethyl acetate (20 μl). FAME (1 μl) was analyzed by gas-liquid chromatography on a Clarus 600 Perkin Elmer system using Famewax RESTEK fused silica capillary columns.

Time-of-Flight Secondary Ion Mass Spectrometry Imaging. *Tissue preparation.* A subset group of patients with the four grades of liver steatosis (3 patients per group) and a group of patients with NASH (4 patients) underwent for ToF-SIMS imaging analyses.

Briefly, each sample was cut at −20 °C with a CM3050-S cryostat (Leica Microsystèmes SAS, France). Tissue sections of 10 μm thickness were deposited on a gold coated glass slide (Mirr IR®, Kevley Technologies, OI, US). Before analysis, tissue sections were placed under vacuum at a pressure of a few hPa during 10 min in order to eliminate water. Before analysis, tissue samples were examined and optical images were recorded with a microscope (Olympus BX 51, Olympus France, SAS, Rungis, France) equipped with a ColorView I camera monitored by Cell^B software (Soft Imaging System GmbH, Münster, Germany). No further sample preparation was required before introduction in the mass spectrometer.

TOF-SIMS imaging acquisition. A TOF-SIMS IV mass spectrometer (ION-TOF GmbH, Münster, Germany), equipped with a Liquid Metal Ion Gun (LMIG) filled with bismuth and allowing delivery of Bi_3^+ cluster ion beam was used to localized lipids of interest directly on the liver tissues as previously described[61].

Briefly, a set of images was acquired without sample stage movement, just by resting the primary ion beam, with a field of view of 500 μm × 500 μm. For these images, the number of pixels was chosen as 256 × 256 to obtain a ~2 μm pixel size. Under these conditions the flow was fixed to 3×10^{11} ions/cm^2 for all the acquisitions, allowing acquisition time of about 10 minutes. Each area was scanned twice in order to record both positive and negative ion images.

Due to the very low initial kinetic energy distribution of the secondary ions, the relationship between the time-of-flight and the square root of m/z is always linear over the whole mass range. Consequently, the mass calibration was made with H^-, C^-, CH^-, CH_2^-, CH_3^-, C_2^-, C_3^-, and C_4H^- ions for the negative ion mode, and H^+, H_2^+, H_3^+, C^+, CH^+, CH_2^+, CH_3^+ and $C_2H_5^+$ for the positive ion mode, respectively. To refine the mass calibration,

ion peaks of cholesterol and vitamin E were used in positive ion mode, and fatty acid carboxylate ions in negative ion mode based on previous studies reported on biological samples[61–64].

Data processing was achieved using Surface Lab 6.2 software (ION-TOF GmbH, Münster, Germany). This software allows extraction of ion spectra and images from the raw data. In order to compare the relative intensity of species in the first set of experiments, a normalization of their respective mass spectrum intensities had to be performed: the intensity of the mass spectrum from each stage scan was normalized against the area of the smallest one, given that all the data had been acquired under the same experimental conditions[61,65–67].

ToF-SIMS data analysis. We selected 3 patients in each group of control, NAFL1, NAFL2 and NAFL3 as well as 4 patients with NASH. The sum of the diacylglycerols (DGs) served to localize the lipid droplets into the liver tissue. C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C20:4 were investigated using ToF-SIMS procedure in different areas of the liver. The size of each patch was $500\,\mu m \times 500\,\mu m$[61].

Statistical Analysis. Lipidomic data of patients with NASH from Paul Brousse Hospital were used as learning dataset (NASH_Lds) whereas patients with NASH L'Archet Hospital were used as validation dataset (NASH_Vds).

In order to be sure that the number of patients per group is acceptable for further statistical analyses, we performed a statistical parametric test, MANOVA (multivariate analysis of variance), using GPower software (version 3.1.9.2) and including the following parameters such as: effect size ($f^2_{(v)} = 0.25$), $\alpha = 0.05$, power ($1\text{-}\beta = 0.95$), and the number of groups ($n = 6$).

All calculations were performed using R v.3.3.1 software[68]. To analyze the homogeneity of the groups of patients with NAFL, recursive partitioning and regression trees ("*rpart*") approach was used to build a regression tree based on the predict values from the lipidomic data leading to obtain a classification analysis and a regression tree (CART). CART was applied on lipid families such as total TG, total DG, Total cholesterol, Total CE, Total SFA, Total USFA, Total MUFA, Total PUFA, Total PC, Total PE, Total PI, Total PS, Total Cer and Total SM (Supplemental Fig. S1).

In order to identify the specific dependent variables (lipids) that contributed to the significant overall effect (between different NAFL groups and between NAFL and NASH groups), a random forests (RF) analysis was used with the following R packages "*randomForest*" and "*varSelRF*" leading to obtain a narrow numbers of markers, as we published recently[18]. Briefly, RF consisted of a collection of tree predictors where each tree depended on the value of a random vector of measured variables sampled independently and with the same distribution for all trees in the forest. RF classified a case by assigning the input vector of variables to each tree of the forest. Each tree gave a classification, *i.e.* a classis voted, and the forest chose the class with the most votes from all the trees in the forest[25,69]. RF analysis was an effective tool in prediction without over-fitting and multiclass classification[25,69–71]. As in many statistical analyses leading to a lot of variables and few groups (as we face here: 104 lipids and 5 groups of patients), a crucial problem was variables not significantly relevant to explain the analyzed phenomenon (*i.e.* occurrence of NASH) and missing values, but could create a random noise which hided the main effects and the relevant predictors[25]. Thus to determine the most discriminant lipids, RF were applied using "*randomForest*" package in R. To determine the best number of predictors (*mtry*) was used for each split of the tree and *tune RF* function was used to determine the lowest *mtry* to the lowest out-of-bag (OOB) error data that was used to get a running unbiased estimate of the classification error as trees were added to the forest melding to determine the confusion matrix (Supplemental Fig. S2a and b). Also *ntree* (number of trees to be built) was set up at 1040 corresponding to the number of the columns (variable) of the matrix multiply by ten. During the analysis, the mean decreased accuracy (MDA) and the mean decreased Giny (MDG) were determined. MDA was determined during the OOB error calculation phase and lipids with a large MDA were more important for classification of the data (Supplemental Fig. S2c and d). In addition, MDG that was a measure of how each variable contributes to the homogeneity of the nodes and leaves in the resulting RF was assessed. Lipids that resulted in nodes with higher purity had a higher MDG (Supplemental Fig. S2d).

Principal component analysis, 95% confident ellipse centre to the mean and lipids of interest were computed using "*FactoMinR*" package. The global p-value was calculated using the critical probability associated with the F- test of the analysis of variance along the axes of the first and the second dimensions ($\alpha = 0.05$). Receiver operating characteristic (ROC) curve was analysed using "*pROC*" and "*Epi*" packages and compared control, NAFL1, NAFL2 and NAFL3 versus NASH patients from Paul Brousse and L'Archet Hospitals based on the 32 lipids identified by random forests. Boxplots were drawn using "*ggplot2*" and "*beeswarm*" packages. "*gplots*" and "*RColorBrewer*" packages were used for graphics.

Individual variables among the different groups of patients were shown as boxplot or barplot (mean ± standard error of the mean or SEM) and tested with analysis of variance (ANOVA-test) followed by unpaired *t*-test. Kruskal-Wallis rank sum test was used to compare gender repartition between the 6 groups of patients. Type I error-set was 5%.

Ethics approval. The institutional review board of each hospital and ethic committee (Paul Brousse Hospital Centre des resources biologiques Paris-Sud and L'Archet Hospital) approved the study and written informed consent was obtained from all patients. Access to this material was in agreement with French ethical laws.

Animal protocols were accepted by the Institutional Animal Care and Use Committee (IACUC) in Main (Jackson Laboratories) and by the "Comité d'Ethique pour l'Expérimentation Animale" registered to the "Comité National de Réflexion Ethique sur l'Expérimentation Animale 05" (Protocol # Ce5/2012/075, Paris, France). In accordance to the animal welfare and in the aim to minimize the number of animals, we used data and samples from our previous publication[18].

References

1. Angulo, P. *et al.* Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology*. **149,** 389–397 e310 (2015).
2. McPherson, S. *et al.* Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: implications for prognosis and clinical management. *J Hepatol*. **62,** 1148–1155 (2015).
3. Byrne, C. D. & Targher, G. NAFLD: a multisystem disease. *J Hepatol*. **62,** S47–64 (2015).
4. Basantani, M. K. *et al.* Pnpla3/Adiponutrin deficiency in mice does not contribute to fatty liver disease or metabolic syndrome. *J Lipid Res*. **52,** 318–329 (2011).
5. (NCD-RisC), N. R. F. C. Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants. *The Lancet*. **387,** 1377–1396 (2016).
6. Loomba, R. & Sanyal, A. J. The global NAFLD epidemic. *Nat Rev Gastroenterol Hepatol*. **10,** 686–690 (2013).
7. Neuschwander-Tetri, B. A. Nontriglyceride hepatic lipotoxicity: the new paradigm for the pathogenesis of NASH. *Curr Gastroenterol Rep*. **12,** 49–56 (2010).
8. Kleiner, D. E. *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. **41,** 1313–1321 (2005).
9. Bedossa, P. *et al.* Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology*. **56,** 1751–1759 (2012).
10. Chiappini, F. *et al.* Exploration of global gene expression in human liver steatosis by high-density oligonucleotide microarray. *Lab Invest*. **86,** 154–165 (2006).
11. Adams, L. A. & Ratziu, V. Non-alcoholic fatty liver - perhaps not so benign. *J Hepatol*. **62,** 1002–1004 (2015).
12. Joshi-Barve, S. *et al.* Palmitic acid induces production of proinflammatory cytokine interleukin-8 from hepatocytes. *Hepatology*. **46,** 823–830 (2007).
13. Pusl, T. *et al.* Free fatty acids sensitize hepatocytes to bile acid-induced apoptosis. *Biochem Biophys Res Commun*. **371,** 441–445 (2008).
14. Tanaka, N., Matsubara, T., Krausz, K. W., Patterson, A. D. & Gonzalez, F. J. Disruption of phospholipid and bile acid homeostasis in mice with nonalcoholic steatohepatitis. *Hepatology*. **56,** 118–129 (2012).
15. Muir, K. *et al.* Proteomic and lipidomic signatures of lipid metabolism in NASH-associated hepatocellular carcinoma. *Cancer Res*. **73,** 4722–4731 (2013).
16. Puri, P. *et al.* A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology*. **46,** 1081–1090 (2007).
17. Yamada, K. *et al.* Characteristics of hepatic fatty acid compositions in patients with nonalcoholic steatohepatitis. *Liver Int*. (2014).
18. Chiappini, F., Desterke, C., Bertrand-Michel, J., Guettier, C. & Le Naour, F. Hepatic and serum lipid signatures specific to nonalcoholic steatohepatitis in murine models. *Sci Rep*. **6,** 31587 (2016).
19. Peng, C. *et al.* Vibrational signatures to discriminate liver steatosis grades. *Analyst* (2015).
20. El-Badry, A. M. *et al.* Assessment of hepatic steatosis by expert pathologists: the end of a gold standard. *Ann Surg*. **250,** 691–697 (2009).
21. Kotronen, A. *et al.* Comparison of lipid and fatty acid composition of the liver, subcutaneous and intra-abdominal adipose tissue, and serum. *Obesity* (*Silver Spring*). **18,** 937–944 (2010).
22. Huang, J. *et al.* Molecular characterization of the role of orphan receptor small heterodimer partner in development of fatty liver. *Hepatology*. **46,** 147–157 (2007).
23. Chiappini, F. *et al.* Relationship between polymerase gamma (POLG) polymorphisms and antiretroviral therapy-induced lipodystrophy in HIV-1 infected patients: a case-control study. *Curr HIV Res*. **7,** 244–253 (2009).
24. Lee, J. H. *et al.* Combination of honokiol and magnolol inhibits hepatic steatosis through AMPK-SREBP-1 c pathway. *Exp Biol Med* (*Maywood*). **240,** 508–518 (2015).
25. Breiman, L. Random forests. *Machine Learning*. **45,** 27 (2001).
26. Lopez-Vicario, C. *et al.* Molecular interplay between Delta5/Delta6 desaturases and long-chain fatty acids in the pathogenesis of non-alcoholic steatohepatitis. *Gut*. **63,** 344–355 (2014).
27. Ferre, P. & Foufelle, F. SREBP-1c transcription factor and lipid homeostasis: clinical perspective. *Horm Res*. **68,** 72–82 (2007).
28. Horton, J. D., Goldstein, J. L. & Brown, M. S. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest*. **109,** 1125–1131 (2002).
29. Jump, D. B. Fatty acid regulation of hepatic lipid metabolism. *Curr Opin Clin Nutr Metab Care*. **14,** 115–120 (2011).
30. Qin, Y., Dalen, K. T., Gustafsson, J. A. & Nebb, H. I. Regulation of hepatic fatty acid elongase 5 by LXRalpha-SREBP-1c. *Biochim Biophys Acta*. **1791,** 140–147 (2009).
31. Reyes-Quiroz, M. E. *et al.* Oleic acid modulates mRNA expression of liver X receptor (LXR) and its target genes ABCA1 and SREBP1c in human neutrophils. *Eur J Nutr*. **53,** 1707–1717 (2014).
32. Wang, Y. *et al.* Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity. *J Lipid Res*. **47,** 2028–2041 (2006).
33. Wang, Y. *et al.* Elevated hepatic fatty acid elongase-5 activity affects multiple pathways controlling hepatic lipid and carbohydrate composition. *J Lipid Res*. **49,** 1538–1552 (2008).
34. Anstee, Q. M. & Goldin, R. D. Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. *Int J Exp Pathol*. **87,** 1–16 (2006).
35. Koteish, A. & Diehl, A. M. Animal models of steatosis. *Semin Liver Dis*. **21,** 89–104 (2001).
36. Rinella, M. E. *et al.* Mechanisms of hepatic steatosis in mice fed a lipogenic methionine choline-deficient diet. *J Lipid Res*. **49,** 1068–1076 (2008).
37. Rinella, M. E. & Green, R. M. The methionine-choline deficient dietary model of steatohepatitis does not exhibit insulin resistance. *J Hepatol*. **40,** 47–51 (2004).
38. Takahashi, Y., Soejima, Y. & Fukusato, T. Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol*. **18,** 2300–2308 (2012).
39. Li, Z. *et al.* The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. *Cell Metab*. **3,** 321–331 (2006).
40. Ling, J., Lewis, J., Douglas, D., Kneteman, N. M. & Vance, D. E. Characterization of lipid and lipoprotein metabolism in primary human hepatocytes. *Biochim Biophys Acta*. **1831,** 387–397 (2013).
41. Browning, J. D. *et al.* Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology*. **40,** 1387–1395 (2004).
42. Brunt, E. M. *et al.* Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology*. **53,** 810–820 (2011).
43. Pelz, S., Stock, P., Bruckner, S. & Christ, B. A methionine-choline-deficient diet elicits NASH in the immunodeficient mouse featuring a model for hepatic cell transplantation. *Exp Cell Res*. **318,** 276–287 (2012).
44. Walle, P. *et al.* Fatty acid metabolism is altered in non-alcoholic steatohepatitis independent of obesity. *Metabolism*: *clinical and experimental*. **65,** 655–666 (2016).

45. Wang, L. *et al.* Fatty acid desaturase 1 gene polymorphisms control human hepatic lipid composition. *Hepatology*. **61,** 119–128 (2015).
46. Araya, J. *et al.* Decreased liver fatty acid delta-6 and delta-5 desaturase activity in obese patients. *Obesity* (*Silver Spring*). **18,** 1460–1463 (2010).
47. Arendt, B. M. *et al.* Altered hepatic gene expression in nonalcoholic fatty liver disease is associated with lower hepatic n-3 and n-6 polyunsaturated fatty acids. *Hepatology*. **61,** 1565–1578 (2015).
48. Guillou, H., Zadravec, D., Martin, P. G. & Jacobsson, A. The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice. *Prog Lipid Res*. **49,** 186–199 (2010).
49. Naudi, A. *et al.* Membrane lipid unsaturation as physiological adaptation to animal longevity. *Front Physiol*. **4,** 372 (2013).
50. van Meer, G., Voelker, D. R. & Feigenson, G. W. Membrane lipids: where they are and how they behave. *Nature reviews. Molecular cell biology*. **9,** 112–124 (2008).
51. Ricchi, M. *et al.* Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *J Gastroenterol Hepatol*. **24,** 830–840 (2009).
52. Maximos, M. *et al.* The role of liver fat and insulin resistance as determinants of plasma aminotransferase elevation in nonalcoholic fatty liver disease. *Hepatology*. **61,** 153–160 (2015).
53. Chiappini, F. *et al.* Ventromedial hypothalamus-specific Ptpn1 deletion exacerbates diet-induced obesity in female mice. *J Clin Invest*. **124,** 3781–3792 (2014).
54. Martinelli, N. *et al.* FADS genotypes and desaturase activity estimated by the ratio of arachidonic acid to linoleic acid are associated with inflammation and coronary artery disease. *Am J Clin Nutr*. **88,** 941–949 (2008).
55. Vessby, B., Gustafsson, I. B., Tengblad, S., Boberg, M. & Andersson, A. Desaturation and elongation of Fatty acids and insulin action. *Ann N Y Acad Sci*. **967,** 183–195 (2002).
56. Aoudjehane, L. *et al.* Interleukin-4 induces human hepatocyte apoptosis through a Fas-independent pathway. *FASEB J*. **21,** 1433–1444 (2007).
57. Podevin, P. *et al.* Production of infectious hepatitis C virus in primary cultures of human adult hepatocytes. *Gastroenterology*. **139,** 1355–1364 (2010).
58. Huang, S. *et al.* Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. *J Lipid Res*. **53,** 2002–2013 (2012).
59. Bligh, E. G. & Dyer, W. J. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. **37,** 911–917 (1959).
60. Barrans, A. *et al.* Hepatic lipase induces the formation of pre-beta 1 high density lipoprotein (HDL) from triacylglycerol-rich HDL2. A study comparing liver perfusion to *in vitro* incubation with lipases. *J Biol Chem*. **269,** 11572–11577 (1994).
61. Debois, D., Bralet, M. P., Le Naour, F., Brunelle, A. & Laprevote, O. *In situ* lipidomic analysis of nonalcoholic fatty liver by cluster TOF-SIMS imaging. *Anal Chem*. **81,** 2823–2831 (2009).
62. Le Naour, F. *et al.* Chemical imaging on liver steatosis using synchrotron infrared and ToF-SIMS microspectroscopies. *PLoS One*. **4,** e7408 (2009).
63. Le Naour, F. *et al. In Situ* Chemical Composition Analysis of Cirrhosis by Combining Synchrotron Fourier Transform Infrared and Synchrotron X-ray Fluorescence Microspectroscopies on the Same Tissue Section. *Anal Chem*. **84,** 10260–10266 (2012).
64. Petit, V. W. *et al.* Multimodal spectroscopy combining time-of-flight-secondary ion mass spectrometry, synchrotron-FT-IR, and synchrotron-UV microspectroscopies on the same tissue section. *Anal Chem*. **82,** 3963–3968 (2010).
65. Touboul, D., Brunelle, A. & Laprevote, O. Mass spectrometry imaging: Towards a lipid microscope? *Biochimie*. **93,** 113–119 (2011).
66. Touboul, D., Laprevote, O. & Brunelle, A. Micrometric molecular histology of lipids by mass spectrometry imaging. *Curr Opin Chem Biol*. **15,** 725–732 (2011).
67. Malmberg, P., Jennische, E., Nilsson, D. & Nygren, H. High-resolution, imaging TOF-SIMS: novel applications in medical research. *Anal Bioanal Chem*. **399,** 2711–2718 (2011).
68. Team, R. C. R: A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing* (2012).
69. Pang, H. *et al.* Pathway analysis using random forests classification and regression. *Bioinformatics*. **22,** 2028–2036 (2006).
70. Furey, T. S. *et al.* Support vector machine classification and validation of cancer tissue samples using microarray expression data. *Bioinformatics*. **16,** 906–914 (2000).
71. Svetnik, V. *et al.* Random forest: a classification and regression tool for compound classification and QSAR modeling. *J Chem Inf Comput Sci*. **43,** 1947–1958 (2003).

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Author Contributions

Designed the study: F.C., F.L.N. Provided clinical samples and information: P.G., A.T., D.S., J.C.D.V., C.G. Performed *in vitro* experiments: F.C., A.C. Murine models and *in vivo* experiments: F.C. Implemented bioinformatics and statistical workflow: F.C., C.D. Performed lipidomic analysis: J.B.M. Performed histological examination: C.G. Performed mass spectrometry imaging: F.C., H.K., D.T., A.B. Analysis and interpretation of data: F.C., A.C., H.K., D.T., J.B.M., A.B., C.G., F.L.N. Wrote the manuscript: F.C., F.L.N.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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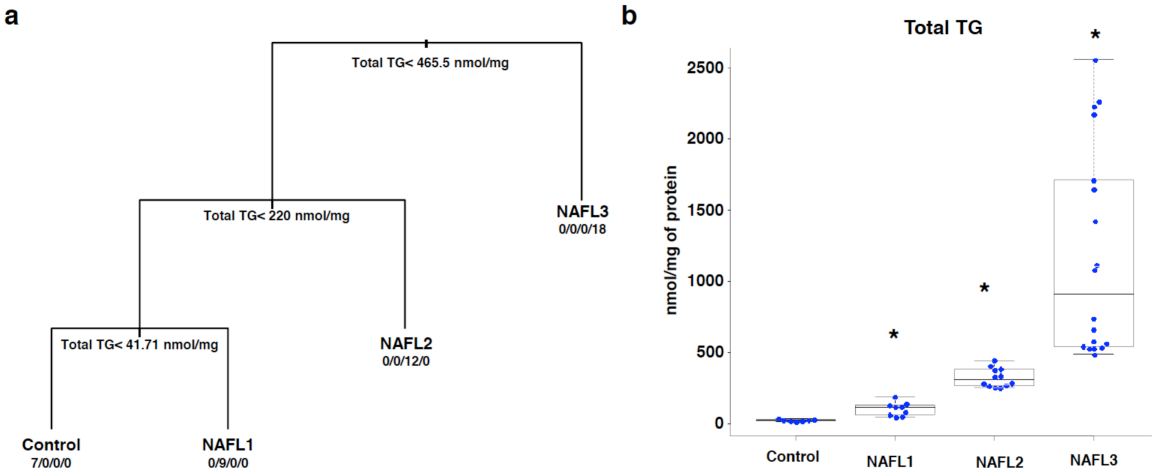
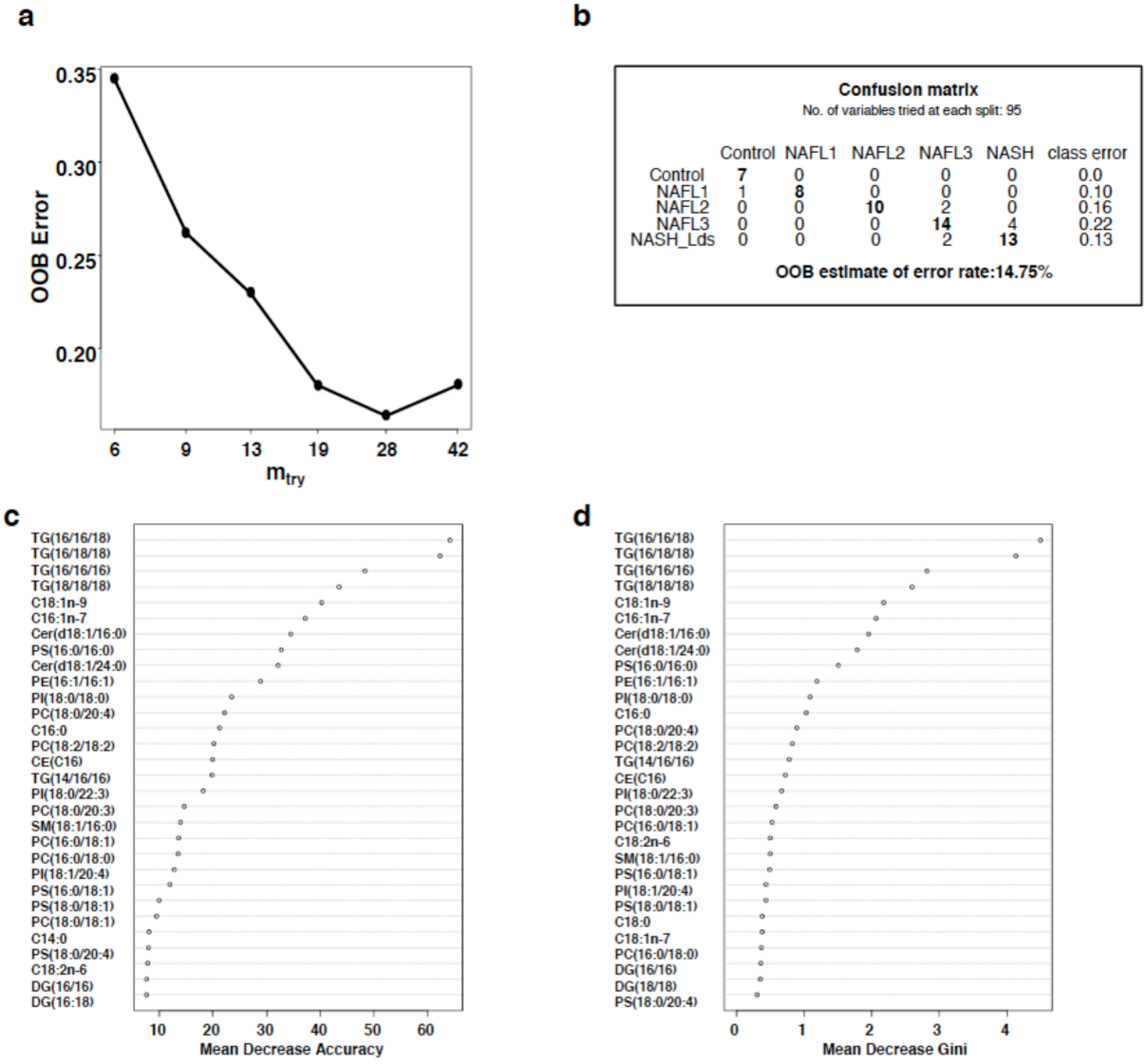
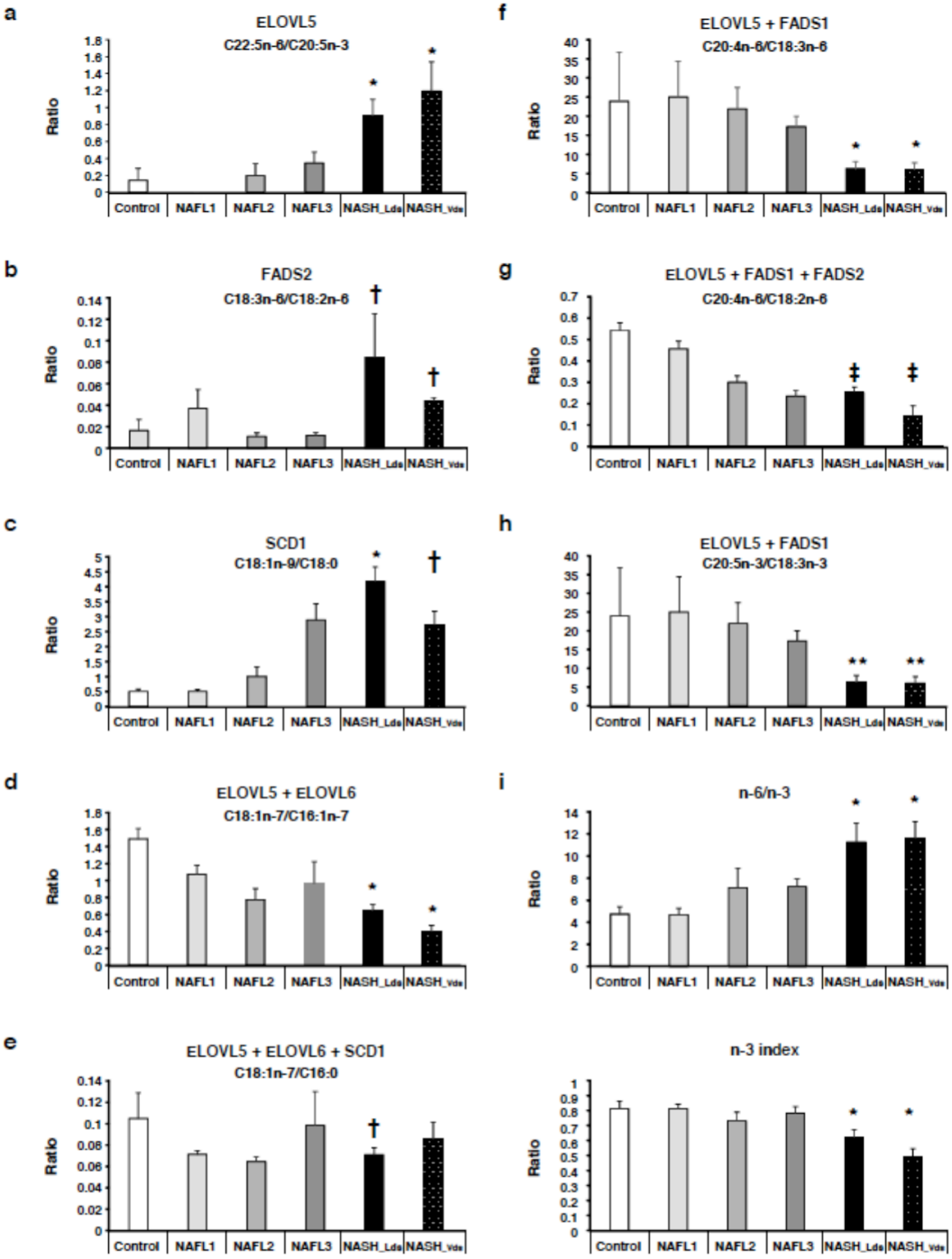


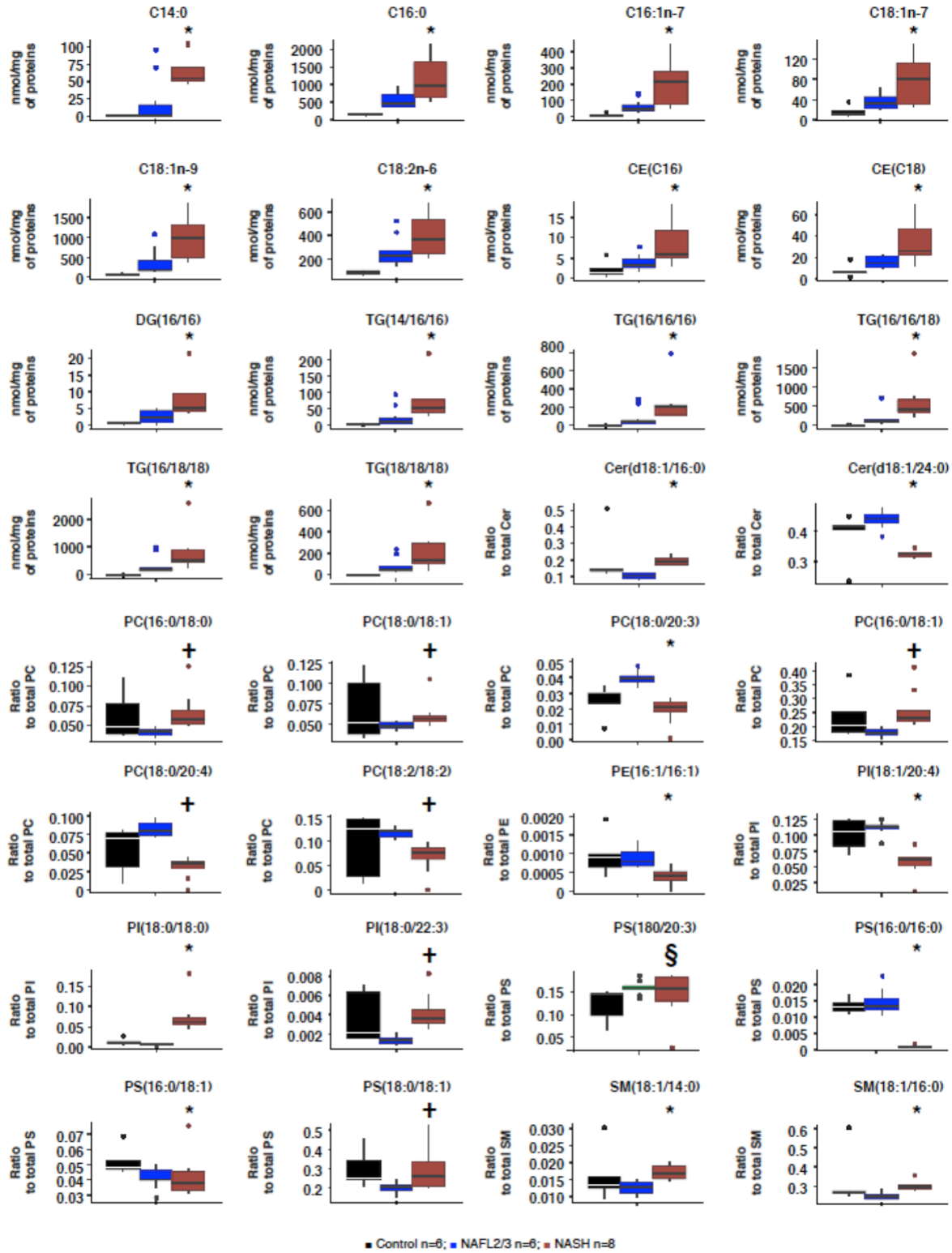
Supplemental Figure S1. Total hepatic triglycerides clustered the four groups of patients with different grades of NAFL based on cluster analysis regression tree (CART). (a) Data are represented as a regression tree where each node of the tree corresponds to the total triglycerides (TG): Ranges of Total TG liver content determine the four groups of patients. **(b)** Total TG content from human liver biopsies in clustered patients based on the CART analysis from (A). Data are represented as boxplot. *p<0.05, by unpaired *t*-test compared to each other groups after ANOVA analysis. Control n=7; NAFL1 n=9; NAFL2 n=12; NAFL3 n=18. NAFL: nonalcoholic fatty liver.



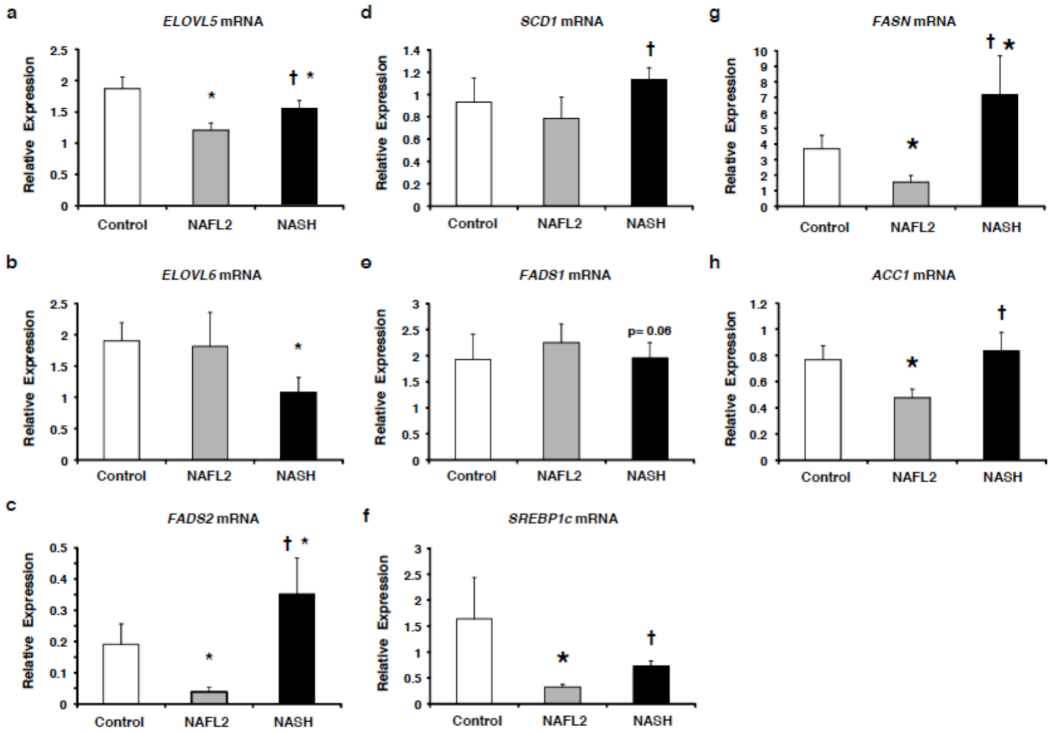
Supplemental Figure S2. Some of lipids selected after random forests analysis clustered NASH patients compared to other groups of patients with different grades of liver steatosis. (a) Determination of the best m_{try} (i.e. the best number of randomly preselected splitting variables) associated to the minimum out-of-bag (OOB) estimate of error rate. **(b)** associated to the confusion matrix The 30 first lipids sorted by random forests analysis and classified based on their **(c)** mean decrease accuracy and **(d)** mean decrease Gini indexes to discriminate NASH patients versus the 4 other groups. Control n=7; NAFL1 n=9; NAFL2 n=12; NAFL3 n=18; NASH_Lds n=15. NAFL: nonalcoholic fatty liver disease; NASH_Lds: nonalcoholic steatohepatitis learning dataset.



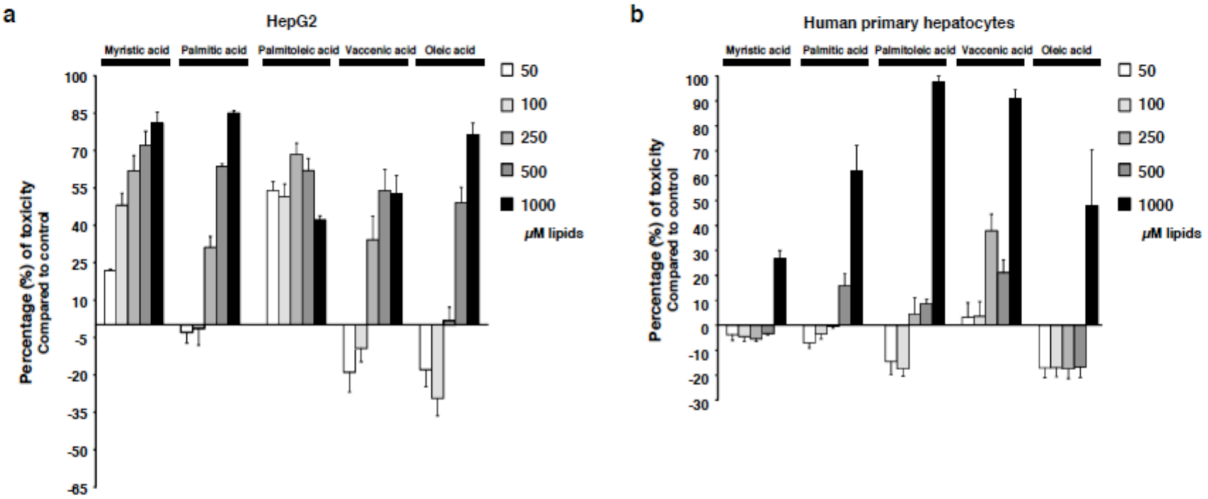
Supplemental Figure S3. Decrease in FADS1 and ELVOL6 index activities in NASH patients. **(a)** Evaluation of ELOVL5 activity using c22:5n-6 to c20:5n-3; **(b)** evaluation of FADS2 activity using c18:3n-6 to c18:2n-6 ratio. **(c)** evaluation of SCD1 using c18:1n-9 to c18:0 ratio **(d)** both ELOVL5 and ELOVL6 activities using c18:1n-7 to c16:1n-7; **(e)** global ELOVL5. ELOVL6 and SCD1 activities using c18:1n-7 to c16:0 ratio; **(f)** both ELOVL5 and FADS1 activities using c20:4n-6 to c18:3n-6 ratio. **(g)** Global ELOVL5. FADS1 and FADS2 activities using 20:4n-6 to c18:2n-6 ratio towards the n-6 pathway, and **(h)** global ELOVL5 and FADS1 activities using 20:5n-3 to c18:3n-3 ratio towards the n-3 pathway. **(i)** Hepatic n-6 to n-3 ratio and n-3 index of the different study groups. in order to evaluate enzyme activities. a ratio between product-to-precursor of each reaction has been used as described before. Data are shown as means ± SEM. *p<0.05 and **p<0.01 by unpaired *t*-test compared to each other groups ‡<0.05 by unpaired *t*-test compared to Control. NAFL1 and NAFL2. +<0.05 by unpaired *t*-test compared to Control and NAFL1. †<0.05 by unpaired *t*-test compared to Control. NAFL2 and NAFL3 after ANOVA analysis. Control n= 7; NAFL1 n=9; NAFL2 n=12; NAFL3 n=18; NASH_Lds n= 15, NASH_Vds n= 7. ELOVL: elongase of very long chain fatty acid; FADS: fatty acid desaturase; NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis; NASH_Lds: learning dataset; NASH_Vds: validation dataset; SCD: stearoyl-CoA desaturase.



Supplemental Figure S4. Hepatic levels of the 32 lipids discriminating NASH group based on random forests analysis from liver biopsies used to look at metabolic gene expression levels involved in *de novo* lipid synthesis. (A) 14 fatty acids including free fatty acids, cholesteryl ester (CE), diglycerides (DG) and triglycerides (TG) "up-regulated" in NASH group. Relative abundance of phospholipids "up-regulated" (n=9) or "down-regulated" (n=9) in NASH group. Data are represented as boxplot. *p<0.05, by unpaired *t*-test compared to Control and NAFL2/3 groups. +p<0.05 by unpaired *t*-test compared NAFL2/3 group. §p<0.05 by unpaired *t*-test compared to Control group. Unpaired *t*-test was done after ANOVA test. ■ Control n=6; ■ NAFL2/3 n=6; ■ NASH n=8. NAFL2/3 group matched age, sex, grade od steatosis to NASH composed by patients from Paul Brousse and Nice hospitals. Cer: Ceramides; NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis; PC: Phosphatidylcholines; PE: Phosphatidylethanolamines; PI: Phosphatidylinositols; PS: Phosphatidylserines; SM: Sphingomyelins



Supplemental Figure S5. Elongases and desaturases involved in LCPUFA synthesis are partially regulated at a transcriptional level. **(a)** *ELOVL5*. **(b)** *ELOVL6*. **(c)** *FADS2*. **(d)** *SCD1*. **(e)** *FADS1*. **(f)** *SREBP1c*. **(g)** *FASN and* **(h)** *ACC1* genes expression from human liver biopsies analyzed by RT-Q-PCR. Data are mean ± SEM. Control patients (n=6). NAFL2 patients (n=6) were matched to NASH patients (n=8).). NASH are matched to NAFL2 regarding to the total lipid content and no difference in age. gender and BMI between the two groups. *p≤ 0.05 compared to Control. †p≤0.05 compared to NAFL2 by unpaired *t*-test. ACC: Acetyl-CoA Carboxylase; ELOVL: elongase of very long-chain; FADS: fatty acid desaturase; FASN: fatty acid synthase; NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis; SCD: stearoyl-CoA desaturase.



SupplementalFigure S6. Hepatic cells exhibit high toxicity after treatment with lipids from the NASH signature. Percentage of toxicity over control cells (non-treated) assessed by total ATP content into HepG2 human hepatoma cell line (left panel) and human primary hepatocytes (right panel). **(a)** HepG2 cell and **(b)** human primary hepatocytes treated with individual lipids at different concentrations. Two independent experiments were done. Data are mean ± SEM. *p<0.05 by unpaired *t*-test compared to Control Mix and NAFL Mix at the same concentration, after ANOVA analysis. NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis.

Supplemental Table S1: List of primers used for Q-RT-PCR and implicated in hepatic human lipogenesis

Genes	Accession number	NCB	Primer Forward 5'-3'	Primer Reverse 5'-3'	TM
ELOVL6_human	AK027031	NM_024090	GCAAACACAAAACCCAAGGC	TGGCTTGCTTTTGTTCTCCC	58,99
ELOVL5_human	AF231981	NM_021814	GGACTCACACTGCTGTCTCT	GTTGTTCTTGCGCAGGATGA	59,1
ELOVL3_human	BC034344	NM_152310	AACCTCATTCCCCATAGCCC	AGCACACGGTTTGCTTTAGG	59,1
SCD1 (delta(9)-desaturase)_human	AF097514	NM_005063	TGAAAGCCAACAACTCTGCC	GCTGGACACTGAGCAAAGAC	59
FADS2 (delta(6)-desaturase)_human	AF126799	NM_004265	TTCCAAGGAGCAGAGAGGTG	CCCTATGAACCCCAAGAGCA	59
FADS1 (delta(5)-desaturase)_human	AF199596	NM_013402	TGCAATGTCCACAAGTCTGC	AGCTGCCCTGACTCCTTTAG	59
SREBP1c_human	AB373959	NM_004176	ACACAGCAACCAGAAACTCAAG	AGTGTGTCCTCCACCTCAGTCT	59
FASN_human	BC063242	NM_004104	CCCTCATCTCCCCACTCATC	CAGCGTCTTCCACACTATGC	59
ACC1_human	AY315627	NM_198834	TTGACTCCTCCATCAACCCC	AATTCCTCCCGCTCCTTCAA	59
Actin Beta_human	X00351	NM_001101	CATCCGCAAAGACCTGTACG	CCTGCTTGCTGATCCACATC	59

Primers were design based on the mRNA sequence found in Uniprot database and using Primer3 software and BLASTed (Basic Local Alignment Search Tool) on https://genome.ucsc.edu genomes

ACC: acetyl CoA carboxylase; ELOVL: elongase of very long chain fatty aid; FADS: fatty acid desaturase; FASN: fatty acid synthesis; SCD: stearoyl CoA desaturase; SREBP; sterol-regulated transcription factors.

3.2 Méthodes

Notre étude a consisté en une analyse lipidomique réalisée par spectrométrie de masse à partir de 68 biopsies de foies humains et 20 murins. Les patients étaient suivis dans deux centres hospitaliers (hôpital Paul Brousse, Villejuif, n = 61 et hôpital L'Archet, Nice, n = 7). Il y avait 7 patients contrôles, 39 patients ayant une stéatose simple et 17 ayant une NASH. Cette analyse a permis l'identification et la quantification relative de la teneur en lipides hépatiques (exprimée en nmol/mg de protéines totales) ou en pourcentage pour chaque catégorie de phospholipides.

Nous avons également utilisé une imagerie par spectrométrie de masse ToF-SIMS pour localiser les lipides d'intérêt sur des coupes de foie congelées. Nous avons réalisé des PCR quantitatives en temps réel de gènes impliqués dans le métabolisme des lipides.

Enfin pour vérifier la toxicité des lipides d'intérêt identifiés, nous les avons utilisé pour traiter des cellules HepG2 ou les hépatocytes primaires humains en culture et mesurer la nécrose et l'apoptose cellulaire.

3.3 Résultats principaux et discussion

Dans cette étude, nous avons établi pour la première fois une signature lipidique de la stéatohépatite non alcoolique sur la base de la quantification de 32 lipides. Aucun des lipides identifiés ne permettait par lui-même de discriminer la stéatose de la NASH, mais en revanche, la signature lipidique globale permettait de distinguer les témoins patients des NAFL et des NASH.

Nous avons également mis en évidence une dérégulation de la voie métabolique impliquée dans la synthèse des acides gras dans la NASH. Cette dysrégulation a été, dans notre étude, observée aussi bien chez l'homme que dans les modèles animaux,. L'augmentation concomitante des LCFA saturés et insaturés avec

la diminution significative des VLCFA polyinsaturés résultant de la diminution de l'activité de FADS1 créée un goulot d'étranglement conduisant à l'accumulation en amont d'acides gras jusqu'à 20 carbones. Ce phénomène est accentué par une augmentation de la synthèse d'acides gras *de novo*, comme en témoigne l'augmentation des niveaux d'expression d'ARNm de l'ACC1 et du FASN. Une diminution de l'expression et de l'activité de ELOVL6 contribue également à l'augmentation des LCFA, en particulier de l'acide myristique (C14:0) dans la NASH. Nos résultats ont démontré que le rapport n-6 sur n-3 était significativement augmenté, associé à une diminution significative n-3 chez l'homme et la souris.

De telles dérégulations métaboliques peuvent aboutir à l'altération de la synthèse des eicosanoïdes et des phospholipides, pouvant influencer la propriété des membranes cellulaires. Notre étude a donc montré le rôle central de FADS1 dans l'homéostasie lipidique et positionné cette désaturase comme une enzyme clé de la NASH.
Enfin, en utilisant les cinq acides gras principaux de la signature de la NASH, nous avons testé la toxicité de ces lipides sur deux lignées cellulaires, une lignée d'hépatome humain HepG2 et une lignée d'hépatocytes primaires.
Bien que chacun des cinq acides gras étudiés aient montré une toxicité individuellement, le mélange correspondant à la composition lipidique observée dans la NASH, sur la base de notre analyse lipidomique, a montré une toxicité significativement plus élevée par rapport aux mélanges liés à la composition observée dans le foie normal ou le foie stéatosique.
Nos résultats suggèrent donc que la lipotoxicité est non seulement liée à la quantité de lipides, mais également à leur composition et à leur proportion. Nous avons identifié 5 lipides hautement toxiques pour les hépatocytes voisins.
En conclusion, nous avons caractérisé une signature lipidique spécifique et sensible, des patients ayant une NASH. Cette étude a mis en évidence des dérégulations de la voie métabolique impliquée dans la synthèse des acides gras et des précurseurs des eicosanoïdes. Notre étude a notamment positionné ELOVL6 et FADS1 comme des acteurs majeurs de la progression vers la NASH. Enfin, la présente étude suggère également un rôle direct des lipides accumulés dans la NASH dans la progression de la pathologie par leur toxicité. Cela ouvre de nouvelles voies pour le développement ultérieur du diagnostic précoce et approches thérapeutiques.

4 Article 2

FABP4 and MMP9 identified as predictive factors of poor prognosis in patients with nonalcoholic fatty liver using data mining approaches and gene expression analysis

4.1 Rationnel et Objectifs du Travail

La stéatopathie non alcoolique ou métabolique (NAFLD) comprend un éventail large de lésions hépatiques allant de la simple stéatose (NAFL) à la stéato-hépatite non alcoolique (NASH) pouvant évoluer vers une fibrose hépatique, une cirrhose et un CHC (175). La NASH est une maladie systémique associée à l'obésité, à l'insulino-résistance, au diabète de type 2 et au syndrome métabolique. L'augmentation spectaculaire de son incidence fait de la NASH l'une des causes les plus fréquentes de maladie chronique du foie et un problème de santé publique majeur dans le monde. En outre, les patients atteints de NASH ont un risque plus élevé de développer une maladie cardiovasculaire, qui est également la principale cause de décès chez ces patients. Par conséquent, il est essentiel de diagnostiquer la NAFL et surtout la NASH à un stade précoce, lorsqu'une réversibilité est encore possible avec une prise en charge thérapeutique adaptée, englobant les règles hygiéno-diététiques au premier plan (182).
Les tests non invasifs permettent de discriminer les patients à un stade avancé de la maladie mais sont souvent mis en défaut pour identifier précocement les malades à risque de développer une cirrhose ou un cancer du foie (183). La biopsie hépatique demeure le gold-standard pour évaluer proprement les lésions histologiques et à aussi un rôle pronostique dans la prise en charge de ces patients.

Nous avons également montré précédemment que les gènes impliqués dans les processus inflammatoires étaient déjà régulés positivement chez les patients atteints de stéatose (176). L'expression génique étant très spécifique et très sensible aux changements environnementaux, l'exploration de l'expression des gènes hépatiques est une approche appropriée pour trouver des marqueurs de progression de la NAFL à la NASH. Le développement d'outils informatiques fiables couplé à la croissance de la puissance informatique a permis la mise en place de techniques de simulation numérique centrées sur la biologie. Les méthodes *in silico* sont complémentaires des études *in vivo* et *in vitro* et permettent aujourd'hui d'identifier de nouveaux axes de recherche, y compris en hépatologie (184).

Dans cette étude, nous voulions utiliser des données d'expression génique (GEO) associés à un algorithme d'analyse de prévision afin de créer une matrice composée de patients virtuels atteints de NAFLD et d'étudier leur génome, à partie de données déjà publiées. L'objectif de notre étude était d'identifier des gènes prédictifs de la transition de la NAFL à la NASH et des formes sévères de NASH incluant les patients ayant développé un CHC.

4.2 L'article

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FABP4 and *MMP9* levels identified as predictive factors for poor prognosis in patients with nonalcoholic fatty liver using data mining approaches and gene expression analysis

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Nonalcoholic fatty liver (NAFLD) may progress to nonalcoholic steatohepatitis (NASH) and ultimately to cirrhosis and hepatocellular carcinoma (HCC). Prognostic markers for these conditions are poorly defined. The aim of this study was to identify predictive gene markers for the transition from NAFL to NASH and then to poorer conditions. Gene expression omnibus datasets associated with a prediction analysis algorithm were used to create a matrix composed of control subject (n = 52), healthy obese (n = 51), obese with NAFL (n = 42) and NASH patients (n = 37) and 19,085 genes in order to identify specific genes predictive of the transition from steatosis to NASH and from NASH to cirrhosis and HCC and thus patients at high risk of complications. A validation cohort was used to validate these results. We identified two genes, fatty acid binding protein-4 (FABP4) and matrix metalloproteinase-9 (MMP9), which respectively allowed distinguishing patients at risk of progression from NAFL to NASH and from NASH to cirrhosis and HCC. Thus, NAFL patients expressing high hepatic levels of FABP4 and NASH patients expressing high hepatic levels of MMP9 are likely to experience disease progression. Therefore, using FABP4 and MMP9 as blood markers could help to predict poor outcomes and/or progression of NAFL during clinical trial follow-up.

Nonalcoholic fatty liver disease (NAFLD) includes a wide spectrum of conditions from nonalcoholic fatty liver (NAFL) or simple steatosis to nonalcoholic steatohepatitis (NASH) which may progress to hepatic fibrosis, cirrhosis and ultimately to hepatocellular carcinoma (HCC)[1–5]. Indeed, 25–30% of NAFL patients will develop liver inflammation and progress to NASH and more than 30% of NASH patients will develop severe fibrosis and/or cirrhosis leading to HCC[1,6,7]. NASH is a liver disorder associated with obesity, insulin resistance, type 2 diabetes mellitus (T2D) and metabolic syndrome[8–9]. The incidence of NASH has dramatically increased and it is now the leading cause of chronic liver disease and a major public health issue worldwide[10–14]. In addition, NASH patients are at higher risk of cardiovascular diseases, which are also the leading cause of mortality in these patients[6]. Therefore, NAFL and NASH should be diagnosed at an early stage during which they may be reversible using lifestyle changes and/or a pharmacological management[15,16].

Guidelines have been recently proposed to determine which patients should be screened for NAFLD such as patients with obesity, T2D and/or metabolic syndrome[17,18]. Non-invasive approaches have been proposed to

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Patients	Control N = 52	Healthy Obese N = 51	NAFL N = 37	NASH N = 42
Gender (F/M)	31/21	44/7	21/16	26/16
Age (year)	56.4 ± 18.2	47.1 ± 9.5**	41.4 ± 8.3**	45.3 ± 10.8**
BMI (kg/m²)	24.2 ± 3.2	41.7 ± 8.0***	41.4 ± 8.4***	49.0 ± 11.9***
Fat (area in %)	0 [0–1]	1 [0–3]	30 [20–52.5]	75 [70–80]
Inflammation	0 [0–0]	0 [0–0]	0 [0–0]	2 [1–2]
NAS	0 [0–0]	0 [0–0]	1.5 [1–2]	5 [4–6]
Fibrosis	0 [0–1]	0 [0–0]	0 [0–1]	1 [0–1]
Leptin level (pg/ml serum)	6.8 ± 6.4	29.1 ± 17.6***	43.4 ± 18.8***	35.7 ± 24.3***
Adiponectin level (pg/ml serum)	12.5 ± 9.6	8.3 ± 3.3	5.8 ± 1.6*	6.9 ± 3.1*

Table 1. Characteristics of patients from the learning dataset (GSE48452 and GSE61260). Data are expressed as a mean ± SEM or a median [interquartile]. Data were collected from previously published GSE48542 and GSE61260 datasets[33,34]. **p-value <0.01 and ***p-value <0.001 *versus* Controls (unpaired *t*-test). Gender distribution across the 4 groups: p-value <0.0001 (Chi-squared test). F: female; M: male; NAFL, nonalcoholic fatty liver; NAS: NAFLD activity score; NASH, nonalcoholic steatohepatitis.

differentiate NAFL from NASH such as the use of ultrasound or a fatty liver index (FLI) which includes the body mass index (BMI), waist circumference, triglyceride levels and serum gamma-glutamyl transferase (γ-GT) levels and to identify liver fibrosis with numerous non-invasive diagnostic tests[19]. In parallel, the primary and secondary causes of steatosis such as viral hepatitis C, autoimmune hepatitis, genetic mutations or polymorphisms, alcohol consumption, medications, total parenteral nutrition, congenital or acquired lipodystrophy, have to be ruled out. Based on the poor or contradictory results of these non-invasive approaches, or in the context of clinical trials, liver biopsy is highly recommended[16–18].

Thus, despite the procedure-related risks of morbidity and mortality, liver biopsy remains the "gold standard" for the diagnosis of NASH. The NASH Clinical Research Network has developed a NAFLD activity score (NAS) based on steatosis grading, the presence of hepatocellular ballooning and lobular inflammation. A NAS below 3 indicates the absence of NASH while a NAS upper 5 supports the diagnosis of NASH. In addition, a fibrosis score may be associated with the NAS[18,20,21]. The diagnosis of a patient with a NAS ranging between 3 and 5 being unclear, this score is mainly used to assess NASH progression in clinical trials, but it cannot really be used as a diagnostic tool to identify NASH patients[16]. Recently, the "Fatty Liver Inhibition of Progression" (FLIP) European Consortium has focused on a diagnostic algorithm for NASH identification (presence of steatosis > 5%, hepatocellular ballooning and lobular inflammation) and has proposed a more accurate and reproducible score separating Steatosis, Activity and Fibrosis (SAF)[22,23]. However, the histological assessment of NAFLD patients remains strongly observer-dependent and is not fully reproducible[24,25]. In addition, it has also been shown that sampling variabilities (*e.g.* right *vs.* left lobe, needle size) may interfere with the diagnosis of NAFLD[24,26–28]. Therefore, the pathologist assessment of NAFLD lesions may be challenging[25,27,29,30] and it may be assumed that the prevalence of NASH and its complications is probably underestimated[1,3,5,10,11,14,19,31]. Based on these observations, we have previously shown the need to confirm the pathological assessment with an analysis of gene expression levels and that genes involved in inflammatory processes are upregulated in patients with steatosis using high-density oligonucleotide microarrays[32].

Despite many years of research to identify non-invasive predictive markers for NASH using sophisticated algorithm approaches, none of the known markers is reliable enough to remove the need to perform liver biopsy due to the large spectrum of disease manifestations ranging from simple steatosis to HCC[8,21,24,25,27,28,30].

Because gene expression is highly specific and very sensitive to environmental changes, exploring comprehensive gene expression levels in liver biopsies may help to identify markers of progression from steatosis to NASH. Thus, the aim of this study was to identify predictive gene markers for the transition from NAFLD to NASH and then to poorer outcomes using hepatic gene expression microarray datasets.

Methods

Learning datasets and statistical analysis. GSE48452 and GSE61260 microarray datasets were selected and downloaded from the genome expression omnibus (GEO) database on NCBI website (http://www.ncbi.nlm.nih.gov/geo/) and were used as learning datasets. Briefly, gene expression levels were measured in human liver biopsies using the same high-density oligonucleotide microarray ([HuGene-1_1-st] Affymetrix® Human Gene 1.1 ST Array [transcript (gene) version]) as previously published. Each array included more than 750,000 unique 25-mer oligonucleotide probes interrogating more than 28,000 genes[33,34]. GSE48452 and GSE61260 respectively included a total of 73 and 109 human liver samples grouped into control samples (n = 14 and 38), healthy obese (HO) samples (n = 27 and 24), obese and NAFL samples (n = 14 and 23) and obese and NASH samples (n = 18 and 24). Thus, a total of 52 control samples, 51 HO samples, 37 obese and NAFL samples and 42 obese and NASH samples could be analyzed when both GSE datasets were combined[33,34]. The characteristics of the patients who provided these samples are summarized in Table 1.

Data were analyzed using R 3.5.2 software[35] following the study flowchart summarized in Fig. 1. First, each matrix of data was normalized with robust multi-array average (RMA). Briefly, RMA is an algorithm used to create an expression matrix from Affymetrix® data. The raw intensity values are corrected for background, log2

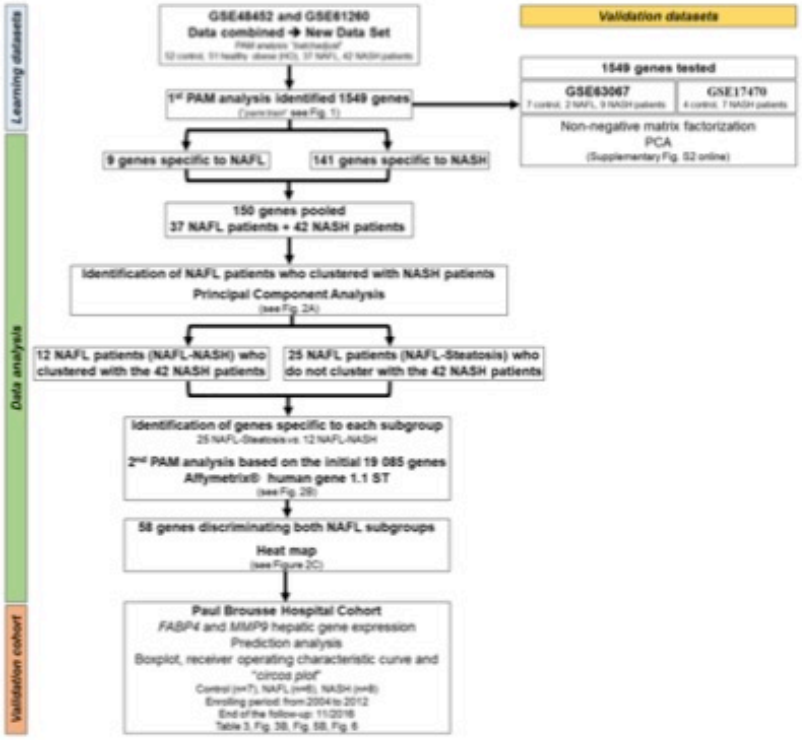

Figure 1. Study flowchart for the identification of 58 genes allowing predicting the subgroup of NAFL patients likely to progress to NASH. The first prediction analysis of microarrays (PAM) performed on the learning datasets (GSE48452 and GSE61260, blue) identified 1,549 genes allowing differentiating the four groups of patients (Controls, heathy obese patients, obese patients with NAFL and obese patients with NASH). The 1,549 genes were then validated using validation datasets (GSE63067 and GSE17470, yellow). Data were then analyzed (green). Nine genes were only specific to obese patients with NAFL and 141 genes were only specific to obese patients with NASH. These 150 genes were pooled, and principal component analyzes were performed in the groups of NAHL and NASH patients. Twelve obese patients with NAFL were misclassified as obese patients with NASH (referred to as NAFL-NASH patients) whereas 25 obese patients with NAFL were not (referred to as NAFL-Steatosis patients). Then, a second PAM of all the 19,085 genes of the microarray chip was performed in the 25 NAFL-Steatosis and 12 NAFL-NASH patients. Fifty-eight genes (54 were upregulated and 4 were downregulated) allowing distinguishing the 12 NAFL-NASH patients and the 25 NAFL-Steatosis patients were identified, as confirmed in the heatmap. Finally, the results were confirmed in the cohort of patients (7 control subjects, 6 NAFL patients and 8 NASH patients) from Paul Brousse Hospital (Validation cohort, orange). The flowchart results are shown in Fig. 2 to Fig. 6 and in Supplementary Fig. S1 to Supplementary Fig. S5.

transformed and then quantile normalized. Then, a linear model is fit to the normalized data to obtain a measure of the gene expression for each probe pair on each array and then combined with structured query language (SQL) request[35]. Then, both batches were normalized using prediction analysis for microarrays (PAM) with "*batchadjust*" implemented in "*PAMR*" for R package at the "*pamr.train*" function[37,38]. Then the threshold was calculated to determine the minimum number of genes allowing distinguishing the four sample groups based on the calculation of the minimum misclassification error for each group leading to a table of true *versus* predicted values (*pamr.confusion*), from a nearest shrunken centroid fit (*pamr.adaptthresh*)[38]. Thereafter, the genes of interest were sorted based on their best score (*pamr.listgenes*) and the gene(s) with the best score which survived the thresholding from the nearest shrunken centroid classifier were plotted (*pamr.geneplot*).

The gene lists generated by the different PAM were compared using the "*Venn*" function in "*gplots*" for R package, indicating the number of overlapping transcripts in each sample group[39].

Validation datasets. Two independent human liver biopsy datasets were selected and downloaded (GSE63067 and GSE17470; http://www.ncbi.nlm.nih.gov/geo/) to validate the genes of interest identified using

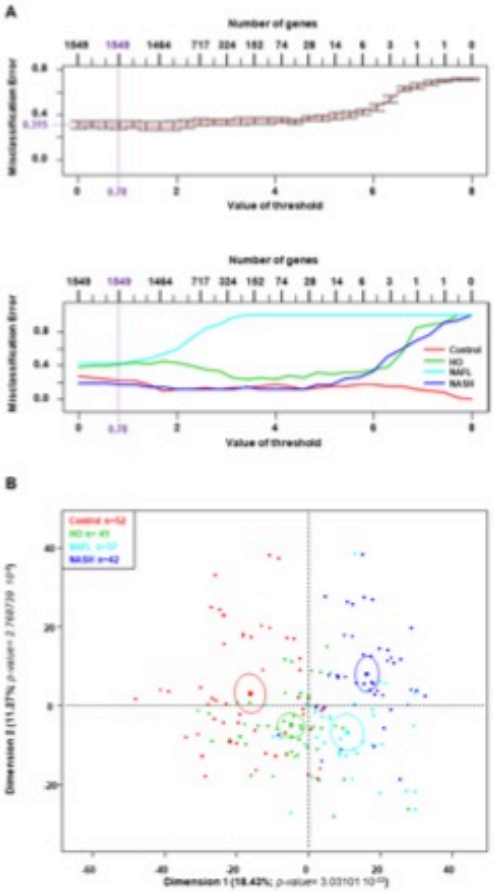

Figure 2. Identification of 1,549 genes differentiating the four groups of patients from the learning datasets (GSE48452 and GSE61260). **(A)** The transcriptome of the four groups of patients from GSE48452 and GSE61260 datasets was analyzed using a prediction analysis of microarrays (PAM) algorithm and 1,549 genes were identified with a threshold of 0.78, corresponding to an overall misclassification error rate of 0.315 (upper panel) and the misclassification error rate for each group of patients is detailed (lower panel). **(B)** Principal component analysis of the four groups of patients differentiated using the 1,549 genes identified. The dots represent each patients from each GSE, the lines are the ellipses centered onto the mean (colored squares) representing the 95% confidence interval, and the *p-value* is the probability associated with the F-test of the analysis of variance along the axes of the first and second dimensions ($\alpha = 0.05$).

the learning datasets (GSE48452 and GSE61260) after the PAM. Here, gene expression levels were measured using HG-U133_Plus_2.0 array (na22 platform, Affymetrix®) and CodeLink Human Whole Genome Bioarray (GE Healthcare/Amersham Biosciences), respectively[40,41]. The GSE63067 dataset included 7 control samples, 2 NAFL samples and 9 NASH samples[41]. The GSE17470 dataset included 4 control samples and 7 NASH samples[40]. The clinical characteristics of the patients who provided these samples were not available. To validate the genes selected in both validation datasets, two independent unsupervised approaches were used, the non-negative matrix factorization (NMF) and the principal component analysis (PCA). The "*NMF*" (R-package) was used here as an unsupervised clustering method for samples using gene expression microarray data from the validation datasets[42]. PCAs were performed using "*FactoMineR*" for R-package. Each sample and the ellipses centered onto the mean representing the 95% confidence interval (CI) were plotted on the PCA. The probabilities associated with the F-test of the variance analysis along the dimension axes ($\alpha = 0.05$) were calculated[43].

Gene set enrichment analysis. The lists of genes identified at each step of the analysis of learning datasets were used as data entries for computing enrichment with existing lists created from prior knowledge organized into gene-set libraries. We used Enrichr, a freely available integrative web-based and mobile software application (http://amp.pharm.mssm.edu/Enrichr/) that includes 17 gene-set libraries, an alternative approach to rank enriched terms, and various interactive visualization approaches to display enrichment results[44,45]. Among the 17 libraries, we searched WikiPathways 2016 and/or KEGG 2016 (Kyoto Encyclopedia Gene and Genome) libraries. The algorithm calculated the p-value, Z-score and combined score for each signaling pathway identified. Using methods based on the Z-score and combined score has been shown to be the best approach to recover a higher number of correct terms[45]. Thus, only the signaling pathways with a significant p-value ($p\text{-value} < 0.05$) were ranked based on their combined score and represented as a bar graph and network. Also, to pin-point certain genes associated with a signaling pathway, data were represented as clustergrams for better clarity.

To identify specific molecular signatures, we also used MSigDB database software v5.2 developed by the Broad Institute (http://software.broadinstitute.org/gsea/msigdb/)[46]. KEGG and Reactome databases were used to identify the genes and signaling pathways significantly related to the NASH group from the learning datasets. Data were represented as enriched set plots.

Unsupervised cluster analyzes. During the identification process of genes specific to a subgroup of samples, to confirm the specific gene expressions in each group, unsupervised cluster analyzes were performed using dendrograms generated from the "*heatmap.2*" function in "*gplots*" for R package. The Z-scores for each gene and sample were calculated and unsupervised cluster analyzes were represented as heat maps.

Validation cohort. A total of 21 patients who consulted at Paul Brousse hospital between 2004 and 2012 and with a follow-up of at least 4 years were included in this study. They were divided as follows: Controls (n = 7), NAFL patients (n = 6) and NASH patients (n = 8). A histological distinction based on the NAS was used to differentiate NAFL from NASH as previously described[20,21]. Patient clinical and biological characteristics, including the general health status, metabolic syndrome and liver function were retrospectively recorded. Exclusion criteria were patients with liver diseases such as viral hepatitis B, viral hepatitis C, primary biliary cirrhosis, sclerosing cholangitis, autoimmune hepatitis, hemochromatosis, Wilson's disease, α1-antitrypsin deficiency, drug-induced liver disease and patients with alcohol consumption greater than 20 g/day for women and 30 g/day for men. Our institutional review board (Paul Brousse hospital-Centre des Ressources Biologiques Paris-SUD, CRB Paris Sud, Bio Banking Number: 0033-00089) approved the study conduct and a written informed consent was obtained from all patients. The study was conducted in accordance with the relevant "Declaration of Helsinki" and "International Conference on Harmonization Good Clinical Practice" guidelines and the French ethical laws.

Quantitative reverse transcription PCR. Total RNA were extracted from frozen liver biopsies using RNA-STAT 60 reagent (AMS Biotechnology Europe LTD). RNA levels and quality were assessed using NanoDrop®-ND1000 (Thermo Scientific). cDNAs were generated using RivertAid® First Strand cDNA Synthesis (Thermo Scientific), and Syber Green from FastStart Essential DNA Green Master mixes (Roche, Life Science) were used to quantify hepatic *fatty acid-binding protein-4* (*FABP4*) and *matrix metallopeptidase-9* (*MMP9*) mRNA levels using the gene-specific primers described in Supplementary Table S1.

Q-RT-PCR was performed using the LightCycler® 96 Instrument (Roche, Life Science). Gene expression levels were normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA levels and data were analyzed using LightCycler® 96 SW 1.1 software (Roche, Life Science). For each sample, the gene of interest level to *GAPDH* level ratio was calculated based on an arbitrary number of copies determined using the standard curve for each gene, as previously described[47].

Q-RT-PCR data analyzes. *FABP4* and *MMP9* hepatic mRNA levels were assessed using a PCA by plotting each patient and the ellipses centered onto the mean representing the 95% CI. The probabilities associated with the F-test of the analysis of variance along the dimension axes ($\alpha = 0.05$) were calculated. To determine the individual and combined sensitivity and specificity of both markers, receiver operating characteristic (ROC) curves, the area under the curve (AUC), negative and positive predictive values (PV) and optimal response cut-off points (Ir.eta) were assessed using "*pROC*" and "*Epi*" for R package. A one-way ANOVA was used to analyze the distribution between the patient groups using "*beeswarm*" for R package and was represented by boxplots. Patient clinical data were analyzed to assess the predictive *FABP4* and *MMP9* mRNA expression levels. NAFL and NASH patients were divided into two subgroups: patients with low *FABP4* and *MMP9* mRNA levels (NAFL_FABP4_MMP9_L and NASH_FABP4_MMP9_L) and patients with high *FABP4* and/or *MMP9* mRNA levels (NAFL_FABP4_MMP9_H and NASH_FABP4_MMP9_H). The clinical events, defined as a worsening of the liver disease, were recorded during the follow-up for each patient. In the Data were analyzed using a Fisher's exact test and represented as circular layouts ("*circos plot*") using "*circlize*" for R package.

Ethics approval and consent to participate. The institutional review board of the hospital (Paul Brousse hospital-Centre des Resources Biologiques Paris-SUD, Bio Banking Number: 0033-00089) approved the study and written informed consent was obtained from all patients. Access to this material and all experiments were performed in accordance with the relevant guidelines and regulation of the French ethical laws.

Results

Clinical characteristics of samples from the learning dataset matrix. For further biostatistical analyzes (Fig. 1), we combined both GSEA (GSE48452 and GSE61260) that have been published previously[33,34] to create a new learning dataset matrix. The clinical data from this learning dataset were based on the clinical data available in the GEO dataset website and included 52 control subjects, 51 HO patients, 37 obese patients

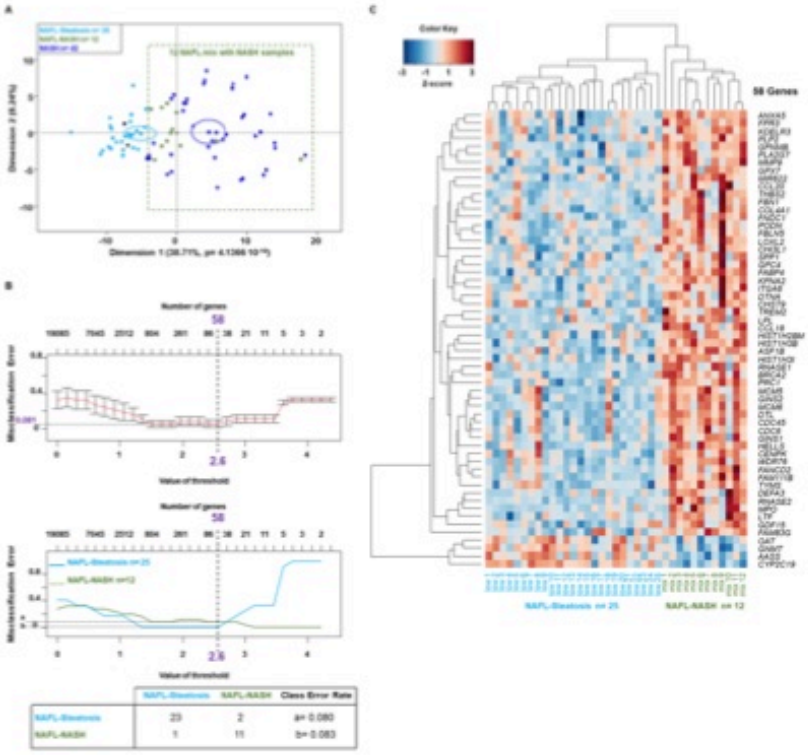

Figure 3. Identification of 58 genes allowing differentiating a subgroup of obese patients with steatosis misclassified as NASH patients. (**A**) Unsupervised analysis of obese patients with NAFL and NASH using a total of 150 genes: 9 genes were specific to NAFL patients and 141 genes were specific to NASH patients compared to the other groups of patients from GSE48452 and GSE61260 datasets. The principal component analysis identified 12 NAFL patients who were misclassified as NASH patients (referred to as NAFL-NASH patients; green dots; dark blue dots) and 25 NAFL patients who were not misclassified as NASH patients (referred to as NAFL-Steatosis patients; light blue dots). The dots represent each patient, the lines are the ellipses centered onto the mean (colored squares) representing the 95% confidence interval, and the p-value is the probability associated with the F-test of the analysis of variance along each axis. Obese patients with NAFL, n = 37; obese patients with NASH, n = 41 according to the original publications. (**B**) The prediction analysis of microarrays identified 58 genes based on the threshold of 2.6 calculated to achieve an optimal misclassification error rate for all groups of 0.081 (upper panel) and for each group of 0.0800 and 0.083 (middle panel, dash lines). The table details the misclassified patients in each subgroup of patients analyzed. (**C**) Heatmap of obese patients with NAFL misclassified in two subgroups of patients based on the 58 genes identified in (**B**) and listed on the right.

with NAFL and 42 obese patients with NASH classified according to their NAFLD-activity score as previously described[20,21]. The clinical characteristics of the different groups of patients are summarized in Table 1. Briefly, control subjects were older than patients who underwent bariatric surgery in the other groups. Women were significantly more represented in the four groups (*p-value* <0.0001, chi-squared test). Importantly, the number of women was higher in control subjects and HO patients than in obese patients with NAFL and NASH (75/28 *versus* 47/32, *p-value* = 0.0581, chi-squared test), but there was no significant difference in gender distribution between obese patients with NAFL and NASH (21/16 *versus* 26/16; *p-value* = 0.641, chi-squared test). As expected, the BMI was significantly higher in the three groups of obese patients compared to control subjects, with significantly higher serum leptin levels and lower serum adiponectin levels. However, no difference in age, BMI, serum leptin and adiponectin levels was observed between the three groups of obese patients.

To confirm the statistical results from the new learning dataset matrix, we used validation datasets (GSE63067 and GSE17470). The clinical characteristics of the patients included in these validation datasets were not available.

	Control	HO	NAFL	NASH	Class Error Rate
Control	**46**	1	3	2	0.1153
HO	13	**29**	6	3	0.4313
NAFL	3	9	**15**	***10***	0.5946
NASH	1	2	2	**37**	0.4313

Table 2. Detailed misclassification errors (confusion matrix) in each group of patients from the learning dataset (GSE48452 and GSE61260) according to the prediction analysis of microarrays (Fig. 2A) performed to analyze the new data frame. HO: healthy obese; GSE48452 and GSE61260[33,34].

Identification of gene signatures and associated signaling pathways according to patient group in the learning and validation datasets. The new learning data matrix combining the four groups of human liver samples from the learning datasets (GSE48452 and GSE61260) was built based on the data from human liver biopsies using the same high-density oligonucleotide microarray version (see Methods)[33,34]. The PCA of the new matrix showed a high variance between the two microarray results of both learning datasets (Supplementary Fig. S1A). Then, the next step was to normalize both datasets to create the learning data matrix. A PAM was then applied to the new matrix using all control subjects from both datasets as "controls" to normalize the variables of the datasets (Fig. 1). We confirmed the homogeneity of the distribution of the patients from both datasets using all genes from the normalized new matrix by performing a PCA (Supplementary Fig. S1B).

Then, a PAM was performed on the standardized and corrected transcriptome arrays including the 182 samples divided into 4 groups. The PAM identified 1,549 genes with a threshold of 0.78, corresponding to an overall low misclassification error rate of 31.5% (Fig. 2A, upper panel). This gene selection allowed separating most samples in each group, as shown by the cross-validated misclassification error curves (Fig. 2A, lower panel). The confusion matrix showed that obese patients with NAFL (n = 37) had a misclassification error rate of 0.5946 (22/37) with three patients classified as "Controls", nine patients classified as HO patients and, interestingly, 10 patients classified as NASH patients (Table 2). These observations suggested that at least 10 obese patients with NAFL could be misclassified and could belong to the group of obese patients with NASH.

The PCA based on the 1,549 genes (Fig. 2B) showed a progression between the four groups of patients based on their gene signature as shown by the first and second dimensions of the PCA, with control subjects on the left panel of the graph followed by HO patients, NAFL patients and NASH patients on the top panel of the graph. These results suggested that among the 1,549 genes identified, some genes could be only specific to a group and not to the other three groups, which could explain the good separation observed in the PCA (Figs. 1, 2B).

Finally, to confirm the signature composed of the 1,549 genes identified, two independent validation datasets (GSE63067 and GSE17470) were used (Fig. 1). A non-negative matrix factorization and a PCA showed that the 1,549 genes also allowed separating each group of patients included in the validation datasets (Supplementary Fig. S2).

Thus, using a batch-adjust normalization approach associated with a gene prediction algorithm to combine two different Affymetrix® microarray datasets, 1,549 genes were selected to distinguish healthy subjects, HO patients, obese patients with NAFL and obese patients with NASH. This gene signature was also confirmed in two other independent GEO datasets. Interestingly, this specific set of genes allowed identifying a subgroup of 10 patients with NAFL among the 37 patients (27.03%) who were classified as NASH patients. These results strongly suggested that these 10 patients with NAFL shared the same specific gene profile as NASH patients and that this specific gene profile could help to identify the process involved in the progression from steatosis to NASH.

Identification of gene expression transitions associated with the inflammatory process, the apoptosis pathway and extracellular matrix remodeling. The PAM (Fig. 2A) identified 186 genes that were only specific to control subjects, 12 genes that were only specific to HO patients, 9 genes that were only specific to obese patients with NAFL and 141 genes that were only specific to obese patients with NASH (Supplementary Dataset 1). Then, "Enrichr" online software was used to identify the pathways associated with the genes identified (Supplementary Fig. 3). The 186 control-specific genes were involved in common signaling pathways such as translation driven by ribosomes, and functions such as estrogen signaling and circadian rhythm (Supplementary Fig. S3A). Interestingly, HO patients also expressed genes (n = 12) involved in estrogen signaling, circadian rhythm (*i.e.* serotonin receptors), nuclear receptors and transcription factors, inflammatory process, and more importantly genes involved in the extracellular matrix remodeling (ECMR) (Supplementary Fig. S3B). In both control subjects and HO patients, the identification of genes involved in the estrogen signaling pathway was in line with the over-represented female gender compared to NAFL and NASH patients (75 to 28 *vs.* 47 to 32, *p-value* = 0.0581, respectively Table 1). Importantly, these results validated the biostatistical workflow chosen and showed the sensitivity of combining such biostatistical approaches and the microarray gene expression technology in a large cohort of patients.

The 9 genes specific to obese patients with NAFL were involved in the JAK/STAT, MAPK and PI3K/AKT/mTOR signaling pathways associated with ECMR pathways (Supplementary Fig. S3C). Finally, the 141 genes specific to obese patients with NASH were also involved in ECMR, apoptosis and carcinogenesis pathways (Supplementary Fig. S3D).

Thus, four sets of genes (186, 21, 9 and 141 genes) that were specific to each group of patients were identified. The sets of genes specific to NAFL and NASH patients (9 and 141 genes, respectively) were involved in ECMR, apoptosis and carcinogenesis pathways.

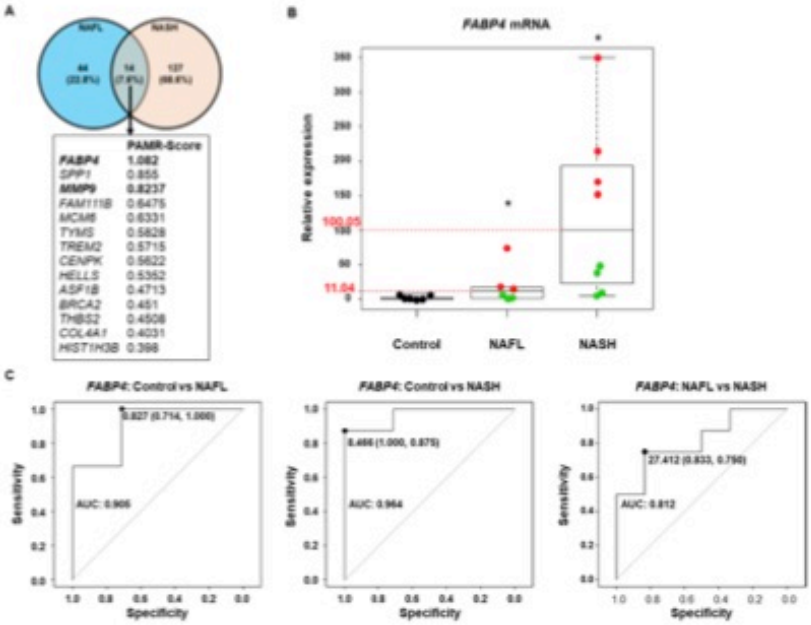

Figure 4. *FABP4* hepatic mRNA expression level distinguished control subjects from patients with NAFL or NASH as well as two subgroups in the NAFL and NASH groups. (**A**) The Venn diagram identified 14 genes among the 58 genes differentiating the two subgroups of NAFL patients (NAFL-Steatosis and NAFL-NASH patients, see Fig. 3C) and among the 141 genes specific to NASH patients. (**B**) Boxplots of *FABP4* hepatic mRNA expression level in Control (n = 7), NAFL (n = 6) and NASH (n = 8) patients from Paul Brousse Hospital (Validation cohort). Identification of two subgroups in NAFL patients and two subgroups in NASH patients based on their median values (11.04 and 100.05, respectively) and represented by green and red dots. *p < 0.05 compared to control patients based on an ANOVA. (**C**) Receiver operating characteristic (ROC) curve using *FABP4* hepatic mRNA expression level comparing control versus NAFL patients (left panel), control versus NASH patients (middle panel) and NAFL versus NASH patients (right panel). The values of the Area Under the Curve (AUC) are shown, as well as the threshold, sensitivity, and specificity for the optimal response cut-off points (black dot).

In addition, the signaling pathways in which the genes identified were involved could be found in each group of patients with an increased expression according to disease the severity, from the inflammatory process to ECMR and oncogenic pathways. Thus, these results also validated the biostatistical workflow approaches used to identify these genes.

Identification of a subgroup of obese patients with NAFL with a gene signature similar to that of obese patients with NASH according to FABP4 expression levels. As shown, the confusion matrix identified 12 obese patients with NAFL classified as control subjects (n = 3) or HO patients (n = 9), and 10 obese patients with NAFL classified as patients with NASH (Table 2). Also, the gene enrichment pathway analysis showed that some pathways were common to the different NAFLD subtypes (Supplementary Fig. S3). These results suggested that a subgroup of NAFL patients (*i.e.* the 10 patients who were misclassified) had already a gene signature similar to that of NASH patients.

To test this assumption, the genes specific to patients with steatosis (n = 9) and NASH (n = 141) were combined to create a new matrix with 150 genes and two groups of patients from the learning datasets (37 + 42 = 79 patients in total, Fig. 1). An unsupervised cluster analysis of this new matrix was then performed and showed that among the 37 obese patients with NAFL, 12 patients were misclassified as obese patients with NASH (Figs. 3A, S4). Then, obese patients with NAFL were split into two groups: patients who were misclassified as obese patients with NASH (NAFL mixed with NASH or NAFL-NASH patients, n = 12) and the remaining NAFL patients (NAFL-Steatosis patients, n = 25). To define a specific gene signature in order to distinguish these two subgroups and to avoid any bias, a PAM was performed using all the genes of the microarrays (n = 19,085). The results showed that the best threshold was 2.6 to achieve a minimum of overall misclassification error rate (0.081) and this threshold allowed identifying 58 genes (Fig. 3B; Supplementary Dataset 2), associated with good results for the misclassification rate as shown by the confusion matrix (Fig. 3B). The unsupervised cluster analysis of the

58 selected genes showed that NAFL livers with a NASH signature (NAFL-NASH) and NAFL (NAFL-Steatosis) patients were clearly distinguishable (Fig. 3C). Among these 58 genes, 54 were upregulated and 4 were downregulated (*OAT*, *GNMT*, *AASS* and *CYP2C19*) in the NAFL-NASH subgroup (Fig. 3C; Supplementary Dataset 2). The 4 genes down-regulated in the NAFL-NASH subgroup were mainly involved in metabolic pathways such as amino acid metabolism which can lead to the synthesis of pyruvate and subsequently acetyl CoA, the precursors of linoleic acid and arachidonic acid synthesis (Supplementary Fig. S5A). This finding was in agreement with what is expected in the fatty liver tissue, where the lipid metabolism is dysregulated[48,49]. The 54 genes distinguishing obese patients with steatosis who were misclassified as NASH patients (NAFL-NASH subgroup) were involved in ECMR, DNA regulation (DNA repair, replication, G1/S cell cycle), inflammatory processes and some were involved in insulin resistance and lipid metabolism (Supplementary Fig. S5B).

Venn diagrams were used to compare the 58 genes that allowed distinguishing the NAFL-NASH and NAFL-steatosis subgroups to the 141 genes specific to obese patients with NASH. Only 14 genes were common to both gene sets (Fig. 4A). The enrichment analysis of these 14 genes showed that they were involved in ECMR, inflammation and oncogenic pathways as expected (Supplementary Fig. S6A,B). Among the 141 genes identified in obese patients with NASH, the top-ranked gene was *FABP4* (+1.082-fold in the NASH group, Supplementary Dataset 1). *FABP4* was also the top-ranked gene among the 58 genes that allowed distinguishing NAFL-NASH patients from NAFL-Steatosis patients according to the PAM results (+1.79-fold, Supplementary Dataset 2, Fig. 2C). Furthermore, *FABP4* was strongly associated with 4 genes involved in ECMR and oncogenic pathways (*BRCA2*, *COL4A1*, *ITGA6* and *MMP9*, Fig. 4A).

NAFL patients with high *FABP4* mRNA expression level (12 out of the 37 obese patients with NAFL) also expressed high mRNA expression levels for genes involved in ECMR and inflammatory pathways (Fig. 3C). Then, to confirm these results, we used a Validation cohort consisting in 21 patients who consulted in our institution and their clinical characteristics were summarized in Table 3. *FABP4* hepatic mRNA expression level was quantified by Q-RT-PCR and allowed distinguishing the three groups of patients (Fig. 4B). *FABP4* mRNA expression level allowed identifying two subgroups both in NAFL and NASH patients based on their median relative gene expression levels (11.04 and 100.05, respectively; Fig. 4B). *FABP4* mRNA expression levels distinguished two subgroups of patients diagnosed by the pathologist as NAFL or NASH patients (Fig. 4B), but the ROC curves based on *FABP4* expression levels also allowed distinguishing Control subjects from NAFL patients (specificity 0.714; sensitivity 1.000; AUC = 0.905) as well as Control subjects from NASH patients (specificity 1.000; sensitivity 0.875; AUC = 0.964) while no significant difference was observed between NAFL and NASH patients (specificity 0.853; sensitivity 0.750; AUC = 0.812, Fig. 4C). The last result could be due to the high variability in *FABP4* mRNA expression in the NAFL and NASH groups (Fig. 4B). Interestingly, these results strongly suggested that *FABP4* mRNA expression level could help to identify NAFL patients likely to belong to the group of NASH patients and also to identify a subgroup of NASH patients likely to progress to cirrhosis and/or HCC.

Thus, we characterized a gene signature allowing predicting what patients with steatosis could progress to NASH.

Identification of a subgroup of NASH patients with a more aggressive gene profile according to MMP9 mRNA levels. From the learning datasets, 13 other genes were associated with high *FABP4* expression levels in NAFL-NASH and NASH patients (Fig. 4A). Among them, four were involved in ECMR: *BRCA2*, *COL4A1*, *ITGA6* and *MMP9*. *MMP9*, a gene encoding for a matrix metalloproteinase, showed the highest scores (fold of +0.8237, Fig. 4A and +1.420, Fig. 5A).

Also, the 14 genes common to NAFL-NASH and NASH patients were involved in PI3K/AKT/mTOR, inflammatory, ECMR and oncogenic pathways (Supplementary Fig. S6) suggesting a progression from NAFL to NASH. Thus, high *MMP9* expression levels could be associated with the progression from steatosis to NASH and possibly with the progression from NASH to HCC or cirrhosis (Figs. 5A and S6). According to epidemiological studies, at least one third of NASH patients would progress to cirrhosis and HCC[3,3–5,41], that is why we investigated *MPP9* mRNA expression level in the four group of patients from the learning datasets (GSE48452 and GSE61260). As expected, *MMP9* mRNA expression levels allowed distinguishing NASH patients from control subjects, and HO patients and NAFL patients (p-value <0.05, ANOVA, Fig. 5B). Furthermore, the boxplot analysis of the 4 groups of patients showed that at least 90% of control subjects, HO patients and NAFL patients expressed low *MMP9* mRNA expression levels, when a threshold corresponding to the third quartile of control subjects was selected. According to this threshold, this analysis showed that *MMP9* mRNA expression levels allowed distinguishing two subgroups of NASH patients, one with high *MMP9* level (23 patients) and another with low *MMP9* level (19 patients, Fig. 5B).

Identification of a subgroup of NASH patients with a more aggressive gene profile according to MMP9 mRNA levels. Then, we focused on the two subgroups of NASH patients distinguished by their *MMP9* mRNA expression level (Fig. 5B). The analysis of the clinical and biological outcomes of the subgroups of NASH patients from the learning datasets showed that the subgroup with high *MMP9* mRNA level had higher hepatic fat content, inflammation, BMI, NAS, leptin and adiponectin levels and this subgroup mainly included female patients (Fig. 5C).

Then, the PAM of both subgroups and the whole transcriptome (n = 19,085 genes) identified 330 predictive genes (229 were upregulated and 101 were downregulated) for NASH patients with high *MMP9* level (Fig. 5D, Supplementary Dataset 3). Using GO-Elite software associated with the Pathway Commons database to analyze the matrix of 330 genes, we showed that the 101 genes down-regulated in NASH patients with high *MMP9* level were related to pathways involved in G2/M DNA damage and cell cycle checkpoints, whereas the 229 genes upregulated in these patients were involved in inflammatory processes including the T-cell receptor signaling (TCR) pathway, MAPK, JNK, p38 activation and leading to CD4+ and CD8+ T cell activation and interleukin (IL)2, IL6, and IL12 secretion, the nuclear factor activated T-cells (NAFT) calcium-calcineurin pathway and

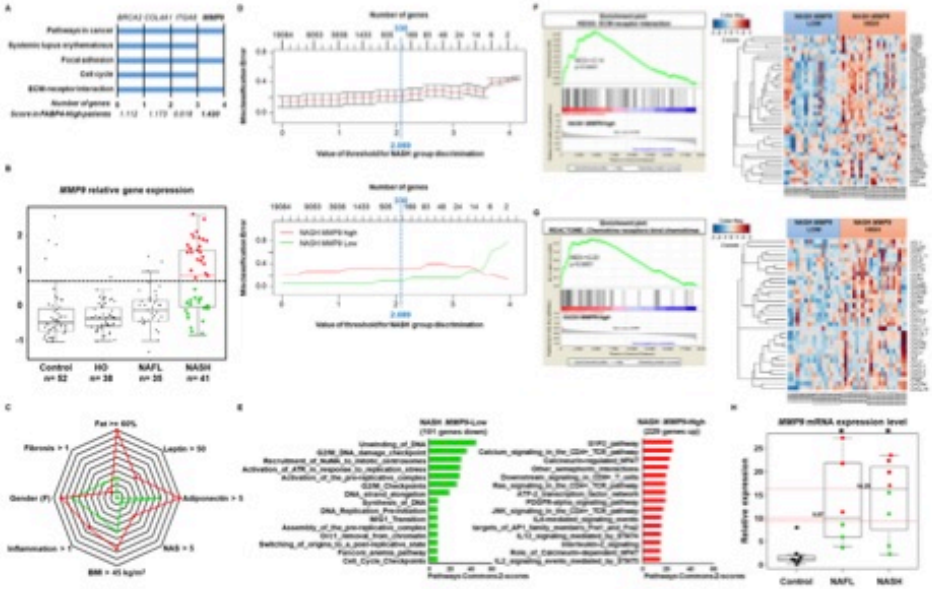

Figure 5. *MMP9* expression level differentiated two subgroups of NASH patients expressing genes predicting a poor disease outcome. (**A**) Gene pathway analysis results based on the 58 genes represented as clustergrams, including the score based on the PAMR analysis for each gene of interest: *BRCA2*, *COL4A1*, *ITGA6* and *MMP9*. (**B**) Relative *MMP9* mRNA expression level in each group of patients from the learning dataset (GSE48452 and GSE61260). Data are represented as boxplots. The threshold represents (dotted line) the 95% confidence interval of the control subjects and identified 23 patients with high relative *MMP9* expression level (red dots) and 19 patients with low relative *MMP9* expression level (green dots). Control subjects, n = 52; HO: healthy obese patients, n = 38; obese patients with NAFL, n = 35; obese patients with NASH, n = 41. ECMR: extracellular matrix remodeling pathways; hsa: homo sapiens. (**C**) Clinical characteristics of the two subgroups of patients expressing high *MMP9* level (red, n = 22) and low *MMP9* level (green, n = 19). (**D**) The prediction analysis of microarrays identified a minimum set of 330 genes (101 were downregulated and 229 were upregulated) based on the optimal misclassification error rate differentiating the two subgroups of patients expressing respectively high *MMP9* level (red line) and low *MMP9* level (green line). (**E**) The genome set pathway analysis showed that the 229 genes upregulated in the subgroup of patients with high *MMP9* level were involved in more aggressive pathways compared to the 101 genes downregulated in the other group of NASH patients. The gene set enrichment analysis of the 229 genes upregulated showed genes involved in (**F**) extracellular matrix remodeling (ECMR) and, (**G**) chemokines and chemokine receptors involved in carcinogenesis in NASH patients with high *MMP9* expression level. Data are presented as enrichment plots using Kyoto encyclopedia of genes and genomes (KEGG) and REACTOME databases in the left panels and as heatmaps with details of genes in the right panels.

hypoxia pathways and genes related to the AP1 complex (*e.g.* FRA1 and FRA2 family members). These results showed that, in the one hand, cell signaling pathways, and especially the G2 DNA damage checkpoint, were inhibited, allowing the resumption of cell cycling and entry into mitosis[30]. On the other hand, inflammatory processes were activated with T-cell and interleukin activation (Fig. 5E, Supplementary Dataset 3).

Also, the gene set enrichment analysis using the KEGG database in NASH patients with high *MMP9* expression levels significantly identified ECMR pathways (NES = +2.14, *p-value* < 0.0001; Fig. 5F, Supplementary Dataset 4) as shown by the unsupervised cluster analysis and represented by the associated heatmap. These ECMR pathways were associated with a significantly increased expression of genes involved in the CXR/CCL chemokine pathway (Fig. 5G), including *CD44* and *CXCR4* which are cancer stem-cell markers. The expression of these genes is high during the epithelial-mesenchymal transition and they may participate in liver stromal remodeling, like during the progression from cirrhosis to liver cancer, as shown by the enrichment plot analysis and by the associated genes shown in Fig. 5G and confirmed by the unsupervised cluster analysis (Supplementary Excel Table S5) and represented by the associated heatmap (Fig. 5G, right panel). These results strongly suggested that NASH patients with high *MMP9* mRNA expression level could also express high levels of genes related to cirrhosis and HCC progression.

To confirm these data, *MMP9* mRNA expression level was quantified in liver biopsies from patients treated at Paul Brousse Hospital and included in the Validation cohort. The data are shown in Fig. 5H. Two subgroups of

	Control n = 7	NAFL n = 6		NASH n = 8		NAFL n = 6		NASH n = 8	
	Low *FABP4* and *MMP9* levels	Low *FABP4* level	High *FABP4* level	Low *FABP4* level	High *FABP4* level	Low *MMP9* level	High *MMP9* level	Low *MMP9* level	High *MMP9* level
Gender F/M	5/2	1/2	1/2	2/2	4/0	0/3	2/1	3/1	3/1
Age (year)	32.4 ± 6.6	54.3 ± 9.4	56.0 ± 8.9	51.3 ± 5.5	60.8 ± 5.9	45.3 ± 7.4	65 ± 4.2	55.7 ± 6.8	57.5 ± 6.1
BMI (kg/m^2)	24.5 ± 0.9	23.1 ± 0.6	27.6 ± 3.1	35.5 ± 2.4	32.9 ± 3.2	24.4 ± 3.1	32.5 ± 2.0	33.5 ± 2.4	34.5 ± 3.3
Glycemia (mol/L)	5.3 ± 0.2	8.1 ± 1.9	6.5 ± 1.2	6.4 ± 0.4	7.2 ± 1.9	5.4 ± 0.6	9.7 ± 0.2	5.5 ± 2.0	8.3 ± 0.4
ALT (IU/L	20.3 ± 2.5	41.5 ± 5.3	32.0 ± 5.2	46.7 ± 5.5	50.8 ± 7.9	30.0 ± 2.3	44.5 ± 3.6	50.8 ± 6.9	46.8 ± 7.2
AST (IU/L)	24 ± 2.2	28.0 ± 6.5	35.3 ± 9.5	40.0 ± 8.0	53.3 ± 3.3	24.0 ± 7.3	45.0 ± 2.6	48.5 ± 2.8	46.3 ± 8.5
γ-GT (IU/L)	29.3 ± 1.9	94.5 ± 61.6	61.3 ± 11.7	57.3 ± 18.5	219.3 ± 127.0	53.3 ± 51.8	106.5 ± 18.6	81.8 ± 36.5	240.7 ± 150.8
ALP (IU/L)	86.2 ± 7.1	89.0 ± 22.1	92.3 ± 16.7	79.6 ± 8.8	122.8 ± 40.3	92.0 ± 21.6	89.5 ± 17.0	85.5 ± 48.0	129.3 ± 11.6
Creatinine (µmol/L)	71.8 ± 5.9	74.5 ± 9.4	82.7 ±	81.0 ±	63.5 ±	71.3 ± 4.5	91.5 ± 8.3	57.3 ± 15.9	89.3 ± 1.1
Steatosis									
S0/S1/S2/S3	7/0/0/0	0/1/2/0	0/1/1/1	0/0/0/4	0/0/1/3	0/0/2/1	0/2/1/0	0/0/0/4	0/0/1/3
NAFLD activity score (NAS)	0.6 ± 0.2	2.3 ± 0.7	3.3 ± 0.7	6.0 ± 0.4	5.8 ± 0.5	2.7 ± 0.9	3.0 ± 0.6	6.0 ± 0.4	5.8 ± 0.5
Fibrosis									
0/1a/1b/1c/2/3	5/2/0/0/0/0	0/1/2/0/0/0	0/1/2/0/0/0	0/2/0/2/0/0	0/4/0/0/0	0/0/3/0/0/0	0/2/1/0/0/0	0/3/0/1/0/0	0/3/0/1/0/0
Follow-up duration (years)	7.7 ± 3.2	9.3 ± 1.3	4.3 ± 0.3	5.0 ± 0.6	4.0 ± 0	8 ± 2.3	5.7 ± 1.2	4.0 ± 0	4.5 ± 0.5
Number of clinical events* during the follow-up	0	1	2	1	3	0	3	1	3

Table 3. Characteristics of patients from Paul Brousse Hospital (*i.e.* Validation cohort) Characteristics of patients from Paul Brousse Hospital (*i.e.* Validation cohort) in whom relative *FABP4* and *MMP9* hepatic mRNA expression levels were assessed by Q-RT-PCR. The cohort of patients was based on frozen liver biopsies available at the time of the study. The follow-up duration ranged from 4 to 12 years (samples were collected from 2004 to 2012 and the follow-up was stopped in 2016). The patients were classified based on their NAFLD activity score (NAS) and the subgroups were classified based on their relative *FABP4* and *MMP9* mRNA expression levels. *In NAFL patients, 2 patients developed steatofibrosis, 1 developed NASH, 1 died from a non-liver cause and the other 3 patients did not experience disease progression. In NASH patients, 1 patient developed cirrhosis, one developed HCC with cirrhosis, 1 developed HCC (one nodule) and 1 developed a cholangiocarcinoma and the other patients did not experience disease progression (no change in NAS). Data are presented as a mean ± SEM. The grey boxes represent the biological data increased compared to the groups with Low *FABP4* level or Low *MMP9* level (p-*value* <0.05; unpaired t-test). ALT: alanine aminotransferase; AST: aspartate aminotransferase; F: female; γ-GT: gamma-glutamyl transferase; m: male; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis.

NAFL patients and two subgroups of NASH patients could be distinguished based on their median *MMP9* mRNA expression levels (9.97-fold and 16.25-fold mRNA expression level, respectively).

High FABP4 and MMP9 mRNA expression levels are associated with a poor prognosis in NAFLD or NASH patients. The analysis of genes identified in NAFL-Steatosis patients and NAFL-NASH patients (n = 58 genes, see Fig. 3C) and in NASH patients (n = 141 genes) with high MMP9 level showed that 4 genes, *FABP4*, *MMP9*, *HELLS* and *TREM2*, were shared between these three groups of patients (Fig. 4A). *HELLS* and *TREM2* are involved in global immune responses. More importantly, *FABP4* has been shown to have pleiotropic effects in steatosis, NAFLD, insulin resistance and metabolic syndrome as well as in cell differentiation and chronic inflammation through macrophage activation[31]. Also, *MMP9* is a protein that induces cancer cell invasion and metastasis. Therefore, *MMP9* expression is also considered as a prognostic marker during cancer progression[32,33]. High *MMP9* mRNA expression levels were associated with the expression of genes involved in cancer progression: *BRCA2*, *COL4A1* and *ITGA6*, and were also associated with high *FABP4* mRNA expression levels (Figs. 4A and 5A).

Then, to determine if *FABP4* and *MMP9* mRNA expression levels could be used as prognostic markers in NAFL and/or NASH patients, we analyzed their expression levels in our Validation cohort (Fig. 1) using a ROC curve analysis. The individual expression levels of *FABP4* and *MMP9* did not allow clearly distinguishing the three groups of patients, in particular NAFL patients from NASH patients (Supplementary Fig. S7). However, when both markers were combined, the unsupervised PCA and ROC curves showed a distinction between Control subjects, NAFL patients and NASH patients (Fig. 6A,B).

The boxplots analyzes of *FABP4* and *MMP9* expression levels from the GSE datasets or the RT-Q-PCR analyzes showed that some NAFL and NASH patients had high or low *FABP4* and *MMP9* mRNA expression levels (Figs. 4B, 5B,H) and a misclassification of patients from the Validation cohort was observed in each group of NAFL and NASH patients as shown by the CART (characteristic and regression tree) analysis (Supplementary Fig. S7C,D, respectively). When *FABP4* and *MMP9* mRNA expression levels were plotted on the same graph

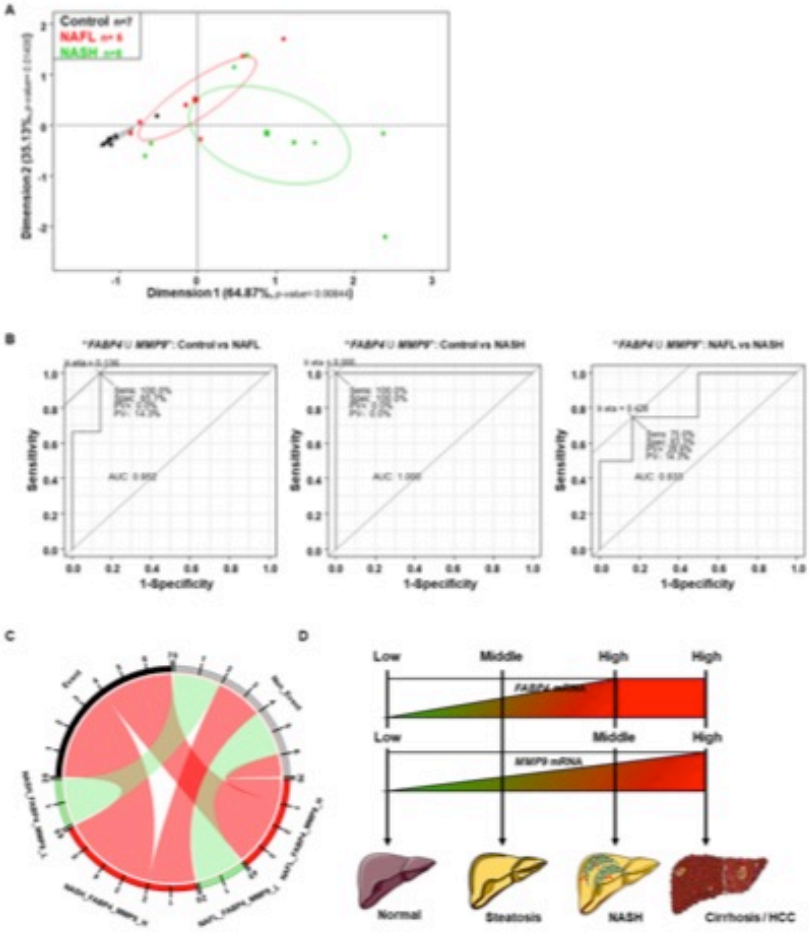

Figure 6. High *FABP4* and *MMP9* hepatic mRNA expression levels predicted poor disease outcomes. (**A**) Principal component analysis of *FABP4* and *MMP9* hepatic mRNA expression levels in three groups of patients from the Validation cohort: 7 control subjects, 6 NAFL patients, and 8 NASH patients. (**B**) Receiver operating characteristic (ROC) curves using *FABP4* and *MMP9* hepatic mRNA expression levels as markers to compare control *versus* NAFL patients (left panel), control *versus* NASH patients (middle panel) and NAFL *versus* NASH patients (right panel). The values of the Area Under the Curve (AUC) are shown, as well as the sensitivity (Sens), specificity (Spec) and predictive values (PV) for the optimal response cut-off points (Ir.eta). (**C**) Circular layout between patients with NAFL or NASH (with at least high *FABP4* and/or *MMP9* mRNA expression levels or with low (L) *FABP4* and *MMP9* mRNA expression levels) and disease worsening (Event) or not (Non-Event). (**D**) Schema showing that patients with steatosis with high *FABP4* hepatic mRNA expression levels were more likely to progress to NASH and that NASH patients with high *MMP9* hepatic mRNA expression levels were more likely to progress to a poorer condition such as cirrhosis and ultimately to hepatocellular carcinoma (HCC).

with thresholds defined by the median value of each group or by a CART analysis, 4 NAFL patients and 6 NASH patients expressed high *FABP4* and/or *MMP9* mRNA levels (Supplementary Fig. S7E).

Interestingly, a retrospective clinical study of these two subgroups of NAFL and NASH patients showed that 3 NAFL patients had poor outcomes (2 had steatofibrosis, 1 had NASH, 1 died but it was not related to the liver failure) and 4 NASH patients had poor outcomes (1 had cirrhosis, 1 had HCC with cirrhosis, 1 had HCC, 1 had cholangiocarcinoma). The other patients did not experience any progression with no change in NAFL score or NAS. The clinical characteristics of these patients are summarized in Table 3, together with their *FABP4* and *MMP9* hepatic mRNA levels and demonstrated by the *Circos* (Fig. 6C).

Thus, we demonstrated that *FABP4* and *MMP9* hepatic mRNA expression levels could be used as predictive markers for clinical outcomes in patients with NAFL and/or NASH and we were able to build a prediction model (Fig. 6D).

Discussion

Using publicly available gene expression data from liver biopsies from NAFLD patients, we identified gene markers for the progression from NAFL to NASH and from NASH to cirrhosis/HCC. For a few years, the use of previously published gene expression omnibus datasets has been an approach to identify more reliable diagnostic and/or prognostic markers in cancer[54–56]. Therefore, our approach avoided conducting a new clinical trial, and it allowed significantly increasing the number of patients per group while having well-defined groups of patients. Indeed, our cohort is one of the largest cohorts of well-defined NAFLD patients investigated so far. Furthermore, we confirmed our data in a smaller cohort of patients, which could be considered a more ethical approach.

Using an unbiased machine-learning approach of prediction analysis of microarray data, we combined two independent GEO datasets from two independent cohorts of patients, GSE48452 and GSE61260[33,34], to create the largest matrix of NAFLD patients associated with gene expression microarray data. Then, a PAMR batch-adjust algorithm was used for both GSE to avoid introducing multiplicative and systematic biases at each step of the microarray experiments and between two or more independent experiments that were performed on the same microarray platform, which led to new biological findings with increased statistical power[57]. This way, we identified 1,549 genes allowing differentiating the four groups of patients and we confirmed these data in two independent human GEO (GSE63067 and GSE17470)[40,41]. Interestingly, the data showed that 27.03% of NAFL patients (10 out of 37 patients) were re-classified in the group of HO patients. As expected, this result confirmed what was already known: the histological scoring system is limited to classify and predict the outcome of NAFL patients. Indeed, we identified 58 genes allowing differentiating two subgroups of patients with NAFL. Fifty-four out of the 58 genes were upregulated in one third of the NAFL patients (12 out of the 37 patients). These genes were involved in inflammatory and ECMR processes as previously reported in NASH patients[32,58–63]. For the first time, we identified 58 genes among which *FABP4* showed the highest expression levels in NAFL patients. *FABP4* was co-expressed with genes involved in NASH progression. Then, these 58 genes expressed in patients with steatosis allowed predicting the outcome of these patients, which could help to improve the follow-up and lead to the implementation of an early therapeutic strategy.

Then, we identified 330 genes specific to a subgroup of NASH patients characterized by high *MMP9* mRNA expression levels. Interestingly, this subgroup of patients had poor outcomes and expressed genes involved in ECMR, inflammation, and carcinogenesis, whereas patients with low *MMP9* mRNA expression levels had gene-expression profiling associated with a better outcome.

Finally, we quantified *FABP4* and *MMP9* hepatic mRNA expression levels in patients who consulted in our institution to confirm if these two genes could be used as prognostic markers. As these prognostic markers were identified in a large cohort of NAFLD patients, this validation cohort was deliberately smaller. Thus, we confirmed retrospectively that *FABP4* and *MMP9* hepatic mRNA expression levels predicted patient clinical outcome. Indeed, elevated *FABP4* hepatic expression levels have been found to positively correlate with NAFLD severity in a human cohort[51,64,65]. Interestingly, high FABP4 serum levels have been reported in NAFLD patients but its use as a prognostic marker in the serum is still controversial[66–70].

For a decade, MMP9 has been involved in the development and progression of human HCC metastasis[71–74]. More recently, *MMP9* polymorphisms have been associated with the risk of NAFLD and obesity[75]. Therefore, our data showed that *MMP9* hepatic mRNA levels could be used earlier as a prognostic marker to identify NASH patients whose disease could progress to cirrhosis and HCC. To note, a higher MMP-9 serum level has been reported in NAFLD patients compared to control patients but it did not allow distinguishing NAFL patients from NASH patients due to the small sample size and because the authors have not studied patient subgroups[76]. However, D'Amico and colleagues have previously shown significantly higher MMP-9 plasma levels in NASH patients compared to hepatitis C-infected patients with liver disease[77]. In addition, MMP-9 levels have been associated with an increase in inflammatory biomarker levels[78]. Thus, FABP4 and MMP-9 serum levels could be used as non-invasive prognostic markers, especially to identify subgroups of NAFL and NASH patients likely to experience disease progression or not. The next step will be to quantify FABP4 and MMP-9 serum levels in a larger cohort of NAFLD patients to confirm whether these two markers could be used as non-invasive markers.

Conclusion

In conclusion, using publicly available GEO datasets and an original machine-learning analysis, we identified gene signatures that could help to determine the outcome of patients with steatosis likely to progress to NASH, and the outcome of patients with NASH likely to progress to cirrhosis and/or HCC. Liver biopsies cannot be avoided, however, the use of predictive markers could strongly reduce the number of biopsies during patient follow-up and enable a better management of the patients. Thus, we identified a predictive gene signature in human liver that could be used in patient clinical follow-up, as well as in clinical trials focused on the development of drugs to treat NASH patients, including two main genes, *FABP4* and *MMP9*, the proteins of which could be quantified in the serum and used as non-invasive prognostic markers for NAFL and NASH progression.

Data availability

GSE48452, GSE61260, GSE63067 and GSE17470 have been selected from genome expression omnibus (GEO) database repository on NCBI website (http://www.ncbi.nlm.nih.gov/geo/) and already published[33,34,40,41].

All data generated or analyzed during this study are included in this published article and its Supplementary Information Files including the Excel tables from the biostatistical analysis.

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References

1. Ascha, M. S. *et al.* The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology* **51**, 1972–1978, https://doi.org/10.1002/hep.23527 (2010).
2. Tiniakos, D. G., Vos, M. B. & Brunt, E. M. Nonalcoholic fatty liver disease: pathology and pathogenesis. *Annu Rev Pathol* **5**, 145–171, https://doi.org/10.1146/annurev-pathol-121808-102132 (2010).
3. Angulo, P. *et al.* Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* **149**, 389–397 e310, https://doi.org/10.1053/j.gastro.2015.04.043 (2015).
4. Dyson, J. *et al.* Hepatocellular cancer: the impact of obesity, type 2 diabetes and a multidisciplinary team. *J Hepatol* **60**, 110–117, https://doi.org/10.1016/j.jhep.2013.08.011 (2014).
5. Younossi, Z. M. *et al.* Association of nonalcoholic fatty liver disease (NAFLD) with hepatocellular carcinoma (HCC) in the United States from 2004 to 2009. *Hepatology* **62**, 1723–1730, https://doi.org/10.1002/hep.28123 (2015).
6. Byrne, C. D. & Targher, G. NAFLD: a multisystem disease. *J Hepatol* **62**, S47–64, https://doi.org/10.1016/j.jhep.2014.12.012 (2015).
7. Chitturi, S. & Farrell, G. C. Etiopathogenesis of nonalcoholic steatohepatitis. *Semin Liver Dis* **21**, 27–41 (2001).
8. Sanyal, A. J. *et al.* Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* **120**, 1183–1192; https://doi.org/10.1053/gast.2001.23256 S0016508501007491 (2001).
9. Rodriguez-Gallego, E. *et al.* Mapping of the circulating metabolome reveals alpha-ketoglutarate as a predictor of morbid obesity-associated non-alcoholic fatty liver disease. *Int J Obes (Lond)*, 279–287, https://doi.org/10.1038/ijo.2014.53 (2014).
10. Loomba, R. & Sanyal, A. J. The global NAFLD epidemic. *Nat Rev Gastroenterol Hepatol* **10**, 686–690, https://doi.org/10.1038/nrgastro.2013.171 (2013).
11. Browning, J. D. *et al.* Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* **40**, 1387–1395, https://doi.org/10.1002/hep.20466 (2004).
12. Ramachandran, A. & Snehalatha, C. Rising burden of obesity in Asia. *J Obes* **2010**, https://doi.org/10.1155/2010/868573 (2010).
13. (NCD-RisC), N. R. F. C. Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants. *The Lancet* **387**, 1377–1396, https://doi.org/10.1016/S0140-6736(16)30054-X (2016).
14. Younossi, Z. M. *et al.* Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* **64**, 73–84, https://doi.org/10.1002/hep.28431 (2016).
15. Coilly, A. & Chiappini, F. Recent Insights in the Treatments of Non-Alcoholic SteatoHepatitis. *J Endocrinol Diab* **3**, 1–11 (2016).
16. de Alwis, N. M., Anstee, Q. M. & Day, C. P. How to Diagnose Nonalcoholic Fatty Liver Disease. *Dig Dis* **34**(Suppl. 1), 19–26, https://doi.org/10.1159/000447277 (2016).
17. Bugianesi, E. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease: disease mongering or call to action? *Diabetologia* **59**, 1145–1147, https://doi.org/10.1007/s00125-016-3930-7 (2016).
18. Bugianesi, E., Rosso, C. & Cortez-Pinto, H. How to diagnose NAFLD in 2016. *J Hepatol* **65**, 643–644, https://doi.org/10.1016/j.jhep.2016.05.038 (2016).
19. Onyekwere, C. A., Ogbera, A. O., Samaila, A. A., Balogun, B. O. & Abdulkareem, F. B. Nonalcoholic fatty liver disease: Synopsis of current developments. *Niger J Clin Pract* **18**, 703–712, https://doi.org/10.4103/1119-3077.163288 (2015).
20. Brunt, E. M. *et al.* Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology* **53**, 810–820, https://doi.org/10.1002/hep.24127 (2011).
21. Kleiner, D. E. *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **41**, 1313–1321, https://doi.org/10.1002/hep.20701 (2005).
22. Bedossa, P. & Consortium, F. P. Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity, and fibrosis (SAF) score in the evaluation of biopsies of nonalcoholic fatty liver disease. *Hepatology* **60**, 565–575, https://doi.org/10.1002/hep.27173 (2014).
23. Bedossa, P. *et al.* Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology* **56**, 1751–1759, https://doi.org/10.1002/hep.25889 (2012).
24. Brunt, E. M. Nonalcoholic Fatty Liver Disease: Pros and Cons of Histologic Systems of Evaluation. *Int J Mol Sci* **17**; https://doi.org/10.3390/ijms17010097 (2016).
25. El-Badry, A. M. *et al.* Assessment of hepatic steatosis by expert pathologists: the end of a gold standard. *Ann Surg* **250**, 691–697, https://doi.org/10.1097/SLA.0b013e3181bcd6dd (2009).
26. Ratziu, V. *et al.* Sampling variability of liver biopsy in nonalcoholic fatty liver disease. *Gastroenterology* **128**, 1898–1906 (2005).
27. Ratziu, V. *et al.* Histological progression of non-alcoholic fatty liver disease: a critical reassessment based on liver sampling variability. *Aliment Pharmacol Ther* **26**, 821–830, https://doi.org/10.1111/j.1365-2036.2007.03425.x (2007).
28. Larson, S. P. *et al.* Histopathologic variability between the right and left lobes of the liver in morbidly obese patients undergoing Roux-en-Y bypass. *Clin Gastroenterol Hepatol* **5**, 1329–1332, https://doi.org/10.1016/j.cgh.2007.06.005 (2007).
29. Group, T. F. M. C. S. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. *Hepatology* **20**, 15–20 (1994).
30. Vuppalanchi, R. *et al.* Effects of liver biopsy sample length and number of readings on sampling variability in nonalcoholic Fatty liver disease. *Clin Gastroenterol Hepatol* **7**, 481–486, https://doi.org/10.1016/j.cgh.2008.12.015 (2009).
31. Bhala, N., Jouness, R. I. & Bugianesi, E. Epidemiology and natural history of patients with NAFLD. *Curr Pharm Des* **19**, 5169–5176 (2013).
32. Chiappini, F. *et al.* Exploration of global gene expression in human liver steatosis by high-density oligonucleotide microarray. *Lab Invest* **86**, 154–165, https://doi.org/10.1038/labinvest.3700374 (2006).
33. Ahrens, M. *et al.* DNA methylation analysis in nonalcoholic fatty liver disease suggests distinct disease-specific and remodeling signatures after bariatric surgery. *Cell Metab* **18**, 296–302, https://doi.org/10.1016/j.cmet.2013.07.004 (2013).
34. Horvath, S. *et al.* Obesity accelerates epigenetic aging of human liver. *Proc Natl Acad Sci USA* **111**, 15538–15543, https://doi.org/10.1073/pnas.1412759111 (2014).
35. Team, R. C. R: A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing* (2012).
36. Irizarry, R. A. *et al.* Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**, 249–264, https://doi.org/10.1093/biostatistics/4.2.249 (2003).
37. Tibshirani, R., Hastie, T., Narasimhan, B. & Chu, G. Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc Natl Acad Sci USA* **99**, 6567–6572, https://doi.org/10.1073/pnas.082099299 (2002).
38. Hastie, T., Tibshirani, R., Narasimhan, B. & Chu, G. pamr: Pam: prediction analysis for microarrays (2014).
39. Thessen Hedreul, M. *et al.* Combining genetic mapping with genome-wide expression in experimental autoimmune encephalomyelitis highlights a gene network enriched for T cell functions and candidate genes regulating autoimmunity. *Hum Mol Genet* **22**, 4952–4966, https://doi.org/10.1093/hmg/ddt343 (2013).
40. Baker, S. S., Baker, R. D., Liu, W., Nowak, N. J. & Zhu, L. Role of alcohol metabolism in non-alcoholic steatohepatitis. *PLoS One* **5**, e9570, https://doi.org/10.1371/journal.pone.0009570 (2010).

41. Frades, I. *et al.* Integrative genomic signatures of hepatocellular carcinoma derived from nonalcoholic Fatty liver disease. *PLoS One* **10**, e0124544, https://doi.org/10.1371/journal.pone.0124544 (2015).
42. Gaujoux, R. & Seoighe, C. A flexible R package for nonnegative matrix factorization. *BMC Bioinformatics* **11**, 367, https://doi.org/10.1186/1471-2105-11-367 (2010).
43. Husson, F., Josse, J., Le, S. & Mazet, J. FactoMineR: Factor Analysis and Data Mining with R. *R package version* **1**, 04 (2007).
44. Chen, E. Y. *et al.* Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics* **14**, 128, https://doi.org/10.1186/1471-2105-14-128 (2013).
45. Kuleshov, M. V. *et al.* Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res* **44**, W90–97, https://doi.org/10.1093/nar/gkw377 (2016).
46. Liberzon, A. *et al.* The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst* **1**, 417–425, https://doi.org/10.1016/j.cels.2015.12.004 (2015).
47. Chiappini, F. *et al.* Ventromedial hypothalamus-specific Ppp1 deletion exacerbates diet-induced obesity in female mice. *J Clin Invest* **124**, 3781–3792, https://doi.org/10.1172/JCI68585 (2014).
48. Neuschwander-Tetri, B. A. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. *Hepatology* **52**, 774–788, https://doi.org/10.1002/hep.23719 (2010).
49. Neuschwander-Tetri, B. A. Nontriglyceride hepatic lipotoxicity: the new paradigm for the pathogenesis of NASH. *Curr Gastroenterol Rep* **12**, 49–56, https://doi.org/10.1007/s11894-009-0083-6 (2010).
50. Calonge, T. M. & O'Connell, M. J. Turning off the G2 DNA damage checkpoint. *DNA Repair (Amst)* **7**, 136–140, https://doi.org/10.1016/j.dnarep.2007.07.017 (2008).
51. Thumser, A. E., Moore, J. B. & Plant, N. J. Fatty acid binding proteins: tissue-specific functions in health and disease. *Curr Opin Clin Nutr Metab Care* **17**, 124–129, https://doi.org/10.1097/MCO.0000000000000031 (2014).
52. Bauvois, B. New facets of matrix metalloproteinases MMP-2 and MMP-9 as cell surface transducers: outside-in signaling and relationship to tumor progression. *Biochim Biophys Acta* **1825**, 29–36, https://doi.org/10.1016/j.bbcan.2011.10.001 (2012).
53. Zarogoulidis, P. *et al.* Interleukin-8 and interleukin-17 for cancer. *Cancer Invest* **32**, 197–205, https://doi.org/10.3109/07357907.2014.898156 (2014).
54. Kwon, M. S. *et al.* Integrative analysis of multi-omics data for identifying multi-markers for diagnosing pancreatic cancer. *BMC Genomics* **16**(Suppl 9), S4, https://doi.org/10.1186/1471-2164-16-S9-S4 (2015).
55. Orsini, M., Travaglione, A. & Capobianco, E. Cancer markers: integratively annotated classification. *Gene* **530**, 257–265, https://doi.org/10.1016/j.gene.2013.07.020 (2013).
56. Zhou, M. *et al.* A potential signature of eight long non-coding RNAs predicts survival in patients with non-small cell lung cancer. *J Transl Med* **13**, 231, https://doi.org/10.1186/s12967-015-0556-3 (2015).
57. Sims, A. H. *et al.* The removal of multiplicative, systematic bias allows integration of breast cancer gene expression datasets - improving meta-analysis and prediction of prognosis. *BMC Med Genomics* **1**, 42, https://doi.org/10.1186/1755-8794-1-42 (2008).
58. Teufel, A. *et al.* Comparison of Gene Expression Patterns Between Mouse Models of Nonalcoholic Fatty Liver Disease and Liver Tissues From Patients. *Gastroenterology* **151**, 513–525 e510, https://doi.org/10.1053/j.gastro.2016.05.051 (2016).
59. Sahini, N. & Borlak, J. Genomics of human fatty liver disease reveal mechanistically linked lipid droplet-associated gene regulations in bland steatosis and nonalcoholic steatohepatitis. *Transl Res* **177**, 41–69, https://doi.org/10.1016/j.trsl.2016.06.003 (2016).
60. Prieur, X., Rouzer, T. & Ricote, M. Lipotoxicity in macrophages: evidence from diseases associated with the metabolic syndrome. *Biochim Biophys Acta* **1801**, 327–337, S1388-1981(09)00230-3, https://doi.org/10.1016/j.bbalip.2009.09.017 (2010).
61. Takaki, A., Kawai, D. & Yamamoto, K. Multiple hits, including oxidative stress, as pathogenesis and treatment target in non-alcoholic steatohepatitis (NASH). *Int J Mol Sci* **14**, 20704–20728, https://doi.org/10.3390/ijms141020704 (2013).
62. Tilg, H. & Moschen, A. R. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* **52**, 1836–1846, https://doi.org/10.1002/hep.24001 (2010).
63. Wallace, K., Burt, A. D. & Wright, M. C. Liver fibrosis. *Biochem J* **411**, 1–18, BJ20071570, https://doi.org/10.1042/BJ20071570 (2008).
64. Auguet, T. *et al.* Altered fatty acid metabolism-related gene expression in liver from morbidly obese women with non-alcoholic fatty liver disease. *Int J Mol Sci* **15**, 22173–22187, https://doi.org/10.3390/ijms151222173 (2014).
65. Greco, D. *et al.* Gene expression in human NAFLD. *Am J Physiol Gastrointest Liver Physiol* **294**, G1281–1287, https://doi.org/10.1152/ajpgi.00074.2008 (2008).
66. Suh, J. B., Kim, S. M., Cho, G. J. & Choi, K. M. Serum AFBP levels are elevated in patients with nonalcoholic fatty liver disease. *Scand J Gastroenterol* **49**, 979–985, https://doi.org/10.3109/00365521.2013.836754 (2014).
67. Shen, J. *et al.* Non-invasive diagnosis of non-alcoholic steatohepatitis by combined serum biomarkers. *J Hepatol* **56**, 1363–1370, https://doi.org/10.1016/j.jhep.2011.12.025 (2012).
68. Kim, Y. C. *et al.* Serum adipocyte-specific fatty acid-binding protein is associated with nonalcoholic fatty liver disease in apparently healthy subjects. *J Nutr Biochem* **22**, 289–292, https://doi.org/10.1016/j.jnutbio.2010.02.007 (2011).
69. Koh, J. H. *et al.* Serum adipocyte fatty acid-binding protein levels are associated with nonalcoholic fatty liver disease in type 2 diabetic patients. *Diabetes Care* **32**, 147–152, https://doi.org/10.2337/dc08-1379 (2009).
70. Milner, K. L. *et al.* Adipocyte fatty acid binding protein levels relate to inflammation and fibrosis in nonalcoholic fatty liver disease. *Hepatology* **49**, 1926–1934, https://doi.org/10.1002/hep.22896 (2009).
71. Shi, G. M. *et al.* CD151 modulates expression of matrix metalloproteinase 9 and promotes neoangiogenesis and progression of hepatocellular carcinoma. *Hepatology* **52**, 183–196, https://doi.org/10.1002/hep.23661 (2010).
72. Li, J. *et al.* Interleukin 23 promotes hepatocellular carcinoma metastasis via NF-kappa B induced matrix metalloproteinase 9 expression. *PLoS One* **7**, e46264, https://doi.org/10.1371/journal.pone.0046264 (2012).
73. Yan, X. L. *et al.* Hepatocellular carcinoma-associated mesenchymal stem cells promote hepatocarcinoma progression: role of the S100A4-miR155-SOCS1-MMP9 axis. *Hepatology* **57**, 2274–2286, https://doi.org/10.1002/hep.26257 (2013).
74. Han, S. *et al.* Kruppellike factor expression and correlation with FAK, MMP9 and Ecadherin expression in hepatocellular carcinoma. *Mol Med Rep* **8**, 81–88, https://doi.org/10.3892/mmr.2013.1471 (2013).
75. Wu, P. *et al.* Interactions of central obesity with rs3918242 on risk of non-alcoholic fat liver disease: a preliminary case-control study. *Int J Clin Exp Pathol* **8**, 4165–4170 (2015).
76. Yilmaz, Y. & Eren, F. Serum biomarkers of fibrosis and extracellular matrix remodeling in patients with nonalcoholic fatty liver disease: association with liver histology. *Eur J Gastroenterol Hepatol* **31**, 43–46, https://doi.org/10.1097/MEG.0000000000001240 (2019).
77. D'Amico, F. *et al.* Liver immunolocalization and plasma levels of MMP-9 in non-alcoholic steatohepatitis (NASH) and hepatitis C infection. *Acta Histochem* **112**, 474–481, https://doi.org/10.1016/j.acthis.2009.05.005 (2010).
78. Okazaki, I. *et al.* Fibrogenesis and Carcinogenesis in Nonalcoholic Steatohepatitis (NASH): Involvement of Matrix Metalloproteinases (MMPs) and Tissue Inhibitors of Metalloproteinase (TIMPs). *Cancers (Basel)* **6**, 1220–1255, https://doi.org/10.3390/cancers6031220 (2014).

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Author contributions

F.C. Designed the study and developed the concept. A.C. Performed the Q-RT-PCR experiments. F.C. and A.C. Acquired, analyzed and interpreted the Q-RT-PCR data. A.C. and C.G. Acquired and interpreted the clinical data and obtained patient consents. C.G. and F.C. Re-performed the histological examination of the liver biopsies. F.C. and C.D. Implemented the bioinformatic analyses, the statistical workflow and the statistical analyses. F.C. designed and draw all the original figures of the manuscript, the tables and the supplementary information. D.S. Obtained funding, consents from the patients and ethical approval from the hospital board. C.G. curated the consents from the patients through the "Centre des Ressources Biologiques-Université Paris-Sud" Bio Banking Number: 0033-00089. F.C. and A.C. Wrote the original draft. F.C. and D.S. Made critical revision of the manuscript for important intellectual content and study supervision. F.C. Wrote, reviewed and edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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4.3 Méthodes

La méthode utilisée dans cette étude peut apparaître complexe au non-initié des analyses bioinformatiques. En simplifiant, nous avons tout d'abord sélectionné deux GEO dont les données complètes étaient en accès libre et ayant fait l'objet de publication (GSE48452 et GSE61260, (185, 186)). Au total, le génome de 52 patients contrôles, 51 patients obèses sans NAFLD, 37 patients ayant une stéatose simple et 42 patients ayant une NASH ont été inclus. Une nouvelle analyse bioinformatique a permis d'identifier une liste de gènes discriminants ces groupes de patients (C Desterke, F Chiappini). Pour affiner l'analyse, deux validations des gènes identifiés ont été réalisées. Une première à l'aide d'une méthode *in silico* une nouvelle fois, en utilisant deux GEO indépendants des premiers et ayant inclus 11 patients contrôles supplémentaires, 2 patients ayant une stéatose simple et 16 patients ayant une NASH (GSE63067 et GSE17470, (187, 188)). Ces méthodes devaient permettre d'identifier une signature moléculaire de la stéatose simple et de la NASH et de reclasser certains patients. La deuxième méthode, plus classique, a consisté en la sélection de patients du centre hépato-biliaire (7 contrôles, 6 ayant une stéatose simple et 8 une NASH) afin de valider par PCR la sur ou sous-expression des gènes d'intérêt, identifiés par les méthodes *in silico*.

4.4 Résultats principaux et discussion

L'analyse par microarray d'expression de gènes a montré 1549 gènes discriminant les patients ayant une stéatopathie, NAFL ou NASH, des obèses sains ou des contrôles. Parmi eux 58 gènes discriminaient la NASH de la stéatose simple. Ces gènes étaient impliqués dans le remodelage de la matrice extracellulaire et l'inflammation. Le gène le plus discriminant était FABP4 (protéine de liaison aux acides gras 4). Parmi les gènes fortement associés à une expression élevée de FABP4, la métalloprotéinase-9 de matrice (MMP9) était surexprimée chez 55% des patients NASH. Nous avons en outre identifié un total de 330 gènes régulés de manière différentielle, dont 229 gènes étaient surexprimés chez des patients NASH présentant un niveau d'expression élevé de MMP9. Ces gènes étaient impliqués dans le remodelage de la matrice extracellulaire, l'inflammation, la prolifération, la progression des cellules souches et l'oncogenèse. En

utilisant les niveaux d'expression génique des gènes FABP4 et MMP9 hépatiques comme indicateurs de la progression de la maladie dans une cohorte indépendante de patients atteints de NAFLD, nous avons identifié les patients atteints de NAFL et NASH susceptibles d'avoir un mauvais pronostic.

Nos résultats sont de plus concordants avec des données publiées. Il a déjà été décrit que MMP9 est impliquée dans le développement et la progression des métastases du carcinome CHC (189-191). Plus récemment, les polymorphismes de la MMP9 ont été associés au risque de NAFLD et d'obésité (192). Il est intéressant de noter que le taux sérique de MMP9 est significativement plus élevé chez les patients ayant une NAFLD par rapport aux contrôles. En revanche, il n'était pas possible de distinguer les patients ayant une stéatose simple et des patients ayant une NASH (193, 194). Aux vues de ses résultats, nous pensons qu'il serait intéressant de coupler les taux sériques de FABP4 et de MMP9 pour évaluer leur rôle pronostique dans la progression de la NAFLD.

Ainsi, en combinant méthode *in silico*, approches statistiques et méthode *in vitro*, nous avons identifié deux biomarqueurs prédictifs potentiels, FABP4 et MMP9, surexprimés au cours de la progression de la NAFL vers la NASH.

5 Article 3

Quantitative assessment of triglycerides by Fourier Transform InfraRed (FTIR) spectroscopy of donor liver helps predicting outcome after liver TH

5.1 Rationnel et Objectifs du Travail

La TH demeure le dernier recours pour les patients ayant une maladie hépatique engageant leur pronostic vital. Cependant, la pénurie d'organes reste une limitation majeure, entraînant une sélection rigoureuse des candidats à la TH et représentant une proportion importante de la mortalité sur liste d'attente (195). Dans ce contexte, l'utilisation des greffons hépatiques provenant de «donneurs à critères étendus» pour augmenter le nombre de greffons est nécessaire dans tous les pays où la TH est réalisée. Il peut s'agir de donneurs âgés, de donneurs décédés après arrêt circulatoire mais aussi de donneurs ayant une stéatose hépatique (196).

La prévalence de la stéatose hépatique dans la population des donneurs est supérieure à 30% (197). Or, la stéatose est considérée comme un facteur de risque de dysfonctionnement du greffon. Le premier rapport l'ayant montré a été publié à la fin des années 1980 et portait sur deux cas de non fonction primaire du greffon dus à une stéatose macrovacuolaire importante (198). Plusieurs rapports ont par la suite montré que la stéatose du greffon était non seulement une cause de dysfonction ou non fonction primaire, mais également une source de résultats défavorables à long terme pour le greffon (166, 168, 199, 200). En effet, ont été cités comme associé à la stéatose macrovacuolaire un sur-risque de complications biliaires, une moins bonne survie des

patients et du greffon mais aussi des durées d'hospitalisation plus longues. Cependant, du fait de la pénurie d'organe, l'utilisation de greffons stéatosiques répondant à certains critères est unanimement acceptée (169, 201, 202).
Une stéatose macrovacuolaire inférieure à 30% a une influence minime sur la fonction du greffon et ce type de foie est généralement accepté. Au contraire, la présence d'une stéatose macrovacuolaire sévère supérieure à 60% contre indique généralement l'utilisation de l'organe pour la TH (203). Entre 30 et 60%, l'acceptation dépend d'autres paramètres pour évaluer la qualité de la greffe (par exemple, l'âge du donneur, la durée de l'ischémie froide, la présence d'une fibrose), mais également des caractéristiques du receveur potentiel afin d'éviter d'accumuler les risques d'échec de la TH. Cependant, l'incidence de dysfonction primaire n'est pas nulle et peut atteindre 15%. De même, une reprise retardée de la fonction du greffon est observée chez 35% des receveurs. La littérature soutenant de telles pratiques reste ancienne, rare et conflictuelle. Il est généralement admis que la stéatose microvésiculaire n'est pas associée à un risque accru de dysfonction du greffon bien que sur ce point encore, plusieurs données sont contradictoires (204-207).

L'une des principales limites est la précision des méthodes actuelles d'évaluation du degré de stéatose. L'inspection par le chirurgien au moment du prélèvement permet de détecter la stéatose, mais la corrélation entre cette évaluation et le degré de stéatose histologique est médiocre, lorsque la stéatose dépasse 35% (208, 209). L'examen anatomopathologique est l'outil de référence pour évaluer la stéatose. Cependant, l'évaluation de la stéatose hépatique est une méthode imparfaite, mal corrélée au contenu lipidique réel du tissu hépatique (210). De plus, un examen demandé en urgence au moment du prélèvement, dit extemporané, rend encore plus difficile l'interprétation.

Ainsi, la disponibilité d'outils permettant une mesure objective et reproductible de la stéatose est plus que souhaitable pour les équipes amenées à accepter ou rejeter les greffons.

Nous avons précédemment publié des données sur l'intérêt de la microspectroscopie à transformée de Fourier en infrarouge (FTIR) pour évaluer avec précision le contenu

lipidique dans la greffe du foie (211). Une coupe de tissu congelé de 5 µm d'épaisseur est nécessaire et déposée sur une lame de verre régulière. L'analyse prend moins d'une minute pour acquérir le spectre, calculer le rapport lipides/protéines et extrapoler la quantité de TG sur la base de la courbe standard établie et donc du pourcentage virtuel de stéatose associé (environ 20%).

Si ce travail préliminaire a permis d'établir la faisabilité et fiabilité de la technique, le but de la présente étude était d'abord de confirmer les résultats en positionnant la FTIR en tant que méthode fiable pour évaluer la stéatose du greffon par rapport au gold standard du dosage lipidique utilisant la spectrométrie de masse sur une série de biopsies avant greffe. Dans un second temps, nous souhaitions comparer l'évaluation de la stéatose par FTIR à l'analyse anatomopathologique. Enfin, nous avons étudié l'influence de la stéatose du greffon obtenue par ces mesures quantitatives et qualitatives à l'évolution clinique des receveurs, en particulier, la fonction du greffon ainsi que la survie du patient et du greffon.

5.2 L'article

Quantitative assessment of triglycerides by Fourier Transform InfraRed (FTIR) spectroscopy of donor liver helps predicting outcome after liver TH

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- Audrey Coilly has no disclosure regarding this topic. She has been a clinical investigator, speaker and/or consultant for Astellas, Gilead Sciences, Intercept Pharma, Novartis, and Abbvie.

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Audrey Coilly, Slávka Kaščáková, Franck Chiappini, François Le Naour, Didier Samuel and Catherine Guettier contributed to the conception and design of this study and to the preparation and finalization of the manuscript. Audrey Coilly, Slávka Kaščáková, Chengyuan Peng recruited patients into the study and participated in data collection and data analysis. Catherine Guettier performed centralized histological reading of liver biopsy specimens. Christophe Desterke and Franck Chiappini contributed to data analysis. All authors critically reviewed the article for important intellectual content and approved the final draft for submission.

Abbreviations

ECD: Extended Criteria Donors

FAME: Fatty Acid Methyl Ester

FFPE: Formalin-Fixed Paraffin-Embedded

FTIR: Fourier Transform-Infrared

HES: Haematoxylin-Eosin-Saffron

ICU: Intensive Care Unit

IR: Infra Red

LT: Liver TH

MaS: Macrovacuolar Steatosis

MELD: Model for End stage Liver Disease

MiS: Microvesicular Steatosis

OvS: Overall Steatosis

PNF: Primary graft Non-Function

TG: Triglycerides

Abstract

Background and Aims: Liver steatosis, especially macrovacuolar steatosis (MaS) of the donor liver is a major predictor of graft dysfunction after transplantation, Current tools to evaluate graft steatosis remains the gross examination of surgeons plus the on-demand histopathological analysis on frozen section. Pathological evaluation of liver steatosis is approximate, pathologist dependent, imperfect in the pretransplant setting and may not reflect the true lipid content. Our aims were 1) to assess the correlation between the triglycerides content of grafts measured by gas-phase chromatography coupled to mass spectrometry (GC/MS) and infrared microspectroscopy (FTIR) to standard histopathological evaluation; 2) to determine if FTIR microspectroscopy could help predicting outcome after liver transplantation.

Methods: Using a cohort of 58 patients, liver transplanted between 02/2012 and 03/2014, we quantified the triglycerides content of the graft using GC/MS and FTIR and compared the results of both methods together and with histological analysis, retrospectively performed on frozen sections by a single expert pathologist. Clinical data and events were also collected.

Results: Fifty-eight patients were included. The mean percentage of macrovacuolar steatosis (MaS) and microvesicular steatosis (MiS) assessed by pathologist, was 2% and 30%. The mean concentration of hepatic triglycerides measured by GC/MS was 214 [10-1045] nmol/mg of hepatic tissue, The estimation of triglyceride contents obtained using FTIR was significantly correlated (r2=0.812) with the GC/MS results. Thirty-four (58%) patients experienced complications defined by a Dindo-Clavien stage ≥2 including 2 primary non-function of the graft and 5 deaths. The most discriminant threshold of triglycerides level to transplant failure were 59.29 and 54.02 nmol/mg of hepatic tissue

using GC/MS and FTIR, respectively. The quantification of hepatic content of triglycerides using GC/MS was significantly associated with patient survival at the end of the follow-up ($p<0.0001$) and transplantation failure ($p<0.0001$). The estimation of hepatic content of triglycerides using FTIR was significantly associated with one-year post-transplant survival ($p<0.0001$).

Conclusion: Our study demonstrates that FTIR-spectroscopy to measure triglycerides content in liver grafts is well correlated to the gold standard histopathological evaluation of steatosis but more importantly, that this content is associated with post-transplantation outcome.

KEY WORDS: liver transplantation, steatosis, donor, spectrometer, infrared, microspectroscopy.

Introduction

Liver transplantation (LT) constitutes a major treatment of patients with acute liver failure, cirrhosis or hepatocellular carcinoma. Despite the development of surgical strategies to expand the liver graft pool as splitting livers, domino procedure, the need for LT exceeds organ availability. The organ shortage remains a major limitation, leading to a drastic selection of candidates for LT and accounting for a large proportion of waiting list mortality [1, 2]. This discrepancy has led most centres worldwide to use livers from 'extended criteria donors' (ECD) to expand the pool of liver grafts with older donors, donors after circulatory death and donors with liver steatosis [3].

The prevalence of liver steatosis in donor population is over 30% of donors [4]. Steatosis is considered a risk factor for graft dysfunction. The first report was published in late 1980s with two cases of primary graft non-function (PNF) due to severe macrovacuolar steatosis (MaS) [5]. Several reports followed showing that steatosis of the graft is not only a cause of PNF but also a source of long-term poorer outcome of the graft [6-9]. Indeed, biliary complications, increased costs and longer stays in hospital, poorer patient and graft survivals have been also associated with MaS. However, with increasing utilisation of ECD organs, there has been acceptance of steatosis liver grafts [10-12]. Mild MaS < 30% has minimal impact on graft function and such donor livers are usually accepted. On the contrary, the presence of a severe MaS > 60% leads to organ discarding [13]. Between 30 and 60%, the acceptance depends on other parameters to evaluate the quality of the graft (e.g. donor age, cold ischemia time) but also on characteristics of the recipients (e.g. indication for LT, severity of liver disease). In this group, the incidence of PNF may reach 15%, and the rate of delayed graft function

approaches 35% [14]. The literature supporting such practices remains former, sparse and conflicting. It is generally admitted that microvesicular steatosis (MiS) is not associated with an increased risk of PNF [15-18].

One main limitation is the accuracy of current methods to assess the degree of steatosis. Inspection at procurement helps detect steatosis but the correlation between surgical assessment and degree of steatosis when steatosis exceeds 35% is poor [19, 20]. Histopathological examination on frozen section is the gold-standard tool to evaluate steatosis. However, the assessment of liver steatosis is an imperfect method with poor inter-observer agreement and is poorly correlated with the true lipid content of the liver tissue [21]. The delay due to frozen section procedure also increases cold ischemia time [22, 23].

To date, the availability of objective and quantifiable tools for graft quality control, especially for the assessment of steatosis would be really helpful for transplantation team [24, 25]. We previously published data on the interest of Fourier Transform InfraRed (FTIR) microspectroscopy to precisely evaluate the lipid content in liver graft [26].

The aim of this study was firstly to definitely validate FTIR microspectroscopy as a reliable method to rate graft steatosis compared to the gold standard of lipidomic assay using mass spectrometry on a series of before clamping graft biopsies, secondly to compare FTIR assessment of steatosis to histopathological analysis, and thirdly to correlate quantitative and qualitative graft steatosis evaluations with graft function, graft loss and patient survival.

Patients and methods

Data source

This study was performed in a tertiary center with extensive experience of LT. Prospectively collected data of adult patients who underwent LT at Paul Brousse Hospital (Villejuif, France) between February 2012 and December 2014 were reviewed. All data were obtained from computerized medical records. This study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practices. All the patients gave written informed consent to participate in the retrospective studies of our centre. We selected recipients for whom frozen tissue of the graft at the time of harvesting have been collected. Liver biopsy specimens were provided by the centre of biological resources of Bicêtre (CRB Paris Sud, Bio Banking number: 0033-00089).

Study population

All the patients received an orthotropic liver transplantation from a heart-beating donor. All the parameters known to impact graft or patient survival were recorded, especially recipient characteristics (age, indication for LT, stage of liver disease, pre-transplant condition), donor characteristics (age, weight, body mass index, cause of death), operative management (cold ischemia time, number of red blood cell transfusion). During follow-up, parameters associated with liver function (including MELD score and Child-Pugh score), kidney function (including creatinine clearance estimated by the Cockcroft and Gault formula), post-operative complications, and immunosuppression regimen. Parameters linked to metabolic syndrome (body mass index, treated diabetes mellitus, treated high blood pressure and treated dyslipidaemia)

were also recorded and graft and patient survival were recorded at Day 0, at intensive care unit (ICU) discharge, at hospitalization discharge, at one year and at the end of the study (May 14th 2016). Complications during hospitalisation stay were defined according to the Dindo-Clavien classification ≥2 [27]. To evaluate patient outcome, we defined the transplantation failure either as the death of the recipient or the need for a re-transplantation.

Pathological assessment

For each LT, surgical biopsies of the graft were systematically harvested before clamping at the time of procurement and after reperfusion at the end of the transplantation procedure, respectively. Biopsy samples were partly snap-frozen and partly fixed in formaldehyde. Fixed specimens were embedded in paraffin and stained with standard haematoxylin-eosin-saffron (HES) and picrosirius stain. The samples were blindly analysed by the team of the pathological unit. Secondarily, all stained sections have been reviewed by a single pathologist blinded to clinical information (CG). MaS and MiS were rated using standardized assessment by an estimation of the percentage of hepatocytes disclosing large or small vacuoles respectively [28]. Assessment of graft steatosis was performed on HES stained frozen sections for before clamping biopsies. Fibrosis stage was also reviewed on formalin-fixed paraffin-embedded (FFPE) sections and ischemia-reperfusion lesions were quantified as slight, moderate or severe on FFPE sections of the reperfusion biopsies. To rate the overall steatosis (OvS), the percentage of MaS was added to the percentage of MiS.

Quantification of hepatic triglycerides content using mass spectrometry

Gas-phase chromatography coupled to mass spectrometry (GC/MS) has been previously used to quantify the overall triglyceride liver content at MetaToul platform [29]. Briefly, lipid extraction from the surgical specimen followed by GC/MS was done for each graft specimen to determine concentration of hepatic triglycerides and to classify specimens into four groups according to the amount of triglycerides (grade 1 to 4) as described previously [30]. The complete method is given in Appendix 1.

Estimation of hepatic triglycerides content using Fourier Transform infrared microspectroscopy

Fourier Transform InfraRed (FTIR) spectroscopy analysis was performed on the frozen liver samples. The principle of FTIR is based on the determination of absorption of infrared (IR) light due to resonance with vibrational motions of functional molecular groups. A frequency will be strongly absorbed if its photon energy coincides with the vibrational energy levels of the molecule sample and all the absorptions bands of lipids, proteins and sugars are seen within the range of IR spectrum [31]. Therefore, FTIR is a very powerful technique that provides fingerprint information on the chemical composition of the tissue, particularly lipid content in this study [32, 33]. A frozen tissue section was deposited on a regular glass slide and the tissue's IR spectrum was recorded using an ATR-FTIR spectrometer (the Nicolet™ iZ10 module) connected to Nicolet™ iN10 infrared microscope. The acquired IR spectrum thus obtained corresponds to an average spectrum of an area 2 x 2 mm2 of the tissue section. The quantification of the triglyceride (TG) content was then addressed from the acquired spectrum by calculating the ratio of integrated intensity of bands attributed to lipids (2800-3100 cm^{-1}) relative to proteins (Amide II: 1485-1595 cm-1) and expressed in nmol/mg as described previously [26]. An experienced researcher measured TG content and mean of three measurements per tissue section was considered as TG content of a patient. Then, the

patients were grouped into four grades according to TG content as G0: <43.66 nmol/mg, G1: 43.66-220.1 nmol/mg, G2: >220.1-465.5 nmol/mg, and G3: >465.5 nmol/mg [30].

Statistical Analysis

In the case of a non-normal distribution, continuous variables were expressed by median and ranges and comparisons used non-parametric tests. In case of a normal distribution, continuous variables were expressed by mean and standard deviation (m ± sd) and parametric test used such as ANOVA and Student t-test. Categorical variables were expressed in terms of the number of patients and percentages, chi-square (χ^2) test used for comparisons and Kaplan- survival analysis performed.

Correlations were presented as scatter plots generated using Pearson product-moment correlation coefficient (r). This method was able to limit the impact of outliers and enable more robust representations.

The systematic bias corresponded to the systematic error that was introduced in all calculations, *i.e.* the precision of the values we obtained.

All statistical tests and 95% confidence intervals (CI 95%) had a significance level *p*-value is less than the type-I error rate 0.05 ($\alpha<0.05$).

Results

Baseline demographic and clinical characteristics

Fifty-eight liver grafts were available for the present study. The characteristics of the donors and recipients are given in Table 1. Liver donor analyses assessed by the pathologist on frozen tissue sections showed that the mean percentage of MaS, was 2% and reached 50% for only one graft. The mean percentage of MiS was 30% and exceeded 50% in 18 grafts. Among the 58 recipients, the main indications of liver transplantation were decompensated cirrhosis in 23 (40%) patients and hepatocellular carcinoma in 11 (19%). The causes of end-stage liver disease were alcohol and non-alcoholic steatohepatitis in 22 (38%) and 7 (12%) patients, respectively. The median MELD score and creatinine clearance at transplantation were 20.4 [6-40] and 53.2 [15-89] mL/min, respectively.

Thirty-four (58%) patients experienced complications during the hospitalisation stay including infections for 23 (40%), biliary strictures for 4 (7%), hepatic artery stenosis for 2 (3%) and PNF for 2 (3%) patients. The median follow-up duration was 30.2 [2-63] months. During the study observation, 5 patients died and 2 were re-transplanted (at day 2 and day 6 post-LT for PNF). The rates of graft and patient survival were both 95% at 20 months (Figure 1).

Quantification of hepatic triglycerides and correlation between assessment methods

Using GC/MS, the median concentration of hepatic triglycerides was 214 [10-1045] nmol/mg of hepatic tissue. The ratio lipids/proteins calculated by FTIR measurement was significantly correlated to the triglycerides assessed by GC/MS (r^2=0.812) as shown in Figure 2.

Both quantitative assessments using GC/MS and FTIR of hepatic triglycerides were positively correlated to MaS, MiS, and OvS determined by histopathological examination (Figure 3). These quantitative approaches were able to divide patients with a rate of OvS between 30 and 60% into two groups (Figure 4).

The overall concentrations of hepatic triglycerides predict outcome after liver transplantation

Considering that the quantitative assessments using GC/MS and FTIR of hepatic triglycerides integrate the overall steatosis of the graft, we evaluated whether these measurements could predict patient outcome. The most discriminant thresholds of triglycerides level to transplant failure were 7.7 and 7.35 Log_2 nmol/mg (whether 59.29 et 54.02 nmol/mg) of hepatic tissue using GC/MS and FTIR, respectively (Figure 4).

The multivariate analysis of factors associated with the quantification of hepatic content of triglycerides using GC/MS and FTIR is shown in Table 2 and Table 3, respectively. The quantification of hepatic content of triglycerides using GC/MS was significantly associated with patient survival at the end of the follow-up and transplantation failure (p-value<0.0001, OR=15.28, 95%CI 1.62-760.02). There is also a trend to predict the occurrence of a treated dyslipidaemia at one year after LT. The estimation of hepatic content of triglycerides using FTIR was significantly associated with one-year post-transplant survival (p<0.0001).

Discussion

Obtaining a reliable and reproducible measurement of steatosis of liver graft is a major issue in liver transplantation. First of all, our study confirmed that it is possible to use FTIR-spectroscopy to measure the graft steatosis on a cohort of liver transplant patients as previously described [26, 30]. Indeed, measurements of TG levels by FTIR are well correlated as previously described by us with GC/MS measurements which represent the gold standard to determine the lipid content in liver. These results are in total agreement with those we obtained in 12 surgical biopsies sampled at harvesting of liver grafts right before clamping, showing that the theoretical amount of TG estimated by FTIR analysis was comparable to the true TG content [26]

In the current study, the correlation between histological assessment of steatosis and TG measurement by GC/MS or FTIR is also pretty good. In a previous study, we have shown conversely that the histological estimation of steatosis is poorly correlated with the true lipid content of the liver tissue after lipid extraction from the same surgical specimen followed by GC-MS [30]. This discrepancy could be explained by differences in liver lipid content; in the current study, the global lipid content is lower as in the previous one as all livers were selected for transplantation.

Quantification of TG liver content by FTIR-spectroscopy was performed here using a Nicolet™ iN10 infrared microscope. The use of an ATR-FTIR connected to a computer makes it possible to implement the technique at the hospital and offers the possibility to quickly analysis the content of liver steatosis in an objective manner. Non-medical but trained staff could use the device. In the context of liver transplantation, results could be obtained without delaying the harvesting procedure and LT. FTIR-spectroscopy offers other advantages than evaluation of steatosis. It could provide complementary

information on the chemical composition of the liver such as glycogen content and collagens linked to fibrosis [29, 31].

Previous clinical studies have shown the feasibility and reliability of evaluating liver steatosis by using spectroscopy [35-37]. In the study of Evers et al., spectroscopic measurements were performed in vivo in 17 patients in liver tissue during LT and ex vivo on liver resection specimens from 41 patients. Correlation between the pathologists' analysis and the spectroscopic measurement was good in both conditions (R2 0.949 and R2 0.854, respectively). In a recent study, Golse et al. demonstrated the accuracy of pocket-sized micro-spectrometers to predict liver graft macrovacuolar steatosis [24]. The main advantage of this latter technique comparing to FTIR method used in our study is the non-invasive and quick measurement of steatosis by this device and which can be transportable as well. The positive and negative predictive values for MaS ≥30% were 100% and 98%, respectively. But this study did not scan any liver graft over 60% of steatosis and failed to demonstrate a correlation for intermediate MaS rate between 30 and 60%, which is an acceptable steatosis rate in some recipients and thus crucial information for determining the translatability of certain graft. Furthermore, neither MiS nor OvS could be predicted by the pocket-sized micro-spectrometer. Finally, contrary to our study, no clinical impact has been reported.

Our study demonstrates that a high TG level in liver graft had a negative impact on the transplant course as it is associated with post-transplant survival and complications based on the Dindo-Clavien stage ≥2 ($p<0.0001$). Our study confirms the large literature regarding the negative impact of steatosis of the liver grafts on the LT outcome. Donor liver steatosis is considered a risk factor for poor post-transplantation outcome, with increased risk of PNF. Todo et al. published two case reports describing PNF following

implantation of two fatty livers in 1980s [5]. These livers showed severe MaS when the pre-transplant biopsy was examined retrospectively. Several studies, mostly single-centre studies, found that severely (>60 %) steatotic grafts are associated with increased risk of poor graft function, whilst moderate–severe (>30 %) steatotic grafts are associated with decreased graft survival [12, 38]. It could be surprising that our study was able to demonstrate an impact of liver with high TG level (defined by a threshold of 59.29 nmol/mg of hepatic tissue) on liver transplant outcome, regarding the small number of patients enrolled and the fact that those liver grafts have been chosen to be transplant. One hypothesis should be that recipients transplanted with steatotic livers have been at higher risk of transplant failure. However, surgeons usually discarded poor grafts for high-risk recipients. Secondly, the two PNF of our study occurred in patients who received grafts with MaS ≤30% whereas the MiS was 30 and 20%, respectively, and the concentration of TG was up to 54.02 nmol/mg of hepatic tissue for both grafts.

This result reopens the debate about the importance of considering MiS as well as MaS. Indeed, MiS is known to be less deleterious than MaS, in particular, enable to worsen ischemia-reperfusion features [39]. But, in several liver diseases, MiS is considered a more serious condition often associated with impaired mitochondrial beta-oxidation and, therefore, a less favourable prognosis [40]. Most studies failed to distinguish the impact of MiS and MaS on graft function as both conditions coexist in the liver grafts. Sharkey et al. identified two subgroups of MiS. High-grade MiS presented many small vesicles that filled the cell cytoplasm with cell enlargement and was more likely to be associated with delayed graft function in the postoperative period [18]. Cieślak et al. did a retrospective analysis of 269 liver transplantations. They found that the risk of PNF was significantly related to MiS (p-value<0.021) [41]. Finally, Yoong et al. analysed the

outcome of 116 patients after re-LT and suggested that severe MiS ≥ 66% of hepatocytes was associated with a significantly higher rate of PNF (p-value<0.01) [15]. More than opposing MiS and MaS, spectroscopic tools make it possible to perform an overall measurement of steatosis, or rather to evaluate the lipid load of hepatocytes. If our study is able to highlight an adverse outcome for fatty livers, it may be precisely because, thanks to spectroscopy, we take into account the overall rate of hepatic lipid content.

In a very next future, it will be of importance to quantify the OvS. Ex situ machine perfusion, a novel method in graft preservation, is showing great promise in providing a tool for the recovery and reconditioning of marginal livers [42]. In the epidemic era of obesity and metabolic syndrome, reconditioning these steatotic livers could increase the number of LT performed. In most studies, standard tools have been used to evaluate the so-called defatting as blood TG levels, markers of graft function [43-45]. We believe that FTIR measurements would provide a quick and precise method to evaluate the transplantability of such reconditionned steatotic livers.

In conclusion, our study shows that FTIR-spectroscopy which is an easy-to-use and fast procedure less than 20mn provides a very good reflection of the true triglyceride content of liver tissue and more importantly, that the TG content assessed by FTIR-spectrosocpy is associated with LT outcome. FTIR could be implemented easily in the liver transplantation process helping transplant teams to choose better graft in term of steatosis. It positions FTIR-spectroscopy as an additional decision-making tool to evaluate a graft before transplantation.

References

[1] Ow MM, Erasmus P, Minto G, Struthers R, Joseph M, Smith A, et al. Impaired functional capacity in potential liver transplant candidates predicts short-term mortality before transplantation. Liver Transpl 2014;20:1081-1088.

[2] Linecker M, Krones T, Berg T, Niemann CU, Steadman RH, Dutkowski P, et al. Potentially inappropriate liver transplantation in the era of the "sickest first" policy - A search for the upper limits. J Hepatol 2018;68:798-813.

[3] Durand F, Renz JF, Alkofer B, Burra P, Clavien PA, Porte RJ, et al. Report of the Paris consensus meeting on expanded criteria donors in liver transplantation. Liver Transpl 2008;14:1694-1707.

[4] Flechtenmacher C, Schirmacher P, Schemmer P. Donor liver histology--a valuable tool in graft selection. Langenbecks Arch Surg 2015;400:551-557.

[5] Todo S, Demetris AJ, Makowka L, Teperman L, Podesta L, Shaver T, et al. Primary nonfunction of hepatic allografts with preexisting fatty infiltration. Transplantation 1989;47:903-905.

[6] Spitzer AL, Lao OB, Dick AA, Bakthavatsalam R, Halldorson JB, Yeh MM, et al. The biopsied donor liver: incorporating macrosteatosis into high-risk donor assessment. Liver Transpl 2010;16:874-884.

[7] Briceno J, Ciria R, Pleguezuelo M, de la Mata M, Muntane J, Naranjo A, et al. Impact of donor graft steatosis on overall outcome and viral recurrence after liver TH for hepatitis C virus cirrhosis. Liver Transpl 2009;15:37-48.

[8] Angele MK, Rentsch M, Hartl WH, Wittmann B, Graeb C, Jauch KW, et al. Effect of graft steatosis on liver function and organ survival after liver TH. Am J Surg 2008;195:214-220.

[9] Zamboni F, Franchello A, David E, Rocca G, Ricchiuti A, Lavezzo B, et al. Effect of macrovescicular steatosis and other donor and recipient characteristics on the outcome of liver transplantation. Clin Transplant 2001;15:53-57.

[10] Dutkowski P, Schlegel A, Slankamenac K, Oberkofler CE, Adam R, Burroughs AK, et al. The use of fatty liver grafts in modern allocation systems: risk assessment by the balance of risk (BAR) score. Ann Surg 2012;256:861-868; discussion 868-869.

[11] Baccarani U, Isola M, Adani GL, Avellini C, Lorenzin D, Rossetto A, et al. Steatosis of the hepatic graft as a risk factor for post-transplant biliary complications. Clin Transplant 2010;24:631-635.

[12] McCormack L, Petrowsky H, Jochum W, Mullhaupt B, Weber M, Clavien PA. Use of severely steatotic grafts in liver transplantation: a matched case-control study. Ann Surg 2007;246:940-946; discussion 946-948.

[13] Ploeg RJ, D'Alessandro AM, Knechtle SJ, Stegall MD, Pirsch JD, Hoffmann RM, et al. Risk factors for primary dysfunction after liver transplantation--a multivariate analysis. Transplantation 1993;55:807-813.

[14] Canelo R, Braun F, Sattler B, Klinge B, Lorf T, Ramadori G, et al. Is a fatty liver dangerous for transplantation? Transplant Proc 1999;31:414-415.

[15] Yoong KF, Gunson BK, Neil DA, Mirza DF, Mayer AD, Buckels JA, et al. Impact of donor liver microvesicular steatosis on the outcome of liver retransplantation. Transplant Proc 1999;31:550-551.

[16] Han S, Ha SY, Park CK, Joh JW, Kwon CH, Kwon GY, et al. Microsteatosis may not interact with macrosteatosis in living donor liver transplantation. J Hepatol 2015;62:556-562.

[17] Nocito A, El-Badry AM, Clavien PA. When is steatosis too much for transplantation? J Hepatol 2006;45:494-499.

[18] Sharkey FE, Lytvak I, Prihoda TJ, Speeg KV, Washburn WK, Halff GA. High-grade microsteatosis and delay in hepatic function after orthotopic liver transplantation. Hum Pathol 2011;42:1337-1342.

[19] Rey JW, Wirges U, Dienes HP, Fries JW. Hepatic steatosis in organ donors: disparity between surgery and histology? Transplant Proc 2009;41:2557-2560.

[20] Yersiz H, Lee C, Kaldas FM, Hong JC, Rana A, Schnickel GT, et al. Assessment of hepatic steatosis by transplant surgeon and expert pathologist: a prospective, double-blind evaluation of 201 donor livers. Liver Transpl 2013;19:437-449.

[21] Gawrieh S, Knoedler DM, Saeian K, Wallace JR, Komorowski RA. Effects of interventions on intra- and interobserver agreement on interpretation of nonalcoholic fatty liver disease histology. Ann Diagn Pathol 2011;15:19-24.

[22] El-Badry AM, Breitenstein S, Jochum W, Washington K, Paradis V, Rubbia-Brandt L, et al. Assessment of hepatic steatosis by expert pathologists: the end of a gold standard. Ann Surg 2009;250:691-697.

[23] Lo IJ, Lefkowitch JH, Feirt N, Alkofer B, Kin C, Samstein B, et al. Utility of liver allograft biopsy obtained at procurement. Liver Transpl 2008;14:639-646.

[24] Golse N, Cosse C, Allard MA, Laurenzi A, Tedeschi M, Guglielmo N, et al. Evaluation of a micro-spectrometer for the real-time assessment of liver graft with mild-to-moderate macrosteatosis: A proof of concept study. J Hepatol 2019;70:423-430.

[25] Schwenzer NF, Springer F, Schraml C, Stefan N, Machann J, Schick F. Non-invasive assessment and quantification of liver steatosis by ultrasound, computed tomography and magnetic resonance. J Hepatol 2009;51:433-445.

[26] Le Naour F, Gadea L, Danulot M, Yousef I, Vibert E, Wavelet M, et al. Quantitative assessment of liver steatosis on tissue section using infrared spectroscopy. Gastroenterology 2015;148:295-297.

[27] Dindo D, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. Ann Surg 2004;240:205-213.

[28] Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 1999;94:2467-2474.

[29] Chiappini F, Coilly A, Kadar H, Gual P, Tran A, Desterke C, et al. Metabolism dysregulation induces a specific lipid signature of nonalcoholic steatohepatitis in patients. Sci Rep 2017;7:46658.

[30] Peng C, Chiappini F, Kascakova S, Danulot M, Sandt C, Samuel D, et al. Vibrational signatures to discriminate liver steatosis grades. Analyst 2015;140:1107-1118.

[31] Diem M, Chiriboga L, Yee H. Infrared spectroscopy of human cells and tissue. VIII. Strategies for analysis of infrared tissue mapping data and applications to liver tissue. Biopolymers 2000;57:282-290.

[32] Petit VW, Refregiers M, Guettier C, Jamme F, Sebanayakam K, Brunelle A, et al. Multimodal spectroscopy combining time-of-flight-secondary ion mass spectrometry, synchrotron-FT-IR, and synchrotron-UV microspectroscopies on the same tissue section. Anal Chem 2010;82:3963-3968.

[33] Le Naour F, Bralet MP, Debois D, Sandt C, Guettier C, Dumas P, et al. Chemical imaging on liver steatosis using synchrotron infrared and ToF-SIMS microspectroscopies. PLoS One 2009;4:e7408.

[34] Le Naour F, Sandt C, Peng C, Trcera N, Chiappini F, Flank AM, et al. In situ chemical composition analysis of cirrhosis by combining synchrotron fourier transform infrared and synchrotron X-ray fluorescence microspectroscopies on the same tissue section. Anal Chem 2012;84:10260-10266.

[35] Reistad N, Nilsson JH, Bergenfeldt M, Rissler P, Sturesson C. Intraoperative liver steatosis characterization using diffuse reflectance spectroscopy. HPB (Oxford) 2019;21:175-180.

[36] Nilsson JH, Reistad N, Brange H, Oberg CF, Sturesson C. Diffuse Reflectance Spectroscopy for Surface Measurement of Liver Pathology. Eur Surg Res 2017;58:40-50.

[37] Evers DJ, Westerkamp AC, Spliethoff JW, Pully VV, Hompes D, Hendriks BH, et al. Diffuse reflectance spectroscopy: toward real-time quantification of steatosis in liver. Transpl Int 2015;28:465-474.

[38] Chu MJ, Dare AJ, Phillips AR, Bartlett AS. Donor Hepatic Steatosis and Outcome After Liver Transplantation: a Systematic Review. J Gastrointest Surg 2015;19:1713-1724.

[39] Andert A, Ulmer TF, Schoning W, Kroy D, Hein M, Alizai PH, et al. Grade of donor liver microvesicular steatosis does not affect the postoperative outcome after liver transplantation. Hepatobiliary Pancreat Dis Int 2017;16:617-623.

[40] Fromenty B, Berson A, Pessayre D. Microvesicular steatosis and steatohepatitis: role of mitochondrial dysfunction and lipid peroxidation. J Hepatol 1997;26 Suppl 1:13-22.

[41] Cieslak B, Lewandowski Z, Urban M, Ziarkiewicz-Wroblewska B, Krawczyk M. Microvesicular liver graft steatosis as a risk factor of initial poor function in relation to suboptimal donor parameters. Transplant Proc 2009;41:2985-2988.

[42] Boteon YL, Boteon A, Attard J, Mergental H, Mirza DF, Bhogal RH, et al. Ex situ machine perfusion as a tool to recondition steatotic donor livers: Troublesome features of fatty livers and the role of defatting therapies. A systematic review. Am J Transplant 2018;18:2384-2399.

[43] Boteon YL, Attard J, Boteon AP, Wallace L, Reynolds G, Hubscher S, et al. Manipulation of lipid metabolism during normothermic machine perfusion: Effect of defatting therapies on donor liver functional recovery. Liver Transpl 2019.

[44] Jamieson RW, Zilvetti M, Roy D, Hughes D, Morovat A, Coussios CC, et al. Hepatic steatosis and normothermic perfusion-preliminary experiments in a porcine model. Transplantation 2011;92:289-295.

[45] Liu Q, Nassar A, Buccini L, Iuppa G, Soliman B, Pezzati D, et al. Lipid metabolism and functional assessment of discarded human livers with steatosis undergoing 24 hours of normothermic machine perfusion. Liver Transpl 2018;24:233-245.

Table 1: Donors and recipients characteristics according to steatosis stage assessed by histopathological analysis

	Overall population	**Overall steatosis ≤30%**	**Overall steatosis >30% to ≤60%**	**Overall steatosis >60%**
Number of patients	**58**	**28**	**9**	**21**
Donors characteristics				
Age (years)	56.5 [18-85]	74.5 [18-85]	55.0 [47-77]	54.0 [33-77]
Male gender	38 (66)	17 (60)	6 (67)	15 (71)
Body mass index (Kg/m2)	22.3 [18-42]	25.5 [20-35]	28.4 [22-40]	26.0 [18-42]
Causes of death				
Vascular	36 (62)	17 (61)	7 (78)	12 (57)
Trauma	15 (26)	8 (28)	1 (11)	6 (29)
Anoxia	7 (12)	3 (11)	1 (11)	3 (14)
AST (IU/L)	35.0 [13-249]	32.0 [15-249]	38.0 [13-121]	34.0 [16-224]
ALT (IU/L)	26.5 [7-270]	31.0 [8-232]	19.0 [8-99]	27.5 [7-270]
GGT (IU/L)	35.0 [6-331]	35.0 [10-331]	28.0 [7-329]	42.0 [6-136]
Macrovesicular steatosis (%)	2 [0-50]	0 [0-30]	2 [0-25]	10 [0-50]
Microvesicular steatosis (%)	30 [0-90]	0 [0-30]	40 [30-60]	80 [40-90]

Table 1: Donors and recipients characteristics according to steatosis stage assessed by histopathological analysis (Suite)

Recipients characteristics and clinical outcome				
Age (years)	55.5 [19-70]	53.5 [19-70]	54.0 [31-61]	58.0 [26-70]
Male gender	39 (67)	23 (81)	5 (56)	11 (52)
Body mass index (kg/m^2)	23.1 [17-45]	27.1 [17-45]	28.4 [22-40]	26.3 [19-37]
Indications of LT				
Decompensated cirrhosis	23 (40)	13 (46)	4 (45)	6 (29)
Hepatocellular carcinoma	11 (19)	6 (22)	2 (22)	3 (14)
Other*	24 (41)	9 (32)	3 (33)	12 (57)
MELD score at listing	20.4 [6-40]	22.0 [6-40]	19.0 [11-34]	20.5 [6-38]
Graft weight to body weight ratio (%)	1.7 [0.9-3.9]	1.7 [0.9-3.9]	2.0 [1.2-3.0]	2.3 [1.1-3.1]
Cold ischemia time (min)	473.5 [150-822]	496 [279-801]	500.0 [150-792]	450.[289-822]
Per-operative transfusion	7.0 [1-19]	7.0 [1-19]	6.0 [1-10]	7.5 [1-17]
Peak within 1 week after surgery				
AST (IU/L)	1158 [42-14414]	1112 [76-6023]	1500 [255-14414]	1534 [42-7087]
ALT (IU/L)	704.[37-12301]	655[37-3656]	704[165-12301]	998.[52-2529]
GGT (IU/L)	103.0 [14-508]	105.0 [18-354]	112.0 [30-376]	69.0 [14-508]
Total bilirubin (µmol/L)	84.0 [12-374]	97.0 [33-374]	84.0 [54-217]	47.0 [12-169]
INR	3.0 [1.1-4.2]	3.5 [1.5-4.2]	3.2 [1.9-3.9]	3.5 [1.1-4.1]
Creatininemia (µmol/L)	91.3 [45-730]	95.3 [47-419]	119.0 [69-158]	85.6 [45-730]
UCI stay (days)	9.0 [2-88]	10.0 [3-35]	5.0 [2-7]	11.0 [3-88]
Hospitalisation stay (days)	15.5 [0-67]	17.0 [0-67]	10.0 [0-22]	14.0 [0-57]
Overall complications during hospitalisation	34 (58)	15 (53)	4 (44)	15 (71)

Quantitative results are expressed as median and interquartiles. Qualitative results are expressed as number and percentages.

* Including fulminant hepatitis (n=7), retransplantation (n=7), cholestatic diseases (n=4), intra-hepatic tumours other than hepatocellular carcinoma (n=3) and metabolic diseases (n=3)

Table 2: Factors significantly associated with the quantification of hepatic content of triglycerides using GC/MS

	Univariate analysis		**Prediction according to the threshold of 7.7**			
		p-value	**Odd Ratio**	**95% Confidence Interval**		**p-value**
				Lower	**Upper**	
Macrovesicular steatosis	2.0 [0-50]	<0.0001				
Microvesicular steatosis	30.0 [0-90]	<0.0001				
Male gender of recipient	39 (67%)	0.04	2.46	0.70	8.95	0.11
Bilirubinemia level at hospitalization discharge >17µmol/L	16.0 [4-180]	0.02	1.14	0.36	4.32	0.82
Complications during hospitalisation stay	34 (58%)	0.05	0.05	0.27	0.075	0.93
Treated dyslipidaemia at the end of follow-up	16 (28%)	0.04	1.28	0.32	5.70	0.79
Transplant failure* at the end of follow-up	**51 (91%)**	**<0.0001**	**15.28**	**1.62**	**760.02**	**<0.0001**
Survival at the end of follow-up	**53 (88%)**	**<0.0001**	**<0.0001**	**<0.0001**	**2.01**	**<0.0001**

*Transplant failure means death and/or retransplantation.

Table 3: Factors significantly associated with the estimation of hepatic content of triglycerides using FTIR

	Univariate analysis		**Prediction according to the threshold of 7.35**			
		p-value	**Odd Ratio**	**95% Confidence Interval**		**p-value**
				Lower	**Upper**	
Macrovesicular steatosis	2.0 [0-50]	<0.0001				
Microvesicular steatosis	30.0 [0-90]	<0.0001				
Male gender of recipient	39 (67.2%)	0.03	2.19	0.61	8.04	0.16
Complications during hospitalisation stay	34 (58%)	0.03	0.73	0.21	2.57	0.57
One year-survival	**55 (95%)**	**0.01**	**<0.0001**	**0.66**	**<0.0001**	**0.03**

Figure legends

Figure 1: Patient and graft survivals (Kaplan-Meier) after liver transplantation among 58 patients

Figure 2: Correlation between GC/MS and FTIR measurements of hepatic content of triglycerides

TG levels were estimated using the lipid/protein ratio obtained from spectrum in FTIR. FTIR: Infrared microspectroscopy; GC/MS: Gas-phase chromatography coupled to mass spectrometry; TG: triglycerides

Figure 3: Distribution of steatosis according to GC/MS and FTIR measurements of hepatic content of triglycerides

TG levels were estimated using the lipid/protein ratio obtained from spectrum in FTIR. Each dot represents one sample and the size of the dot represents the percentages of steatosis, macrovacuolar, microvesicular and overall steatosis, respectively. To better identified the distribution of samples, percentages of steatosis <30% are represented in red, percentages of steatosis ≥30% and <60% are represented in green and percentages of steatosis ≥60% are represented in blue. FTIR: Infrared microspectroscopy; GC/MS: Gas-phase chromatography coupled to mass spectrometry; TG: triglycerides.

Figure 4: Distribution of clinical events according to GC/MS and FTIR measurements of hepatic content of triglycerides

TG levels were estimated using the lipid/protein ratio obtained from spectrum in FTIR. Each dot represents one sample and the size of the dot represents the percentages of the

overall steatosis, macrovacuolar plus microvesicular. Seven patients experienced a transplant failure during follow-up, 5 patients died (represented in red) and 2 patients were retransplanted (represented in green). FTIR: Infrared microspectroscopy; GC/MS: Gas-phase chromatography coupled to mass spectrometry; TG: triglycerides. The most discriminant threshold of triglycerides level to transplant failure was 7.7 Log_2 nmol/mg using GC/MS.

Appendix 1

Method to quantify and estimate hepatic triglycerides content using mass spectrometry

Liver samples

Tissues were collected during a surgical procedure. Then, they were fixed in formalin for routine pathological assessment and one specimen of was immediately snap frozen in liquid nitrogen and stored at −80 °C until use.

Tissue section

Serial sections were cut from frozen specimens with 6 µm thickness at −20 °C with a CM3050-S cryostat (Leica Microsystèmes SAS, France) and alternately deposited on glass slide for histological control or on a gold coated sample holder mirrIR (Tientascience, Indianapolis, IN) for Fourier transform-infrared (FTIR) microspectroscopy. Sections for histology were stained with haematoxylin, eosin and safran. Sections for microspectroscopy were dried at room temperature.

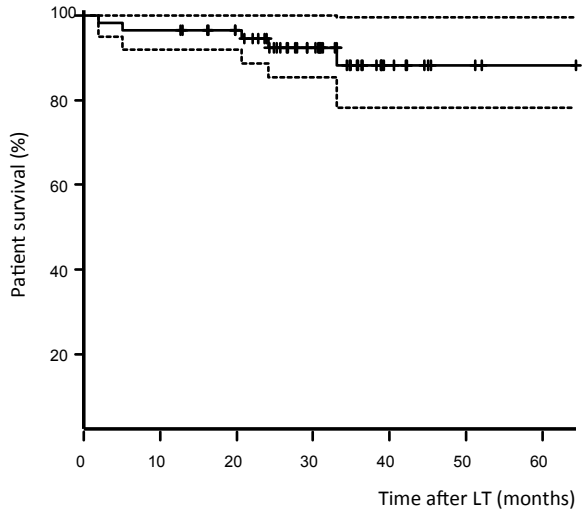
Lipid profiling

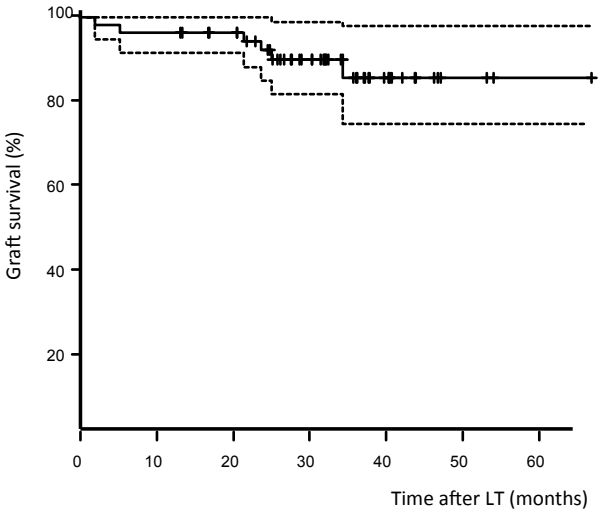
Lipidomic analysis was performed on the platform MetaToul at IFR150 (Toulouse, France). Liver biopsies (5–10 mg) were homogenized in 2 ml of methanol containing 5 mM EGTA (2 : 1 v/v) with FAST-PREP (MP Biochemicals). The equivalents of 0.5 mg of tissue were evaporated, the dry pellets were dissolved in 0.25 ml of NaOH (0.1 M) overnight and proteins were measured with the Bio-Rad assay. The quantification of the lipids is expressed in nmol per mg of total proteins.

Lipids corresponding to an equivalent of 1 mg of tissue were extracted according to Bligh and Dyer in dichloromethane–methanol–water (2.5 : 2.5 : 2.1, v/v/v), in the

presence of 15 µg of glyceryl triheptadecanoate as an internal standard. The dichloromethane phase was evaporated to dryness and dissolved in 20 µl of ethyl acetate. 1 µl of the lipid extract was analysed by gas-liquid chromatography on a FOCUS Thermo Electron system using an Zebron-1 Phenomenex fused silica capillary column (5 m x 0.32 mm i.d., 0.50 µm film thickness). Oven temperature was programmed from 200 °C to 350 °C at a rate of 5 °C per min and the carrier gas was hydrogen (0.5 bar). The injector and the detector were set at 315 °C and 345 °C respectively. For fatty acid methyl ester (FAME) analysis such as for saturated, unsaturated and polyunsaturated fatty acids as well as total C16 and C18, homogenates were extracted as neutral lipids in the presence of the internal standards glyceryl triheptadecanoate (2 µg), and transmethylated for 1 h in boron trifluoride methanol solution (10%) at 55 °C. After addition of water (1 ml) to the crude, FAMEs were extracted with hexane
(3 ml), evaporated to dryness and dissolved in ethyl acetate (20 µl). FAMEs (1 µl) were analysed by gas-liquid chromatography on a Claus 600 Perkin Elmer system using a Famewax RESTEK fused silica capillary column.

Figure 1: Patient and graft survivals after liver transplantation among the 58 patients





Patient survival				
Months	0	20	40	60
N	58	49	9	1
%	100	95	88	88

Graft survival				
Months	0	20	40	60
N	58	49	9	1
%	100	95	86	86

Figure 2: Correlation between GC/MS and FTIR measurements of hepatic content of triglycerides

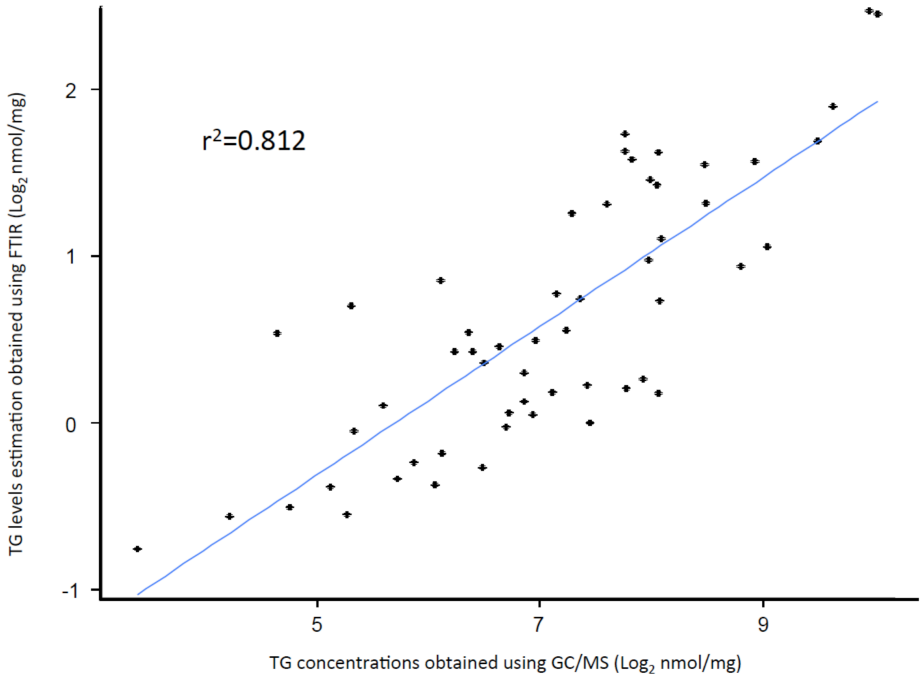


Figure 3: Distribution of steatosis according to GC/MS and FTIR measurements of hepatic content of triglycerides

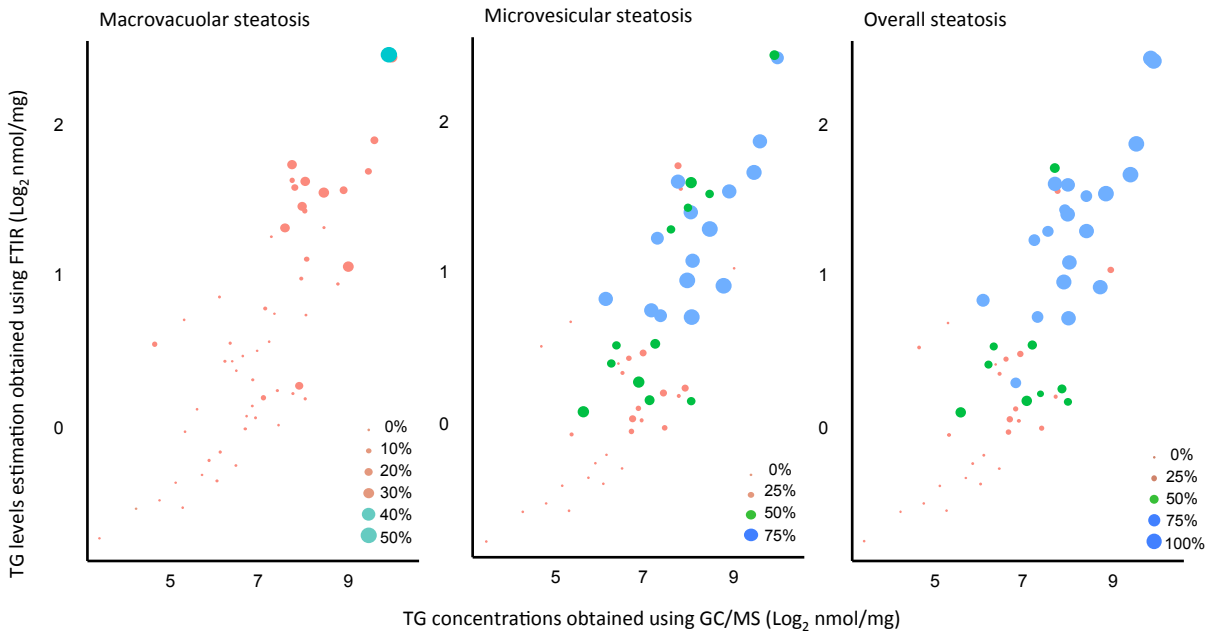
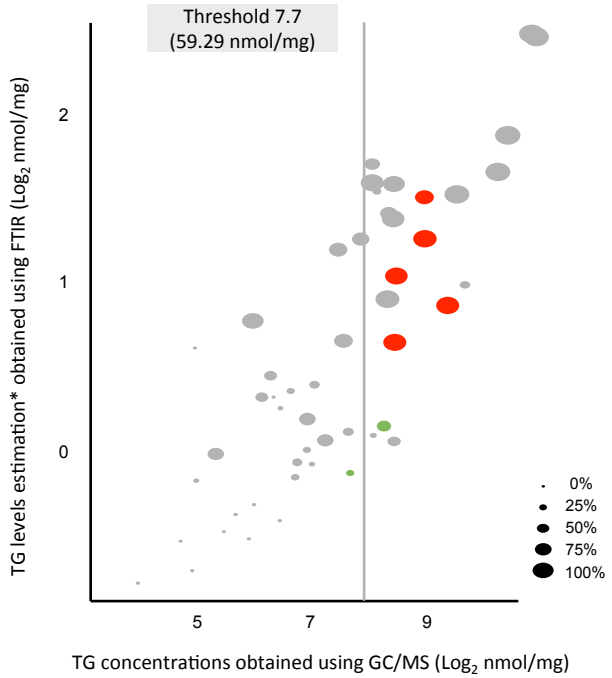


Figure 4: Projection of events on GC/MS and FTIR measurements of hepatic triglycerides content



5.3 Méthodes

Nous avons mené une étude rétrospective, monocentrique, ayant inclus 58 patients transplantés hépatiques, au centre hépato-biliaire de 2012 à 2014.

Les conditions pour être inclus étaient que la mesure du contenu lipidique par FTIR soit disponible, de même que la mesure par spectroscopie de masse (Dr Kaščáková) et l'analyse anatomopathologique (Pr Guettier). L'évaluation anatomopathologique de la stéatose était réalisée en aveugle des résultats de la FTIR. La stéatose globale, macrovacuolaire et microvésiculaire était décrite et quantifiée en pourcentage.

La chromatographie en phase gazeuse couplée à la spectrométrie de masse a été utilisée pour quantifier le contenu en triglycérides du foie sur la plateforme MetaToul. En bref, une extraction lipidique de l'échantillon chirurgical suivie d'une chromatographie en phase gazeuse couplée à la spectrométrie de masse a été effectuée pour chaque échantillon de greffon afin de déterminer la concentration en triglycérides hépatique et de classer les échantillons en quatre groupes en fonction de la quantité de triglycérides (grades 1 à 4). Nous avions déjà décrit cette technique précédemment (212, 213).

Une analyse FTIR a été effectuée sur des échantillons de foie congelés à l'aide d'un spectromètre ATR-FTIR (module Nicolet ™ iZ10) connecté au microscope infrarouge Nicolet ™ iN10. Le spectre infrarouge acquis ainsi obtenu correspond à un spectre moyen d'une zone de 2 x 2 mm^2 de la coupe de tissu. La quantification de la teneur en triglycérides a ensuite été obtenue à partir du spectre acquis en calculant le rapport d'intensité intégrée des bandes attribuées aux lipides par rapport aux protéines et exprimé en nmol/mg (211).

Enfin, l'ensemble des données clinico-biologiques pertinentes pré- et post-transplantation ont été recueillies de façon rétrospective.

5.4 Résultats principaux et discussion

Parmi les 58 prélèvements de greffons provenant des 58 patients inclus, le pourcentage moyen de stéatose macrovacuolaire et de stéatose microvésiculaire, évalué par le pathologiste, était de 2% à 30%, respectivement. La concentration moyenne en triglycérides hépatiques mesurée par chromatographie couplée en phase gazeuse à la

spectrométrie était de 214 [10-1045] nmol/mg de tissu hépatique. L'estimation de la teneur en triglycérides obtenue par FTIR était significativement corrélée (r2 = 0,812) avec les résultats de la concentration moyenne en triglycérides hépatiques mesurée par chromatographie couplée en phase gazeuse à la spectrométrie. Trente-quatre (58%) patients ont présenté des complications définies par un stade Dindo-Clavien ≥2, dont 2 non-fonction primaire du greffon et 5 décès. Le seuil le plus discriminant entre le niveau de triglycérides et l'échec de la TH était de 59,29 et 54,02 nmol/mg de tissu hépatique obtenu par spectrométrie et FTIR, respectivement. La quantification du contenu hépatique en triglycérides par GC / MS était significativement associée à la survie du patient à la fin du suivi (p<0,0001) et à un échec de la transplantation (p <0,0001). L'estimation du contenu hépatique en triglycérides à l'aide de FTIR était significativement associée à la survie après greffe d'un an (p<0,0001).

Notre étude a confirmé qu'il était possible d'utiliser la FTIR pour mesurer la stéatose du greffon sur une cohorte de patients transplantés du foie comme décrit précédemment. En effet, les mesures des taux de TG par FTIR sont bien corrélées, comme nous l'avons précédemment décrit, avec les mesures obtenues par spectrométrie de masse qui représentent le gold standard pour déterminer le contenu lipidique dans le foie.

Dans notre étude, la corrélation entre l'évaluation histologique de la stéatose et la mesure des TG par FTIR ou spectrométrie est également assez bonne. Dans une étude précédente, nous avions montré au contraire que l'estimation histologique de la stéatose était faiblement corrélée au contenu en lipides réels du tissu hépatique après extraction des lipides du même échantillon chirurgical suivi d'une spectrométrie de masse (213). Cette différence pourrait être expliquée par les différences de contenu lipidique du foie; dans la présente étude, la teneur globale en lipides est plus basse, tous les foies ayant été sélectionnés pour une transplantation, ce qui n'était pas le cas dans l'étude précédente.

L'utilisation d'un spectroscope FTIR connecté à un ordinateur permet de mettre en œuvre la technique à l'hôpital et offre la possibilité d'analyser rapidement le contenu de la stéatose hépatique de manière objective. Un personnel non médical mais formé pourrait utiliser l'appareil. Dans le contexte de la transplantation hépatique, des résultats pourraient être obtenus sans retarder la procédure de prélèvement. Cette technique pourrait aussi permettre de fournir des informations complémentaires sur la composition chimique du foie, telles que la teneur en glycogène et les collagènes liés à la fibrose.

Notre étude n'est pas la seule à montrer la faisabilité et la fiabilité de l'évaluation de la stéatose hépatique par spectroscopie (214-216). En particulier, dans une étude récente, Golse et al. ont démontré la précision des micro-spectromètres de poche pour prédire la stéatose macrovacuolaire du greffon du foie (217). Le principal avantage de cette dernière technique par rapport à la méthode FTIR utilisée dans notre étude est la mesure non invasive et rapide de la stéatose par ce dispositif, qui peut également être transportable. Mais cette méthode n'a pas démontré de corrélation pour une stéatose macrovacuolaire entre 30 et 60%, taux de stéatose acceptable chez certains receveurs et donc essentiel pour déterminer la transplantabilité de certains organes. En outre, le spectromètre de poche n'a pas été comparé à la mesure globale de la stéatose. Enfin, contrairement à notre étude, aucune influence clinique n'a été rapportée.
Notre étude démontre qu'un taux élevé de TG dans la greffe du foie a eu un impact négatif sur le déroulement de la greffe car il est associé à la survie après la greffe et à des complications basées sur le stade Dindo-Clavien ≥2 ($p < 0,0001$). Notre étude confirme l'importante littérature concernant l'impact négatif de la stéatose du greffon sur le résultat de la TH. Les deux non-fonction primaires de notre étude sont survenues chez des patients ayant reçu un greffon ayant une stéatose macrovacuolaire ≤ 30%, alors que la valeur de la stéatose microvésiculaire était de 30 et 20%, respectivement, et que la concentration en TG atteignait 54,02 nmol/mg de tissu hépatique pour les deux greffons. Ce résultat relance le débat sur l'importance de prendre en compte la stéatose globale. En effet, la stéatose microvésiculaire est connue pour être moins délétère que la stéatose macrovacuolaire, mais pourrait aggraver les signes d'ischémie-reperfusion et altérer la bêta-oxydation mitochondriale (171, 173). Les outils spectroscopiques permettent d'effectuer une mesure globale de la stéatose, ou plutôt d'évaluer la charge lipidique des hépatocytes. Si notre étude est en mesure de mettre en évidence une issue défavorable pour ces foies gras, c'est peut-être précisément parce que, grâce à la spectroscopie, nous prenons en compte le taux global du contenu lipidique hépatique.
Dans un très proche avenir, il sera important de quantifier rapidement et de façon répétée la stéatose. Les machines de perfusion du greffon semblent être prometteuses pour optimiser voire reconditionner des foies marginaux. En période d'épidémie d'obésité et de syndrome métabolique, le reconditionnement de ces foies stéatosiques pourrait augmenter le nombre de TH réalisées, procédé nommé "defatting". En conclusion, notre étude montre que la spectroscopie FTIR, une procédure rapide et

facile à utiliser, reflète très bien le contenu réel en triglycérides du tissu hépatique et, plus important encore, que le contenu en TG évalué par spectroscopie FTIR est associé aux résultats de TH. FTIR pourrait être facilement implémenté dans le processus de transplantation hépatique en aidant les équipes de transplantation à choisir le meilleur greffon en termes de stéatose. Il positionne la spectroscopie FTIR comme un outil de prise de décision supplémentaire pour évaluer les greffons avant transplantation.

6 Conclusions générales et perspectives

L'objectif de ce travail de thèse était d'identifier de nouveaux outils diagnostiques ou pronostiques de la NAFLD, dans le contexte général, comme celui de la TH. Ce travail s'inscrit dans un contexte d'augmentation de la prévalence et de l'incidence de la NAFLD à travers le monde, mais également des travaux scientifiques s'y rapportant. En particulier, alors qu'à ce jour, aucun médicament n'a obtenu la mise sur le marché pour traiter la NASH, plusieurs molécules sont en développement dont une, l'acide obéticholique, dont les résultats en Phase III devrait permettre un accès à court terme pour les patients dans cette indication. Ainsi, développer des outils non invasifs pour discriminer les patients candidats aux traitements s'avère nécessaire. A ce jour, seule l'histologie permet de poser formellement le diagnostic. Le nombre de patients à prendre en charge ne permettra pas de conserver ce gold-standard.

Grâce aux résultats de la première partie de cette thèse, nous disposons d'une signature lipidique de la NASH qui permet de distinguer les témoins et les patients ayant une stéatose simple des patients ayant une NASH. Ce travail permet aussi de confirmer que la lipotoxicité jouent un rôle central dans la physiopathologie de la NAFLD. Nous avons confirmé dans cette étude une dérégulation de la voie métabolique impliquée dans la synthèse des acides gras dans la NASH. Nos résultats ont démontré également que le rapport n-6 sur n-3 était significativement augmenté, associé à une diminution significative n-3 chez l'homme et la souris. La concentration mais également la composition lipidique hépatique peuvent être centrales pour induire les lésions de la NASH. En effet, en utilisant les cinq acides gras principaux de la signature de la NASH, nous avons démontré que la signature de la NASH était capable d'induire plus de mort cellulaire que les signatures du foie sain ou de la stéatose simple sur deux modèles cellulaires d'hépatocytes.

Ces résultats pourraient ouvrir de nouvelles voies pour le développement ultérieur du diagnostic précoce et approches thérapeutiques. En effet, une perspective serait de valider cette signature sur une cohorte de validation. Il serait intéressant de regarder si une signature à 5 lipides serait suffisante pour discriminer la NASH. Enfin, la limite de cette signature est qu'il s'agit d'une signature hépatique donc nécessite la réalisation

d'une biopsie hépatique. Il serait dans ce contexte intéressant de comparer cette signature aux lipides circulants qui composent cette signature.

La deuxième partie de la thèse avait pour objectif d'exploiter des données existantes en effectuant de nouvelles analyses bioinformatiques afin de mettre en évidence un profil d'expression génique de la progression de la NAFLD. Cette analyse *in silico* a montré 1549 gènes discriminants. Ces gènes étaient impliqués dans le remodelage de la matrice extracellulaire, l'inflammation, la prolifération, la progression des cellules souches et l'oncogenèse. Le gène le plus discriminant était FABP4. Parmi les gènes fortement associés à une expression élevée de FABP4, la MMP9 était surexprimée chez 55% des patients NASH. Nous avons validé le niveau d'expression génique des gènes FABP4 et MMP9 hépatiques comme indicateurs de la progression de la maladie dans une cohorte indépendante de patients atteints de NAFLD. Nos résultats sont de plus concordants avec des données publiées. Nos résultats permettent de faire émerger deux gènes possiblement liés au pronostic de la NAFLD. Nous pensons qu'il serait intéressant d'étudier l'immunomarquage de ces protéines mais aussi d'évaluer leur taux sérique comme marqueur pronostique de la progression de la NAFLD.

Enfin, il était important dans notre centre de s'intéresser aux enjeux que représente la NAFLD en TH. La stéatose des donneurs est une préoccupation maintenant quotidienne des équipes de transplantation et compte tenu de l'augmentation du nombre de greffons stéatosiques proposés, les cliniciens sont amenés à faire des choix difficiles en acceptant parfois des greffons stéatosiques fautes pour pallier à la pénurie de greffon. Nous pensons que ce choix doit maintenant s'appuyer sur d'autres outils que l'expérience du chirurgien et l'analyse extemporanée du greffon, qui ont tous deux des limites. Ainsi, nous avons souhaité étudier la quantification globale de la stéatose pour FTIR et surtout évaluer l'association entre cette mesure et les résultats de la transplantation.

La troisième partie de cette thèse a permis de montrer que l'estimation de la teneur en triglycérides des greffons obtenue par FTIR était significativement corrélée à la concentration moyenne en triglycérides mesurée par chromatographie couplée en phase gazeuse à la spectrométrie. Plus important, un seuil de triglycérides de 54,02 nmol/mg de tissu hépatique obtenu par était significativement associé aux échecs de la TH. Même si cette étude comprend des biais, il serait intéressant de la confirmer de façon prospective. L'utilisation d'un spectroscope FTIR connecté à un ordinateur permet de mettre en œuvre la technique à l'hôpital et offre la possibilité d'analyser rapidement le

contenu de la stéatose hépatique de manière objective. Un personnel non médical mais formé pourrait utiliser l'appareil. C'est d'autant plus important que de nouveaux concepts de préservation émergent notamment les machines de perfusion. Dans un avenir proche, il sera possible de dégraisser les greffons sur machine avant leur implantation. Cependant, ces techniques auront un coût et établir quels greffons bénéficieront de la mise sur machine est un enjeu important. L'estimation de la concentration en triglycérides par FTIR permettrait aussi d'évaluer l'efficacité du « defatting ».

En conclusion, ces travaux proposent des nouveaux outils tant diagnostiques que pronostiques qui pourraient s'insérer dans le champ nouveau de la prise en charge de la NAFLD.

7 Références

1. Ludwig J, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin Proc 1980;55:434-438.
2. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 1999;94:2467-2474.
3. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-1321.
4. Younossi Z, Tacke F, Arrese M, Sharma BC, Mostafa I, Bugianesi E, Wong VW, et al. Global Perspectives on Non-alcoholic Fatty Liver Disease and Non-alcoholic Steatohepatitis. Hepatology 2018.
5. European Association for the Study of the L, European Association for the Study of D, European Association for the Study of O. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol 2016;64:1388-1402.
6. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. Gastroenterology 2012;142:1592-1609.
7. Bellentani S, Saccoccio G, Masutti F, Croce LS, Brandi G, Sasso F, Cristanini G, et al. Prevalence of and risk factors for hepatic steatosis in Northern Italy. Ann Intern Med 2000;132:112-117.
8. Persico M, Iolascon A. Steatosis as a co-factor in chronic liver diseases. World J Gastroenterol 2010;16:1171-1176.
9. Leandro G, Mangia A, Hui J, Fabris P, Rubbia-Brandt L, Colloredo G, Adinolfi LE, et al. Relationship between steatosis, inflammation, and fibrosis in chronic hepatitis C: a meta-analysis of individual patient data. Gastroenterology 2006;130:1636-1642.

10. Lin CW, Huang XL, Liu HL, Wang Y. Interactions of Hepatitis B Virus Infection with Nonalcoholic Fatty Liver Disease: Possible Mechanisms and Clinical Impact. Dig Dis Sci 2015;60:3513-3524.
11. Frith J, Day CP, Henderson E, Burt AD, Newton JL. Non-alcoholic fatty liver disease in older people. Gerontology 2009;55:607-613.
12. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology 2016;64:73-84.
13. Lau K, Baumeister SE, Lieb W, Meffert PJ, Lerch MM, Mayerle J, Volzke H. The combined effects of alcohol consumption and body mass index on hepatic steatosis in a general population sample of European men and women. Aliment Pharmacol Ther 2015;41:467-476.
14. Younossi ZM, Stepanova M, Rafiq N, Henry L, Loomba R, Makhlouf H, Goodman Z. Nonalcoholic steatofibrosis independently predicts mortality in nonalcoholic fatty liver disease. Hepatol Commun 2017;1:421-428.
15. Golabi P, Otgonsuren M, de Avila L, Sayiner M, Rafiq N, Younossi ZM. Components of metabolic syndrome increase the risk of mortality in nonalcoholic fatty liver disease (NAFLD). Medicine (Baltimore) 2018;97:e0214.
16. Younossi ZM, Otgonsuren M, Venkatesan C, Mishra A. In patients with non-alcoholic fatty liver disease, metabolically abnormal individuals are at a higher risk for mortality while metabolically normal individuals are not. Metabolism 2013;62:352-360.
17. Estes C, Anstee QM, Arias-Loste MT, Bantel H, Bellentani S, Caballeria J, Colombo M, et al. Modeling NAFLD disease burden in China, France, Germany, Italy, Japan, Spain, United Kingdom, and United States for the period 2016-2030. J Hepatol 2018;69:896-904.
18. Machado M, Marques-Vidal P, Cortez-Pinto H. Hepatic histology in obese patients undergoing bariatric surgery. J Hepatol 2006;45:600-606.
19. Lassailly G, Caiazzo R, Buob D, Pigeyre M, Verkindt H, Labreuche J, Raverdy V, et al. Bariatric Surgery Reduces Features of Nonalcoholic Steatohepatitis in Morbidly Obese Patients. Gastroenterology 2015;149:379-388; quiz e315-376.
20. Stefan N, Haring HU, Hu FB, Schulze MB. Metabolically healthy obesity: epidemiology, mechanisms, and clinical implications. Lancet Diabetes Endocrinol 2013;1:152-162.

21. Firneisz G. Non-alcoholic fatty liver disease and type 2 diabetes mellitus: the liver disease of our age? World J Gastroenterol 2014;20:9072-9089.
22. Ioannou GN, Weiss NS, Kowdley KV, Dominitz JA. Is obesity a risk factor for cirrhosis-related death or hospitalization? A population-based cohort study. Gastroenterology 2003;125:1053-1059.
23. Chou TC, Liang WM, Wang CB, Wu TN, Hang LW. Obstructive sleep apnea is associated with liver disease: a population-based cohort study. Sleep Med 2015;16:955-960.
24. Corey KE, Misdraji J, Gelrud L, King LY, Zheng H, Malhotra A, Chung RT. Obstructive Sleep Apnea Is Associated with Nonalcoholic Steatohepatitis and Advanced Liver Histology. Dig Dis Sci 2015;60:2523-2528.
25. Labarca G, Cruz R, Jorquera J. Continuous Positive Airway Pressure in Patients With Obstructive Sleep Apnea and Non-Alcoholic Steatohepatitis: A Systematic Review and Meta-Analysis. J Clin Sleep Med 2018;14:133-139.
26. Soderberg C, Stal P, Askling J, Glaumann H, Lindberg G, Marmur J, Hultcrantz R. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. Hepatology 2010;51:595-602.
27. Ong JP, Pitts A, Younossi ZM. Increased overall mortality and liver-related mortality in non-alcoholic fatty liver disease. J Hepatol 2008;49:608-612.
28. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Aliment Pharmacol Ther 2011;34:274-285.
29. Giannini EG, Bodini G, Furnari M, Marabotto E. NASH-related and cryptogenic cirrhosis similarities extend beyond cirrhosis. J Hepatol 2018;69:972-973.
30. Thuluvath PJ, Kantsevoy S, Thuluvath AJ, Savva Y. Is cryptogenic cirrhosis different from NASH cirrhosis? J Hepatol 2018;68:519-525.
31. Charlotte F, Le Naour G, Bernhardt C, Poynard T, Ratziu V, Group LS. A comparison of the fibrotic potential of nonalcoholic fatty liver disease and chronic hepatitis C. Hum Pathol 2010;41:1178-1185.
32. Anstee QM, Targher G, Day CP. Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. Nat Rev Gastroenterol Hepatol 2013;10:330-344.
33. Fracanzani AL, Valenti L, Bugianesi E, Andreoletti M, Colli A, Vanni E, Bertelli C, et al. Risk of severe liver disease in nonalcoholic fatty liver disease with normal

aminotransferase levels: a role for insulin resistance and diabetes. Hepatology 2008;48:792-798.

34. Ratziu V, Bonyhay L, Di Martino V, Charlotte F, Cavallaro L, Sayegh-Tainturier MH, Giral P, et al. Survival, liver failure, and hepatocellular carcinoma in obesity-related cryptogenic cirrhosis. Hepatology 2002;35:1485-1493.

35. Liu B, Balkwill A, Reeves G, Beral V, Million Women Study C. Body mass index and risk of liver cirrhosis in middle aged UK women: prospective study. BMJ 2010;340:c912.

36. Singal AG, Manjunath H, Yopp AC, Beg MS, Marrero JA, Gopal P, Waljee AK. The effect of PNPLA3 on fibrosis progression and development of hepatocellular carcinoma: a meta-analysis. Am J Gastroenterol 2014;109:325-334.

37. Harrison SA. Nonalcoholic fatty liver disease and fibrosis progression: the good, the bad, and the unknown. Clin Gastroenterol Hepatol 2015;13:655-657.

38. Singh S, Allen AM, Wang Z, Prokop LJ, Murad MH, Loomba R. Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a systematic review and meta-analysis of paired-biopsy studies. Clin Gastroenterol Hepatol 2015;13:643-654 e641-649; quiz e639-640.

39. Satapathy SK, Sanyal AJ. Epidemiology and Natural History of Nonalcoholic Fatty Liver Disease. Semin Liver Dis 2015;35:221-235.

40. Berzigotti A, Garcia-Tsao G, Bosch J, Grace ND, Burroughs AK, Morillas R, Escorsell A, et al. Obesity is an independent risk factor for clinical decompensation in patients with cirrhosis. Hepatology 2011;54:555-561.

41. Sanyal AJ, Banas C, Sargeant C, Luketic VA, Sterling RK, Stravitz RT, Shiffman ML, et al. Similarities and differences in outcomes of cirrhosis due to nonalcoholic steatohepatitis and hepatitis C. Hepatology 2006;43:682-689.

42. Bhala N, Angulo P, van der Poorten D, Lee E, Hui JM, Saracco G, Adams LA, et al. The natural history of nonalcoholic fatty liver disease with advanced fibrosis or cirrhosis: an international collaborative study. Hepatology 2011;54:1208-1216.

43. Ekstedt M, Hagstrom H, Nasr P, Fredrikson M, Stal P, Kechagias S, Hultcrantz R. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. Hepatology 2015;61:1547-1554.

44. Younossi ZM, Stepanova M, Rafiq N, Makhlouf H, Younoszai Z, Agrawal R, Goodman Z. Pathologic criteria for nonalcoholic steatohepatitis: interprotocol

agreement and ability to predict liver-related mortality. Hepatology 2011;53:1874-1882.

45. White DL, Kanwal F, El-Serag HB. Association between nonalcoholic fatty liver disease and risk for hepatocellular cancer, based on systematic review. Clin Gastroenterol Hepatol 2012;10:1342-1359 e1342.

46. Siegel AB, Zhu AX. Metabolic syndrome and hepatocellular carcinoma: two growing epidemics with a potential link. Cancer 2009;115:5651-5661.

47. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N Engl J Med 2003;348:1625-1638.

48. Polesel J, Zucchetto A, Montella M, Dal Maso L, Crispo A, La Vecchia C, Serraino D, et al. The impact of obesity and diabetes mellitus on the risk of hepatocellular carcinoma. Ann Oncol 2009;20:353-357.

49. Oh SW, Yoon YS, Shin SA. Effects of excess weight on cancer incidences depending on cancer sites and histologic findings among men: Korea National Health Insurance Corporation Study. J Clin Oncol 2005;23:4742-4754.

50. Samanic C, Chow WH, Gridley G, Jarvholm B, Fraumeni JF, Jr. Relation of body mass index to cancer risk in 362,552 Swedish men. Cancer Causes Control 2006;17:901-909.

51. Borena W, Strohmaier S, Lukanova A, Bjorge T, Lindkvist B, Hallmans G, Edlinger M, et al. Metabolic risk factors and primary liver cancer in a prospective study of 578,700 adults. Int J Cancer 2012;131:193-200.

52. Schlesinger S, Aleksandrova K, Pischon T, Fedirko V, Jenab M, Trepo E, Boffetta P, et al. Abdominal obesity, weight gain during adulthood and risk of liver and biliary tract cancer in a European cohort. Int J Cancer 2013;132:645-657.

53. Turati F, Talamini R, Pelucchi C, Polesel J, Franceschi S, Crispo A, Izzo F, et al. Metabolic syndrome and hepatocellular carcinoma risk. Br J Cancer 2013;108:222-228.

54. Welzel TM, Graubard BI, Quraishi S, Zeuzem S, Davila JA, El-Serag HB, McGlynn KA. Population-attributable fractions of risk factors for hepatocellular carcinoma in the United States. Am J Gastroenterol 2013;108:1314-1321.

55. Larsson SC, Wolk A. Overweight, obesity and risk of liver cancer: a meta-analysis of cohort studies. Br J Cancer 2007;97:1005-1008.

56. Chen Y, Wang X, Wang J, Yan Z, Luo J. Excess body weight and the risk of primary liver cancer: an updated meta-analysis of prospective studies. Eur J Cancer 2012;48:2137-2145.
57. Chen CL, Yang HI, Yang WS, Liu CJ, Chen PJ, You SL, Wang LY, et al. Metabolic factors and risk of hepatocellular carcinoma by chronic hepatitis B/C infection: a follow-up study in Taiwan. Gastroenterology 2008;135:111-121.
58. El-Serag HB, Richardson PA, Everhart JE. The role of diabetes in hepatocellular carcinoma: a case-control study among United States Veterans. Am J Gastroenterol 2001;96:2462-2467.
59. Naveau S, Giraud V, Borotto E, Aubert A, Capron F, Chaput JC. Excess weight risk factor for alcoholic liver disease. Hepatology 1997;25:108-111.
60. Hart CL, Morrison DS, Batty GD, Mitchell RJ, Davey Smith G. Effect of body mass index and alcohol consumption on liver disease: analysis of data from two prospective cohort studies. BMJ 2010;340:c1240.
61. Hassan MM, Hwang LY, Hatten CJ, Swaim M, Li D, Abbruzzese JL, Beasley P, et al. Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. Hepatology 2002;36:1206-1213.
62. Hassan MM, Abdel-Wahab R, Kaseb A, Shalaby A, Phan AT, El-Serag HB, Hawk E, et al. Obesity Early in Adulthood Increases Risk but Does Not Affect Outcomes of Hepatocellular Carcinoma. Gastroenterology 2015;149:119-129.
63. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology 2004;126:460-468.
64. Adami HO, Chow WH, Nyren O, Berne C, Linet MS, Ekbom A, Wolk A, et al. Excess risk of primary liver cancer in patients with diabetes mellitus. J Natl Cancer Inst 1996;88:1472-1477.
65. Hassan MM, Curley SA, Li D, Kaseb A, Davila M, Abdalla EK, Javle M, et al. Association of diabetes duration and diabetes treatment with the risk of hepatocellular carcinoma. Cancer 2010;116:1938-1946.
66. Balkau B, Kahn HS, Courbon D, Eschwege E, Ducimetiere P, Paris Prospective S. Hyperinsulinemia predicts fatal liver cancer but is inversely associated with fatal cancer at some other sites: the Paris Prospective Study. Diabetes Care 2001;24:843-849.

67. Rapp K, Schroeder J, Klenk J, Ulmer H, Concin H, Diem G, Oberaigner W, et al. Fasting blood glucose and cancer risk in a cohort of more than 140,000 adults in Austria. Diabetologia 2006;49:945-952.

68. Rao Kondapally Seshasai S, Kaptoge S, Thompson A, Di Angelantonio E, Gao P, Sarwar N, Whincup PH, et al. Diabetes mellitus, fasting glucose, and risk of cause-specific death. N Engl J Med 2011;364:829-841.

69. Adams LA, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A, Angulo P. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. Gastroenterology 2005;129:113-121.

70. Baffy G, Brunt EM, Caldwell SH. Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace. J Hepatol 2012;56:1384-1391.

71. Bengtsson B, Stal P, Wahlin S, Bjorkstrom NK, Hagstrom H. Characteristics and outcome of hepatocellular carcinoma in patients with NAFLD without cirrhosis. Liver Int 2019;39:1098-1108.

72. Sanyal A, Poklepovic A, Moyneur E, Barghout V. Population-based risk factors and resource utilization for HCC: US perspective. Curr Med Res Opin 2010;26:2183-2191.

73. Yasui K, Hashimoto E, Komorizono Y, Koike K, Arii S, Imai Y, Shima T, et al. Characteristics of patients with nonalcoholic steatohepatitis who develop hepatocellular carcinoma. Clin Gastroenterol Hepatol 2011;9:428-433; quiz e450.

74. Farges O, Ferreira N, Dokmak S, Belghiti J, Bedossa P, Paradis V. Changing trends in malignant transformation of hepatocellular adenoma. Gut 2011;60:85-89.

75. Mottillo S, Filion KB, Genest J, Joseph L, Pilote L, Poirier P, Rinfret S, et al. The metabolic syndrome and cardiovascular risk a systematic review and meta-analysis. J Am Coll Cardiol 2010;56:1113-1132.

76. Targher G, Bertolini L, Padovani R, Rodella S, Tessari R, Zenari L, Day C, et al. Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. Diabetes Care 2007;30:1212-1218.

77. Targher G, Bertolini L, Padovani R, Rodella S, Zoppini G, Pichiri I, Sorgato C, et al. Prevalence of non-alcoholic fatty liver disease and its association with cardiovascular disease in patients with type 1 diabetes. J Hepatol 2010;53:713-718.

78. Dam-Larsen S, Franzmann M, Andersen IB, Christoffersen P, Jensen LB, Sorensen TI, Becker U, et al. Long term prognosis of fatty liver: risk of chronic liver disease and death. Gut 2004;53:750-755.

79. Lazo M, Hernaez R, Bonekamp S, Kamel IR, Brancati FL, Guallar E, Clark JM. Non-alcoholic fatty liver disease and mortality among US adults: prospective cohort study. BMJ 2011;343:d6891.
80. Stepanova M, Younossi ZM. Independent association between nonalcoholic fatty liver disease and cardiovascular disease in the US population. Clin Gastroenterol Hepatol 2012;10:646-650.
81. Fraser A, Harris R, Sattar N, Ebrahim S, Smith GD, Lawlor DA. Gamma-glutamyltransferase is associated with incident vascular events independently of alcohol intake: analysis of the British Women's Heart and Health Study and Meta-Analysis. Arterioscler Thromb Vasc Biol 2007;27:2729-2735.
82. Nascimbeni F, Pais R, Bellentani S, Day CP, Ratziu V, Loria P, Lonardo A. From NAFLD in clinical practice to answers from guidelines. J Hepatol 2013;59:859-871.
83. Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology 2018;67:328-357.
84. Barritt ASt, Gitlin N, Klein S, Lok AS, Loomba R, Malahias L, Powell M, et al. Design and rationale for a real-world observational cohort of patients with nonalcoholic fatty liver disease: The TARGET-NASH study. Contemp Clin Trials 2017;61:33-38.
85. Wright AP, Desai AP, Bajpai S, King LY, Sahani DV, Corey KE. Gaps in recognition and evaluation of incidentally identified hepatic steatosis. Dig Dis Sci 2015;60:333-338.
86. Kowdley KV, Belt P, Wilson LA, Yeh MM, Neuschwander-Tetri BA, Chalasani N, Sanyal AJ, et al. Serum ferritin is an independent predictor of histologic severity and advanced fibrosis in patients with nonalcoholic fatty liver disease. Hepatology 2012;55:77-85.
87. Vuppalanchi R, Gould RJ, Wilson LA, Unalp-Arida A, Cummings OW, Chalasani N, Kowdley KV, et al. Clinical significance of serum autoantibodies in patients with NAFLD: results from the nonalcoholic steatohepatitis clinical research network. Hepatol Int 2012;6:379-385.
88. Ryan CK, Johnson LA, Germin BI, Marcos A. One hundred consecutive hepatic biopsies in the workup of living donors for right lobe liver transplantation. Liver Transpl 2002;8:1114-1122.

89. Sberna AL, Bouillet B, Rouland A, Brindisi MC, Nguyen A, Mouillot T, Duvillard L, et al. European Association for the Study of the Liver (EASL), European Association for the Study of Diabetes (EASD) and European Association for the Study of Obesity (EASO) clinical practice recommendations for the management of non-alcoholic fatty liver disease: evaluation of their application in people with Type 2 diabetes. Diabet Med 2018;35:368-375.

90. de Ledinghen V, Vergniol J, Capdepont M, Chermak F, Hiriart JB, Cassinotto C, Merrouche W, et al. Controlled attenuation parameter (CAP) for the diagnosis of steatosis: a prospective study of 5323 examinations. J Hepatol 2014;60:1026-1031.

91. Eddowes PJ, Sasso M, Allison M, Tsochatzis E, Anstee QM, Sheridan D, Guha IN, et al. Accuracy of FibroScan Controlled Attenuation Parameter and Liver Stiffness Measurement in Assessing Steatosis and Fibrosis in Patients With Nonalcoholic Fatty Liver Disease. Gastroenterology 2019;156:1717-1730.

92. Cusi K, Chang Z, Harrison S, Lomonaco R, Bril F, Orsak B, Ortiz-Lopez C, et al. Limited value of plasma cytokeratin-18 as a biomarker for NASH and fibrosis in patients with non-alcoholic fatty liver disease. J Hepatol 2014;60:167-174.

93. Chen J, Zhu Y, Zheng Q, Jiang J. Serum cytokeratin-18 in the diagnosis of non-alcoholic steatohepatitis: A meta-analysis. Hepatol Res 2014;44:854-862.

94. Vuppalanchi R, Jain AK, Deppe R, Yates K, Comerford M, Masuoka HC, Neuschwander-Tetri BA, et al. Relationship between changes in serum levels of keratin 18 and changes in liver histology in children and adults with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol 2014;12:2121-2130 e2121-2122.

95. Bedossa P, Consortium FP. Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity, and fibrosis (SAF) score in the evaluation of biopsies of nonalcoholic fatty liver disease. Hepatology 2014;60:565-575.

96. Kaswala DH, Lai M, Afdhal NH. Fibrosis Assessment in Nonalcoholic Fatty Liver Disease (NAFLD) in 2016. Dig Dis Sci 2016;61:1356-1364.

97. Boursier J, Vergniol J, Guillet A, Hiriart JB, Lannes A, Le Bail B, Michalak S, et al. Diagnostic accuracy and prognostic significance of blood fibrosis tests and liver stiffness measurement by FibroScan in non-alcoholic fatty liver disease. J Hepatol 2016;65:570-578.

98. Musso G, Gambino R, Cassader M, Pagano G. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. Ann Med 2011;43:617-649.
99. Angulo P, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, Enders F, et al. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology 2007;45:846-854.
100. Imajo K, Kessoku T, Honda Y, Tomeno W, Ogawa Y, Mawatari H, Fujita K, et al. Magnetic Resonance Imaging More Accurately Classifies Steatosis and Fibrosis in Patients With Nonalcoholic Fatty Liver Disease Than Transient Elastography. Gastroenterology 2016;150:626-637 e627.
101. Boursier J, Anty R, Vonghia L, Moal V, Vanwolleghem T, Canivet CM, Michalak S, et al. Screening for therapeutic trials and treatment indication in clinical practice: MACK-3, a new blood test for the diagnosis of fibrotic NASH. Aliment Pharmacol Ther 2018;47:1387-1396.
102. Wong VW, Vergniol J, Wong GL, Foucher J, Chan HL, Le Bail B, Choi PC, et al. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. Hepatology 2010;51:454-462.
103. Boursier J, Zarski JP, de Ledinghen V, Rousselet MC, Sturm N, Lebail B, Fouchard-Hubert I, et al. Determination of reliability criteria for liver stiffness evaluation by transient elastography. Hepatology 2013;57:1182-1191.
104. Castera L, Foucher J, Bernard PH, Carvalho F, Allaix D, Merrouche W, Couzigou P, et al. Pitfalls of liver stiffness measurement: a 5-year prospective study of 13,369 examinations. Hepatology 2010;51:828-835.
105. Kanwal F, Kramer JR, Mapakshi S, Natarajan Y, Chayanupatkul M, Richardson PA, Li L, et al. Risk of Hepatocellular Cancer in Patients with Non-alcoholic Fatty Liver Disease. Gastroenterology 2018.
106. Dyson J, Jaques B, Chattopadyhay D, Lochan R, Graham J, Das D, Aslam T, et al. Hepatocellular cancer: the impact of obesity, type 2 diabetes and a multidisciplinary team. J Hepatol 2014;60:110-117.
107. Vongsuvanh R, van der Poorten D, Iseli T, Strasser SI, McCaughan GW, George J. Midkine Increases Diagnostic Yield in AFP Negative and NASH-Related Hepatocellular Carcinoma. PLoS One 2016;11:e0155800.

108. Liu YL, Patman GL, Leathart JB, Piguet AC, Burt AD, Dufour JF, Day CP, et al. Carriage of the PNPLA3 rs738409 C >G polymorphism confers an increased risk of non-alcoholic fatty liver disease associated hepatocellular carcinoma. J Hepatol 2014;61:75-81.

109. Vilar-Gomez E, Martinez-Perez Y, Calzadilla-Bertot L, Torres-Gonzalez A, Gra-Oramas B, Gonzalez-Fabian L, Friedman SL, et al. Weight Loss Through Lifestyle Modification Significantly Reduces Features of Nonalcoholic Steatohepatitis. Gastroenterology 2015;149:367-378 e365; quiz e314-365.

110. Rodriguez B, Torres DM, Harrison SA. Physical activity: an essential component of lifestyle modification in NAFLD. Nat Rev Gastroenterol Hepatol 2012;9:726-731.

111. Bugianesi E, Gentilcore E, Manini R, Natale S, Vanni E, Villanova N, David E, et al. A randomized controlled trial of metformin versus vitamin E or prescriptive diet in nonalcoholic fatty liver disease. Am J Gastroenterol 2005;100:1082-1090.

112. Tiikkainen M, Hakkinen AM, Korsheninnikova E, Nyman T, Makimattila S, Yki-Jarvinen H. Effects of rosiglitazone and metformin on liver fat content, hepatic insulin resistance, insulin clearance, and gene expression in adipose tissue in patients with type 2 diabetes. Diabetes 2004;53:2169-2176.

113. Bhalla K, Hwang BJ, Dewi RE, Twaddel W, Goloubeva OG, Wong KK, Saxena NK, et al. Metformin prevents liver tumorigenesis by inhibiting pathways driving hepatic lipogenesis. Cancer Prev Res (Phila) 2012;5:544-552.

114. Zhang ZJ, Zheng ZJ, Shi R, Su Q, Jiang Q, Kip KE. Metformin for liver cancer prevention in patients with type 2 diabetes: a systematic review and meta-analysis. J Clin Endocrinol Metab 2012;97:2347-2353.

115. Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. N Engl J Med 2010;362:1675-1685.

116. Armstrong MJ, Hull D, Guo K, Barton D, Hazlehurst JM, Gathercole LL, Nasiri M, et al. Glucagon-like peptide 1 decreases lipotoxicity in non-alcoholic steatohepatitis. J Hepatol 2016;64:399-408.

117. Armstrong MJ, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, Hazlehurst JM, et al. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. Lancet 2016;387:679-690.

118. Ratziu V, de Ledinghen V, Oberti F, Mathurin P, Wartelle-Bladou C, Renou C, Sogni P, et al. A randomized controlled trial of high-dose ursodesoxycholic acid for nonalcoholic steatohepatitis. J Hepatol 2011;54:1011-1019.
119. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, Chalasani N, et al. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. Lancet 2015;385:956-965.
120. Ekstedt M, Franzen LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, Kechagias S. Long-term follow-up of patients with NAFLD and elevated liver enzymes. Hepatology 2006;44:865-873.
121. Wong VW, Wong GL, Choi PC, Chan AW, Li MK, Chan HY, Chim AM, et al. Disease progression of non-alcoholic fatty liver disease: a prospective study with paired liver biopsies at 3 years. Gut 2010;59:969-974.
122. McPherson S, Hardy T, Henderson E, Burt AD, Day CP, Anstee QM. Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: implications for prognosis and clinical management. J Hepatol 2015;62:1148-1155.
123. Puri P, Baillie RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O, Sargeant C, et al. A lipidomic analysis of nonalcoholic fatty liver disease. Hepatology 2007;46:1081-1090.
124. Yamaguchi K, Yang L, McCall S, Huang J, Yu XX, Pandey SK, Bhanot S, et al. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. Hepatology 2007;45:1366-1374.
125. Richardson MM, Jonsson JR, Powell EE, Brunt EM, Neuschwander-Tetri BA, Bhathal PS, Dixon JB, et al. Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction. Gastroenterology 2007;133:80-90.
126. Angulo P, Kleiner DE, Dam-Larsen S, Adams LA, Bjornsson ES, Charatcharoenwitthaya P, Mills PR, et al. Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. Gastroenterology 2015;149:389-397 e310.
127. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J Clin Invest 2005;115:1343-1351.

128. Li ZZ, Berk M, McIntyre TM, Feldstein AE. Hepatic lipid partitioning and liver damage in nonalcoholic fatty liver disease: role of stearoyl-CoA desaturase. J Biol Chem 2009;284:5637-5644.
129. Wang C, Zhao Y, Gao X, Li L, Yuan Y, Liu F, Zhang L, et al. Perilipin 5 improves hepatic lipotoxicity by inhibiting lipolysis. Hepatology 2015;61:870-882.
130. Hirsova P, Ibrahim SH, Gores GJ, Malhi H. Lipotoxic lethal and sublethal stress signaling in hepatocytes: relevance to NASH pathogenesis. J Lipid Res 2016;57:1758-1770.
131. Akazawa Y, Cazanave S, Mott JL, Elmi N, Bronk SF, Kohno S, Charlton MR, et al. Palmitoleate attenuates palmitate-induced Bim and PUMA up-regulation and hepatocyte lipoapoptosis. J Hepatol 2010;52:586-593.
132. El-Badry AM, Moritz W, Contaldo C, Tian Y, Graf R, Clavien PA. Prevention of reperfusion injury and microcirculatory failure in macrosteatotic mouse liver by omega-3 fatty acids. Hepatology 2007;45:855-863.
133. Sanyal AJ, Abdelmalek MF, Suzuki A, Cummings OW, Chojkier M, Group E-AS. No significant effects of ethyl-eicosapentanoic acid on histologic features of nonalcoholic steatohepatitis in a phase 2 trial. Gastroenterology 2014;147:377-384 e371.
134. Han MS, Park SY, Shinzawa K, Kim S, Chung KW, Lee JH, Kwon CH, et al. Lysophosphatidylcholine as a death effector in the lipoapoptosis of hepatocytes. J Lipid Res 2008;49:84-97.
135. Raichur S, Wang ST, Chan PW, Li Y, Ching J, Chaurasia B, Dogra S, et al. CerS2 Haploinsufficiency Inhibits beta-Oxidation and Confers Susceptibility to Diet-Induced Steatohepatitis and Insulin Resistance. Cell Metab 2014;20:919.
136. Ioannou GN. The Role of Cholesterol in the Pathogenesis of NASH. Trends Endocrinol Metab 2016;27:84-95.
137. Bellanti F, Mitarotonda D, Tamborra R, Blonda M, Iannelli G, Petrella A, Sanginario V, et al. Oxysterols induce mitochondrial impairment and hepatocellular toxicity in non-alcoholic fatty liver disease. Free Radic Biol Med 2014;75 Suppl 1:S16-17.
138. Begriche K, Massart J, Robin MA, Bonnet F, Fromenty B. Mitochondrial adaptations and dysfunctions in nonalcoholic fatty liver disease. Hepatology 2013;58:1497-1507.
139. Serviddio G, Bellanti F, Tamborra R, Rollo T, Romano AD, Giudetti AM, Capitanio N, et al. Alterations of hepatic ATP homeostasis and respiratory chain during

development of non-alcoholic steatohepatitis in a rodent model. Eur J Clin Invest 2008;38:245-252.

140. Cortez-Pinto H, Chatham J, Chacko VP, Arnold C, Rashid A, Diehl AM. Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. JAMA 1999;282:1659-1664.

141. Guo S. Insulin signaling, resistance, and the metabolic syndrome: insights from mouse models into disease mechanisms. J Endocrinol 2014;220:T1-T23.

142. Puri P, Sanyal AJ. The Intestinal Microbiome in Nonalcoholic Fatty Liver Disease. Clin Liver Dis 2018;22:121-132.

143. Khambu B, Yan S, Huda N, Liu G, Yin XM. Autophagy in non-alcoholic fatty liver disease and alcoholic liver disease. Liver Res 2018;2:112-119.

144. Cholankeril G, Wong RJ, Hu M, Perumpail RB, Yoo ER, Puri P, Younossi ZM, et al. Liver Transplantation for Nonalcoholic Steatohepatitis in the US: Temporal Trends and Outcomes. Dig Dis Sci 2017;62:2915-2922.

145. Charlton MR, Burns JM, Pedersen RA, Watt KD, Heimbach JK, Dierkhising RA. Frequency and outcomes of liver transplantation for nonalcoholic steatohepatitis in the United States. Gastroenterology 2011;141:1249-1253.

146. Wong RJ, Aguilar M, Cheung R, Perumpail RB, Harrison SA, Younossi ZM, Ahmed A. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States. Gastroenterology 2015;148:547-555.

147. Felmlee DJ, Coilly A, Chung RT, Samuel D, Baumert TF. New perspectives for preventing hepatitis C virus liver graft infection. Lancet Infect Dis 2016;16:735-745.

148. Kardashian AA, Dodge JL, Roberts J, Brandman D. Weighing the risks: Morbid obesity and diabetes are associated with increased risk of death on the liver transplant waiting list. Liver Int 2018;38:553-563.

149. Stine JG, Argo CK, Pelletier SJ, Maluf DG, Caldwell SH, Northup PG. Advanced non-alcoholic steatohepatitis cirrhosis: A high-risk population for pre-liver transplant portal vein thrombosis. World J Hepatol 2017;9:139-146.

150. Durand F, Antoine C, Soubrane O. Liver Transplantation in France. Liver Transpl 2019;25:763-770.

151. Afzali A, Berry K, Ioannou GN. Excellent posttransplant survival for patients with nonalcoholic steatohepatitis in the United States. Liver Transpl 2012;18:29-37.

152. van den Berg EH, Douwes RM, de Meijer VE, Schreuder T, Blokzijl H. Liver transplantation for NASH cirrhosis is not performed at the expense of major post-operative morbidity. Dig Liver Dis 2018;50:68-75.
153. Malik SM, deVera ME, Fontes P, Shaikh O, Ahmad J. Outcome after liver transplantation for NASH cirrhosis. Am J Transplant 2009;9:782-793.
154. Wang X, Li J, Riaz DR, Shi G, Liu C, Dai Y. Outcomes of liver transplantation for nonalcoholic steatohepatitis: a systematic review and meta-analysis. Clin Gastroenterol Hepatol 2014;12:394-402 e391.
155. Vallin M, Guillaud O, Boillot O, Hervieu V, Scoazec JY, Dumortier J. Recurrent or de novo nonalcoholic fatty liver disease after liver transplantation: natural history based on liver biopsy analysis. Liver Transpl 2014;20:1064-1071.
156. Narayanan P, Mara K, Izzy M, Dierkhising R, Heimbach J, Allen AM, Watt KD. Recurrent or De Novo Allograft Steatosis and Long-term Outcomes After Liver Transplantation. Transplantation 2019;103:e14-e21.
157. Berenguer M, Ferrell L, Watson J, Prieto M, Kim M, Rayon M, Cordoba J, et al. HCV-related fibrosis progression following liver transplantation: increase in recent years. J Hepatol 2000;32:673-684.
158. Bhati C, Idowu MO, Sanyal AJ, Rivera M, Driscoll C, Stravitz RT, Kohli DR, et al. Long-term Outcomes in Patients Undergoing Liver Transplantation for Nonalcoholic Steatohepatitis-Related Cirrhosis. Transplantation 2017;101:1867-1874.
159. Everhart JE, Lombardero M, Lake JR, Wiesner RH, Zetterman RK, Hoofnagle JH. Weight change and obesity after liver transplantation: incidence and risk factors. Liver Transpl Surg 1998;4:285-296.
160. Tsochatzis E, Coilly A, Nadalin S, Levistky J, Tokat Y, Ghobrial M, Klinck J, et al. International Liver Transplantation Consensus Statement on End-stage Liver Disease Due to Nonalcoholic Steatohepatitis and Liver Transplantation. Transplantation 2019;103:45-56.
161. Choudhary NS, Saigal S, Saraf N, Soin AS. Predictors of De Novo Nonalcoholic Fatty Liver Disease After Liver Transplantation and Associated Fibrosis. Liver Transpl 2019;25:967-968.
162. Sprinzl MF, Weinmann A, Lohse N, Tonissen H, Koch S, Schattenberg J, Hoppe-Lotichius M, et al. Metabolic syndrome and its association with fatty liver disease after orthotopic liver transplantation. Transpl Int 2013;26:67-74.

163. Rinella ME, Alonso E, Rao S, Whitington P, Fryer J, Abecassis M, Superina R, et al. Body mass index as a predictor of hepatic steatosis in living liver donors. Liver Transpl 2001;7:409-414.
164. Selzner M, Rudiger HA, Sindram D, Madden J, Clavien PA. Mechanisms of ischemic injury are different in the steatotic and normal rat liver. Hepatology 2000;32:1280-1288.
165. Selzner N, Selzner M, Jochum W, Amann-Vesti B, Graf R, Clavien PA. Mouse livers with macrosteatosis are more susceptible to normothermic ischemic injury than those with microsteatosis. J Hepatol 2006;44:694-701.
166. Spitzer AL, Lao OB, Dick AA, Bakthavatsalam R, Halldorson JB, Yeh MM, Upton MP, et al. The biopsied donor liver: incorporating macrosteatosis into high-risk donor assessment. Liver Transpl 2010;16:874-884.
167. de Graaf EL, Kench J, Dilworth P, Shackel NA, Strasser SI, Joseph D, Pleass H, et al. Grade of deceased donor liver macrovesicular steatosis impacts graft and recipient outcomes more than the Donor Risk Index. J Gastroenterol Hepatol 2012;27:540-546.
168. Briceno J, Ciria R, Pleguezuelo M, de la Mata M, Muntane J, Naranjo A, Sanchez-Hidalgo J, et al. Impact of donor graft steatosis on overall outcome and viral recurrence after liver transplantation for hepatitis C virus cirrhosis. Liver Transpl 2009;15:37-48.
169. McCormack L, Petrowsky H, Jochum W, Mullhaupt B, Weber M, Clavien PA. Use of severely steatotic grafts in liver transplantation: a matched case-control study. Ann Surg 2007;246:940-946; discussion 946-948.
170. Kulik U, Lehner F, Klempnauer J, Borlak J. Primary non-function is frequently associated with fatty liver allografts and high mortality after re-transplantation. Liver Int 2017;37:1219-1228.
171. Fromenty B, Berson A, Pessayre D. Microvesicular steatosis and steatohepatitis: role of mitochondrial dysfunction and lipid peroxidation. J Hepatol 1997;26 Suppl 1:13-22.
172. Cieslak B, Lewandowski Z, Urban M, Ziarkiewicz-Wroblewska B, Krawczyk M. Microvesicular liver graft steatosis as a risk factor of initial poor function in relation to suboptimal donor parameters. Transplant Proc 2009;41:2985-2988.
173. Andert A, Ulmer TF, Schoning W, Kroy D, Hein M, Alizai PH, Heidenhain C, et al. Grade of donor liver microvesicular steatosis does not affect the postoperative outcome after liver transplantation. Hepatobiliary Pancreat Dis Int 2017;16:617-623.

174. El-Badry AM, Breitenstein S, Jochum W, Washington K, Paradis V, Rubbia-Brandt L, Puhan MA, et al. Assessment of hepatic steatosis by expert pathologists: the end of a gold standard. Ann Surg 2009;250:691-697.
175. Younossi ZM, Loomba R, Anstee QM, Rinella ME, Bugianesi E, Marchesini G, Neuschwander-Tetri BA, et al. Diagnostic modalities for nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, and associated fibrosis. Hepatology 2018;68:349-360.
176. Chiappini F, Barrier A, Saffroy R, Domart MC, Dagues N, Azoulay D, Sebagh M, et al. Exploration of global gene expression in human liver steatosis by high-density oligonucleotide microarray. Lab Invest 2006;86:154-165.
177. Joshi-Barve S, Barve SS, Amancherla K, Gobejishvili L, Hill D, Cave M, Hote P, et al. Palmitic acid induces production of proinflammatory cytokine interleukin-8 from hepatocytes. Hepatology 2007;46:823-830.
178. Pusl T, Wild N, Vennegeerts T, Wimmer R, Goke B, Brand S, Rust C. Free fatty acids sensitize hepatocytes to bile acid-induced apoptosis. Biochem Biophys Res Commun 2008;371:441-445.
179. Tanaka N, Matsubara T, Krausz KW, Patterson AD, Gonzalez FJ. Disruption of phospholipid and bile acid homeostasis in mice with nonalcoholic steatohepatitis. Hepatology 2012;56:118-129.
180. Muir K, Hazim A, He Y, Peyressatre M, Kim DY, Song X, Beretta L. Proteomic and lipidomic signatures of lipid metabolism in NASH-associated hepatocellular carcinoma. Cancer Res 2013;73:4722-4731.
181. Chiappini F, Desterke C, Bertrand-Michel J, Guettier C, Le Naour F. Hepatic and serum lipid signatures specific to nonalcoholic steatohepatitis in murine models. Sci Rep 2016;6:31587.
182. Younossi ZM, Loomba R, Rinella ME, Bugianesi E, Marchesini G, Neuschwander-Tetri BA, Serfaty L, et al. Current and future therapeutic regimens for nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Hepatology 2018;68:361-371.
183. Bugianesi E, Rosso C, Cortez-Pinto H. How to diagnose NAFLD in 2016. J Hepatol 2016;65:643-644.
184. Poloznikov A, Gazaryan I, Shkurnikov M, Nikulin S, Drapkina O, Baranova A, Tonevitsky A. In vitro and in silico liver models: Current trends, challenges and opportunities. ALTEX 2018;35:397-412.

185. Horvath S, Erhart W, Brosch M, Ammerpohl O, von Schonfels W, Ahrens M, Heits N, et al. Obesity accelerates epigenetic aging of human liver. Proc Natl Acad Sci U S A 2014;111:15538-15543.
186. Ahrens M, Ammerpohl O, von Schonfels W, Kolarova J, Bens S, Itzel T, Teufel A, et al. DNA methylation analysis in nonalcoholic fatty liver disease suggests distinct disease-specific and remodeling signatures after bariatric surgery. Cell Metab 2013;18:296-302.
187. Baker SS, Baker RD, Liu W, Nowak NJ, Zhu L. Role of alcohol metabolism in non-alcoholic steatohepatitis. PLoS One 2010;5:e9570.
188. Frades I, Andreasson E, Mato JM, Alexandersson E, Matthiesen R, Martinez-Chantar ML. Integrative genomic signatures of hepatocellular carcinoma derived from nonalcoholic Fatty liver disease. PLoS One 2015;10:e0124544.
189. Shi GM, Ke AW, Zhou J, Wang XY, Xu Y, Ding ZB, Devbhandari RP, et al. CD151 modulates expression of matrix metalloproteinase 9 and promotes neoangiogenesis and progression of hepatocellular carcinoma. Hepatology 2010;52:183-196.
190. Li J, Lau G, Chen L, Yuan YF, Huang J, Luk JM, Xie D, et al. Interleukin 23 promotes hepatocellular carcinoma metastasis via NF-kappa B induced matrix metalloproteinase 9 expression. PLoS One 2012;7:e46264.
191. Yan XL, Jia YL, Chen L, Zeng Q, Zhou JN, Fu CJ, Chen HX, et al. Hepatocellular carcinoma-associated mesenchymal stem cells promote hepatocarcinoma progression: role of the S100A4-miR155-SOCS1-MMP9 axis. Hepatology 2013;57:2274-2286.
192. Wu P, Hua Y, Tan S, Li M, Shu Y, Fang G. Interactions of central obesity with rs3918242 on risk of non-alcoholic fat liver disease: a preliminary case-control study. Int J Clin Exp Pathol 2015;8:4165-4170.
193. Yilmaz Y, Eren F. Serum biomarkers of fibrosis and extracellular matrix remodeling in patients with nonalcoholic fatty liver disease: association with liver histology. Eur J Gastroenterol Hepatol 2019;31:43-46.
194. D'Amico F, Consolo M, Amoroso A, Skarmoutsou E, Mauceri B, Stivala F, Malaponte G, et al. Liver immunolocalization and plasma levels of MMP-9 in non-alcoholic steatohepatitis (NASH) and hepatitis C infection. Acta Histochem 2010;112:474-481.
195. Linecker M, Krones T, Berg T, Niemann CU, Steadman RH, Dutkowski P, Clavien PA, et al. Potentially inappropriate liver transplantation in the era of the "sickest first" policy - A search for the upper limits. J Hepatol 2018;68:798-813.

196. Durand F, Renz JF, Alkofer B, Burra P, Clavien PA, Porte RJ, Freeman RB, et al. Report of the Paris consensus meeting on expanded criteria donors in liver transplantation. Liver Transpl 2008;14:1694-1707.
197. Flechtenmacher C, Schirmacher P, Schemmer P. Donor liver histology--a valuable tool in graft selection. Langenbecks Arch Surg 2015;400:551-557.
198. Todo S, Demetris AJ, Makowka L, Teperman L, Podesta L, Shaver T, Tzakis A, et al. Primary nonfunction of hepatic allografts with preexisting fatty infiltration. Transplantation 1989;47:903-905.
199. Angele MK, Rentsch M, Hartl WH, Wittmann B, Graeb C, Jauch KW, Loehe F. Effect of graft steatosis on liver function and organ survival after liver transplantation. Am J Surg 2008;195:214-220.
200. Zamboni F, Franchello A, David E, Rocca G, Ricchiuti A, Lavezzo B, Rizzetto M, et al. Effect of macrovescicular steatosis and other donor and recipient characteristics on the outcome of liver transplantation. Clin Transplant 2001;15:53-57.
201. Dutkowski P, Schlegel A, Slankamenac K, Oberkofler CE, Adam R, Burroughs AK, Schadde E, et al. The use of fatty liver grafts in modern allocation systems: risk assessment by the balance of risk (BAR) score. Ann Surg 2012;256:861-868; discussion 868-869.
202. Baccarani U, Isola M, Adani GL, Avellini C, Lorenzin D, Rossetto A, Curro G, et al. Steatosis of the hepatic graft as a risk factor for post-transplant biliary complications. Clin Transplant 2010;24:631-635.
203. Ploeg RJ, D'Alessandro AM, Knechtle SJ, Stegall MD, Pirsch JD, Hoffmann RM, Sasaki T, et al. Risk factors for primary dysfunction after liver transplantation--a multivariate analysis. Transplantation 1993;55:807-813.
204. Nocito A, El-Badry AM, Clavien PA. When is steatosis too much for transplantation? J Hepatol 2006;45:494-499.
205. Yoong KF, Gunson BK, Neil DA, Mirza DF, Mayer AD, Buckels JA, McMaster P. Impact of donor liver microvesicular steatosis on the outcome of liver retransplantation. Transplant Proc 1999;31:550-551.
206. Han S, Ha SY, Park CK, Joh JW, Kwon CH, Kwon GY, Kim G, et al. Microsteatosis may not interact with macrosteatosis in living donor liver transplantation. J Hepatol 2015;62:556-562.

207. Sharkey FE, Lytvak I, Prihoda TJ, Speeg KV, Washburn WK, Halff GA. High-grade microsteatosis and delay in hepatic function after orthotopic liver transplantation. Hum Pathol 2011;42:1337-1342.
208. Yersiz H, Lee C, Kaldas FM, Hong JC, Rana A, Schnickel GT, Wertheim JA, et al. Assessment of hepatic steatosis by transplant surgeon and expert pathologist: a prospective, double-blind evaluation of 201 donor livers. Liver Transpl 2013;19:437-449.
209. Rey JW, Wirges U, Dienes HP, Fries JW. Hepatic steatosis in organ donors: disparity between surgery and histology? Transplant Proc 2009;41:2557-2560.
210. Gawrieh S, Knoedler DM, Saeian K, Wallace JR, Komorowski RA. Effects of interventions on intra- and interobserver agreement on interpretation of nonalcoholic fatty liver disease histology. Ann Diagn Pathol 2011;15:19-24.
211. Le Naour F, Gadea L, Danulot M, Yousef I, Vibert E, Wavelet M, Kascakova S, et al. Quantitative assessment of liver steatosis on tissue section using infrared spectroscopy. Gastroenterology 2015;148:295-297.
212. Le Naour F, Bralet MP, Debois D, Sandt C, Guettier C, Dumas P, Brunelle A, et al. Chemical imaging on liver steatosis using synchrotron infrared and ToF-SIMS microspectroscopies. PLoS One 2009;4:e7408.
213. Peng C, Chiappini F, Kascakova S, Danulot M, Sandt C, Samuel D, Dumas P, et al. Vibrational signatures to discriminate liver steatosis grades. Analyst 2015;140:1107-1118.
214. Reistad N, Nilsson JH, Bergenfeldt M, Rissler P, Sturesson C. Intraoperative liver steatosis characterization using diffuse reflectance spectroscopy. HPB (Oxford) 2019;21:175-180.
215. Nilsson JH, Reistad N, Brange H, Oberg CF, Sturesson C. Diffuse Reflectance Spectroscopy for Surface Measurement of Liver Pathology. Eur Surg Res 2017;58:40-50.
216. Evers DJ, Westerkamp AC, Spliethoff JW, Pully VV, Hompes D, Hendriks BH, Prevoo W, et al. Diffuse reflectance spectroscopy: toward real-time quantification of steatosis in liver. Transpl Int 2015;28:465-474.
217. Golse N, Cosse C, Allard MA, Laurenzi A, Tedeschi M, Guglielmo N, Fernandez-Sevilla E, et al. Evaluation of a micro-spectrometer for the real-time assessment of liver graft with mild-to-moderate macrosteatosis: A proof of concept study. J Hepatol 2019;70:423-430.

8 Articles en annexe

8.1 Article 4

Symbiosis

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Recent Insights into Treatment of Non-Alcoholic Steatohepatitis

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Abstract

Non-Alcoholic Fatty Liver Disease (NAFLD) represents a major public health issue worldwide. The main characteristic of NAFLD is the accumulation of lipids in hepatocytes to form lipid droplets. The spectrum of NAFLD ranges from non-alcoholic fatty liver (i.e., steatosis) to Non-Alcoholic Steatohepatitis (NASH), a progressive condition increasing in Western Countries and leading to cirrhosis, liver failure and hepatocellular carcinoma. The prevention of NAFLD requires strategies for careful management and monitoring of patients with obesity, diabetes and other components of metabolic syndrome. There is no currently approved treatment that can reverse NASH once it is established. There is no evidence that pioglitazone or vitamin E can improve fibrosis. Life-style changes and bariatric surgery may improve hepatic histology in some patients with NASH. Currently, a few new drugs targeting pathways that have recently been implicated in the development of NAFLD are under development Therefore, treatment of NASH should be approached as a complex therapy that would take into account etiology of the disease and patient's history. This review summarizes recent insights into the treatment of NASH.

Keywords: NAFLD; NASH; Metabolic syndrome; Obeticholic acid; Treatment

Introduction

For the last two decades, Non-Alcoholic Fatty Liver Disease (NAFLD) and especially Non-Alcoholic Steatohepatitis (NASH) have been the main cause of chronic liver disease in the Western Countries as well as in Middle East, Asia, Africa and South America [1-4]. The increasing prevalence of NAFLD and NASH in developing countries correlates with changes in lifestyle such as consumption of fast-food, soft soda sugar-sweetened beverages and sedentary lifestyle, leading to the increased prevalence of obesity. NAFLD is usually associated with obesity, insulin resistance and type 2 diabetes, dyslipidemia and metabolic syndrome, which affect both adult and pediatric populations [1-11]. NAFLD also increases the risk for development of Cardiovascular Disease (CVD) independently to other risk factors [12,13], making NAFLD not only a liver disease but also a "systemic disease".

NAFLD is a condition ranging from simple Non-Alcoholic Fatty Liver (NAFL) to NASH, steatofibrosis and cryptogenic cirrhosis. The main characteristic of these lesions is fat accumulation in the form of diacylglycerols and triglycerides contained in hepatic lipid droplets. NAFL is usually considered as benign and reversible whereas NASH may progress to liver failure, cirrhosis and hepatocellular carcinoma [2,6,7,9,14].

The diagnosis of NAFLD is made by histology that still remains the gold standard. Diagnosis of NASH is a little more complicated because it encompasses hepatic steatosis, ballooning of hepatocytes, Mallory's body and inflammation, which may or may not be associated fibrosis. For the past years, some groups worked on better defining NASH by developing different scores such as the NASH Activity Score (NAS) or the Steatosis, Activity and Fibrosis (SAF) score [15-18].

In attempts to better understand the onset and progression of NASH and find therapeutic targets for its treatment, a number of different mechanisms have been described [14,19-21]. Mitochondrial dysfunction (e.g. decreased β-oxidation and increased oxidative stress), impaired lipid metabolism (e.g. nuclear receptors and transcription factors implicated in de novo lipogenesis) and excretion (e.g. bile acids synthesis and lipoproteins homeostasis) have been raised to play a role [11,14,19-25], but so far, no mechanism specifically linked to NAFLD, and especially NASH, has been pinpointed to explain accumulation of the lipids in the liver and predict the progression of the disease.

Therefore, because prevalence and severity of NASH associated with its hepatic and systemic complications are dramatically increasing worldwide, there is an urgent need to find remedies to decrease or even stop its progression. The first line of intervention is the lifestyle changes. But, academic and pharmacological laboratories are also focused on developing drug therapies. In this review, we will focus on the recent advances in NASH treatments, the failures and the hopes.

Life style modifications

Diet: Lipid overload in the liver is mainly due to high-caloric diets also known as Western Country diets. The high content of lipids such as cholesterol saturated and n-6 unsaturated fatty acids (i.e., decrease in n-3 to n-6 polyunsaturated fatty acids ratio) combined with high sugar content (mainly fructose) has been implicated in the occurrence and progression of NASH [26]. The obvious goal would be to modify the diet by decreasing cholesterol, Saturated Fatty Acid (SFA) and fructose intake, and increase n-3 Polyunsaturated Fatty Acid (PUFA) intake. The whole caloric intake has to be decreased by 25% in calories from the normal diet

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based on patient's sex and age according to the World Gastroenterology Organization Global Guidelines [27-30].

Small observational studies showed that a weight loss exceeding 7% of body weight over 1 year improves histology in patients with biopsy-proven NASH [31,32]. More durable weight loss can be achieved in patients with NAFLD by combining diet and exercise for a period longer than one year [33]. It is now recommended that hypocaloric diet should provide 1000-1200 calories per day for women and 1200-1500 calories per day for men with a goal to achieve a weight loss of 0.5-1.0 kg per week based on published guidelines [34].

It has been shown that moderately calorie-restricted diet with changes in macronutrient composition leads to better results compared to a very-low-caloric diet with 5% to 10% weight loss as the goal [35]. Furthermore, low-fat diet associated with weight loss program improves body weight, Body Mass Index (BMI), fatty liver, insulin sensitivity and plasma triglycerides [36]. Importantly, heavy alcohol consumption should be avoided in NAFLD patients [37].

Mediterranean diet due to preparation with olive oil, rich in n-3 PUFA, might be of additional help to modify hepatic lipid content and decrease cardiovascular risks [38]. Recently, a randomized controlled trial named Mediterranean Dietary Intervention for Adults with Non Alcoholic Fatty Liver Disease (MEDINA) enrolling 94 patients with NAFLD associated with insulin resistance and receiving either Mediterranean diet or low-caloric diet during 3 months, showed that Mediterranean diet can result in significant benefits in liver fat and Insulin Resistance (IR), independent of weight loss. These changes are sustained at 12 months. However, liver status was only assessed using magnetic resonance spectroscopy. Hence, this study failed to explore further NAFLD progression [39]. Another study directly assessed effects of Mediterranean diet on liver histology and showed that Mediterranean diet was associated with lower probability of development of high grade of hepatic steatosis and NASH [40,41].

Other studies showed that diets enriched in vegetable oils such as canola, olive pomace and olive oils might improve the grade of hepatic steatosis and insulin sensitivity, especially the combination of olive and canola oils [42]. Indeed, the impact of n-3 PUFA has been investigated in the randomized WELCOME trial and showed that high dose of n-3 PUFA (docosahexaenoic + eicosapentaenoic, Omacor®) did not improve microvascular function but was associated with a decrease in hepatic fat content and improvement in NAFLD severity [43-45].

Other studies have been conducted to test different nutrients enriched in Monounsaturated Fatty Acids (MUFA) and n-3 PUFA such as oily fish/fish oil, nuts and avocado and showed improvement in liver fat and steatosis grading, liver function and insulin sensitivity [46-49]. Some recommendations have been made about the optimal consumption of each nutrient (e.g. avocado, nuts, and olive oil) and the amount per day [48].

Some groups have also studied effects of tea, coffee and caffeine consumption on hepatic steatosis, NASH and fibrosis and showed that there was a negative correlation between caffeine consumption and hepatic fibrosis in overweight or obese patients with NASH [50-52]. In addition, coffee may reduce cardiovascular risk factors by favorably affecting inflammation, insulin sensitivity and decreasing hypertension [53,54]. Thus, caffeine and its derivatives might have a potential to slow down fibrogenesis during NASH progression but further investigation has to be done to assess their effects on NASH development.

In conclusion, the optimal diet for NAFLD patients is still undetermined. However, patients with NAFLD may benefit from a moderate- to low-carbohydrate (40%-45% of total calories) diet, coupled with high MUFA, high n-3 PUFA, low SFA, and low-cholesterol diet.

Physical activity: Associated with the unhealthy diet, sedentary lifestyle contributes to the occurrence of obesity and NAFLD. Changes in lifestyle by increasing physical activity have been shown to improve triglyceride turnover and liver fat accumulation leading to increased hepatic insulin sensitivity and whole lipid oxidation in the body and decreased hepatic free fatty acids uptake independently of weight-loss [26,30]. But in both cases, the crucial issue is the motivation. Indeed, the first significant weight-loss improvement might appear after 6 months of training, which may discourage the patients. Therefore, cognitive-behavioral therapy is needed [41,55,56]. A systematic review of data from various randomized controlled trials showed that exercise reduced hepatic fat content [57]. The European guidelines suggest at least 150 min per week of moderate-intensity physical activity and at least 75 min per week of vigorous-intensity physical activity, with additional muscle strengthening exercise twice a week [58].

Surgery: Based on the idea that NAFLD/NASH progresses due to the excessive food intake and loss of satiety feeling (e.g. leptin resistance), bariatric surgery may improve NAFLD by reducing food intake [59]. This therapeutic approach is recommended for patients with morbid obesity (>40 kg/ m^2) or severe obesity (>35 kg/ m^2) associated with complications [60,61]. This surgery is offered to the patients that were not able to lose weight after a period of non-surgical treatment [60,61]. Several techniques have been reported including sleeve gastrectomy, gastric band and Roux-en-Y gastric bypass. Currently, mini-invasive procedures are favored such as laparoscopic approach [62]. Bariatric surgery has been shown to improve insulin sensitivity, dyslipidemia, systemic hypertension, decrease CVD risks and improve the histological and biochemical parameters of NAFL and NASH [63]. Bariatric surgery is also associated with the most rapid, effective and sustained weight loss [64,65]. A recent study showed that bariatric surgery-induced the disappearance of NASH from nearly 85% of 109 morbidly obese patients [66]. However, recently Goossens et al. showed that obese patients with NASH undergoing bariatric surgery had an increased risk of death compared to obese patients without NASH before surgery. This study emphasizes the importance of the systemic perioperative liver biopsy in obese patients undergoing bariatric surgery as a diagnostic and prognostic assessment of the death risk [67], and also suggests that NASH should be managed, in addition to changes in lifestyle, with drug therapy to decrease the death risk for these patients. At the same time another study has found no correlation between NASH and death after bariatric surgery, so the exact relationship between the two, and potential risk factors are still not clear [68].

Drug therapy

Because in addition to obesity NASH progression is associated with IR/type 2 diabetes and dyslipidemia, the goal for drug therapy is to improve general conditions by increasing the whole body insulin sensitivity, decreasing lipid absorption, hepatic *de novo lipid* synthesis and promoting lipid oxidation in skeletal muscle and liver. In this chapter, we will focus on the recent advances in drug therapies of NASH.

Insulin sensitizers: The first idea to treat NASH was to use insulin sensitizers metformin and thiazolidinediones (e.g. pioglitazone, rosiglitazone), which are used extensively to treat IR/type 2 diabetes. Results of the studies of potential effects of metformin on the development of NASH are contradictory. Recently, a meta-analysis showed no benefit of metformin in improving serum aminotransferases or liver histology among NAFLD patients [69]. Thiazolidinediones (TZDs) are selective peroxisome proliferator–activated receptor-gamma agonists. Pioglitazone demonstrated the benefit with histological improvements in NASH patients such as hepatic steatosis, lobular inflammation and ballooning degeneration [70,71]. Furthermore, two recent meta-analyses confirmed the histological improvement of NASH in patients treated with pioglitazone [72,73]. In addition, pioglitazone demonstrated mortality

reduction related to CVD. However, pioglitazone has received black box warning by the Food and Drug Administration due to reports of congestive heart failure. Previously, rosiglitazone has been prohibited in Europe and highly restricted in USA [74-76]. Despite their effects on reducing hepatic gluconeogenesis, intestinal lipid absorption, lipogenesis, lowering serum lipid concentration, improved liver enzymes and increasing global insulin sensitivity, these molecules failed assessments for long-term benefit as they are associated with weight gain, edema, heart failure, risk of CVD and cancer (e.g. bladder cancer) [74-76].

Antioxidants: Because NASH development and progression are associated with oxidative stress, another treatment line is the use of antioxidants. Currently, the most commonly used antioxidant is vitamin E supplementation given at 800 IU per day. It improves inflammation and fibrosis induced by suppression of lipid peroxidation and oxidative stress [77-79]. Several studies suggest that vitamin E improves liver enzymes, hepatic steatosis, and liver injury in NASH patients but robust data including prospective evaluation are currently lacking. Furthermore, deleterious effects and an increase in mortality rate have been suggested in a long-term exposure [80]. Further studies are required to clarify the beneficial role of vitamin E monotherapy in NASH patients.

Despite these effects, several studies have evaluated vitamin E. One important study compared effects of vitamin E and insulin sensitizers. 247 biopsy-proven NASH patients without diabetes were randomized to receive pioglitazone 30 mg, vitamin E 800 IU, or placebo for 96 weeks [79]. Vitamin E was superior to placebo with a significantly higher rate of improvement in NASH (43% vs. 19%, $p = 0.001$) whereas pioglitazone was not but showed significant benefits in some of the secondary outcomes (decrease in aminotransferase, hepatic steatosis and lobular inflammation).

Pentoxifylline is another antioxidant that has shown benefit through decreasing oxidative stress. In a randomized trial enrolling 55 biopsy-proven NASH patients, it improved histological features of NASH compared to placebo [81]. This result has been confirmed in a recent meta-analysis [82]. Although these results are encouraging, their validation in a large cohort with a longer follow-up is mandatory.

Cholesterol-lowering drugs: Hepatic cholesterol overload due to increasing in hepatic cholesterol synthesis and in intestinal cholesterol absorption participate strongly in the development and progression of NASH [11,12]. A therapeutic strategy is to antagonize both de novo hepatic cholesterol synthesis and/or cholesterol absorption by the gut.

Statins inhibit cholesterol synthesis by targeting the hydroxy-methyl-glutaryl-coenzyme A (HMG-CoA) reductase. Therefore, randomized controlled trials have been conducted in patients with NAFLD. Although statins decreased lipid levels, simvastatin did not induce significant improvement in serum aminotransferase levels, hepatic steatosis, necroinflammatory activity, or stage of fibrosis in NASH patients when liver biopsies were conducted [83].

Recently, a preliminary report from 6 patients showed that rosuvastatin could improve NASH within a year of treatment in patients with dyslipidemia [84]. The next step of this study will be to do randomize control trial in a larger cohort of NAFLD/NASH patients with more than a year of follow-up to assess if these effects on NASH improvement are transient and to assess the side effects of the statins (statin-associated muscle symptoms) that may lead to discontinuation of treatment [85].

Statins combination therapy with ezetimibe, an inhibitor of intestinal cholesterol absorption, has been used to decrease the dose of statin and led to a significant decrease in hepatic cholesterol and plasma LDL level. However, the effect of ezetimibe alone or combined with diet is still controversial in terms of improvement of NASH [86-89]. Thus, the new randomized-control study in a larger cohort of patients using ezetimibe alone or combined with diet and/or statins needs to be conducted to assess the beneficial effect on NASH features.

FXR agonists: Farnesoid X Receptor (FXR) is a nuclear receptor implicated in the regulation of different genes involved mainly in glucose, bile acid and lipid metabolism. Because the main causes of lipid accumulation in NASH are the excess of lipids and carbohydrates from diet, increase in de novo lipid synthesis and decrease in lipid oxidation associated with a decrease in hepatic lipid excretion, FXR is becoming one of the most interesting drug targets.

FXR is a bile acid sensor that recognizes specific DNA response elements and binds to them as a heterodimer with retinoid X receptor. Binding of bile acids to FXR leads to the repression of expression of rate-limiting enzymes in the synthesis of bile acids such as cytochrome P450 (CYP) 7A1 and CYP8B1. FXR inhibits de novo lipid synthesis by inducing repression of hepatic sterol regulatory element binding protein 1c (SREBP1c), a transcription factor important for the synthesis of fatty acids and triglycerides. The FXR also inhibits the transcriptional activity of Carbohydrate Response Element Binding Protein (ChREBP), a transcriptional factor implicated in gluconeogenesis and triglyceride synthesis. In addition, FXR interacts with and inhibits directly ChREBP and hepatocyte nuclear factor 4 alpha (HNF4α) proteins [23]. HNF4α is also implicated in the synthesis of bile acids by increasing CYP7A1 and CYP8B1 [23,90].

Fibroblast Growth Factor 21 (FGF21), one of the FXR target genes mainly expressed in the liver, increases fatty acid oxidation, adiponectin secretion and decreases leptin levels. FGF21 also inhibits lipogenesis by repressing SREBP1c and decreases triglyceride and blood glucose levels [91]. Therefore, FGF21 actions have positive effects on plasma lipid levels and hepatic steatosis [92,93].

At the same time, FXR activates expression of hepatic genes involved in lipoprotein clearance from the plasma such as LDL receptor, SR-B1 (i.e., HDL receptor) and also molecules that regulate lipoprotein lipase [63,94,95]. Therefore, bile acids and derivatives have been investigated as the treatment for NASH.

Ursodeoxycholic Acid (UDCA) is a secondary hydrophilic bile acid usually made by the intestinal microbiota. UDCA is used to treat primary biliary cirrhosis [96]. Then, in several studies UDCA has been tested to treat NASH and controversial conclusions about the efficacy of the treatment to reduce NASH lesions and to improve clinical parameters have been drawn. Some studies have shown that after at least one year of treatment, UDCA improved hepatic steatosis and enzymes and the high dose of UDCA treatment also improved serum fibrosis markers, glycemia and IR [97,98]. On the other hand, a study by Leuschner, et al. [99] did not show any beneficial effect of high-dose of UDCA on overall histology features of NASH. Recently, a long-term study combining UDCA with vitamin E showed improvement in liver function (AST, ALT, γ-GT) among the 101 patients enrolled. Out of those, 10 patients underwent a biopsy and histology were performed before and after treatment. Five patients did not improve their NAS score (0 or-1), one patient had an increased NAS score, and 4 patients showed a decreased of at least -2 points of NAS score. Also, during the study period eighteen (n = 18) patients stopped the treatment because of diarrhea, nausea, pruritus, ineffectiveness, or spontaneously (n = 9) and the other (n = 9) because of pregnancy or normalization of biochemical parameters [78]. These results do not seem very conclusive, which is also supported by a few other papers debating on the subject [100-102]. A larger cohort is needed to understand and to better identify the responder and non-responder patient populations. At this point, UDCA is not recommended for the treatment of NAFLD or NASH by the Food and Drug Administration in USA [37].

Nonetheless, bile acid conjugates have recently been tested such as Ursodeoxycholyl lysophosphatidylethanolamide (UDCA-LPE) that has been used on high-fat diet mouse model and showed a a modification in

fatty acid metabolism, hepatoprotective and anti-inflammatory effects [103-106]. Moreover, it has been shown that UDCA-LPE modulates flawed fatty acid metabolism in mice fed High-Fat Diet (HFD) thus restoring altered lipid profiles and has pronounced anti-inflammatory effects [107]. Further investigations have to be done in patients with NASH.

Obeticholic acid, a synthetic bile acid acting as a ligand of FXR, showed efficacy in animal models [108]. Therefore, a multicenter, double-blind, placebo-controlled (1:1), randomized phase II clinical trial has been conducted by NASH Clinical Research Network in non-cirrhotic and non-alcoholic steatohepatitis (FLINT) [109]. The study showed improvement in histological features of NASH in 50% of 141 patients. These encouraging results have been balanced by side effects as 33 (23%) of 141 patients in the obeticholic acid developed pruritus compared with nine (6%) of 142 in the placebo group. Also, another study showed that obeticholic acid treatment had the same results on NAFLD activity score (-1.7 SD 1.8) as vitamin E (-1.9 SD 2.1) and pioglitazone (-0.7 SD 1.8) [79]. Less than 50% of patients are responders, mainly patients with diabetes, leaving a substantial proportion of patients with NASH without an effective treatment [110].

Singh, et al. [111] performed a Bayesian network meta-analysis combining treatment comparison to assess the efficacy of pharmacological compounds for the treatment of NASH. All studies included in the analysis used biopsy-proven NASH and compared effects of vitamin E, TZDs, pentoxifylline or obeticholic acid to each other or placebo. Interestingly, they showed that depending on the drugs or combination of drugs, improvement of histological features was not the same. Pentoxifylline and Obeticholic acid improved fibrosis, whereas vitamin E, TZDs and Obeticholic acid improved ballooning degeneration. Further investigation using combinations of drugs has to be done to increase efficacy (i.e., every patient with NASH improved) and decrease side effects (i.e., decrease digestive perturbation and pruritus). Another FXR agonist, the newly renamed GS 9674 is currently being evaluated in a phase I study (ClinicalTrials.gov NCT02654002). Hence, bile acid derivatives seem promising for NASH treatments.

Prebiotics, probiotics and "synbiotics" as treatments of NASH

For more than a decade, microbiota has been in the focus of attention of the scientific community especially when it comes to its role in the development and progression of chronic liver diseases such as NAFLD, cirrhosis and HCC [112-116]. Several studies have suggested that alterations in intestinal microbiota and inflammatory response might play a central role in the development and progression of NASH [117-131]. Following this idea, the goal is to modify gut microbiota by using either specific nutrients, or directly by ingesting specific mix of microbes or by using both approaches also known as prebiotics, probiotics or "synbiotics", respectively.

Prebiotic treatments: Prebiotics are defined as a group of nutrients composed mainly of non-digestible carbohydrates (e.g. fibers, fructo-oligosaccharides) that beneficially affect the host by altering the composition and thus the activity of the gut microbiota [132]. Mouse model of NASH treated with Fructo-Oligosaccharides (FOS) showed changes in their gut microbiota and a decrease in n-3 PUFA synthesis modulating hepatic steatosis toward changing in gene expression in the liver [133]. In rats fed HFD, FOS supplementation prevented deleterious effects of HFD such as alterations in lipid profile and hepatic morphologic changes [134]. Also, mice fed HFD supplemented with 6% hydroxypropyl methylcellulose, usually used as emulsifier, thickening and suspending agent, showed improvement on intestinal permeability, insulin resistance, hepatic lipid accumulation, glucocorticoid-related bile acid recycling, oxidative stress, and weight gain [135]. Because thiazolidinedione treatment leads to weight gain, Alligier, et al. [136] used prebiotic treatment to counteract the side effects of TZDs in animal models. Inulin-type fructan prebiotics decreased adiposity and improved the metabolic response in HFD-fed mice treated with TZDs.

In human clinical trials, FOS has been used to treat NAFLD but did not show efficacy in NASH improvement as observed in animal models. This lack of efficiency in treated patients might be due to power, randomized, controlled clinical trials, involving various centers and the population of different origin, but also to assessing NASH improvement mainly by measuring ALT, a non-specific marker of NASH, and using different doses of prebiotics [137,138]. Recently, a double blinded, placebo controlled, parallel group study, adults (BMI ≥ 25 kg/ m^2) with confirmed NAFLD randomized to either a 16 g/day prebiotic or isocaloric diet, was conducted. However, the study is still in progress and no results are available yet [139].

Probiotic treatments: A direct approach to modifing intestinal microbiota are probiotics -live microorganisms- which, when administered properly, confer beneficial effects on the host [140]. Therefore using probiotics to modulate gut microbiota and to improve NASH have been considered for a decade [141,142]. Interestingly, studies on mouse models of NASH showed that probiotic VSL#3®, a mixture of eight probiotics, modulated liver fibrosis but did not decrease steatosis and inflammation in liver [143], whereas in ApoE deficient mice, VSL#3® corrected insulin resistance in liver and adipose tissues and protected against development of steatohepatitis [144]. On the other hand, mouse fed high-fat/ high sucrose diet and treated with *Lactobacillus paracasei* showed attenuation of hepatic steatosis and increased M2-dominant Kupffer cells in a NASH model [145]. In rat models of NASH a specific probiotic inducing butyrate production, *Clostridium butyricum* (MIYAIRI 588), showed a beneficial effect in the prevention of NAFLD progression [146]. Supplementation on sodium butyrate in mice fed Western diet were also protected from inflammation in the liver and thus from the development of NASH [147].

Despite the controversial results in animal models, clinical trials using probiotics have been recently conducted with promising results. A first pilot study was conducted on 28 patients with histology-proven NAFLD, who were analyzed in a double-blind randomized clinical trial. Patients were randomized to one of the following treatments during 3 months: group I, treated with one tablet per day with 500 million of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* and group II, treated with one placebo tablet (120 mg of starch) showed improvement on liver aminotransferases levels in patients with NAFLD but no data are available on liver histology [148]. Another pilot study including patients with NASH assessed by histology was randomized to receive probiotics (n = 10) or usual care (n = 10) during 6 months. The Lepicol® probiotic formula contains *Lactobacillus plantarum*, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* combined with fiber (Psyllium husks) and inulin. This proof of concept study showed promising results in reducing liver fat and AST level in NASH patients [149]. The same group conducted a study with a larger group of patients (n= 22 controls and n=16 NASH patients) and looked at gut microbiota. They showed that NASH patients had fecal dysbiosis and changes in microbiota correlate with improvement in hepatic steatosis [150].

Synbiotic treatments: Combining both prebiotics and probiotics approaches (i.e., synbiotics) might further improve the effect of each treatment given alone. Based on this idea, a larger study was conducted by another group including random trials that involved patients with NASH assessed by histology before and after the end of the study. Patients were divided into two groups one of which received *Bifidobacterium longum* with FOS and lifestyle modification (i.e., diet and exercise) while the other had lifestyle modification alone. The group of patients receiving *Bifidobacterium longum* with FOS and lifestyle modification had decreased inflammation, serum AST, HOMA-IR, serum endotoxin, hepatic steatosis, and the NASH activity index compared to the lifestyle modification

group. Importantly, a decrease in BMI was observed in both groups of patients suggesting that pre- and/or probiotics have to be associated with a different therapeutic effect [151]. Recently, a randomized, double-blinded, placebo-controlled clinical trial was conducted as a pilot study on 52 patients with NAFLD diagnosed on the basis of the presence of hepatic steatosis on ultrasound examination, fibrosis score determined by transient elastography, and with a persistently elevated ALT concentration (60 U/L) during 6 months before the study. The patients were randomized and the follow-up was conducted at 7, 14, 21 and 28 weeks. Data showed that synbiotic supplementation, in addition to lifestyle modification, has a better outcome compared to lifestyle modification alone for the treatment of NAFLD, at least partially through attenuation of serum inflammatory markers. These effects were seen at the beginning of week 14, and this trend was sustained until the end of the study [152]. The main downsides of this study are that liver histology was not performed, making it difficult to make a conclusion about the improvement of NASH, and uncertainty as to whether these effects will be sustained with longer treatment durations.

Taken together these results underscore the fact that more randomized studies with larger cohorts have to be conducted. Also, more combinations of bacteria need to be tested on a larger number of patients during a longer time period, which will allow to completely changing their gut microbiota. This treatment also has to be associated/ combined with other approaches (e.g. control of diet, lifestyle changes) and drug treatments.

Other drugs under evaluation

The elafibranor, a potent agonist of PPARα/δ, recently completed a phase IIb in 276 patients with NASH in Europe and USA [153]. This drug has shown significant activity on the regression of NASH and on markers of liver fibrosis, as well as significant improvement in cardiovascular risk. Elafibranor should start phase III clinical trials in 2016.

C-C chemokine receptors type 2 and 5 (CCR2 and CCR5, respectively) were shown to be implicated in inflammation and fibrosis. Cenicriviroc, an antagonist of CCR2 and CCR5, is in phase IIb development in a multicenter study. This study included patients with NASH and fibrosis. The goal of this study is to treat patients with cenicriviroc to improve NASH activity score without worsening fibrosis [154].

Finally, simtuzumab and apoptosis signal-regulating kinase 1 (ASK1) inhibitor (GS 4997), are also being evaluated alone or in combination (NCT02466516). ASK1 is implicated in apoptosis process and inflammation. Using ASK1 inhibitor might decrease and limit inflammation during NASH progression [155].

Next step approaches

Adiponectin receptor agonists: Adiponectin is a hormone secreted from adipose tissue and binds to adiponectin receptors AdipoR1 and AdipoR2 to activate LKB1-AMPK and PPARα, respectively. Adiponectin binding to AdipoR1 decreases neoglucogenesis, fasting glucose and SREBPc activation whereas binding to AdipoR2, it increases fatty acid oxidation and energy expenditure. Thus, adiponectin has antidiabetic effects and limits steatosis development [156].

Recently, a synthetic small molecule named AdipoRon has been tested on obese rodent model db/db mice. Data showed that db/db mice treated orally by AdipoRon have an insulin resistance index and plasma glucose level decreased by acting on white adipose tissue, muscle and liver. In addition, mice fed high fat and treated daily at 30 mg per kg body weight with AdipoRon showed an increase in longevity. Therefore, AdipoRon could achieve the same outcome much like caloric restriction and exercise. Moreover, AdipoRon showed an anti-inflammatory effect [157].

Taken together, small-molecule AdipoR agonists are very promising candidates for NASH treatment. Further experiments have to be done in rodent models that developed NASH to assess their efficacy.

Honokiol therapy: Honokiol, a major phenolic constituent isolated from *Magnolia officinalis* extracts, has been reported to have several pharmacological effects, including anti-inflammatory, anti-thrombosis, antioxidant effects and anti-cancerous effects. For few years now honokiol has been shown to have liver protective effects, especially by decreasing fatty liver through the decrease in SREB1c activity and AMPK-LKB1 pathway [158,159]. Therefore, honokiol significantly inhibited SREBP1c maturation and the transcription of lipogenic genes such as Stearoyl-Coa Desaturase-1 (SCD-1) and Fatty Acid Synthases (FAS) in fatty liver [159]. Also, in high-fat diet mouse model of NAFLD, honokiol and magnolol (also a phenolic constituent isolated from *Magnolia officinalis*) showed a significant decrease in fatty acid accumulation in the liver through the activation of AMPK leading to the inhibition of LXRα-SREBP-1 c pathway [160]. These drugs might be interesting to control fatty liver accumulation but further investigations need to be done, especially regarding efficacy and safety (i.e., assessing side effects) in patients with NASH.

Nicotinamide riboside drug approach: One of fatty acid overload mechanisms in patients with hepatic steatosis and NASH is mitochondrial dysfunction leading to a decrease in fatty acid β-oxidation and oxidative phosphorylation, decrease in $NADH/H^+$ oxidation into NAD^+ (i.e., NAD^+ repletion) by the mitochondrial respiratory chain complex leading to the leak of electrons and overproduction of Reactive Oxygen Species (ROS) and lipid peroxidation damaging the mitochondria and leading to a vicious circle [14,19-22,24,25,161]. Indeed, NAD^+ is implicated in numerous physiological processes such as caloric restriction, muscle contraction/heart beating, exercise, circadian rhythms, senescence, kidney, and liver functions.

Recently, Gariani, et al. [162] by using existing liver tissue datasets and high-fat/high-sucrose diet in animal models demonstrated that reduction in hepatic mitochondrial content, function and ATP levels associated with NAD^+ depletion leads to an increase in liver weight, lipid content and lipid peroxidation. Therefore, using a precursor of NAD^+ biosynthesis (Nicotinamide Riboside) as preventive or therapeutic strategy, the authors demonstrated the prevention or the reversion of the NAFLD toward activating sirtuin-1 and -3 pathway leading to an increase in β-oxidation, mitochondrial content and activity These data led to an increased use of nicotinamide riboside to boost NAD^+ biosynthesis to manage the development or progression of NAFLD in clinical trials [163]. In addition, nicotinamide riboside will target and stimulate the mitochondria in the liver to catalyze the excess of free fatty acids, ergo nicotinamide riboside will also increase ROS production, a second hit during the progression of NASH [14,19,21]. These might be a limitation for long-term treatment even if the drug is associated with ROS scavenger such as α-tocopherol (i.e., tocopheryl acetate or vitamin E). Because nicotinamide riboside also is not tissue-specific, long-term treatment might have systemic consequences and then side effects. Therefore, further investigations have to be done before using nicotinamide riboside in double-blind, randomized clinical trial to assess efficacy, side effects and safety (i.e., toxicity on other organs).

Conclusion

NASH associated with metabolic syndrome can progress to advanced fibrosis and cirrhosis. Weight loss and lifestyle modification have been shown to improve NASH. Other medications used for weight loss and metabolic syndrome have been evaluated such as metformin and thiazolidinediones. Alternative regimens using ursodeoxycholic acid, statins and probiotics as well as bariatric surgery have been evaluated but have not been recommended as first-line treatment for NASH. Vitamin E for NASH patients without diabetes seems to be promising. Many molecules are in the pipeline of the development for the treatment

of NASH, the most advanced being the FXR agonists. The lack of effective treatment for NASH suggests the heterogeneity of patients presenting with the NASH phenotype. Indeed, NASH has many underlying causes of genetic (i.e., differences between individuals) to environment (i.e., geography, diets, socio-cultural differences) with similar histological features. Therefore, no single treatment exists. The best treatment strategy for these patients may be to identify their pathogenic target and develop personalized treatment protocols.

But most importantly, the difficulty in diagnosing NASH without biopsy and therefore, the absence of noninvasive biomarkers makes it difficult to enroll patients in large clinical trials limiting the development of the new molecules.

References

1. Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, et al. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology. 2004;40(6):1387-95.doi: 10.1002/hep.20466.
2. Loomba R, Sanyal AJ. The global NAFLD epidemic. Nat Rev Gastroenterol Hepatol. 2013;10(11):686-90. doi: 10.1038/nrgastro.2013.171.
3. Ong JP, Younossi ZM. Epidemiology and natural history of NAFLD and NASH. Clin Liver Dis. 2007;11(1):1-16, vii.
4. Ramachandran A, Snehalatha C. Rising burden of obesity in Asia. J Obes. 2010;2010. pii: 868573. doi: 10.1155/2010/868573.
5. Arora A, Sharma P. Non-invasive Diagnosis of Fibrosis in Non-alcoholic Fatty Liver Disease. J Clin Exp Hepatol. 2012;2(2):145-55. doi: 10.1016/S0973-6883(12)60103-0.
6. Baffy G, Brunt EM, Caldwell SH. Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace. J Hepatol. 2012;56(6):1384-91. doi: 10.1016/j.jhep.2011.10.027.
7. Bugianesi E, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, et al. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology. 2002;123(1):134-40.
8. Lam DW, LeRoith D. The worldwide diabetes epidemic. Curr Opin Endocrinol Diabetes Obes. 2012;19(2):93-6. doi: 10.1097/MED.0b013e328350583a.
9. Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. Hepatology. 2010;51(5):1820-32. doi: 10.1002/hep.23594.
10. Wanless IR, Lentz JS. Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors. Hepatology. 1990;12(5):1106-10.
11. Zámbó V, Simon-Szabó L, Szelényi P, Kereszturi E, Bánhegyi G, Csala M. Lipotoxicity in the liver. World J Hepatol. 2013;5(10):550-7. doi: 10.4254/wjh.v5.i10.550.
12. Pascale A, Pais R, Ratziu V. An overview of nonalcoholic steatohepatitis: past, present and future directions. J Gastrointestin Liver Dis. 2010;19(4):415-23.
13. Fracanzani AL, Tiraboschi S, Pisano G, Consonni D, Baragetti A, Bertelli C, et al. Progression of carotid vascular damage and cardiovascular events in non-alcoholic fatty liver disease patients compared to the general population during 10 years of follow-up. Atherosclerosis. 2016;246:208-13. doi: 10.1016/j.atherosclerosis.2016.01.016.
14. Basaranoglu M, Basaranoglu G, Sentürk H. From fatty liver to fibrosis: a tale of "second hit". World J Gastroenterol. 2013;19(8):1158-65. doi: 10.3748/wjg.v19.i8.1158.
15. Bedossa P, FLIP Pathology Consortium. Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity, and fibrosis (SAF) score in the evaluation of biopsies of nonalcoholic fatty liver disease. Hepatology. 2014;60(2):565-75. doi: 10.1002/hep.27173.
16. Bedossa P, Poitou C, Veyrie N, Bouillot JL, Basdevant A, Paradis V, et al. Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. Hepatology. 2012;56(5):1751-9. doi: 10.1002/hep.25889.
17. Brunt EM, Kleiner DE, Wilson LA, Belt P, Neuschwander-Tetri BA, NASH Clinical Research Network (CRN). Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. Hepatology. 2011;53(3):810-20. doi: 10.1002/hep.24127.
18. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41(6):1313-21.
19. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology. 1998;114(4):842-5.
20. Pessayre D, Berson A, Fromenty B, Mansouri A. Mitochondria in steatohepatitis. Semin Liver Dis. 2001;21(1):57-69.
21. Takaki A, Kawai D, Yamamoto K. Multiple hits, including oxidative stress, as pathogenesis and treatment target in non-alcoholic steatohepatitis (NASH). Int J Mol Sci. 2013;14(10):20704-28. doi: 10.3390/ijms141020704.
22. Araseki M, Kobayashi H, Hosokawa M, Miyashita K. Lipid peroxidation of a human hepatoma cell line (HepG2) after incorporation of linoleic acid, arachidonic acid, and docosahexaenoic acid. Biosci Biotechnol Biochem. 2005;69(3):483-90.
23. Caron S, Huaman Samanez C, Dehondt H, Ploton M, Briand O, Lien F, et al. Farnesoid X receptor inhibits the transcriptional activity of carbohydrate response element binding protein in human hepatocytes. Mol Cell Biol. 2013;33(11):2202-11. doi: 10.1128/MCB.01004-12.
24. Chiappini F, Barrier A, Saffroy R, Domart MC, Dagues N, Azoulay D, et al. Exploration of global gene expression in human liver steatosis by high-density oligonucleotide microarray. Lab Invest. 2006;86(2):154-65.
25. Powell EE, Cooksley WG, Hanson R, Searle J, Halliday JW, Powell LW. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. Hepatology. 1990;11(1):74-80.
26. Marchesini G, Petta S, Dale Grave R. Diet, weight loss, and liver health in nonalcoholic fatty liver disease: Pathophysiology, evidence, and practice. Hepatology. 2015. doi: 10.1002/hep.28392.
27. Utzschneider KM, Bayer-Carter JL, Arbuckle MD, Tidwell JM, Richards TL, Craft S. Beneficial effect of a weight-stable, low-fat/low-saturated fat/low-glycaemic index diet to reduce liver fat in older subjects. Br J Nutr. 2013;109(6):1096-104. doi: 10.1017/S0007114512002966.
28. Rosqvist F, Iggman D, Kullberg J, Cedernaes J, Johansson HE, Larsson A, et al. Overfeeding polyunsaturated and saturated fat causes distinct effects on liver and visceral fat accumulation in humans. Diabetes. 2014;63(7):2356-68. doi: 10.2337/db13-1622.
29. Wouters K, van Gorp PJ, Bieghs V, Gijbels MJ, Duimel H, Lütjohann D, et al. Dietary cholesterol, rather than liver steatosis, leads to hepatic

inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. Hepatology. 2008;48(2):474-86. doi: 10.1002/hep.22363.

30. Review Team, LaBrecque DR, Abbas Z, Anania F, Ferenci P, Khan AG, et al. World Gastroenterology Organisation global guidelines: Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. J Clin Gastroenterol. 2014;48(6):467-73. doi: 10.1097/MCG.0000000000000116.

31. Huang MA, Greenson JK, Chao C, Anderson L, Peterman D, Jacobson J, et al. One-year intense nutritional counseling results in histological improvement in patients with non-alcoholic steatohepatitis: a pilot study. Am J Gastroenterol. 2005;100(5):1072-1081. doi: 10.1111/j.1572-0241.2005.41334.x.

32. Ueno T, Sugawara H, Sujaku K, Hashimoto O, Tsuji R, Tamaki S, et al. Therapeutic effects of restricted diet and exercise in obese patients with fatty liver. J Hepatol. 1997;27(1):103-107. doi: 9252081.

33. Wu T, Gao X, Chen M, van Dam RM. Long-term effectiveness of diet-plus-exercise interventions vs. diet-only interventions for weight loss: a meta-analysis. Obes Rev. 2009;10(3):313-323. doi: 10.1111/j.1467-789X.2008.00547.x.

34. Nascimbeni F, Pais R, Bellentani S, Day CP, Ratziu V, Loria P, et al. From NAFLD in clinical practice to answers from guidelines. J Hepatol. 2013;59(4):859-871. doi: 10.1016/j.jhep.2013.05.044.

35. Madero M, Arriaga JC, Jalal D, Rivard C, McFann K, Pérez-Méndez O, et al. The effect of two energy-restricted diets, a low-fructose diet versus a moderate natural fructose diet, on weight loss and metabolic syndrome parameters: a randomized controlled trial. 2011;60(11):1551-1559. doi: 10.1016/j.metabol.2011.04.001.

36. Chan DC, Watts GF, Gan S, Wong AT, Ooi EM, Barrett PH. Nonalcoholic fatty liver disease as the transducer of hepatic oversecretion of very-low-density lipoprotein-apolipoprotein B-100 in obesity. Arterioscler Thromb Vasc Biol. 2010;30(5):1043-1050. doi: 10.1161/ATVBAHA.109.202275.

37. Chalasani N, Younossi Z, Lavine J E, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. Gastroenterology. 2012;142(7):1592-1609. doi: 10.1053/j.gastro.2012.04.001.

38. Estruch R, Ros E, Salas-Salvado J, Maria-Isabel Covas, Dolores Corella, Fernando Arós, et al. Primary prevention of cardiovascular disease with a Mediterranean diet. N Engl J Med. 2013;368(14):1279-1290. doi: 10.1056/NEJMoa1200303.

39. Papamiltiadous ES, Roberts SK, Nicoll AJ, Marno C. Ryan, Catherine Itsiopoulos, Agus Salim, et al. A randomised controlled trial of a Mediterranean Dietary Intervention for Adults with Non Alcoholic Fatty Liver Disease (MEDINA): study protocol. BMC Gastroenterol. 2016;16(1):14. doi: 10.1186/s12876-016-0426-3.

40. Aller R, Izaola O, De la Fuente B, De Luis Roman DA. Mediterranean Diet Is Associated with Liver Histology in Patients with Non Alcoholic Fatty Liver Disease. Nutr Hosp. 2015;32(n06):2518-2524.

41. Abenavoli L, Milic N, Peta V, Alfieri F, De Lorenzo A, Bellentani S. Alimentary regimen in non-alcoholic fatty liver disease: Mediterranean diet. World J Gastroenterol. 2014;20(45):16831-16840. doi: 10.3748/wjg.v20.i45.16831.

42. Nigam P, Bhatt S, Misra A, Chadha Davinder S, Vaidya Meera, Dasgupta Jharna, et al. Effect of a 6-month intervention with cooking oils containing a high concentration of monounsaturated fatty acids (olive and canola oils) compared with control oil in male Asian Indians with nonalcoholic fatty liver disease. Diabetes Technol Ther. 2014;16(4):255-261. doi: 10.1089/dia.2013.0178.

43. Cormick KG, Scorletti E, Bhatia L, Philip C. Calder, Michael J. Griffin, Geraldine F. Clough, et al. Impact of high dose n-3 polyunsaturated fatty acid treatment on measures of microvascular function and vibration perception in non-alcoholic fatty liver disease: results from the randomised WELCOME trial. Diabetologia. 2015;58(8):1916-1925. doi: 10.1007/s00125-015-3628-2.

44. Scorletti E, Bhatia L, McCormick KG, Geraldine F. Clough, Kathryn Nash, Leanne Hodson, et al. Effects of purified eicosapentaenoic and docosahexaenoic acids in nonalcoholic fatty liver disease: results from the Welcome* study. Hepatology. 2014;60(4):1211-1221. doi: 10.1002/hep.27289.

45. Bhatia L, Scorletti E, Curzen N, Clough GF, Calder PC, Byrne CD. Improvement in non-alcoholic fatty liver disease severity is associated with a reduction in carotid intima-media thickness progression. Atherosclerosis. 2016;246(13-20). doi: 10.1016/j.atherosclerosis.2015.12.028.

46. Barrera F, George J. The role of diet and nutritional intervention for the management of patients with NAFLD. Clin Liver Dis. 2014;18(1):91-112. doi: 10.1016/j.cld.2013.09.009.

47. Parker HM, Johnson NA, Burdon CA, Cohn JS, O'Connor HT, George J. Omega-3 supplementation and non-alcoholic fatty liver disease: a systematic review and meta-analysis. J Hepatol. 2012;56(4):944-951. doi: 10.1016/j.jhep.2011.08.018.

48. Pahua-Ramos ME, Garduno-Siciliano L, Dorantes-Alvarez L, German Chamorro-Cevallos, Julieta Herrera-Martínez, Obed Osorio-Esquivel, et al. Reduced-calorie avocado paste attenuates metabolic factors associated with a hypercholesterolemic-high fructose diet in rats. Plant Foods Hum Nutr. 2014;69(1):18-24. doi: 10.1007/s11130-013-0395-4.

49. Gupta V, Mah XJ, Garcia MC, Antonypillai C, van der Poorten D. Oily fish, coffee and walnuts: Dietary treatment for nonalcoholic fatty liver disease. World J Gastroenterol. 2015;21(37):10621-10635. doi: 10.3748/wjg.v21.i37.10621.

50. Molloy JW, Calcagno CJ, Williams CD, Jones FJ, Torres DM, Harrison SA. Association of coffee and caffeine consumption with fatty liver disease, nonalcoholic steatohepatitis, and degree of hepatic fibrosis. Hepatology. 2012;55(2):429-436. doi: 10.1002/hep.24731.

51. Sakata R, Nakamura T, Torimura T, Ueno T, Sata M. Green tea with high-density catechins improves liver function and fat infiltration in non-alcoholic fatty liver disease (NAFLD) patients: a double-blind placebo-controlled study. Int J Mol Med. 2013;32(5):989-994. doi: 10.3892/ijmm.2013.1503.

52. Anty R, Marjoux S, Iannelli A, Stéphanie Patouraux, Anne-Sophie Schneck, Stéphanie Bonnafous, et al. Regular coffee but not espresso drinking is protective against fibrosis in a cohort mainly composed of morbidly obese European women with NAFLD undergoing bariatric surgery. J Hepatol. 2012;57(5):1090-1096. doi: 10.1016/j.jhep.2012.07.014.

53. Loftfield E, Freedman ND, Graubard BI, Kristin A. Guertin, Amanda Black, Wen-Yi Huang, et al. Association of Coffee Consumption With Overall and Cause-Specific Mortality in a Large US Prospective Cohort Study. Am J Epidemiol. 2015;182(12):1010-1022. doi: 10.1093/aje/kwv146.

54. O'Keefe JH, Bhatti SK, Patil HR, DiNicolantonio JJ, Lucan SC, Lavie CJ.

Effects of habitual coffee consumption on cardiometabolic disease, cardiovascular health, and all-cause mortality. J Am Coll Cardiol. 2013;62(12):1043-1051. doi: 10.1016/j.jacc.2013.06.035.

55. Fabricatore AN. Behavior therapy and cognitive-behavioral therapy of obesity: is there a difference? J Am Diet Assoc. 2007;107(1):92-99. doi: 10.1016/j.jada.2006.10.005.

56. Jensen MD, Ryan DH, Apovian CM, Jamy D, Anthony G. Comuzzie, Frank B. Hu,et al. 2013 AHA/ACC/TOS guideline for the management of overweight and obesity in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and The Obesity Society. Circulation. 2014;129(25 Suppl 2):102-138. doi: 10.1161/01.cir.0000437739.71477.ee.

57. Keating SE, Hackett DA, George J, Johnson NA. Exercise and non-alcoholic fatty liver disease: a systematic review and meta-analysis. J Hepatol. 2012;57(1):157-166. doi: 10.1016/j.jhep.2012.02.023.

58. Ratziu V, Bellentani S, Cortez-Pinto H, Day C, Marchesini G. A position statement on NAFLD/NASH based on the EASL 2009 special conference. J Hepatol. 2010;53(2):372-384. doi: 10.1016/j.jhep.2010.04.008.

59. Terra X, Auguet T, Guiu-Jurado E, Alba Berlanga, Josep Maria Orellana-Gavaldà, Mercè Hernández, et al. Long-term changes in leptin, chemerin and ghrelin levels following different bariatric surgery procedures: Roux-en-Y gastric bypass and sleeve gastrectomy. Obes Surg. 2013;23(11):1790-1798. doi: 10.1007/s11695-013-1033-9.

60. American Diabetes A. Standards of medical care in diabetes--2014. Diabetes Care. 2014;37(1):14-80. doi: 10.2337/dc14-S014.

61. Poirier P, Cornier MA, Mazzone T, Stiles S, Cummings S, Klein S, et al. Bariatric surgery and cardiovascular risk factors: a scientific statement from the American Heart Association. Circulation. 2011;123(15):1683-701. doi: 10.1161/CIR.0b013e3182149099.

62. Jerome Dargent. Novel Endoscopic Management of Obesity. Clin Endosc. 2016;49(1):30–36. Doi: 10.5946/ce.2016.49.1.30.

63. Aguilar-Olivos NE, Almeda-Valdes P, Aguilar-Salinas CA, Uribe M, Méndez-Sánchez N. The role of bariatric surgery in the management of nonalcoholic fatty liver disease and metabolic syndrome. Metabolism. 2015. pii: S0026-0495(15)00258-9. Doi: 10.1016/j.metabol.2015.09.004.

64. Sjöström L, Peltonen M, Jacobson P, Sjöström CD, Karason K, Wedel H, et al. Bariatric surgery and long-term cardiovascular events. JAMA. 2012;307(1):56-65. Doi: 10.1001/jama.2011.1914.

65. Hutter MM, Schirmer BD, Jones DB, Ko CY, Cohen ME, Merkow RP, et al. First report from the American College of Surgeons Bariatric Surgery Center Network: laparoscopic sleeve gastrectomy has morbidity and effectiveness positioned between the band and the bypass. Ann Surg. 2011;254(3):410-20; discussion 420-2. Doi: 10.1097/SLA.0b013e31822c9dac.

66. Lassailly G, Caiazzo R, Buob D, Pigeyre M, Verkindt H, Labreuche J et al. Bariatric Surgery Reduces Features of Nonalcoholic Steatohepatitis in Morbidly Obese Patients. Gastroenterology. 2015;149(2):379-88; quiz e15-6. Doi: 10.1053/j.gastro.2015.04.014.

67. Goossens N, Hoshida Y, Song WM, Jung M, Morel P, Nakagawa S, et al. Nonalcoholic Steatohepatitis Is Associated With Increased Mortality in Obese Patients Undergoing Bariatric Surgery. Clin Gastroenterol Hepatol. 2015. pii: S1542-3565(15)01406-8. Doi: 10.1016/j.cgh.2015.10.010.

68. Weingarten TN, Mantilla CB, Swain JM, Kendrick ML, Oberhansley JM, Burcham RJ, et al. Nonalcoholic steatohepatitis in bariatric patients with a diagnosis of obstructive sleep apnea. Obes Facts. 2012;5(4):587-96. Doi: 10.1159/000342677.

69. Rakoski MO1, Singal AG, Rogers MA, Conjeevaram H. Meta-analysis: insulin sensitizers for the treatment of non-alcoholic steatohepatitis. Aliment Pharmacol Ther. 2010;32(10):1211-21. doi: 10.1111/j.1365-2036.2010.04467.x.

70. Aithal GP, Thomas JA, Kaye PV, Lawson A, Ryder SD, Spendlove I, et al. Randomized, placebo-controlled trial of pioglitazone in nondiabetic subjects with nonalcoholic steatohepatitis. Gastroenterology. 2008;135(4):1176-84. doi: 10.1053/j.gastro.2008.06.047.

71. Belfort R, Harrison SA, Brown K, Darland C, Finch J, Hardies J, et al. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. N Engl J Med. 2006;355(22):2297-307. doi: 10.1056/NEJMoa060326

72. Musso G, Cassader M, Rosina F, Gambino R. Impact of current treatments on liver disease, glucose metabolism and cardiovascular risk in non-alcoholic fatty liver disease (NAFLD): a systematic review and meta-analysis of randomised trials. Diabetologia. 2012;55(4):885-904. Doi: 10.1007/s00125-011-2446-4.

73. Boettcher E, Csako G, Pucino F, Wesley R, Loomba R.Meta-analysis: pioglitazone improves liver histology and fibrosis in patients with non-alcoholic steatohepatitis. Aliment Pharmacol Ther. 2012;35(1):66-75. doi: 10.1111/j.1365-2036.2011.04912.x.

74. Ratziu V, Charlotte F, Bernhardt C, Giral P, Halbron M, Lenaour G, et al. Long-term efficacy of rosiglitazone in nonalcoholic steatohepatitis: results of the fatty liver improvement by rosiglitazone therapy (FLIRT 2) extension trial. Hepatology. 2010;51(2):445-53. Doi: 10.1002/hep.23270.

75. Milic S, Mikolasevic I, Krznaric-Zrnic I, Stanic M, Poropat G, Stimac D,et al. Nonalcoholic steatohepatitis: emerging targeted therapies to optimize treatment options. Drug Des Devel Ther. 2015;9:4835-45. Doi: 10.2147/DDDT.S64877.

76. Takahashi Y, Sugimoto K, Inui H, Fukusato T. Current pharmacological therapies for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World J Gastroenterol. 2015 ;21(13):3777-85. doi: 10.3748/wjg.v21.i13.3777.

77. Lavine JE. Vitamin E treatment of nonalcoholic steatohepatitis in children: a pilot study. J Pediatr. 2000;136(6):734-8. doi: 10839868.

78. Pietu F, Guillaud O, Walter T, Vallin M, Hervieu V, Scoazec JY, et al. Ursodeoxycholic acid with vitamin E in patients with nonalcoholic steatohepatitis: long-term results. Clin Res Hepatol Gastroenterol. 2012;36(2):146-55. doi: 10.1016/j.clinre.2011.10.011.

79. Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. N Engl J Med. 2010;362(18):1675-1685. doi: 10.1056/NEJMoa090792.

80. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. JAMA. 2007;297(8):842-57. doi: 10.1001/jama.297.8.842.

81. Zein CO, Yerian LM, Gogate P, Lopez R, Kirwan JP, Feldstein AE, et al. Pentoxifylline improves nonalcoholic steatohepatitis: a randomized placebo-controlled trial. Hepatology. 2011;54(5):1610-1619. doi: 10.1002/hep.24544.

82. Du J, Ma YY, Yu CH, Li YM. Effects of pentoxifylline on nonalcoholic fatty liver disease: a meta-analysis. World J Gastroenterol. 2014;20(2):569-577. doi: 10.3748/wjg.v20.i2.569.

83. Nelson A, Torres DM, Morgan AE, Fincke C, Harrison SA. A pilot study using simvastatin in the treatment of nonalcoholic steatohepatitis: A randomized placebo-controlled trial. J Clin Gastroenterol. 2009;43(10):990-994. doi: 10.1097/MCG.0b013e31819c392e.

84. Kargiotis K, Katsiki N, Athyros VG, Giouleme O, Patsiaoura K, Katsiki E, et al. Effect of rosuvastatin on non-alcoholic steatohepatitis in patients with metabolic syndrome and hypercholesterolaemia: a preliminary report. Curr Vasc Pharmacol. 2014;12(3):505-511. doi: 24805248.

85. Laufs U, Scharnagl H, Marz W. Statin intolerance. Curr Opin Lipidol. 2015;26(6):492-501. doi: 10.1097/MOL.0000000000000236.

86. Loomba R, Sirlin CB, Ang B, Bettencourt R, Jain R, Salotti J, et al. Ezetimibe for the treatment of nonalcoholic steatohepatitis: assessment by novel magnetic resonance imaging and magnetic resonance elastography in a randomized trial (MOZART trial). Hepatology. 2015;61(4):1239-1250. doi: 10.1002/hep.27647.

87. Takeshita Y, Takamura T, Honda M, Kita Y, Zen Y, Kato K, et al. The effects of ezetimibe on non-alcoholic fatty liver disease and glucose metabolism: a randomised controlled trial. Diabetologia. 2014;57(5):878-890. doi: 10.1007/s00125-013-3149-9.

88. Park H, Shima T, Yamaguchi K, et al. Efficacy of long-term ezetimibe therapy in patients with nonalcoholic fatty liver disease. J Gastroenterol. 2011;46(1):101-107. doi: 10.1007/s00535-010-0291-8.

89. Enjoji M, Machida K, Kohjima M, Kato M, Kotoh K, Matsunaga K, et al. NPC1L1 inhibitor ezetimibe is a reliable therapeutic agent for non-obese patients with nonalcoholic fatty liver disease. Lipids Health Dis. 2010;9(29. doi: 10.1186/1476-511X-9-29.

90. Abrahamsson A, Gustafsson U, Ellis E, Nilsson LM, Sahlin S, Björkhem I, et al. Feedback regulation of bile acid synthesis in human liver: importance of HNF-4alpha for regulation of CYP7A1. Biochem Biophys Res Commun. 2005;330(2):395-399. doi: 10.1016/j.bbrc.2005.02.170.

91. Nakamura MT, Yudell BE, Loor JJ. Regulation of energy metabolism by long-chain fatty acids. Prog Lipid Res. 2014;53:124-144. doi: 10.1016/j.plipres.2013.12.001.

92. Markan KR, Potthoff MJ. Metabolic fibroblast growth factors (FGFs): Mediators of energy homeostasis. Semin Cell Dev Biol. 2015. doi: 10.1016/j.semcdb.2015.09.021.

93. Kim KH, Lee MS. FGF21 as a mediator of adaptive responses to stress and metabolic benefits of anti-diabetic drugs. J Endocrinol. 2015;226(1):R1-16. doi: 10.1530/JOE-15-0160.

94. Aguilar-Olivos NE, Carrillo-Córdova D, Oria-Hernández J, Sánchez-Valle V, Ponciano-Rodríguez G, et al. The nuclear receptor FXR, but not LXR, up-regulates bile acid transporter expression in non-alcoholic fatty liver disease. Ann Hepatol. 2015;14(4):487-493. doi: 26019035.

95. Sanyal AJ. Use of farnesoid X receptor agonists to treat nonalcoholic fatty liver disease. Dig Dis. 2015;33(3):426-432. doi: 10.1159/000371698.

96. Zhu GQ, Shi KQ, Huang S, Huang GQ, Lin YQ, Zhou ZR, et al. Network meta-analysis of randomized controlled trials: efficacy and safety of UDCA-based therapies in primary biliary cirrhosis. Medicine (Baltimore). 2015;94(11):e609. doi: 10.1097/MD.0000000000000609.

97. Laurin J, Lindor KD, Crippin JS, Gossard A, Gores GJ, Ludwig J, et al. Ursodeoxycholic acid or clofibrate in the treatment of non-alcohol-induced steatohepatitis: a pilot study. Hepatology. 1996;23(6):1464-1467. doi: 10.1002/hep.510230624.

98. Ratziu V, de Ledinghen V, Oberti F, Mathurin P, Wartelle-Bladou C, Renou C, et al. A randomized controlled trial of high-dose ursodesoxycholic acid for nonalcoholic steatohepatitis. J Hepatol. 2011;54(5):1011-1019. doi: 10.1016/j.jhep.2010.08.030.

99. Leuschner UF, Lindenthal B, Herrmann G, Arnold JC, Rössle M, Cordes HJ, et al. High-dose ursodeoxycholic acid therapy for nonalcoholic steatohepatitis: a double-blind, randomized, placebo-controlled trial. Hepatology. 2010;52(2):472-479. doi: 10.1002/hep.23727.

100. Liechti F, Dufour JF. Treatment of NASH with ursodeoxycholic acid: cons. Clin Res Hepatol Gastroenterol. 2012;36 Suppl 1: S46-52. doi: 10.1016/S2210-7401(12)70021-9.

101. Ratziu V. Treatment of NASH with ursodeoxycholic acid: pro. Clin Res Hepatol Gastroenterol. 2012;36 Suppl 1: S41-45. doi: 10.1016/S2210-7401(12)70020-7.

102. Vajro P, Paolella G, Pierri L, D'Aniello R. Treatment of NASH with ursodeoxycholic acid: pros and cons. More information in children. Clin Res Hepatol Gastroenterol. 2013;37(3):e93-94. doi: 10.1016/j.clinre.2013.02.012.

103. Chamulitrat W, Liebisch G, Xu W, et al. Ursodeoxycholyl lysophosphatidylethanolamide inhibits lipoapoptosis by shifting fatty acid pools toward monosaturated and polyunsaturated fatty acids in mouse hepatocytes. Mol Pharmacol. 2013;84(5):696-709. doi: 10.1124/mol.113.088039.

104. Chamulitrat W, Zhang W, Xu W, Pathil A, Setchell K, Stremmel W. Hepatoprotectant ursodeoxycholyl lysophosphatidylethanolamide increasing phosphatidylcholine levels as a potential therapy of acute liver injury. Front Physiol. 2012;3:24. doi: 10.3389/fphys.2012.00024.

105. Pathil A, Mueller J, Warth A, Chamulitrat W, Stremmel W. Ursodeoxycholyl lysophosphatidylethanolamide improves steatosis and inflammation in murine models of nonalcoholic fatty liver disease. Hepatology. 2012;55(5):1369-1378. doi: 10.1002/hep.25531.

106. Chamulitrat W, Burhenne J, Rehlen T, Pathil A, Stremmel W. Bile salt-phospholipid conjugate ursodeoxycholyl lysophosphatidylethanolamide as a hepatoprotective agent. Hepatology. 2009;50(1):143-154. doi: 10.1002/hep.22955.

107. Pathil A, Liebisch G, Okun JG, Chamulitrat W, Schmitz G, Stremmel W. Ursodeoxycholyl Lysophosphatidylethanolamide modifies aberrant lipid profiles in NAFLD. Eur J Clin Invest. 2015;45(9):925-931. doi: 10.1111/eci.12486.

108. Kunne C, Acco A, Duijst S, de Waart DR, Paulusma CC, Gaemers I, et al. FXR-dependent reduction of hepatic steatosis in a bile salt deficient mouse model. Biochim Biophys Acta. 2014;1842(5):739-746. doi: 10.1016/j.bbadis.2014.02.004.

109. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, et al. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. Lancet. 2015;385(9972):956-965. doi: 10.1016/S0140-6736(14)61933-4.

110. Musso G, Cassader M, Gambino R. Trials of obeticholic acid for non-alcoholic steatohepatitis. Lancet. 2015;386(9988):27. doi: 10.1016/S0140-6736(15)61198-9.

111. Singh S, Khera R, Allen AM, Murad MH, Loomba R. Comparative effectiveness of pharmacological interventions for nonalcoholic

steatohepatitis: A systematic review and network meta-analysis. Hepatology. 2015;62(5):1417-1432. doi: 10.1002/hep.27999.

112. Son G, Kremer M, Hines IN. Contribution of gut bacteria to liver pathobiology. Gastroenterol Res Pract. 2010; 2010. doi: 10.1155/2010/453563.

113. Henao-Mejia J, Elinav E, Thaiss CA, Flavell RA. The intestinal microbiota in chronic liver disease. Adv Immunol. 2013;117:73-97. doi: 10.1016/B978-0-12-410524-9.00003-7.

114. Lade A, Noon LA, Friedman SL. Contributions of metabolic dysregulation and inflammation to nonalcoholic steatohepatitis, hepatic fibrosis, and cancer. Curr Opin Oncol. 2014;26(1):100-107. doi: 10.1097/CCO.0000000000000042.

115. De Minicis S, Rychlicki C, Agostinelli L, Saccomanno S, Candelaresi C, Trozzi L, et al. Dysbiosis contributes to fibrogenesis in the course of chronic liver injury in mice. Hepatology. 2014;59(5):1738-1749. doi: 10.1002/hep.26695.

116. Boursier J, Mueller O, Barret M, Machado M, Fizanne L, Araujo-Perez F, et al. The severity of nonalcoholic fatty liver disease is associated with gut dysbiosis and shift in the metabolic function of the gut microbiota. Hepatology. 2016;63(3):764-775. doi: 10.1002/hep.28356.

117. Dumas ME, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, et al. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. Proc Natl Acad Sci USA. 2006;103(33):12511-12516. doi: 10.1073/pnas.0601056103.

118. Abu-Shanab A, Quigley EM. The role of the gut microbiota in nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol. 2010;7(12):691-701. doi: 10.1038/nrgastro.2010.172.

119. Spencer MD, Hamp TJ, Reid RW, Fischer LM, Zeisel SH, Fodor AA. Association between composition of the human gastrointestinal microbiome and development of fatty liver with choline deficiency. Gastroenterology. 2011;140(3): 976-986. doi: 10.1053/j.gastro.2010.11.049.

120. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature. 2012;482(7384):179-185. doi: 10.1038/nature10809.

121. Wood NJ. Microbiota: Dysbiosis driven by inflammasome deficiency exacerbates hepatic steatosis and governs rate of NAFLD progression. Nat Rev Gastroenterol Hepatol. 2012;9(3):123. doi: 10.1038/nrgastro.2012.21.

122. Compare D, Coccoli P, Rocco A, Nardone OM, De Maria S, Cartenì M, et al. Gut--liver axis: the impact of gut microbiota on non alcoholic fatty liver disease. Nutr Metab Cardiovasc Dis. 2012;22(6):471-476. doi: 10.1016/j.numecd.2012.02.007.

123. Machado MV, Cortez-Pinto H. Gut microbiota and nonalcoholic fatty liver disease. Ann Hepatol. 2012;11(4):440-449.

124. Zhu L, Baker SS, Gill C, Liu W, Alkhouri R, Baker RD,, et al. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. Hepatology. 2013;57(2):601-609. doi: 10.1002/hep.26093.

125. Alisi A, Ceccarelli S, Panera N, Nobili V. Causative role of gut microbiota in non-alcoholic fatty liver disease pathogenesis. Front Cell Infect Microbiol. 2012;2:132. doi: 10.3389/fcimb.2012.00132.

126. Le Roy T, Llopis M, Lepage P, Bruneau A, Rabot S, Bevilacqua C, Martin P, et al. Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice. Gut. 2013;62(12):1787-1794. doi: 10.1136/gutjnl-2012-303816.

127. Moschen AR, Kaser S, Tilg H. Non-alcoholic steatohepatitis: a microbiota-driven disease. Trends Endocrinol Metab. 2013;24(11):537-545. doi: 10.1016/j.tem.2013.05.009.

128. Aron-Wisnewsky J, Gaborit B, Dutour A, Clement K. Gut microbiota and non-alcoholic fatty liver disease: new insights. Clin Microbiol Infect. 2013;19(4):338-348. doi: 10.1111/1469-0691.12140.

129. Mouzaki M, Comelli EM, Arendt BM, Bonengel J, Fung SK, Fischer SE, et al. Intestinal microbiota in patients with nonalcoholic fatty liver disease. Hepatology. 2013;58(1):120-127. doi: 10.1002/hep.26319.

130. Sanyal AJ. An integrated view of liver injury and disease progression in nonalcoholic steatohepatitis. Hepatol Int. 2013;7 Suppl 2: 800-805. doi: 10.1007/s12072-013-9479-3.

131. Imajo K, Yoneda M, Ogawa Y, Wada K, Nakajima A. Microbiota and nonalcoholic steatohepatitis. Semin Immunopathol. 2014;36(1):115-132. doi: 10.1007/s00281-013-0404-6.

132. Parnell JA, Raman M, Rioux KP, Reimer RA. The potential role of prebiotic fibre for treatment and management of non-alcoholic fatty liver disease and associated obesity and insulin resistance. Liver Int. 2012;32(5) 701-711. doi: 10.1111/j.1478-3231.2011.02730.x.

133. Pachikian BD, Essaghir A, Demoulin JB, Catry E, Neyrinck AM, Dewulf EM, et al. Prebiotic approach alleviates hepatic steatosis: implication of fatty acid oxidative and cholesterol synthesis pathways. Mol Nutr Food Res. 2013;57(2):347-359. doi: 10.1002/mnfr.201200364.

134. Correia-Sa I, de-Sousa-Lopes H, Martins MJ, Azevedo I, Moura E, Vieira-Coelho MA. Effects of raftilose on serum biochemistry and liver morphology in rats fed with normal or high-fat diet. Mol Nutr Food Res. 2013;57(8):1468-1472. doi: 10.1002/mnfr.201200693.

135. Kim H, Bartley GE, Young SA, Seo KH, Yokoyama W. Altered hepatic gene expression profiles associated with improved fatty liver, insulin resistance, and intestinal permeability after hydroxypropyl methylcellulose (HPMC) supplementation in diet-induced obese mice. J Agric Food Chem. 2013;61(26):6404-6411. doi: 10.1021/jf400545w.

136. Alligier M, Dewulf EM, Salazar N, Mairal A, Neyrinck AM, Cani PD, et al. Positive interaction between prebiotics and thiazolidinedione treatment on adiposity in diet-induced obese mice. Obesity (Silver Spring). 2014;22(7):1653-1661. doi: 10.1002/oby.20733.

137. Tarantino G, Finelli C. Systematic review on intervention with prebiotics/probiotics in patients with obesity-related nonalcoholic fatty liver disease. Future Microbiol. 2015;10(5):889-902. doi: 10.2217/fmb.15.13.

138. Buss C, Valle-Tovo C, Miozzo S, Alves de Mattos A. Probiotics and synbiotics may improve liver aminotransferases levels in non-alcoholic fatty liver disease patients. Ann Hepatol. 2014;13(5):482-488. doi: 25152979.

139. Lambert JE, Parnell JA, Eksteen B Raman M, Bomhof MR, Rioux KP, et al. Gut microbiota manipulation with prebiotics in patients with non-alcoholic fatty liver disease: a randomized controlled trial protocol. BMC Gastroenterol. 2015;15:169. doi: 10.1186/s12876-015-0400-5.

140. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11(8):506-514. doi: 10.1038/nrgastro.2014.66.

141. Nardone G, Rocco A. Probiotics: a potential target for the prevention and treatment of steatohepatitis. J Clin Gastroenterol. 2004;38(6 Suppl): S121-122.

142. Medina J, Fernandez-Salazar LI, Garcia-Buey L, Moreno-Otero R. Approach to the pathogenesis and treatment of nonalcoholic steatohepatitis. Diabetes Care. 2004;27(8):2057-2066.

143. Velayudham A, Dolganiuc A, Ellis M, Petrasek J, Kodys K, Mandrekar P, et al. VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced nonalcoholic steatohepatitis model in mice. Hepatology. 2009;49(3):989-997. doi: 10.1002/hep.22711.

144. Mencarelli A, Cipriani S, Renga B, et al. VSL#3 resets insulin signaling and protects against NASH and atherosclerosis in a model of genetic dyslipidemia and intestinal inflammation. PLoS One. 2012;7(9):e45425. doi: 10.1371/journal.pone.0045425.

145. Sohn W, Jun DW, Lee KN, Lee HL, Lee OY, Choi HS, et al. *Lactobacillus paracasei* Induces M2-Dominant Kupffer Cell Polarization in a Mouse Model of Nonalcoholic Steatohepatitis. Dig Dis Sci. 2015;60(11):3340-3350. doi: 10.1007/s10620-015-3770-1.

146. Endo H, Niioka M, Kobayashi N, Tanaka M, Watanabe T. Butyrate-producing probiotics reduce nonalcoholic fatty liver disease progression in rats: new insight into the probiotics for the gut-liver axis. PLoS One. 2013;8(5):e63388. doi: 10.1371/journal.pone.0063388.

147. Jin CJ, Sellmann C, Engstler AJ, Ziegenhardt D, Bergheim I. Supplementation of sodium butyrate protects mice from the development of non-alcoholic steatohepatitis (NASH). Br J Nutr. 2015;114(11):1745-1755. doi: 10.1017/S0007114515003621.

148. Aller R, De Luis DA, Izaola O, Conde R, Gonzalez Sagrado M, Primo D, et al. Effect of a probiotic on liver aminotransferases in nonalcoholic fatty liver disease patients: a double blind randomized clinical trial. Eur Rev Med Pharmacol Sci. 2011;15(9):1090-1095.

149. Wong VW, Won GL, Chim AM, Chu WC, Yeung DK, Li KC, et al. Treatment of nonalcoholic steatohepatitis with probiotics. A proof-of-concept study. Ann Hepatol. 2013;12(2):256-262.

150. Wong VW, Tse CH, Lam TT, Wong GL, Chim AM, Chu WC, et al. Molecular characterization of the fecal microbiota in patients with nonalcoholic steatohepatitis--a longitudinal study. PLoS One. 2013;8(4):e62885. doi: 10.1371/journal.pone.0062885.

151. Malaguarnera M, Vacante M, Antic T, Giordano M, Chisari G, Acquaviva R, et al. *Bifidobacterium longum* with fructo-oligosaccharides in patients with non alcoholic steatohepatitis. Dig Dis Sci. 2012;57(2):545-53. doi: 10.1007/s10620-011-1887-4.

152. Eslamparast T, Poustchi H, Zamani F, Sharafkhah M, Malekzadeh R, Hekmatdoost A. Synbiotic supplementation in nonalcoholic fatty liver disease: a randomized, double-blind, placebo-controlled pilot study. Am J Clin Nutr. 2014;99(3):535-42. doi: 10.3945/ajcn.113.068890.

153. Ratziu V, Harrison S, Francque S, Bedossa P, Lehert P, Serfaty LS, et al. Elafibranor, an Agonist of the Peroxisome Proliferator-activated Receptor-alpha and -delta, Induces Resolution of Nonalcoholic Steatohepatitis Without Fibrosis Worsening. Gastroenterology. 2016. doi: 10.1053/j.gastro.2016.01.038.

154. Friedman S, Sanyal A, Goodman Z, Lefebvre E, Gottwald M, Fischer L, et al. Efficacy and safety study of cenicriviroc for the treatment of non-alcoholic steatohepatitis in adult subjects with liver fibrosis: Contemp Clin Trials. 2016;47:356-365. doi: 10.1016/j.cct.2016.02.012.

155. Brenner C, Galluzzi L, Kepp O, Kroemer G. Decoding cell death signals in liver inflammation. J Hepatol. 2013;59(3):583-94. doi: 10.1016/j.jhep.2013.03.033.

156. Okada-Iwabu M, Iwabu M, Ueki K, Yamauchi T, Kadowaki T. Perspective of Small-Molecule AdipoR Agonist for Type 2 Diabetes and Short Life in Obesity. Diabetes Metab J. 2015;39(5):363-72. doi: 10.4093/dmj.2015.39.5.363.

157. Okada-Iwabu M, Yamauchi T, Iwabu M, Honma T, Hamagami K, Matsuda K, et al. A small-molecule AdipoR agonist for type 2 diabetes and short life in obesity. Nature. 2013;503(7477):493-499. doi: 10.1038/nature12656.

158. Nagalingam A, Arbiser JL, Bonner MY, Saxena NK, Sharma D. Honokiol activates AMP-activated protein kinase in breast cancer cells via an LKB1-dependent pathway and inhibits breast carcinogenesis. Breast Cancer Res. 2012; 14(1):R35. doi: 10.1186/bcr3128.

159. Seo MS, Kim JH, Kim HJ, Chang KC, Park SW. Honokiol activates the LKB1-AMPK signaling pathway and attenuates the lipid accumulation in hepatocytes. Toxicol Appl Pharmacol. 2015;284(2):113-124. doi: 10.1016/j.taap.2015.02.020.

160. Lee JH, Jung JY, Jang EJ, Jegal KH, Moon SY, Ku SK, et al. Combination of honokiol and magnolol inhibits hepatic steatosis through AMPK-SREBP-1 c pathway. Exp Biol Med (Maywood). 2015;240(4):508-518. doi: 10.1177/1535370214547123.

161. Day CP, Yeaman SJ. The biochemistry of alcohol-induced fatty liver. Biochim Biophys Acta. 1994;1215(1-2):33-48.

162. Gariani K, Menzies KJ, Ryu D, Wegner CJ, Wang X, Ropelle ER, et al. Eliciting the mitochondrial unfolded protein response by nicotinamide adenine dinucleotide repletion reverses fatty liver disease in mice. Hepatology. 2016;63(4):1190-204. doi: 10.1002/hep.28245.

163. Gual P, Postic C. Therapeutic potential of nicotinamide adenine dinucleotide for nonalcoholic fatty liver disease. Hepatology. 2016;63(4):1074-7. doi: 10.1002/hep.28383.

8.2 Article 5

International Liver Transplantation Consensus Statement on End-stage Liver Disease Due to Nonalcoholic Steatohepatitis and Liver Transplantation

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Abstract Nonalcoholic steatohepatitis NASH)-related cirrhosis has become one of the most common indications for liver transplantation LT), particularly in candidates older than 65 years. Typically, NASH candidates have concurrent obesity, metabolic, and cardiovascular risks, which directly impact patient evaluation and selection, waitlist morbidity and mortality, and eventually posttransplant outcomes. The purpose of these guidelines is to highlight specific features commonly observed in NASH candidates and strategies to optimize pretransplant evaluation and waitlist survival. More specifically, the working group addressed the following clinically relevant questions providing recommendations based on the Grading of Recommendation, Assessment, Development and Evaluation GRADE) system supported by rigorous systematic reviews and consensus: 1) Is the outcome after LT similar to that of other etiologies of liver disease? 2) Is the natural history of NASH-related cirrhosis different from other etiologies of end-stage liver disease? 3) How should cardiovascular risk be assessed in the candidate for LT? Should the assessment differ from that done in other etiologies? 4) How should comorbidities hypertension, diabetes, dyslipidemia, obesity, renal dysfunction, etc.) be treated in the candidate for LT? Should treatment and monitoring of these comorbidities differ from that applied in other etiologies? 5) What are the therapeutic strategies recommended to improve the cardiovascular and nutritional status of a NASH patient in the waiting list for LT? 6) Is there any circumstance where obesity should contraindicate LT? 7) What is the optimal time for bariatric surgery: before, during, or after LT? 8) How relevant is donor steatosis for LT in NASH patients?

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n the United States, nonalcoholic steatohepatitis (NASH) cirrhosis has become the second most common indication for liver transplantation (LT) waitlisting, and the third indication for LT, particularly in candidates older than 65 years.[1-10] In addition, NASH is the most rapidly growing indication for simultaneous liver-kidney (SLK) transplantation also in the United States.[7] Similar trends, but still not reaching the numbers of US registries, have been reported elsewhere. Because of frequent comorbidities that increase the risk of cardiovascular (CV) disease, the outcome and management of NASH candidates may differ from that of other indications.[1-11]

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2018, comprised of a global panel of expert hepatologists and transplant surgeons, to develop guidelines on key aspects of NASH in relation to liver transplantation. This is one of the 6 articles that have been put together by the various working groups and focuses on end stage liver disease and liver transplantation related to NASH. There were 8 predefined questions that were addressed by the consensus panel. These questions were addressed through critical literature review, followed by working group proposals and subsequent consensus, which was reviewed by the whole group. The guidelines are presented using the Grading of Recommendations Assessment Development and Evaluation approach.[1] This method includes consideration of the quality of evidence, benefits and harms, values and preferences, resource use, and cost effectiveness. Quality of the evidence was rated as very low, low, moderate, or high. The strength of the recommendation was rated as strong, moderate, or conditional (weak) and reflects confidence that adherence to guidance will result in more good than harm.

1 IS THE OUTCOME AFTER LT SIMILAR TO THAT OF OTHER ETIOLOGIES OF LIVER DISEASE?

Recommendations

The outcome of LT in patients with NASH-related cirrhosis with or without hepatocellular carcinoma (HCC) does not differ from that of other liver transplant etiologies as posttransplant survival is similar (quality of evidence high level; strength of recommendation, strong).

Background

A systematic review published in 2014 which included 9 publications with a total of 717 transplants in NASH patients and 3520 transplants in non-NASH indications found that survival at 1 (odds ratio [OR], 0.77; 95 confidence interval [CI], 0.59-1; $P = 0.05$), 3 (OR, 0.97; 95 CI, 0.67-1.40; $P = 0.86$), and 5 (OR, 1.09; 95 CI, 0.77-1.65; $P = 0.63$) years postliver transplantation was similar between these 2 groups.[2] The same study demonstrated a higher mortality due to CV causes (OR, 1.65; 95 CI, 1.01-2.70; $P = 0.05$) and sepsis (OR, 1.71; 95 CI, 1.17-2.50; $P = 0.006$) in NASH indications. However, patients with NASH were at lower risk of graft failure compared with patients without NASH (OR, 0.21; 95 CI, 0.05-0.89; $P = 0.03$).

Interestingly, NASH-cirrhotic patients had distinctive features at time of transplantation compared to the non-NASH candidates including older age, higher body mass index (BMI) and greater frequency of diabetes mellitus (DM), arterial hypertension, dyslipidemia, obesity, and history of CV events; in addition, the prevalence of women was higher and that of concurrent HCC lower. Importantly, model for end-stage liver disease (MELD) score at transplantation was similar between the 2 groups.

Two large studies using large North American Registries also found similar survival rates after LT. In the first study by Charlton et al[3] based on the Scientific Registry of Transplant Recipients (SRTR), both graft and patient survival at 3 years posttransplantation did not differ between the 1959 NASH patients and 33822 non-NASH patients ($P = 0.67$) undergoing transplantation from 2001 to 2009. Patient survival estimates at 1 and 3 years after LT for NASH were 84 and 78 , respectively, compared with 86 and 79 for cryptogenic cirrhosis and 87 and 78 for other indications ($P = 0.67$). Patient and graft survival after LT for recipients with NASH was similar to that of other indications in multivariate analysis after adjusting for creatinine level, gender, age, and BMI.

In the study by Afzali et al,[4] the authors used data provided by the United Network for Organ Sharing (UNOS) for first-time adult deceased donor LT performed in the United States between 1997 and 2010. Posttransplant survival of patients with NASH (n = 1810) at 1 year (87.6), 3 years (82.2), and 5 years (76.7) was superior to the survival of patients with HCC, hepatitis C virus (HCV), alcoholic liver disease, acute hepatic necrosis, hemochromatosis, or cryptogenic liver disease and was inferior to the survival of only 4 groups of patients (those with primary biliary cirrhosis, primary sclerosing cholangitis, autoimmune hepatitis, or hepatitis B virus [HBV]), with results not differing after adjusting for both donor and recipient characteristics.

More recent analyses of US registries[5,6] confirm previous findings. In the recent cohort study utilizing the UNOS and Organ Procurement and Transplantation (OPTN) 2003 to 2014 database, the outcome of 63061 adult patients undergoing LT from 2003 to 2014 was evaluated.[5] The study included 20782 HCV (32.96), 9470 alcoholic liver disease (15.02), and 8262 NASH (13.11) patients. Posttransplant survival in NASH was significantly higher compared to HCV (5-year survival: NASH, 77.81 ; 95 CI 76.37-79.25 vs HCV, 72.15 , 95 CI 71.37-72.93, $P < 0.001$). In the multivariate Cox proportional hazards model, NASH demonstrated significantly higher posttransplant survival compared with HCV (HR, 0.75; 95 CI 0.71-0.79, $P < 0.001$). As in previous studies, patients with NASH were more likely to be women, had higher BMI and a higher prevalence of diabetes and history of cardiac disease.

Similar findings have been reported in NASH patients with additional comorbidities, such as severe renal disease requiring SLK transplantation,[7] or coexistence of HCC.[8] In 1 registry study based on UNOS database (2002-2011),[7] of the 38533 liver transplants performed during that study period, about 5.6 (N = 2162) received SLK transplantation with 584 (6.2), 320 (8.7), and 1258 (5) belonging to immune- or alcohol-related indications (primary biliary cholangitis, primary sclerosing cholangitis or alcoholic cirrhosis (group I), NASH, and cryptogenic cirrhosis with BMI greater than 30 (group II), and HCV with and without alcohol, HBV, and HCC (group III), respectively. Five-year outcomes were similar comparing the NASH group (group II) versus group I for liver graft (78 vs 74 , $P = 0.14$) and patient survival (81 vs 76 , $P = 0.07$). In contrast, kidney graft outcome was worse for group II (70 vs 79 , $P = 0.002$). Risk of kidney graft loss was over 1.5-fold higher among group II SLK recipients compared with group I after controlling for recipient characteristics.

Using a 2-center retrospective design, Sadler et al[8] analyzed the outcome of all patients from 2004 to 2014 that received LT for HCC and compared the outcome of those transplanted for HCC on top of NASH (60/929, 6.5) versus the remainder non-NASH patients. There were no significant differences between groups for pretransplant or explant tumor characteristics. The actuarial 1-, 3- and 5-year overall survival was 98 , 96 , and 80 , respectively, in NASH versus 95 , 84 , and 78 , respectively, in non-NASH ($P = 0.1$).

In summary, in most studies NASH patients have been shown to have similar survival rates compared with patients

without NASH even though their profile is consistent with a high-risk candidate (older, greater rate of obesity, more likely to be diabetic, more likely to have prior history of CV events).[1-17] It is likely that these results may be explained in part, by the lower risk of graft failure compared with other indications, particularly HCV. These results may change with the introduction of the extremely effective oral antiviral drugs against HCV. Alternatively, extensive screening for CV disease in patients with NASH-related cirrhosis may have led to an exclusion of the "poor NASH candidates (with significant CV disease)" allowing the inclusion in the LT waiting list of those considered "the best NASH candidates". Interestingly though, both single-center studies, studies from large registries and systematic reviews have demonstrated that CV deaths constitute a higher proportion of deaths among NASH patients compared to non-NASH transplant recipients.[1-17] In addition, mean follow-up in many of these studies is less than 5 years. It is still unknown if results will change with longer follow-up, as more CV disease develops in NASH-patients.

2 IS THE NATURAL HISTORY OF NASH-RELATED CIRRHOSIS DIFFERENT FROM OTHER ETIOLOGIES OF END-STAGE LIVER DISEASE?

Statements

Limited data are available. Patients with NASH-related cirrhosis have increased CV morbidity and mortality compared to patients with cirrhosis of other etiologies. Patients with NASH cirrhosis have lower mortality rates in the compensated state (Child Pugh A) but similar mortality in the decompensated state (Child Pugh B and C) compared to HCV-related cirrhosis.

Background

In a prospective follow-up of 256 adult patients with compensated NASH-related cirrhosis, 49 (19) subjects experienced liver-related clinical events after a follow-up of 26.7 months.[18] At 24 months, event free survival was 92 in patients with a hepatic venous portal gradient (HVPG) < 10 mm Hg compared with 75 in those with HVPG of 10 mm Hg or greater. In the multivariate analysis, independent predictors of clinical events were higher baseline HVPG, greater change in HVPG over time and lower baseline albumin.

In a case control study from Australia that included 23 patients with NASH cirrhosis and 46 patients with HCV cirrhosis, the prognosis of patients with NASH cirrhosis was similar to or better than that of HCV-related cirrhosis.[19] In a case control study from Japan that included 68 patients with NASH-related cirrhosis and 69 patients with HCV-related cirrhosis, the 5-year HCC rate was higher in HCV cirrhosis (30.5 in HCV vs 11.3 in NASH) but the 5-year survival rates were similar (73.8 in HCV vs 75.2 in NASH).[20] The authors of these 2 studies did not perform comparisons according to Child Pugh class. In a US case-control study of 152 patients with NASH-related cirrhosis and 150 patients with HCV-related cirrhosis, patients with Child class A NASH cirrhosis had lower mortality compared with Child class A HCV cirrhosis (3/74 vs 15/75, $P < 0.004$), whereas there was no difference in mortality in Child Pugh B/C cirrhosis.[21] Patients with Child class A cirrhosis due to NASH also had a significantly lower risk of decompensation ($P < 0.07$). Patients with NASH had higher cardiac mortality (8/152 vs 1/150, $P < 0.03$).[21]

In a follow-up of 218 patients with NASH cirrhosis listed for LT, NASH-patients were older and had more comorbidities despite a similar MELD score compared with patients with HCV cirrhosis.[22] Patients with NASH cirrhosis and MELD of 15 or less were less likely to receive LT and more likely to die or delisted from the waitlist because of comorbidities compared with patients with HCV-related cirrhosis. The median progression rate among patients with NASH was 1.3 MELD points per year versus 3.2 MELD points per year for the HCV group ($P = 0.003$). Among patients with MELD scores greater than 15, there were no differences between groups in percentage that received transplants or rate of MELD score progression.[22]

In a study from the UNOS database, among US adults with HCC listed for LT, patients with NASH-HCC were significantly less likely to have active MELD exceptions compared with HCV-HCC, and those without active exception had a lower likelihood of receiving LT.[23] The authors postulated that this could be due to a higher rate of comorbid conditions in patients with NASH and/or better hepatic function and slower progression of cirrhosis in the NASH-HCC group.

3 HOW SHOULD CV RISK BE ASSESSED IN THE NASH-CANDIDATE FOR LT? SHOULD THE ASSESSMENT DIFFER FROM THAT DONE IN OTHER ETIOLOGIES?

Recommendations

1- Liver transplant candidates with NASH should be considered at high risk of developing CV events before and after transplantation (quality of evidence, high; strength of recommendation, strong).
2- The accumulation of CV risk factors should be carefully assessed by a multidisciplinary team, which should include a cardiologist and anesthesiologist with special interest in transplantation (quality of evidence, low; strength of recommendation, strong).
3- Although NASH is considered an independent risk factor for CV events similar to other traditional risk factors, there is not enough evidence to support a different approach to the pre-LT CV assessment. (quality of evidence, moderate; strength of recommendation, strong).
4- There is insufficient evidence to recommend a specific CV risk algorithm for NASH patients undergoing liver transplantation evaluation. The algorithm, and particularly the place of stress tests, will be determined in part by local expertise (quality of evidence, moderate; strength of recommendation, moderate IIa)

Background

Nonalcoholic Fatty Liver Disease and CV Disease

Typically, NASH patients have a metabolic profile compatible with high CV risk, which makes them more likely to present with silent CV disease.[2,10-14,24] Indeed, a growing body of evidence supports the existence of a bidirectional relationship between NAFLD and the metabolic syndrome, particularly hypertension and type II DM.[24] Importantly, the metabolic syndrome, at the heart of NASH, encompasses well-known CV risk factors, including central obesity, atherogenic dyslipidemia, together with hypertension and type II DM.

It should not be a surprise then that NASH is strongly linked to an increased risk of developing fatal and nonfatal CV events. Whether NAFLD by itself (though multiple pathophysiological derangements, including chronic inflammation, hypercoagulation, chronic kidney disease, etc.) independently contributes to the development of CV disease is still a topic of debate but increasingly data points toward that independent effect.

The spectrum of CV complications associated with NAFLD is very wide ranging from premature atherosclerosis to aortic valve sclerosis and left ventricular dysfunction/hypertrophy leading to congestive heart failure and cardiac arrhythmias (mainly atrial fibrillation and QTc interval prolongation).[24] In a recent meta-analysis including almost 34 000 individuals from 16 observational studies, the authors concluded that the presence of NAFLD (diagnosed through imaging or histology) was associated with a 65 increased risk of developing fatal and nonfatal CV events over a medium follow-up of almost 7 years.[25] Importantly, the authors also showed how this risk increased with increased severity of the liver disease.[25] Based on this strong link between the presence and severity of NAFLD and the increased risk of CV disease, most Societies have suggested that NAFLD by itself, regardless of other known risk factors, identifies a subset of patients with a higher risk of CV disease mortality and morbidity over time, and thus recommend a thorough CV risk assessment.[26] It is important to highlight that cirrhosis is the end-spectrum of NAFLD, representing those with the most severe form of liver disease, and as such, in theory at least, at highest risk of CV disease. Cohort studies as well as systematic reviews and meta-analysis have shown that waitlist patients with NASH are typically older compared to those with other etiologies, more likely to be female, more likely to be diabetics and obese and with worse renal function.[2,6] Compared to other cirrhotic patients, NASH-cirrhosis is associated with a higher risk of CV events after LT, particularly in the early postoperative period.[14] In addition, several studies have also documented that patients with end-stage liver disease due to NASH have higher rates of coronary artery disease (CAD) than those of other etiologies.[27-31]

CV Risk Assessment of the LT Candidate

The stress inherent to transplantation, including surgery itself, and complications that may occur in the postoperative period, can transform silent CV disease into serious CV events, eventually increasing mortality. Therefore, it is essential that the pretransplant evaluation includes a thorough CV evaluation both structural and functional in conditions of rest and stress to reveal (and correct if possible) any significant CV disease. Unfortunately, defining what should be the best approach to adequately assess the CV risk in LT candidates is a focus of intense and moving debate. Recognizing the hemodynamic challenges encountered by LT patients in the perioperative period and how these responses can be exacerbated by underlying cardiac pathology is critical in developing recommendations for the preoperative risk assessment and management of these patients.[32] Overall, CV assessment should reveal subclinical CAD, portopulmonary hypertension and myocardial disease and should be used to either treat abnormal findings and/or deny transplantation to those where the risk is too high and who are nontreatable. Patients with overt heart failure due to cardiac disease will most likely not benefit from LT and have significant postoperative morbidity and mortality related to worsening heart failure.[32] In addition, patients with moderate to severe portopulmonary hypertension who do not respond to vasodilator therapy should not be considered for LT.[33] Finally, high-risk patients with CAD not amenable to revascularization or those with concurrent left ventricular dysfunction will also not likely benefit from LT.[31]

Unfortunately, despite a clear understanding that CV events represent a source of morbidity and mortality, risk stratification approaches and performance characteristics of different cardiac testing modalities remain unclear. In a recent systematic review aimed at characterizing the incidence and risk factors for CV events post-LT, which included 29 studies representing 57 493 patients, definitions of CV outcomes were highly inconsistent. Incidence rates were widely variable: 1 to 41 for outcomes at 6 months or shorter and 0 to 31 for outcomes longer than 6 months. Multivariate analyses demonstrated that older age and history of cardiac disease were the most consistent predictors of CV events posttransplant. Unfortunately, the predictive capacity of various cardiac imaging modalities was also discrepant. Based on these data, the authors concluded that the true incidence of CV outcomes post-LT remains unknown in large part due to lack of consensus regarding outcome definition.[34]

Of note, patients with established CAD who undergo LT have worse outcomes than patients without CAD. In 1 study, 1-year mortality rate of about 40 was reported,[31] although improvements in screening and perioperative care have reduced early posttransplant deaths substantially. More recent reports indicate mortality hazard ratios in patients with clinical CAD of 2.0 to 3.9 against 1-year all-cause mortality rates of 4 to 5 , suggesting a risk of 8 to 18 .[34-38] Because severe multivessel disease or inducible ischemia may justify intervention in these patients, a strategy of stress testing and coronary angiography (CAG) is generally accepted.

Addressing the impact and management of patients with clinically silent disease is less clear.[39] The prevalence of angiographically demonstrated CAD in transplant candidates is known to be similar to that reported in the general population and higher in NASH,[40,41] but functional impairment caused by encephalopathy, sarcopenia, fluid retention and/or acute decompensation often prevents clinical assessment of cardiac reserve.

Tools to Evaluate CV Risk in Liver Transplant Candidates

lassical Noninvasive Tools

The CV evaluations are challenging in LT candidates. The majority of these patients cannot undergo cardiopulmonary exercise testing due to deconditioning, malnutrition-associated muscle weakness, ascites, or anemia. Cardiac evaluation with electrocardiography and echocardiography is done on a routine basis in most centers. A prolonged QTc is not a contraindication to LT, but should prompt a search for reversible causes, such as electrolyte disturbance (eg, hypokalemia or hypomagnesemia) or the use of QT interval-prolonging drugs.[34,35] Transthoracic echocardiography with Doppler is recommended for all LT candidates to assess left and right ventricular size and function, valvular function, and pulmonary artery pressure. Generally, when an abnormal finding

is detected using these methods, further investigations are recommended, particularly through noninvasive techniques (cardiopulmonary exercise testing, dobutamine stress echocardiography [DSE], myocardial perfusion imaging by single-photon emission computed tomography [SPECT] and/or cardiac computed tomography), positive findings typically leading to the use of invasive CAG.[42] This has been assumed to enhance risk prediction and outcomes by identifying both candidates with disease so severe as to preclude transplant and those suitable for risk-reducing intervention. However, neither of these potential benefits has been demonstrated beyond reasonable doubt.

First, noninvasive functional testing for ischemia has limited predictive value for obstructive CAD in this population. Most patients cannot undergo exercise testing. In addition, although previous meta-analysis has suggested that DSE detects CAD with a high degree of sensitivity and specificity in the general population,[42] its performance is clearly reduced in the LT setting where poor sensitivity has been reported, possibly secondary to an inability to achieve target heart rate and peak double product (heart rate multiplied by blood pressure).[43] The use of -blocking agents for the prevention of esophageal variceal bleeding has been found to be a common cause of failure to achieve the target heart rate in DSE. In a retrospective study of 105 cirrhotic patients who underwent both DSE and CAG, DSE was found to have a sensitivity of 13 and negative predictive value (NPV) of 75 for obstructive CAD.[44] In another analysis of LT candidates, DSE compared with CAG had 75 sensitivity and 57 specificity in detecting CAD.[45] Another series reported 9 sensitivity, 33 positive predictive value (PPV), and 89 NPV for predicting early cardiac events after LT.[46] In a large retrospective study of 400 LT patients, preoperative DSE had a PPV of only 27 for the identification of posttransplant cardiac events (death/nonfatal myocardial infarction) within 1 month after LT but the NPV reached 89 .[37] The majority of patients in this study though had relatively low MELD scores. In a quantitative systematic review assessing DSE's use in detecting CAD and predicting perioperative and long term cardiac events in patients undergoing LT, based on 7 studies, including a total of 580 patients, the authors confirmed the limited accuracy of DSE for the detection of CAD in candidates for LT. However, among patients selected for LT, the NPV of DSE for both perioperative and long term cardiac events was found to be high.[47,48] Similarly, nuclear SPECT stress imaging cannot be effective because of the relatively low sensitivity to detect CAD in LT candidates due to the chronic vasodilatory state exhibited by patients with end-stage liver disease.[49,50] Overall, in 2 recent systematic reviews,[34,51] the authors concluded that DSE and SPECT do not satisfactorily predict increased risk of perioperative major CV events or all-cause mortality among cirrhotic patients listed for LT, among small and heterogenous studies. In summary, noninvasive stress imaging has been shown to predict major adverse cardiac events no more effectively than conventional clinical risk scoring.

Invasive Tools

In response to the low sensitivity of noninvasive stress imaging in the LT setting, many US centers have adopted CAG as a primary investigation in up to 80 of prospective recipients, with or without noninvasive testing.[50,52] Comparable 1-year posttransplant outcomes have been reported in retrospective studies of patients undergoing CAG compared to recipients with no CV disease, but these make no distinction between those investigated on the basis of CAD history or symptoms and those with risk factors alone.[52-54] Therefore, although the authors interpreted their outcomes as evidence of the effectiveness of aggressive investigation and revascularization, it is also possible that a low mortality risk in a high proportion of recipients with untreated silent disease masked a lack of benefit in those undergoing intervention. Furthermore, 1 recent study questions routine angiography and intervention, reporting 50 posttransplant mortality in revascularized recipients.[55] In this study, among 13 patients with severe CAD, 3 underwent percutaneous coronary intervention (PCI), and 6 underwent coronary artery bypass grafting (CABG). Overall, 50 of patients who underwent either intervention died of cardiac-related causes, whereas no patient died of a cardiac-related cause after undergoing neither intervention. Some clinicians, especially outside the US, argue that an emphasis on angiographic findings of obstructive disease may underestimate the role of nonobstructive plaque and microvascular dysfunction as causes of major cardiac events in this setting. Both are common in NAFLD, and impaired microvascular perfusion is often present in the absence of obstructive epicardial disease.[56] The view that CAG and revascularization may not be beneficial in silent disease is supported by randomized studies showing that, outside the context of acute coronary syndromes, PCI offers no survival advantage in the community, nor in major vascular surgery.[42] The latter is especially significant because vascular surgery is associated with higher perioperative cardiac mortality than LT. Given the risks and delays incurred by intervention, and the absence of diagnostic randomized controlled trials (RCTs), current American Heart Association (AHA)/American College of Cardiology (ACC) guidelines do not recommend routine preoperative stress-testing, PCI or CABG in asymptomatic patients in other noncardiac surgical settings.[42] Moreover, the current AHA/ACC scientific statement on cardiac evaluation of LT candidates states only that noninvasive testing in LT candidates 'may be considered in the presence of multiple (3 or more) risk factors (particularly diabetics aged >50 years), rating the evidence as class IIb, level C.[42] Despite its low sensitivity and PPV, DSE is the tool recommended by these associations because of its high NPV.[39]

Newer Noninvasive Tools

Newer alternative modalities and strategies have recently been proposed in pretransplant cardiac evaluation, including coronary artery calcium score (CACS) and coronary computed tomography angiography (CCTA), cardiac magnetic resonance (MRA) and contrast-enhanced DSE.[38,41,56-60] There is again debate about whether these new tools should be considered routinely for all LT candidates due to the low a priori probability of detecting severe stenosis. For instance, in a large study of 1045 asymptomatic cirrhotic patients (no history of chest pain or CAD), CCTA revealed a similar frequency of obstructive CAD in the cirrhotic (7.9) and healthy (7.2) cohorts.[41] In addition, although observational studies of some of these confirm threshold values of test-generated variables associated with increased risk of cardiac events and mortality,[56,60] none has been reported to be associated with hazard ratios that are prohibitive as a single factor (that is, a hazard

ratio > 7, which when multiplied by average risk yields an early mortality risk >30). Again, none has been assessed in a diagnostic RCT in LT.

Finally, it is arguable that the scale of the problem of early CV mortality in LT has been overstated. 30-day CV mortality in LT was reported as 1.16 in a recent analysis of 54697 liver recipients in the UNOS database, a low figure despite the inclusion of fatal stroke, thromboembolism and intraoperative cardiac arrests, which are often multifactorial and not conclusively cardiac.[61] Dating from 2002 to 2012, this cohort must have included a high proportion of candidates with subclinical CAD who did not undergo CAG, but mortality was equal or lower than that seen in other high-risk surgical groups.[42]

On the basis of this limited evidence, and of unpublished reports of fatal complications of CAG in this setting,[62] some units are reluctant to pursue angiography and intervention on the basis of risk factors alone. Given the perceived deficiencies of noninvasive tests, some will choose to follow current AHA/ACC guidelines for noncardiac surgery and request these only in candidates with poor functional status and multiple CAD risk factors, particularly diabetics older than 50 years.[31,63,64] In addition, in the absence of a recognized indication for revascularization, a finding of inducible ischemia is treated as an additional risk factor, which may tip the balance against transplant without recourse to angiography.[56] The same reasoning can be applied to CCTA, CACS, and MRA, which may be useful in determining significant added risk in support of a decision not to list.

In essence, no gold standard has yet been developed for cardiac evaluation in LT candidates. LT candidates are at risk of developing a variety of cardiac-related complications, particularly those related to cardiomyopathy or CAD. However, in the US population, reported early CV mortality in LT is similar to that seen in other major procedures, for which aggressive investigation and intervention in subclinical disease is not recommended in AHA/ACC guidelines.

Routine noninvasive stress imaging may not be sufficient alone for preoperative testing as these tests do not accurately predict early cardiac risk, do not quantify plaque burden and are confounded by microvascular dysfunction in end-stage liver disease. Newer noninvasive modalities have not yet been adequately assessed in this population. To date, none has revealed new parameters reliably indicating prohibitive risk, nor has any been shown to be of value in a diagnostic RCT in other surgical settings. However, findings on these may contribute to an overall clinical judgment of risk. These new tools seem to be reliable screening options for preoperative noninvasive evaluation of CAD in selected patients, such as those with DM or ≥ 2 traditional risk factors for CAD (age > 45 years for male or > 55 years for female, hypercholesterolemia, hypertension, tobacco use, and family history of early CAD) but further work is needed. Whether NASH patients alone without the consideration of these additional risk factors should undergo these noninvasive techniques for pre-LT evaluation is still unclear, although increasing data support the concept that it should be considered a traditional risk factor.

An abnormal noninvasive test (such as coronary artery stenosis ≥50 on CCTA or CACS >400) or a high pretest probability of CAD should prompt consideration for CAG and coronary revascularization should be considered in LT candidates with obstructive CAD if the extent of CAD contraindicates transplantation. However, to date, there are no diagnostic RCTs in LT demonstrating superior outcomes with any preoperative screening strategy in patients with clinical but particularly subclinical CAD, and furthermore in other types of high-risk surgery, RCTs of noninvasive testing followed by PCI or CABG show no benefit and a potential for added risk associated with delayed surgery. Whether revascularization results in enhanced LT outcomes requires also further investigation. In units not advocating routine stress testing and CAG in candidates with silent disease, local guidelines may advocate a case-by case multidisciplinary approach, ideally involving a cardiologist with a special interest in this field.

4 HOW SHOULD COMORBIDITIES HYPERTENSION, DIABETES, DYSLIPIDEMIA, OBESITY, RENAL DYSFUNCTION, etc.) BE MANAGED IN THE CANDIDATE FOR LT? SHOULD TREATMENT AND MONITORING OF THESE COMORBIDITIES DIFFER FROM THAT APPLIED IN OTHER ETIOLOGIES?

Recommendations

- A multidisciplinary approach is recommended to establish a risk minimization plan (endocrinology and nutrition, psychology, cardiology, hepatology, surgery, anesthetist) (Quality of evidence: Moderate; Strength of recommendation: Strong).
- Appropriate screening for hypertension, diabetes, and dyslipidemia is recommended in NASH-patients with indication for LT and medical optimization is strongly recommended (quality of evidence, moderate; strength of recommendation, strong).
- NASH is an independent risk factor for pre and post-LT renal dysfunction; appropriate screening and management of kidney disease is highly recommended in this patient population (quality of evidence, high; strength of recommendation: strong).
- There is no data to support a different approach for the treatment and monitoring of comorbidities in NASH patients compared to other etiologies.

Background

Age, severity of liver disease, CAD, DM, obesity, hypertension and renal failure are individual risk predictors of poor postoperative and late outcomes after LT. Among LT candidates, patients with NASH represent a particularly challenging group because they are most likely to have these risk factors, which may contribute in both an independent and additive manner to patient selection and outcomes after LT.[2,10-15,20,25,31,43] There are currently no specific guidelines for preoperative assessment in this population or regarding specific treatment and monitoring strategies.

Arterial Hypertension Dyslipidemia and DM

Each traditional risk factor, such as DM, hypertension, or dyslipidemia, should be treated and medical strategies maximized. In addition to diet and physical activity that could have a beneficial impact on each condition, adequate pharmacotherapy is strongly encouraged.

For DM in compensated cirrhosis, pioglitazone could be considered, as it has both demonstrated efficacy in DM treatment as well as improvement of NASH-histological features, but concerns remain regarding potential adverse effects such as weight gain, bladder cancer or cardiac events. Glucagon-like peptide 1 agonists represent a promising therapeutic class but data remain insufficient to recommend these agents as

first-line treatment.[65] The potential efficacy of pioglitazone and glucagon-like peptide 1 agonists has been demonstrated in noncirrhotic NASH and data in cirrhosis is lacking. For decompensated cirrhosis, insulin is the first-line treatment.

For dyslipidemia, statin therapy should be considered as first-line treatment. The potential rare occurrence of drug-induced liver injury needs to be balanced with the beneficial impact on preventing CAD but also its effects on the natural history of cirrhosis, portal hypertension, and HCC prevention.[66] Furthermore, a cross-sectional study evaluated the effect of statins in 1201 high-risk NAFLD patients (age 50, severe obesity, 50 DM) without cirrhosis who underwent liver biopsy. Prior statin therapy for at least 6 months was associated with less steatosis (OR, 0.09), less inflammation according to NAS and less risk of advanced fibrosis stage F2-F4.[67] Fibrates have also been studied as they may promote hepatic fatty acid oxidation and reduce hepatic triglyceride synthesis and very low density lipoprotein production and export through their action as PPAR- agonists. However, mixed effects have been observed on liver histology, with 1 study showing improvement only in ballooning while another study showed no effect with fibrates.[68]

For hypertension, non-cardioselective beta-blockers are probably the best option to treat both hypertension and portal hypertension when recommended, although evidence supporting this recommendation is lacking. When beta-blockers are indicated to prevent or treat CAD, cardioselective beta-blockers can be used (see question 5). The second line option is angiotensin-converting enzyme inhibitors or angiotensin-receptor blockers. There is some evidence suggesting that blocking the renin-angiotensin system may impact on NAFLD histology including fibrosis.[69]

Renal Dysfunction

NASH is an important risk factor for renal dysfunction both pre and post-LT.[70] Renal dysfunction in this setting is multifactorial due to other comorbidities (hypertension, DM) but also related to the severity of liver disease. Importantly, renal dysfunction is a risk factor for posttransplant CV disease and mortality.[71,72] Even mild renal disease at the time of LT has been shown in 1 study to be a risk factor for posttransplant all-cause and CV mortality.[72] In 1 study, more rapid declines in estimated glomerular filtration rates soon after LT correlated with risk of adverse CV outcomes, highlighting the need to study whether early renal preservation interventions also reduce CV complications.[72] Consequently, the main therapeutic goal is to prevent kidney function deterioration by treating risk factors and consider SLK transplantation when needed.

Differences With Other Etiologies

To date, there are no data to support a different approach for the management of comorbidities in NASH patients compared to other etiologies. The peculiarity of patients with NASH lies in the fact that the number of comorbidities[2,10-15,70] seems greater and their age more advanced than for other LT indications. It is difficult to establish groups of patients based on their risk from literature data. A multidisciplinary approach is necessary to optimize the management of these patients whose complex and often contradictory pathologies are intricate.

5 WHAT ARE THE THERAPEUTIC STRATEGIES RECOMMENDED TO IMPROVE THE CV AND NUTRITIONAL STATUS OF A NASH PATIENT IN THE WAITING LIST FOR LT?

Recommendations

- Patients with Child A/B NASH cirrhosis and CV comorbidities can be considered for a cardioselective beta-blocker and statin (Quality of evidence: low, Strength of recommendation: Moderate IIb)
- A protocol of moderate exercise is recommended with the dual objective of losing weight and improving muscle mass (quality of evidence, low-moderate; strength of recommendation, moderate)

Background

CV disease remains a leading cause of death in LT recipients, with the highest rates occurring immediately after transplantation. Pretransplant hypertension, diabetes and atrial fibrillation are all risk factors that contribute to post-LT CV morbidity.[73] Patients with NASH cirrhosis are at increased risk of posttransplant CV events independent of traditional cardiac risk factors.[14,25] Therefore, CV comorbidities such as obesity, hypertension, DM and hyperlipidemia need to be assessed and adequately controlled in the pre and posttransplant setting[74,75] (see questions 3 and 4).

Beta-blockers and statins improve CV outcomes in patients with CV risk factors undergoing noncardiac surgery, but data on the transplant setting are missing. In a randomized controlled trial involving 1066 intermediate cardiac risk patients, patients randomized to bisoprolol at least 7 days before surgery had a lower incidence of perioperative cardiac death and nonfatal myocardial infarction than those randomized to bisoprolol-control (hazard ratios, 0.34; 95 CI, 0.17-0.67).[76] In a randomized controlled study of 8351 patients at risk of atherosclerotic disease undergoing noncardiac surgery, extended release metoprolol started in the perioperative period was associated with less CV deaths, nonfatal myocardial infarction and nonfatal cardiac arrest at the expense of an increased incidence of stroke.[77] Therefore, beta-blockers, if used, should be started and titrated well before the perioperative period.

In a meta-analysis of 15 trials, statin use perioperatively reduced mortality by 44 in noncardiac surgery.[78] Several studies have established the safety of statins in patients with liver disease, including those with compensated cirrhosis.[79] Thus, if needed for hyperlipidemia, statins may be used in patients with NASH-cirrhosis in the waiting list for LT, but data in the decompensated patient is missing, and some guidelines contraindicate their use in NASH patients with decompensated cirrhosis.[65] In 1 study, statins were started in 19 (23) of LT candidates with CAD, while aspirin was used in 30 (36). Use of statin therapy was not linked to hepatic decompensation, hospitalization or rise in MELD.[80] If needed after LT, pravastatin is the statin of choice as it does not interact with calcineurin inhibitors.

Patients with NASH are frequently obese and/or have diabetes, and both conditions are associated with an increased risk of mortality before and after LT due to CV events or sepsis.[81,82] Screening and treating diabetes on the waiting list is mandatory, preferentially using insulin sensitizers which could have beneficial effect in both insulin resistance and

NASH. There is no evidence for a histological efficacy of metformin in NASH based on 3 randomized studies, therefore metformin is not currently recommended for the treatment of NASH in the European Association for the Study of the Liver (EASL)-European Association for the Study of Diabetes (EASD)- European Association for the Study of Obesity (EASO) and American Association of the Study of Liver Disease Clinical Practice Guidelines.[83-85] Pioglitazone, a PPARg agonist, showed improvement in all histological features except for fibrosis and achieved resolution of NASH more often than placebo in 3 randomized controlled trials.[86] This option is currently the one with the strongest evidence to treat both NASH and diabetes but might increase weight gain and also increases the risk of bladder cancer. Other medications are emerging, such as liraglutide, an incretin mimetic that acts as an agonist of glucagon-like peptide-1 receptor.[87]

Nutrition is an integral part of patient care before LT. Nutrition status has been associated with various factors which are related to the success of LT such as morbidity, mortality, and length of hospital stay.[88] A high-calorie diet is associated with NAFLD. High fructose consumption may increase the risk of NASH and advanced fibrosis but data are controversial. While lifestyle correction measures are mandatory in all NASH patients, there does not seem to be any specific weight loss requirements for patients with end-stage liver disease or on the waiting list. In overweight/obese patients, a 7 to 10 weight loss is the target of most lifestyle interventions and may result in improvement of liver enzymes and histology.[89] Pragmatic approaches combine dietary restriction together with a progressive increase in aerobic exercise/resistance training. In a recent prospective, multicenter, uncontrolled pilot study, 16 weeks of diet and moderate exercise (personalized hypocaloric normoproteic diet and 60 min/wk of supervised physical activity) were found to be safe with reduction of portal pressure documented in 50 obese patients with cirrhosis and portal hypertension (from 13.9 ± 5.6 mm Hg to 12.3 ± 5.2 mm Hg; $P < 0.0001$).[90]

The main challenge in the pretransplant area is to diagnose malnutrition in NASH patients, even if obese. Several studies have demonstrated that around 25 of obese patients suffer from malnutrition.[89,91] It should be underlined that exercising under inadequate nutrients and proteins intake could be dangerous in patients with decompensated cirrhosis, given that it could promote further protein catabolism and loss of muscle mass. Therefore, a proper nutritional assessment and supplementation are indicated before initiating low-calorie diet and physical activity in this population.[89] A personalized, adapted physical activity program based on cycloergometry plus muscle strengthening according to ventilatory threshold for 12 weeks demonstrated to be safe and feasible in patients awaiting LT, improving peak VO_2, maximum power, ventilator threshold power, 6 minutes walking distance, and strength of knee extensor muscles.[92] A previous controlled pilot study demonstrated similar results in 9 cirrhotic patients who did 8-weeks of supervised exercise on a cycle ergometer 3 days/week.[93]

Finally, increasing evidence is now available supporting the presence of low bone mineral density and low vitamin D in patients with NAFLD as well as in cirrhotic patients.[94] Screening and surveillance of skeletal system regarding osteoporosis/osteomalacia in patients with NASH cirrhosis should be considered an important goal.

6 IS THERE ANY CIRCUMSTANCE WHERE OBESITY SHOULD CONTRAINDICATE LIVER TRANSPLANTATION?

Recommendations

Class I-III obesity alone does not constitute a contraindication for liver transplantation. However, in the presence of medical comorbidities, particularly concurrent diabetes, rigorous patient selection is strongly recommended. (Quality of evidence: Moderate; Strength of recommendation: Strong).

Background

One of the largest studies conducted by Nair et al[81] showed that "morbid obesity should be considered a relative contraindication to LT". In this SRTR-based review including over 23 000 recipients, morbid obesity was an independent predictor of mortality. However, this pre-MELD era study was criticized due to overestimation of obesity in the setting of ascites. In a prospective multicenter study including 1300 patients, corrected BMI after ascites volume removal was not found to be independently predictive of both patient and graft survival.[95] In each weight class, no difference was observed regarding postoperative complications and hospital stay. In a registry study based on the UNOS database (2003-2012),[96] of 57 255 LT performed during the study period, patients in all obesity classes had similar survival. Interestingly, overweight and class 1 obese patient had better survival compared to those with normal BMI values even after adjusting the data for both ascites and albumin levels. Presence of diabetes at the time of LT but not obesity was found to be an independent predictive factor for worse posttransplant survival (HR 1.29; CI 1.21-1.36). In addition, posttransplant survival among class I and II obese patients with concurrent diabetes was lower compared to patients with the same class obesity but without DM (for BMI ≥30 kg/m^2, 69 vs 75 , $P < 0.001$). CV cause of death and recurrent HCV and malignancy were more common in DM patients compared to non-DM patients. In another UNOS database[97] study that included 73 583 adult LT performed from 1987 to 2007, Dick et al reported that underweight status and class III obesity were associated with significantly lower posttransplant survival. One study also reported highest rate of waitlist dropout in these patients.[98] Finally, in a systematic literature search from 1990 until July 2013 where the main outcome was to evaluate the impact of obesity on survival in adult LT recipients, and where 13 studies with a total 2275 obese and 72 212 nonobese patients were included, BMI did not specifically impact patient survival.[99] Moreover, no differences in mortality were noted in subgroup analysis comparing different BMI thresholds. There were also no differences in survival when BMI was adjusted for ascites or in studies where the liver disease severity was similar.

In more recent reviews[100] investigating the impact of obesity on posttransplant outcome, there were conflicting data considering BMI cutoff values and outcome parameters. While 5 studies reported significant posttransplant mortality, particularly in patients with BMI ≥40 kg/m^2,[81,97,101,102] the remaining studies reported similar posttransplant outcome regardless of BMI cutoff (BMI > 35 kg/m^2 or BMI ≥ 40 kg/m).[8,95,96,103,104] Given the conflicting results about cutoff of BMI to determine the posttransplantation risk in obese patients, Barone et al suggested that BMI is not a satisfactory tool to stratify

the risk of obesity, and that visceral adipose tissue and muscle mass should be the parameters that should be added to complete an adequate pretransplantation evaluation.[100]

Interestingly, in the recent observational, retrospective population-based study using the UNOS/OPTN database that included 84 254 liver transplant candidates (2002-2013),[98] in addition to Class II (BMI, 35-39.9 kg/m^2) and III (BMI ≥40 kg/m^2) obesity, DM was also identified as a predictor of poor waitlist outcome. However, NASH etiology was not associated with a greater dropout risk (HR, 0.96; 95 CI, 0.84-1.09). The authors attributed these results to a selection bias considering that NASH patients were more frequently "ideal" candidates who presumably had undergone careful waitlist selection excluding those with CV disease.

In the study by Younossi et al,[105] the authors used data from the SRTR database between 1994 and 2013 that included over 80000 adult LT recipients. There was no association between BMI values and posttransplant mortality but the DM status of both patient (pretransplant [HR, 1.21; 95 CI, 1.12-1.30] or posttransplant [HR, 1.06; 95 CI, 1.02-1.11]) and donor (HR, 1.10; 95 CI, 1.02-1.19)] impacted posttransplant outcomes. Pretransplant DM was found to be associated with CV mortality. This study has limitations due to its retrospective design and incomplete clinical data. Another single-center retrospective design study by Dare et al[106] investigated the adequacy of using BMI to assess obesity in patients with end-stage liver disease. In addition, the authors also evaluated the potential impact of comorbidities, including obesity and DM, on outcome. Body fat percentage and BMI were compared, and BMI was found to be an adequate tool to determine obesity-associated risks in LT. On the other hand, obesity with concomitant DM was the strongest predictor of posttransplant event rates (CR, 1.75; $P < 0.001$).

In essence, most studies investigating the effect of obesity on posttransplant survival have found that outcome is similar in all classes of obesity. All but 1 out of 5 studies that showed significant increase of posttransplant mortality underscored that the negative effects were only observed with BMI of 40 kg/m^2 or greater. Most studies though, considering variable cutoff values of BMI, have reported similar posttransplant patient survival across all BMI categories in the absence of concurrent comorbidities. However, most studies are limited by lack of DM-specific data, or ascites status of the patients to provide corrected BMI and therefore, it is not plausible to draw definite conclusions regarding these associations. In addition, most studies are retrospective and/or include an unmatched patient population. It is still unclear whether different posttransplant outcomes will be achieved by performing immediate transplantation or, alternatively, undergoing optimal control of comorbidities such as obesity and diabetes before transplantation.

7 OPTIMAL TIME FOR BARIATRIC SURGERY: BEFORE, DURING, OR AFTER LIVER TRANSPLANTATION?

Recommendations

Bariatric surgery (BS) seems to be feasible and effective in morbid obese patients in the setting of liver transplantation, though associated to high postoperative complication rate; however, comparative data on long-term outcomes regarding optimal timing and type of bariatric procedure are lacking. Sleeve gastrectomy is currently the preferred approach. We suggest a tailored approach based on stringent selection criteria (quality of evidence: low, strength of recommendation: weak).

Background

Patients with morbid obesity have more infectious and surgical complications after LT[107,108] (see question 6). However, BS, which is performed to solve this problem, may also complicate posttransplantation period. It is still unknown "when is the optimal time to perform" BS and which BS procedure is best for this specific patient population, as all of them have some pros and cons to be considered.

Timing of the BS includes 3 options: *Bariatric first approach* for appropriate patients with low-MELD score will fulfill, in theory at least, the primary aim of this intervention and potentially improve the outcome of LT. Takata and Lin[109,110] reported promising results concerning metabolic comorbidities but significantly higher postoperative complication rates compared to the general population. Likewise, complication rates up to 35 were reported in patients incidentally diagnosed with cirrhosis after BS.[111] Secondly, *concomitant LT BS* procedure should only be performed in very selected patients, particularly with high MELD scores that are not appropriate for pretransplantation BS. In addition to increased operative time and complexity of the procedure (requiring both bariatric and transplant surgeon), early immunosuppressive therapy and poor nutritional status of the patients may complicate and limit the use of this approach.[112] The third option is *post transplantation BS*.[113,114] It will however not solve the problem of morbid obesity during waitlisting or in the immediate posttransplant period. Its only advantage is the proper selection of the patient requiring BS. However, disadvantages include difficult access to the abdomen and high postoperative morbidity and reoperation rates reported in the literature.

Technical feasibility and plausible posttransplant complications should be considered when choosing the type of bariatric procedure. In many studies, the most common procedure has been SG[115-119]—up to 100 in some series (excluding case reports). There are several advantages compared to Roux-en-Y gastric bypass (RYGB): it can be performed with minimal additional operative time and does not require intestinal anastomosis, it does maintain adequate immunosuppression levels without altering the absorption of medications and allows endoscopic access to the biliary system for management of posttransplant biliary complications. Although, long-term outcome regarding durability of SG is not available, reported series have demonstrated steady and gradual EWL . In the study by Takata et al including both RYGB and SG, SG reported acceptable EWL (25-75) but lower compared to RYGB.[109] Efficacy of gastric banding is limited to case reports. Disadvantages include placement of a foreign body in an immunosuppressed patient with a risk of gastric wall erosion and relative difficulty to access the gastrointestinal system.[115-119]

The NASH patients constitute a very specific group of increasing LT candidacy. Because these patients have already metabolic syndrome and other comorbidities, such as CV problems, bariatric first or LT-SG combined approach might be reasonable for these patients to manage these modifiable risk factors and improve both pre and posttransplant outcome (see questions 3-6). Strong recommendations cannot be made since most of the studies are case reports, small-sized, with a retrospective design and short mean follow-up, generally

less than 5 years.[115] Importantly, 1 small recent prospective study comparing LT alone to LT-SG demonstrated that patients who underwent LT + SG maintained a significantly higher percentage of total body weight loss after 3 years of follow-up. They also had a lower prevalence of hypertension, insulin resistance, and hepatic steatosis and required fewer antihypertensive medications and lipid agents at last follow-up.[120] In the light of the limited available data, pretransplantation BS might be a reasonable approach for obese patients with low MELD score, whereas concomitant/posttransplantation BS might be considered for highly selected patients. Bariatric first or concomitant approach might be reasonable for NASH patients who have pre-LT comorbidities including metabolic and CV problems that may complicate the post-transplant period. The optimal type of BS remains unclear, but SG seems to be the preferred approach by most surgeons.[115-117,120]

8 DONOR STEATOSIS: HOW RELEVANT IS IT FOR LT IN NASH PATIENTS

Recommendation

While steatosis, particularly moderate to severe macrosteatosis, is considered an independent risk factor for post-transplant worse outcome, there is not enough evidence to support a different approach to donor steatosis in NASH as opposed to non-NASH candidates. (Quality of evidence: low; Strength of recommendation: weak IIb).

Background

Increased fatty liver disease in the donor population is an indirect effect of the increasing rates of NAFLD in the worldwide population with prevalence rates estimated to be around 25 with significant geographic variability. Hepatic steatosis was seen on biopsy in 76 of potential living liver donors with a BMI greater than 28.[121] In a recent study evaluating 612 living-related liver donor candidates between 2001 and 2017, 196 (32) liver biopsies had pathological findings, of which fatty changes was the commonest found in 86 livers (44).[122] There are insufficient data on the impact of donor steatosis in patients with NASH-related cirrhosis who receive a LT. As with other etiologies, it is expected that donor steatosis will disappear soon after LT and the main impact is perceived to be in the immediate posttransplant period.[123] Steatotic donor livers, particularly those with greater than 60 of steatosis, are associated with poor graft function due to ischemia-reperfusion injury.[124] The outcomes of transplants with donor liver steatosis 30 to 60 varies and depends on recipient factors as well, with acceptable outcomes only when the cumulative risk at transplant is low.[125] Existing evidence does not support a different selection process or approach to recipients with NASH cirrhosis.

REFERENCES

1. Atkins D, Best D, Briss PA, et al. Grading quality of evidence and strength of recommendations. *BMJ*. 2004;328:1490.
2. Wang X, Li J, Riaz DR, et al. Outcomes of liver transplantation for nonalcoholic steatohepatitis: a systematic review and meta-analysis. *Clin Gastroenterol Hepatol*. 2014;12:394 402.e391.
3. Charlton MR, Burns JM, Pedersen RA, et al. Frequency and outcomes of liver transplantation for nonalcoholic steatohepatitis in the United States. *Gastroenterology*. 2011;141:1249 1253.
4. Afzali A, Berry K, Ioannou GN. Excellent posttransplant survival for patients with nonalcoholic steatohepatitis in the United States. *Liver Transpl*. 2012; 18:29 37.
5. Cholankeril G, Wong RJ, Hu M, et al. Liver transplantation for nonalcoholic steatohepatitis in the US: temporal trends and outcomes. *Dig Dis Sci*. 2017;62:2915 2922.
6. Wong RJ, Chou C, Bonham CA, et al. Improved survival outcomes in patients with non-alcoholic steatohepatitis and alcoholic liver disease following liver transplantation: an analysis of 2002-2012 United Network for Organ Sharing data. *Clin Transpl*. 2014;28:713 721.
7. Singal AK, Hasanin M, Kaif M, et al. Nonalcoholic steatohepatitis is the most rapidly growing indication for simultaneous liver kidney transplantation in the United States. *Transplantation*. 2016;100:607 612.
8. Sadler EM, Mehta N, Bhat M, et al. Liver transplantation for NASH-related hepatocellular carcinoma versus non-NASH etiologies of hepatocellular carcinoma. *Transplantation*. 2018;102:640 647.
9. Kemmer N, Neff GW, Franco E, et al. Nonalcoholic fatty liver disease epidemic and its implications for liver transplantation. *Transplantation*. 2013;96:860 862.
10. Agopian VG, Kaldas FM, Hong JC, et al. Liver transplantation for nonalcoholic steatohepatitis: the new epidemic. *Ann Surg*. 2012;256:624 633.
11. Barritt AS 4th, Dellon ES, Kozlowski T, et al. The influence of nonalcoholic fatty liver disease and its associated comorbidities on liver transplant outcomes. *J Clin Gastroenterol*. 2011;45:372 378.
12. Houlihan DD, Armstrong MJ, Davidov Y, et al. Renal function in patients undergoing transplantation for nonalcoholic steatohepatitis cirrhosis: time to reconsider immunosuppression regimens? *Liver Transpl*. 2011; 17:1292 1298.
13. Park CW, Tsai NT, Wong LL. Implications of worse renal dysfunction and medical comorbidities in patients with NASH undergoing liver transplant evaluation: impact on MELD and more. *Clin Transplant*. 2011;25:E606 E611.
14. Vanwagner LB, Bhave M, Te HS, et al. Patients transplanted for nonalcoholic steatohepatitis are at increased risk for postoperative cardiovascular events. *Hepatology*. 2012;56:1741 1750.
15. Bhagat V, Mindikoglu AL, Nudo CG, et al. Outcomes of liver transplantation in patients with cirrhosis due to nonalcoholic steatohepatitis versus patients with cirrhosis due to alcoholic liver disease. *Liver Transpl*. 2009; 15:1814 1820.
16. Kennedy C, Redden D, Gray S, et al. Equivalent survival following liver transplantation in patients with non alcoholic steatohepatitis compared with patients with other liver diseases. *HPB Oxford)*. 2012;14:625 634.
17. Malik SM, deVera ME, Fontes P, et al. Outcome after liver transplantation for NASH cirrhosis. *Am J Transplant*. 2009;9:782 793.
18. Bosch J, Harrison SA, Abdelmalek MF, et al. Hepatic venous pressure gradient HVPG) predicts clinical disease progression in patients with compensated cirrhosis due to NASH. *Hepatology*. 2017;66:244A.
19. Hui JM, Kench JG, Chitturi S, et al. Long-term outcomes of cirrhosis in nonalcoholic steatohepatitis compared with hepatitis C. *Hepatology*. 2003;38:420 427.
20. Yatsuji S, Hashimoto E, Tobari M, et al. Clinical features and outcomes of cirrhosis due to non-alcoholic steatohepatitis compared with cirrhosis caused by chronic hepatitis C. *J Gastroenterol Hepatol*. 2009;24:248 254.
21. Sanyal AJ, Banas C, Sargeant C, et al. Similarities and differences in outcomes of cirrhosis due to nonalcoholic steatohepatitis and hepatitis C. *Hepatology*. 2006;43:682 689.
22. O'Leary JG, Landaverde C, Jennings L, et al. Patients with NASH and cryptogenic cirrhosis are less likely than those with hepatitis C to receive liver transplants. *Clin Gastroenterol Hepatol*. 2011;9:700 704.e1.
23. Young K, Aguilar M, Gish R, et al. Lower rates of receiving model for end-stage liver disease exception and longer time to transplant among non-alcoholic steatohepatitis hepatocellular carcinoma. *Liver Transpl*. 2016; 22:1356 1366.
24. Lonardo A, Nascimbeni F, Mantovani A, et al. Hypertension, diabetes, atherosclerosis and NASH: cause or consequence? *J Hepatol*. 2018; 68:335 352.
25. Targher G, Byrne CD, Lonardo A, et al. Non-alcoholic fatty liver disease and risk of incident cardiovascular disease: a meta-analysis. *J Hepatol*. 2016;65:589 600.
26. Manoushagian S, Meshkov A. Evaluation of solid organ transplant candidates for coronary artery disease. *Am J Transplant*. 2014;14:2228 2234.
27. Targher G, Bertolini L, Padovani R, et al. Increased prevalence of cardiovascular disease in Type 2 diabetic patients with nonalcoholic fatty liver disease. *Diabet Med*. 2006;23:403 409.
28. Arslan U, Turkoglu S, Balcioglu S, et al. Association between nonalcoholic fatty liver disease and coronary artery disease. *Coron Artery Dis*. 2007;18:433 436.

29. Choi SY, Kim D, Kim HJ, et al. The relation between non-alcoholic fatty liver disease and the risk of coronary heart disease in Koreans. *Am J Gastroenterol*. 2009;104:1953 1960.
30. Lu H, Zeng L, Liang B, et al. High prevalence of coronary heart disease in type 2 diabetic patients with non-alcoholic fatty liver disease. *Arch Med Res*. 2009;40:571 575.
31. Plotkin JS, Johnson LB, Rustgi V, et al. Coronary artery disease and liver transplantation: the state of the art. *Liver Transpl*. 2000;6 Suppl 1):53 56.
32. Yotti R, Ripoll C, Bermejo J, et al. Cardiac function, a key component in evaluation for liver transplant. *Liver Transpl*. 2018;24:7 8.
33. Krowka MJ, Fallon MB, Kawut SM, et al. International Liver Transplant Society Practice Guidelines: diagnosis and management of hepatopulmonary syndrome and portopulmonary hypertension. *Transplantation*. 2016;100: 1440 1452.
34. Konerman MA, Fritze D, Weinberg RL, et al. Incidence of and risk assessment for adverse cardiovascular outcomes after liver transplantation: a systematic review. *Transplantation*. 2017;101:1645 1657.
35. Darstein F, Hoppe-Lotichius M, Vollmar J, et al. Pretransplant coronary artery disease is a predictor for myocardial infarction and cardiac death after liver transplantation. *Eur J Intern Med*. 2017;S0953-6205:30484 30483.
36. Tovikkai C, Charman SC, Praseedom RK, et al. Time-varying impact of comorbidities on mortality after liver transplantation: a national cohort study using linked clinical and administrative data. *BMJ Open*. 2015;5: e006971.
37. Safadi A, Homsi M, Maskoun W, et al. Perioperative risk predictors of cardiac outcomes in patients undergoing liver transplantation surgery. *Circulation*. 2009;120:1189 1194.
38. Fouad TR, Abdel-Razek WM, Burak KW, et al. Prediction of cardiac complications after liver transplantation. *Transplantation*. 2009;87:763 770.
39. Filì D, Vizzini G, Biondo D, et al. Clinical burden of screening asymptomatic patients for coronary artery disease prior to liver transplantation. *Am J Transplant*. 2009;9:1151 1157.
40. Patel SS, Nabi E, Guzman L, et al. Coronary artery disease in decompensated patients undergoing liver transplantation evaluation. *Liver Transpl*. 2018;24:333 342.
41. An J, Shim JH, Kim SO, et al. Prevalence and prediction of coronary artery disease in patients with liver cirrhosis: a registry-based matched case-control study. *Circulation*. 2014;130:1353 1362.
42. Fleisher LA, Fleischmann KE, Auerbach AD, et al. 2014 ACC/AHA guideline on perioperative cardiovascular evaluation and management of patients undergoing noncardiac surgery: executive summary a report of the American College of Cardiology/American Heart Association task force on practice guidelines. *Circulation*. 2014;130:2215 2245.
43. Ripoll C, Yotti R, Bermejo J, et al. The heart in liver transplantation. *J Hepatol*. 2011;54:810 822.
44. Harinstein ME, Flaherty JD, Ansari AH, et al. Predictive value of dobutamine stress echocardiography for coronary artery disease detection in liver transplant candidates. *Am J Transplant*. 2008;8:1523 1528.
45. Donovan CL, Marcovitz PA, Punch JD, et al. Two-dimensional and dobutamine stress echocardiography in the preoperative assessment of patients with end-stage liver disease prior to orthotopic liver transplantation. *Transplantation*. 1996;61:1180 1188.
46. Nicolau-Raducu R, Gitman M, Ganier D, et al. Adverse cardiac events after orthotopic liver transplantation: a cross-sectional study in 389 consecutive patients. *Liver Transpl*. 2015;21:13 21.
47. Nguyen P, Plotkin J, Fishbein TM, et al. Dobutamine stress echocardiography in patients undergoing orthotopic liver transplantation: a pooled analysis of accuracy, perioperative and long term cardiovascular prognosis. *Int J Cardiovasc Imaging*. 2013;29:1741 1748.
48. Snipelisky D, Levy M, Shapiro B. Utility of dobutamine stress echocardiography as part of the pre-liver transplant evaluation: an evaluation of its efficacy. *Clin Cardiol*. 2014;37:468 472.
49. Davidson CJ, Gheorghiade M, Flaherty JD, et al. Predictive value of stress myocardial perfusion imaging in liver transplant candidates. *Am J Cardiol*. 2002;89:359 360.
50. Bhutani S, Tobis J, Gevorgyan R, et al. Accuracy of stress myocardial perfusion imaging to diagnose coronary artery disease in end stage liver disease patients. *Am J Cardiol*. 2013;111:1057 1061.
51. Soldera J, Camazzola F, Rodríguez S, et al. Dobutamine stress echocardiography, myocardial perfusion scintigraphy, invasive coronary angiography, and post-liver transplantation events: systematic review and meta-analysis. *Clin Transplant*. 2018;32:e13222.
52. Maddur H, Bourdillon PD, Liangpunsakul S, et al. Role of cardiac catheterization and percutaneous coronary intervention in the preoperative assessment and management of patients before orthotopic liver transplantation. *Liver Transpl*. 2014;20:664 672.
53. Wray C, Scovotti JC, Tobis J, et al. Liver transplantation outcome in patients with angiographically proven coronary artery disease: a multi-institutional study. *Am J Transplant*. 2013;13:184 191.
54. Satapathy SK, Vanatta JM, Helmick RA, et al. Outcome of liver transplant recipients with revascularized coronary artery disease: a comparative analysis with and without cardiovascular risk factors. *Transplantation*. 2017; 101:793 803.
55. Snipelisky DF, McRee C, Seeger K, et al. Coronary interventions before liver transplantation might not avert postoperative cardiovascular events. *Tex Heart Inst J*. 2015;42:438 442.
56. Baibhav B, Mahabir CA, Xie F, et al. Predictive value of dobutamine stress perfusion echocardiography in contemporary end-stage liver disease. *J Am Heart Assoc*. 2017;6:e005102.
57. Choi JM, Kong YG, Kang JW, et al. Coronary computed tomography angiography in combination with coronary artery calcium scoring for the preoperative cardiac evaluation of liver transplant recipients. *Biomed Res Int*. 2017;2017:4081525.
58. McAvoy NC, Kochar N, McKillop G, et al. Prevalence of coronary artery calcification in patients undergoing assessment for orthotopic liver transplantation. *Liver Transpl*. 2008;14:1725 1731.
59. Jodocy D, Abbrederis S, Graziadei IW, et al. Coronary computer tomographic angiography for preoperative risk stratification in patients undergoing liver transplantation. *Eur J Radiol*. 2012;81:2260 2264.
60. Kong YG, Kang JW, Kim YK, et al. Preoperative coronary calcium score is predictive of early postoperative cardiovascular complications in liver transplant recipients. *Br J Anaesth*. 2015;114:437 443.
61. VanWagner LB, Lapin B, Levitsky J, et al. High early cardiovascular mortality after liver transplantation. *Liver Transpl*. 2014;20:1306 1316.
62. Keeffe BG, Valantine H, Keeffe EB. Detection and treatment of coronary artery disease in liver transplant candidates. *Liver Transpl*. 2001;7: 755 761.
63. Appleton CP, Hurst RT. Reducing coronary artery disease events in liver transplant patients: moving toward identifying the vulnerable patient. *Liver Transpl*. 2008;14:1691 1693.
64. Carey WD, Dumot JA, Pimentel RR, et al. The prevalence of coronary artery disease in liver transplant candidates over age 50. *Transplantation*. 1995;59:859 864.
65. Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of nonalcoholic fatty liver disease: practice guidance from the American Association for the Study of Liver Diseases. *Hepatology*. 2018;67:328 357.
66. Vargas JI, Arrese M, Shah VH, et al. Use of statins in patients with chronic liver disease and cirrhosis: current views and prospects. *Curr Gastroenterol Rep*. 2017;19:43.
67. Dongiovanni P, Petta S, Mannisto V, et al. Statin use and non-alcoholic steatohepatitis in at risk individuals. *J Hepatol*. 2015;63:705 712.
68. Fernandez-Miranda C, Perez-Carreras M, Colina F, et al. A pilot trial of fenofibrate for the treatment of non-alcoholic fatty liver disease. *Dig Liver Dis*. 2008;40:200 205.
69. Goh GB, Pagadala MR, Dasarathy J, et al. Renin-angiotensin system and fibrosis in non-alcoholic fatty liver disease. *Liver Int*. 2015;35:979 985.
70. Musso G, Gambino R, Tabibian JH, et al. Association of non-alcoholic fatty liver disease with chronic kidney disease: a systematic review and meta-analysis. *PLoS Med*. 2014;11:e1001680.
71. Di Maira T, Rubin A, Puchades L, et al. Framingham score, renal dysfunction, and cardiovascular risk in liver transplant patients. *Liver Transpl*. 2015;21:812 822.
72. VanWagner LB, Montag S, Zhao L, et al. Cardiovascular disease outcomes related to early stage renal impairment following liver transplantation. *Transplantation*. Published online March 20, 2018. DOI:10.1097/TP.0000000000002175.
73. VanWagner LB, Ning H, Whitsett M, et al. A point-based prediction model for cardiovascular risk in orthotopic liver transplantation: The CAR-OLT score. *Hepatology*. 2017;66:1968 1979.
74. Alvarez-Sotomayor D, Satorres C, Rodríguez-Medina B, et al. Controlling diabetes after liver transplantation: room for improvement. *Transplantation*. 2016;100:e66 e73.
75. Martínez-Saldivar B, Prieto J, Berenguer M, et al. Control of blood pressure in liver transplant recipients. *Transplantation*. 2012;93:1031 1037.
76. Dunkelgrun M, Boersma E, Schouten O, et al. Bisoprolol and fluvastatin for the reduction of perioperative cardiac mortality and myocardial infarction in intermediate-risk patients undergoing noncardiovascular surgery: a randomized controlled trial DECREASE-IV). *Ann Surg*. 2009;249:921 926.
77. Devereaux PJ, Yang H, Yusuf S, et al. Effects of extended-release metoprolol succinate in patients undergoing non-cardiac surgery POISE trial): a randomised controlled trial. *Lancet*. 2008;371:1839 1847.

78. Hindler K, Shaw AD, Samuels J, et al. Improved postoperative outcomes associated with preoperative statin therapy. *Anesthesiology*. 2006;105: 1260 1272; quiz 1289 1290.
79. Lewis JH, Mortensen ME, Zweig S, et al. Efficacy and safety of high-dose pravastatin in hypercholesterolemic patients with well-compensated chronic liver disease: results of a prospective, randomized, double-blind, placebo-controlled, multicenter trial. *Hepatology*. 2007;46:1453 1463.
80. Patel SS, Guzman L, Lin FP, et al. Utilization of aspirin and statin in management of coronary artery disease in patients with cirrhosis undergoing liver transplant evaluation. *Liver Transpl*. Published online April 6, 2018. DOI:10.1002/lt.25067.
81. Nair S, Verma S, Thuluvath PJ. Obesity and its effect on survival in patients undergoing orthotopic liver transplantation in the United States. *Hepatology*. 2002;35:105 109.
82. Yoo HY, Thuluvath PJ. The effect of insulin-dependent diabetes mellitus on outcome of liver transplantation. *Transplantation*. 2002;74:1007 1012.
83. Haukeland JW, Konopski Z, Eggesbo HB, et al. Metformin in patients with non-alcoholic fatty liver disease: a randomized, controlled trial. *Scand J Gastroenterol*. 2009;44:853 860.
84. Shields WW, Thompson KE, Grice GA, et al. The Effect of metformin and standard therapy versus standard therapy alone in nondiabetic patients with insulin resistance and nonalcoholic steatohepatitis NASH): a pilot trial. *Therap Adv Gastroenterol*. 2009;2:157 163.
85. European Association for the Study of the L, European Association for the Study of D, European Association for the Study of O. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol*. 2016;64:1388 1402.
86. Sanyal AJ, Chalasani N, Kowdley KV, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med*. 2010;362:1675 1685.
87. Armstrong MJ, Gaunt P, Aithal GP, et al. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. *Lancet*. 2016;387: 679 690.
88. McCullough AJ, Bugianesi E. Protein-calorie malnutrition and the etiology of cirrhosis. *Am J Gastroenterol*. 1997;92:734 738.
89. Berzigotti A, Saran U, Dufour JF. Physical activity and liver diseases. *Hepatology*. 2016;63:1026 1040.
90. Berzigotti A, Albillos A, Villanueva C, et al. Effects of an intensive lifestyle intervention program on portal hypertension in patients with cirrhosis and obesity: the SportDiet study. *Hepatology*. 2017;65:1293 1305.
91. Leibovitz E, Giryes S, Makhline R, et al. Malnutrition risk in newly hospitalized overweight and obese individuals: Mr NOI. *Eur J Clin Nutr*. 2013;67: 620 624.
92. Debette-Gratien M, Tabouret T, Antonini MT, et al. Personalized adapted physical activity before liver transplantation: acceptability and results. *Transplantation*. 2015;99:145 150.
93. Zenith L, Meena N, Ramadi A, et al. Eight weeks of exercise training increases aerobic capacity and muscle mass and reduces fatigue in patients with cirrhosis. *Clin Gastroenterol Hepatol*. 2014;12:1920 1926.e1922.
94. Luger M, Kruschitz R, Kienbacher C, et al. Prevalence of liver fibrosis and its association with non-invasive fibrosis and metabolic markers in morbidly obese patients with vitamin D deficiency. *Obes Surg*. 2016;26:2425 2432.
95. Leonard J, Heimbach JK, Malinchoc M, et al. The impact of obesity on long-term outcomes in liver transplant recipients-results of the NIDDK liver transplant database. *Am J Transpl*. 2008;8:667 672.
96. Wong RJ, Cheung R, Perumpail RB, et al. Diabetes mellitus, and not obesity, is associated with lower survival following liver transplantation. *Dig Dis Sci*. 2015;60:1036 1044.
97. Dick AA, Spitzer AL, Seifert CF, et al. Liver transplantation at the extremes of the body mass index. *Liver Transpl*. 2009;15:968 977.
98. Dodge JL, Roberts J, Brandman D. Weighing the risks: morbid obesity and diabetes are associated with increased risk of death on the liver transplant waiting list. *Liver Int*. 2018;38:553 563.
99. Saab S, Lalezari D, Pruthi P, et al. The impact of obesity on patient survival in liver transplant recipients: a meta-analysis. *Liver Int*. 2015;35:164 170.
100. Barone M, Viggiani MT, Avolio AW, et al. Obesity as predictor of postoperative outcomes in liver transplant candidates: review of the literature and future perspectives. *Dig Liver Dis*. 2017;49:957 966.
101. Rustgi VK, Marino G, Rustgi S. Impact of body mass index on graft failure and overall survival following liver transplantation. *Clin Transplant*. 2004; 18:634 637.
102. Conzen KD, Vachharajani N, Collins KM, et al. Morbid obesity in liver transplant recipients adversely affects longterm graft and patient survival in a single-institution analysis. *HPB Oxford)*. 2015;17:251 257.
103. Pelletier SJ, Maraschio MA, Schaubel DE, et al. Survival benefit of kidney and liver transplantation for obese patients on the waiting list. *Clin Transpl*. 2003;17:77 88.
104. Singhal A, Wilson GC, Wima K, et al. Impact of recipient morbid obesity on outcomes after liver transplantation. *Transpl Int*. 2015;28:148 155.
105. Younossi ZM, Stepanova M, Saab S, et al. The impact of type 2 diabetes and obesity on the long-term outcomes of more than 85000 liver transplant recipients in the US. *Aliment Pharmacol Ther*. 2014;40:686 694.
106. Dare AJ, Plank LD, Phillips AR, et al. Additive effect of pre- transplantation obesity, diabetes, and cardiovascular risk factors on outcomes after liver transplantation. *Liver Transpl*. 2014;20:281 290.
107. Hakeem AR, Cockbain AJ, Raza SS, et al. Increased morbidity in overweight and obese liver transplant recipients: a single-center experience of 1325 patients from the United Kingdom. *Liver Transpl*. 2013;19:551 562.
108. LaMattina JC, Foley DP, Fernandez LA, et al. Complications associated with liver transplantation in the obese recipient. *Clin Transplant*. 2012; 26:910 918.
109. Takata MC, Campos GM, Ciovica R, et al. Laparoscopic bariatric surgery improves candidacy in morbidly obese patients awaiting transplantation. *Surg Obes Relat Dis*. 2008;4:159 164.
110. Lin MYC, Mehdi Tavakol M, Sarin A, et al. Laparoscopic sleeve gastrectomy is safe and efficacious for pretransplant candidates. *Surg Obes Relat Dis*. 2013;9:653 658.
111. Braunfeld MY, Chan S, Pregler J, et al. Liver transplantation in the morbidly obese. *J Clin Anesth*. 1996;8:585 590.
112. Heimbach JK, Watt KD, Poterucha JJ, et al. Combined liver transplantation and gastric sleeve resection for patients with medically complicated obesity and end-stage liver disease. *Am J Transplant*. 2013;13:363 368.
113. Butte J, Devaud N, Jarufe NP, et al. Sleeve gastrectomy as treatment for severe obesity after orthotopic liver transplantation. *Obes Surg*. 2007;17: 1517 1519.
114. Lin MY, Tavakol MM, Sarin A, et al. Safety and feasibility of sleeve gastrectomy in morbidly obese patients following liver transplantation. *Surg Endosc*. 2013;27:81 85.
115. Lazzati A, Iannelli A, Schneck AS, et al. Bariatric surgery and liver transplantation: a systematic review a new frontier for bariatric surgery. *Obes Surg*. 2015;25:134 142.
116. Lazzati A, Guy-Lachuer R, Delaunay V, et al. Bariatric surgery trends in France: 2005 2011. *Surg Obes Relat Dis*. 2014;10:328 334.
117. Shimizu H, Phuong V, Maia M, et al. Bariatric surgery in patients with liver cirrhosis. *Surg Obes Relat Dis*. 2013;9:1 6.
118. Dallal RM, Mattar SG, Lord JL, et al. Results of laparoscopic gastric bypass in patients with cirrhosis. *Obes Surg*. 2004;14:47 53.
119. Tariq N, Moore LW, Sherman V. Bariatric surgery and end-stage organ failure. *Surg Clin North Am*. 2013;96:1359 1371.
120. Zamora-Valdes D, Watt KD, Kellogg TA, et al. Long-term outcomes of patients undergoing simultaneous liver transplantation and sleeve gastrectomy. *Hepatology*. Published online February 19, 2018. DOI:10. 1002/hep.29848.
121. Rinella ME, Alonso E, Rao S, et al. Body mass index as a predictor of hepatic steatosis in living liver donors. *Liver Transpl*. 2001;7:409 414.
122. Ayvazoglu Soy EH, Boyvat F, Ozdemir BH, et al. Liver biopsy results in potential donor evaluation in living related liver transplant. *Exp Clin Transplant*. 2018;16 Suppl 1):35 37.
123. EASL Clinical Practice Guidelines: Liver transplantation. *J Hepatol*. 2016; 64:433 485.
124. Chu MJ, Dare AJ, Phillips AR, et al. Donor hepatic steatosis and outcome after liver transplantation: a systematic review. *J Gastrointest Surg*. 2015;19:1713 1724.
125. Dutkowski P, Schlegel A, Slankamenac K, et al. The use of fatty liver grafts in modern allocation systems: risk assessment by the balance of risk BAR) score. *Ann Surg*. 2012;256:861 868; discussion 868 869.

Résumé

Titre : Marqueurs diagnostiques et pronostiques de la stéatose et stéatohépatite métabolique.

Mots clés : lipides, carcinome hépatocellulaire, stéatohépatite

L'obésité est un problème majeur de santé publique en France puisque 50% de la population est en surpoids ou obèse. Plusieurs complications hépatiques de l'obésité existent dont la NASH, pathologie caractérisée par l'association de lésions histologiques de stéatose hépatique et d'hépatite, d'anomalies des tests hépatiques et par l'absence de maladie hépatique connue, en particulier toxique (alcool) ou virale. Chez un tiers des patients, la NASH conduit à l'apparition d'une fibrose puis d'une cirrhose. Elle favorise également l'apparition du carcinome hépatocellulaire. La physiopathologie de la NASH est caractérisée par une dérégulation du métabolisme lipidique qui conduit à l'accumulation de lipides au niveau des hépatocytes. Cette accumulation de lipides est toxique et une des raisons de l'insulinorésistance et du développement d'un diabète de type II. Toutes les étapes du métabolisme lipidique sont affectées par une accumulation de triglycérides, une augmentation de la lipogenèse hépatique et une diminution de la ß-oxydation. La composition et le rôle des lipides comme promoteur de la NASH sont de plus en plus étudiés. Le premier volet de la thèse concerne la mise en évidence de marqueurs diagnostiques de la NASH. Dans cette étude, nous avons établi pour la première fois une signature lipidique de la stéatohépatite non alcoolique sur la base de la quantification de 32 lipides.Aucun des lipides identifiés ne permettait par lui-même de discriminer la stéatose de la NASH, mais en revanche, la signature lipidique globale permettait de distinguer les témoins patients des NAFL et des NASH. Nous avons également mis en évidence une dérégulation de la voie métabolique impliquée dans la synthèse des acides gras dans la NASH. Cette dérégulation a été observée, dans notre étude, aussi bien chez l'homme que dans les modèles animaux. Le deuxième volet avait pour objectif l'identification de nouveaux marqueurs pronostiques hépatiques de la NASH. L'analyse par microarray d'expression de gènes a montré 1549 gènes discriminant les patients ayant une stéatopathie, NAFL ou NASH, des obèses sains ou des contrôles. Parmi eux, 58 gènes discriminaient la NASH de la stéatose simple. Ces gènes étaient impliqués dans le remodelage de la matrice extracellulaire et l'inflammation. Le gène le plus discriminant était FABP4 (protéine de liaison aux acides gras 4). Parmi les gènes fortement associés à une expression élevée de FABP4, la métalloprotéinase-9 de matrice (MMP9) était surexprimée chez 55% des patients NASH. Nous avons identifié un total de 330 gènes régulés de manière différentielle, dont 229 gènes étaient surexprimés chez des patients NASH présentant un niveau d'expression élevé de MMP9. En utilisant les niveaux d'expression génique des gènes FABP4 et MMP9 hépatiques comme indicateurs de la progression de la maladie dans une cohorte indépendante de patients atteints de NAFLD, nous avons identifié les patients atteints de NAFL et NASH susceptibles d'avoir un mauvais pronostic.

Enfin, dans le troisième volet, nous nous sommes intéressés à la valeur diagnostique et pronostique de la stéatose des greffons hépatiques, mesurée par FTIR (Microspectroscopie à Transformée de Fourier en Infrarouge). En effet, la stéatose en particulier lorsqu'elle dépasse 60% et macrovacuolaire, est connue pour impacter significativement la fonction et la survie des greffons hépatiques. Dans notre étude, parmi 58 prélèvements de greffons, le pourcentage moyen de stéatose macrovacuolaire et de stéatose microvésiculaire, évalué par le pathologiste, était de 2% à 30%, respectivement. La concentration moyenne en triglycérides hépatiques mesurée par chromatographie couplée en phase gazeuse à la spectrométrie était de 214 [10-1045] nmol/mg de tissu hépatique. L'estimation de la teneur en triglycérides obtenue par FTIR était significativement corrélée (r2 = 0,812) avec les résultats de la concentration moyenne en triglycérides hépatiques mesurée par chromatographie couplée en phase gazeuse à la spectrométrie. Trente-quatre (58%) patients ont présenté des complications définies par un stade Dindo-Clavien ≥2, dont 2 non-fonction primaire du greffon et 5 décès. Le seuil le plus discriminant entre le niveau de triglycérides et l'échec de la TH était de 59,29 et 54,02 nmol/mg de tissu hépatique obtenu par spectrométrie et FTIR, respectivement. La quantification du contenu hépatique en triglycérides par GC / MS était significativement associée à la survie du patient à la fin du suivi ($p<0,0001$) et à un échec de la transplantation ($p <0,0001$). L'estimation du contenu hépatique en triglycérides à l'aide de FTIR était significativement associée à la survie après greffe d'un an ($p<0,0001$).

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Summary

Title : Diagnostic and prognostic markers of NASH

Keywords : lipids, hepatocellular carcinoma, steatohepatitis

Obesity is a major public health problem in France since 50% of the population has overweight. Several hepatic complications of obesity exist including NASH, pathology characterized by the combination of histological lesions of hepatic steatosis and hepatitis, liver test abnormalities and the absence of known liver disease, particularly toxic (alcohol) or virus. In one third of patients, NASH leads to fibrosis and then cirrhosis. It also promotes the development of hepatocellular carcinoma.

The pathophysiology of NASH is characterized by a deregulation of lipid metabolism that leads to the accumulation of lipids in the hepatocytes. This accumulation of lipids is toxic and one of the causes of insulin resistance and the development of diabetes mellitus. All stages of lipid metabolism are affected by an accumulation of triglycerides, an increase in hepatic lipogenesis and a decrease in ß-oxidation. The composition and the role of lipids as a promoter of NASH is being increasingly studied.

The first part of the thesis concerns the detection of diagnostic markers of NASH. In this study, we established for the first time a lipid signature of nonalcoholic steatohepatitis by quantification of 32 lipids. The overall lipid signature allowed distinguishing controls from NAFL and NASH. We have also demonstrated a deregulation of the metabolic pathway involved in the synthesis of fatty acids in NASH. This deregulation has been observed in both humans and animal models in our study.

The second part aimed to identify new hepatic prognostic markers of NASH. Microarray analysis of gene expression showed 1549 genes discriminating patients with NAFL or NASH, healthy obese or controls. Among them, 58 genes discriminated NASH from simple steatosis. These genes were involved in extracellular matrix remodeling and inflammation. The most discriminating gene was FABP4 (fatty acid binding protein 4). Among genes strongly associated with high expression of FABP4, matrix metalloproteinase-9 (MMP9) was overexpressed in 55% of NASH patients. We identified a total of 330 differentially regulated genes, of which 229 genes were overexpressed in NASH patients with high levels of MMP9 expression. Using the gene expression levels of the liver FABP4 and MMP9 genes as indicators of disease progression in an independent cohort of NAFLD patients, we identified patients with NAFL and NASH who may have a poor prognosis.

Finally, in the third part, we looked at the diagnostic and prognostic value of steatosis of liver grafts, measured by FTIR (Infrared Fourier Transform Microspectroscopy). Indeed, steatosis, when it exceeds 60% and is macrovacuolar, is known to significantly impact the function and survival of liver grafts. In our study, among 58 graft samples, the average percentage of macrovacuolar steatosis and microvesicular steatosis assessed by the pathologist was 2% to 30%, respectively. The average concentration of liver triglycerides measured by gas chromatography-spectrometry was 214 [10-1045] nmol/mg liver tissue. The FTIR triglyceride content estimate was significantly correlated ($r2=0.812$) with the results of the average hepatic triglyceride concentration measured by gas chromatography-spectrometry. Thirty-four (58%) patients had complications defined by a Dindo-Clavien stage ≥2, including 2 non-primary graft function and 5 deaths. The most discriminating threshold between triglyceride level and TH failure was 59.29 and 54.02 nmol/mg hepatic tissue obtained by spectrometry and FTIR, respectively. Quantification of hepatic triglyceride content by GC/MS was significantly associated with patient survival at the end of follow-up ($p < 0.0001$) and failure of transplantation ($p<0.0001$). Estimating hepatic triglyceride content using FTIR was significantly associated with one-year post-transplant survival ($p<0.0001$).