Abstract

Les hépatopathies alcoolique et non-alcoolique sont responsables d'une importante morbidité et mortalité. L'absence d'un traitement efficace prouvé pour ces maladies découle d'une compréhension imparfaite de leur pathogenèse. Les mécanismes physiopathologiques menant à ces hépatopathies sont l'objet d'intenses recherches expérimentales et cliniques. Dans ce contexte, un concept ayant évolué depuis plus d'un siècle a abouti, récemment, à une approche révolutionnaire du problème. Ce travail examine l'assertion fascinante que des altérations dans la composition de la flore microbienne intestinale, engendrées par l'alcool et/ou un régime riche en graisses, jouent un rôle déterminant dans l'apparition et la progression de ces affections. Une microflore « dysbiotique » répand un nombre accru d'endotoxines qui, lorsque la perméabilité intestinale est augmentée, atteignent le foie où elles activent les cellules de Kupffer, engendrant une cascade nécro-inflammatoires lésant le tissu hépatique. Cette théorie, permettant l'identification de nouvelles cibles thérapeutiques, offre la possibilité de [...]
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"DE LA MICROFLORE INTESTINALE AUX MALADIES HEPATIQUES"

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La Faculté de médecine, sur le préavis de Monsieur Antoine Hadengue, professeur ordinaire au Département de médecine interne, autorise l'impression de la présente thèse, sans prétendre par là émettre d'opinion sur les propositions qui y sont énoncées.

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REFERENCES
Références bibliographiques :

« DE LA MICROFLORE INTESTINALE AUX MALADIES HEPATIQUES»

Résumé :
Les hépatopathies alcoolique et non-alcoolique sont responsables d’une importante morbidité et mortalité. L’absence d’un traitement efficace prouvé pour ces maladies découle d’une compréhension imparfaite de leur pathogénèse. Les mécanismes physiopathologiques menant à ces hépatopathies sont l’objet d’intenses recherches expérimentales et cliniques. Dans ce contexte, un concept ayant évolué depuis plus d’un siècle a abouti, récemment, à une approche révolutionnaire du problème. Ce travail examine l’assertion fascinante que des altérations dans la composition de la flore microbienne intestinale, engendrées par l’alcool et/ou un régime riche en graisses, jouent un rôle déterminant dans l’apparition et la progression de ces affections. Une microflore « dysbiotique » répand un nombre accru d’endotoxines qui, lorsque la perméabilité intestinale est augmentée, atteignent le foie où elles activent les cellules de Kupffer, engendrant une cascade nécro-inflammatoire lésant le tissu hépatique. Cette théorie, permettant l’identification de nouvelles cibles thérapeutiques, offre la possibilité de développer de nouvelles thérapies pour ces hépatopathies.
II. SUMMARY IN FRENCH

Les hépatopathies alcoolique (alcoholic liver disease (ALD)) et non-alcoolique (non-alcoholic fatty liver disease (NAFLD)) sont des affections caractérisées par un même spectre de lésions histologiques dont la lésion initiale, la stéatose simple, peut évoluer en stéatohépatite puis en cirrhose [348]. Ces deux pathologies, dont l'incidence est en nette augmentation, sont responsables d'une importante morbidité et mortalité [81], [137], [170]. L'absence d'un traitement efficace prouvé pour ces maladies [89], [203] découle probablement d'une compréhension imparfaite de leur pathogénèse respective [137], [117]. Ainsi, les mécanismes menant à ces hépatopathies sont l'objet d'intenses recherches expérimentales et cliniques. Dans ce contexte, un concept datant de plus d'un siècle [257] a récemment suscité un intérêt croissant et a abouti à une approche révolutionnaire du problème.

L'hypothèse fascinante que la flore microbienne de l'intestin puisse influencer le développement des maladies hépatiques alcoolique et non-alcoolique expliquerait bien des aspects obscurs de leur pathogénèse. Cette idée est soutenue par un nombre grandissant de données scientifiques [257]. Si cette théorie attise l'espoir de déchiffrer certains mécanismes pathogéniques, elle comporte en revanche encore beaucoup d'interrogations. Ce travail examine les tenants et aboutissants validant l'assertion que des altérations de la composition de la flore intestinale jouent un rôle dans l'apparition de la stéatose hépatique et son évolution vers la stéatohépatite et la cirrhose.

Le tractus gastro-intestinal accueille une population diverse et complexe de microorganismes connue sous le nom de « flore intestinale » et qui englobe trois domaines : les bactéries, qui prédominent, les eucaryotes et les archées [94]. Cette communauté microbienne, qui habite surtout le colon, entretient une relation symbiotique avec l'hôte humain [63]. Elle compte approximativement $10^{13}$ à $10^{14}$ cellules bactériennes, dépassant de dix fois le nombre de cellules constituant le corps humain et représentant plus de cent fois le génome humain [409].

La composition de la flore microbienne de l'intestin est plastique, modifiée par des facteurs tels que le régime alimentaire, la consommation d'alcool, d'antibiotiques ou d'anti-inflammatoires [72], [208]. Ces facteurs peuvent rendre la flore intestinale « dysbiotique », c'est-à-dire qu'ils réduisent la population de bactéries bénéfiques pour la santé de l'hôte, telles que les Bifidobactéries et les Lactobacilles, et induisent la prolifération d'espèces nocives [208]. Une telle flore dysbiotique a été observée chez des consommateurs chroniques d'alcool [54], [150], [190], des sujets obèses [208] et des patients atteints d'hépatopathie non-alcoolique [257].

Un autre exemple de changement dans la composition de la flore commensale est la pullulation bactérienne de l'intestin grêle (« small intestine bacterial overgrowth » ou SIBO, en anglais). Il s'agit d'un phénomène fréquent caractérisé par un nombre excessif de bactéries dans l'intestin grêle résultant d'une prolifération de bactéries coliques (typiquement à gram négatif), conduisant à des symptômes digestifs et/ou une malabsorption [206], [285]. L'incidence de cette pullulation bactérienne est spécialement élevée chez les sujets souffrant de maladie alcoolique [52] et non-alcoolique [257], [403] du foie, probablement en raison des troubles moteurs gastro-intestinaux qu'impliquent ces pathologies [306], [403].

Les conséquences de ces altérations de la composition de la microflore sur la santé humaine sont de plus en plus acceptées. La dysbiose semble participer au développement de certaines pathologies non-infectieuses [119], [139] telles que l'obésité, le syndrome métabolique [341] ainsi que les maladies hépatiques étudiées dans ce travail. Néanmoins, la perturbation de cet écosystème de bactéries commensales ne pourrait influencer des organes internes comme le foie sans la présence d'une seconde altération ; la dysfonction de la barrière muqueuse intestinale. En effet, cette dernière est considérée comme la condition sine qua non de l'implication de la flore microbienne intestinale dans les maladies internes et a été identifiée chez des patients diagnostiqués avec une maladie hépatique alcoolique ou non-alcoolique [257], [307].
Grâce à sa structure élaborée et à une régulation complexe, la muqueuse du tractus digestif constitue une barrière sélective qui empêche l’absorption de substances toxiques et de microorganismes tout en permettant l’assimilation de nutriments et d’autres éléments essentiels. Les deux composants de cette barrière épithéliale sont la membrane apicale des entérocytes et l’espace paracellulaire, situé entre les cellules épithéliales et contrôlé par des jonctions intercellulaires dynamiques: les jonctions serrées (zonula occludens) et les jonctions adhérentes (zonula adhaerens) [16], [156]. Ces jonctions jouent un rôle-clé dans la régulation de la perméabilité de la muqueuse intestinale [263].

La dysfonction de la barrière muqueuse, c’est-à-dire une augmentation de la perméabilité intestinale [156], peut être modulée par divers stimuli qui agissent sur les jonctions serrées. Ces stimuli comprennent, entre autres, des signaux de type humoral ou neuronal ainsi que des médiateurs inflammatoires, mais aussi un régime riche en graisses, l’alcool et certains médicaments anti-inflammatoires [16]. Il existe plusieurs maladies dans lesquelles l’augmentation de la perméabilité gastro-intestinale joue un rôle pathogénique déterminant. C’est le cas pour le diabète de type I, le syndrome métabolique, la maladie de Crohn, la cœliaquie, le syndrome du colon irritable [16], [59], [292] et, très probablement, les hépatopathies alcoolique et non-alcoolique. Chez des patients souffrant de ces pathologies, l’évaluation clinique de la perméabilité intestinale est utile pour apprécier la réponse à un traitement, confirmer certains diagnostics mais aussi, éventuellement, pour prédire l’évolution de l’affection [17], [42], [156]. Les méthodes permettant une telle évaluation sont basées sur le principe d’excrétion urinaire différentielle de substances-test telles que des sucrés, des chélateurs radio-marqués non-dégradables ou des polymères [29], [42].

Il a été mentionné que des altérations de la barrière intestinale ont été constatées chez des patients avec une hépatopathie alcoolique [307]. L’augmentation de la perméabilité intestinale chez ces sujets était d’abord considérée comme une conséquence de la maladie hépatique, plus précisément de l’hypertension portale qui y est associée [149]. Or, de plus en plus d’études expérimentales suggèrent qu’elle représente une condition préalable au développement de lésions hépatiques [122], [182], [183] et engendrée par l’alcool. En effet, l’acétaldéhyde, métabolite le plus toxique du métabolisme de l’éthanol, endommage la barrière muqueuse en provoquant notamment la redistribution des protéines composant les jonctions serrées et adhérentes vers des compartiments intracellulaires [21], [301], [336], [339]. Il est intéressant de noter que, puisque les bactéries commensales sont responsables de plus de 50% de l’activité métabolique de l’alcool dans le tube digestif, elles contribuent à la dysfonction de la barrière muqueuse induite par l’alcool en influençant la quantité d’acétaldéhyde s’accumulant dans la lumière intestinale [278]. Un autre moyen par lequel l’alcool augmente la perméabilité intestinale est le stress oxydatif local [124]. Des mécanismes additionnels doivent sans doute encore être identifiés. Aussi, certaines questions demeurent sans réponse, comme par exemple de déterminer si l’alcool influence la perméabilité gastro-intestinale ou intestinale seulement et si cette augmentation de la perméabilité est transitoire ou permanente [306].

Chez des patients atteints de la maladie non-alcoolique du foie, une augmentation importante de la perméabilité intestinale a également été observée. Cette augmentation semble être corollée à la sévérité de la stéatose et à la prévalence du syndrome métabolique [257]. Cependant, si le mécanisme principal responsable de l’exacerbation de la perméabilité intestinale est bien défini pour l’hépatopathie alcoolique, il demeure incertain pour ce qui est de la maladie hépatique non-alcoolique. Les hypothèses actuelles indiquent que la réaction de peroxydation lipidique et le stress oxydatif intestinal pourraient être impliqués dans ce mécanisme. L’hyperinsulinémie, ainsi que les hauts taux circulants de cytokines représentent aussi des facteurs potentiels pouvant altérer la fonction de la barrière muqueuse [59], [283], [416]. Ainsi, des investigations supplémentaires sont nécessaires.
L’augmentation de la perméabilité de l’intestin joue un rôle crucial dans le développement des maladies hépatiques alcoolique et non-alcoolique en facilitant l’accès de certaines molécules nocives dérivées d’une flore intestinale dysbiotique à la circulation porte et au foie [257], [403]. Les endotoxines sont des lipopolysaccharides (LPS) provenant de la paroi de bactéries à gram négatif résidant normalement dans le tube digestif. Lorsque la barrière muqueuse est dysfonctionnelle, une quantité importante de ces toxines passe de la lumière intestinale au tissu hépatique où elles sont phagocytées par les cellules de Kupffer, des cellules immunitaires appartenant à la lignée des macrophages. Si la quantité de LPS dépasse la capacité phagocytaire de ces cellules, ces molécules gagnent la circulation systémique, une situation désignée « endotoxémie » [306].

Les facteurs contribuant à l’endotoxémie comprennent, outre l’augmentation de la perméabilité intestinale, la présence d’une flore microbienne dysbiotique qui délivre plus de LPS qu’une microflore saine. C’est le cas, en particulier, de la pullulation bactérienne de l’intestin grêle [312], mais ceci n’a pas encore été clairement démontré. Un régime alimentaire riche en graisses ainsi que la consommation d’alcool influencent également les taux plasmatiques d’endotoxines. En plus d’engendrer une élévation de la perméabilité intestinale, ils provoquent un accroissement du nombre de microorganismes à gram négatif dans la flore gastro-intestinale [65], [72]. D’autre part, les lipoprotéines facilitent le transport des LPS de l’intestin vers la circulation sanguine [387].

Dans le foie, les LPS qui ne sont pas neutralisés se lient aux cellules de Kupffer par la LPS-binding protein (LBP) et activent ces cellules par deux types de récepteurs : le « cluster of differentiation-14 » (CD-14) et le « toll-like receptor-4 » (TLR-4) [301]. En conséquence, des cascades de signalisation mènent à l’activation du facteur de transcription NF-κB [398], ce qui déclenche la production, par les cellules de Kupffer, de nombreux médiateurs tels que des espèces réactives oxygénées (« reactive oxygen species » ou ROS, en anglais), comme le superoxyde, le peroxyde d’hydrogène et des radicaux hydroxyles, et des cytokines (en particulier TNF-α) [268], [341], [394], [399]. Le stress oxydatif et la réponse inflammatoire qui s’ensuivent sont responsables d’un processus nécro-inflammatoire et, à plus long terme, fibrotique, lésant le tissu hépatique [72], [301], [398].

Il est intéressant de noter que l’endotoxine n’est pas la seule substance capable d’activer les cellules de Kupffer. D’autres ligands comme le peptidoglycan, la flagelline [301] ou l’acide lipoteichoique [8] se lient aux TLR-2 ou TLR-5 et activent les cellules immunes, impliquant les bactéries à gram positif dans ce processus pathologique (puisque l’épaisseur du peptidoglycan est plus importante chez ces microorganismes) [301] et indiquant que d’autres ligands ou récepteurs, encore non-identifiés, pourraient y participer. De même, dans le foie, les cellules-cible des LPS n’incluent pas seulement les cellules de Kupffer mais aussi les hépatocytes, les neutrophiles [154], les cellules endothéliales sinusoidales [106] et les cellules étoilées, responsables de la fibrose hépatique [301].

Un nombre croissant de données indique que l’endotoxine joue un rôle majeur dans le déclenchement et la progression de la maladie alcoolique du foie [301]. En effet, les taux plasmatiques de LPS sont significativement plus élevés chez des patients atteints des divers stades de cette pathologie, en comparaison avec des sujets témoins [129], [289], [306], [331]. Ceci est vraisemblablement dû à la dysfonction de la barrière intestinale ainsi qu’à la modification de la flore bactérienne (y compris la pullulation bactérienne de l’intestin grêle), toutes deux induites par l’alcool [306]. De plus, la fonction phagocytaire des cellules de Kupffer est amoindrie par l’alcool, ce qui contribue encore à l’endotoxémie [315].
Une véritable corrélation entre le degré d'endotoxémie et la sévérité de la maladie n'a pu encore être démontrée [272]. Néanmoins, l'élévation des valeurs d'endotoxémie chez ces patients semble coïncider avec le développement de l'atteinte hépatique [306]. En outre, le passage de la stéatose alcoolique à la stéatohépatite apparaît dépendant de la présence de LPS qui est le facteur déclenchant de la cascade nécro-inflammatoire [182]. Il est maintenant reconnu que l'alcool exerce ses effets néfastes sur le foie en collaboration avec les endotoxines [182], [306]. Un point intéressant de cette synergie est la sensibilisation, par l'alcool, des hépatocytes, des cellules de Kupffer et d'autres cellules résidant dans le foie aux effets du LPS [152], [363], [386], [398], créant ainsi un cercle vicieux entretenant le processus nécro-inflammatoire dans le tissu hépatique [182]. Les mécanismes responsables de ce phénomène de sensibilisation ne sont pas entièrement élucidés mais semblent impliquer les mitochondries hépatocytaires [199].

A l'instar de la maladie alcoolique du foie, les études expérimentales et cliniques qui soutiennent un rôle déterminant de l'endotoxine dans le développement de l'hépatopathie non-alcoolique sont de plus en plus nombreuses [2], [59], [211], [213]. La présence de hauts taux d'endotoxémie chez les patients souffrant de cette affection [112] découle probablement, comme pour l'hépatopathie alcoolique, de l'exacerbation de la perméabilité intestinale associée à cette maladie. La pullulation bactérienne de l'intestin grêle, très fréquente chez ces patients, y contribue certainement aussi, tant par l'accumulation de bactéries Gram-négatif, sources de LPS, que par l'augmentation de la perméabilité intestinale qu'elle semble induire [59], [318], [403]. Il est important de noter que les facteurs responsables de l'endotoxémie sont communs aux deux hépatopathies discutées. Ainsi, ces deux pathologies, qui partagent déjà leurs lésions histologiques, paraissent avoir une pathogénèse très semblable.

Les patients souffrant d'hépatopathie non-alcoolique présentent des taux plasmatiques d'endotoxines et de TNF-α spécialement élevés [403]. Les endotoxines, via la production de TNF-α qu'elles induisent, jouent un rôle crucial dans le passage de la stéatose à la stéatohépatite non-alcoolique [171], [365], [403], [416]. En effet, le TNF-α, en augmentant l'activité de la stearoyl-CoA desaturase-1 (SCD-1), une enzyme lipogénique impliquée dans le métabolisme des acides gras, semble amplifier la stéatose [210]. De plus, pour ce qui est du phénomène de sensibilisation, des expériences in vitro démontrent que les hépatocytes stéatosiques exposés au TNF-α deviennent hyper-réactifs à cette même cytokine [422]. Le mécanisme qui régit ce cercle vicieux n'est pas encore compris. De fait, cette cytokine influence non seulement la stéatose hépatique non-alcoolique, de par son effet sur la SCD-1, mais aussi le développement de la stéatohépatite, via ses propriétés pro-apoptotique et pro-inflammatoire sur des hépatocytes rendus plus vulnérables.

Considérées dans leur ensemble, ces données soutiennent l'existence d'une influence déterminante de la flore microbienne de l'intestin sur les lésions hépatiques des maladies alcoolique et non-alcoolique du foie. En effet, les bactéries commensales et les endotoxines qu'elles répandent, la dysfonction de la barrière muqueuse, les cellules de Kupffer, le TNF-α et d'autres cytokines ainsi que le stress oxydatif tissent un réseau complexe impliquant des interactions plastiques et influencé par de nombreux facteurs. Ce concept pathogénique semble commun aux deux pathologies. Ainsi, la découverte d'un mécanisme physiopathologique pour l'une profite certainement aux investigations propres à l'autre. Un jour peut-être, avec les connaissances apportées par la recherche, ces deux entités pourront être considérées comme une seule affection comprenant deux étiologies distinctes. Aussi, la révélation de ces interactions pathologiques a permis l'identification de nouvelles cibles thérapeutiques et offre donc la possibilité de développer de nouvelles thérapies pour ces hépatopathies.

L'augmentation de l'incidence des maladies alcoolique et non-alcoolique du foie, ces dernières années, est préoccupante, surtout en l'absence d'un traitement efficace. Les thérapies actuelles pour la maladie du foie liée à l'alcool et à l'obésité sont basées sur l'abstinence et la perte de poids, respectivement [365]. Cependant, jusqu'à présent, aucune n'a pu prouver un résultat satisfaisant ni a atteint un consensus parmi les hépatologues [267].
La modification de la flore microbienne du tractus digestif par des pro-, pré- et synbiotiques est une approche sûre et révolutionnaire [94]. Les probiotiques sont des compléments alimentaires constitués de bactéries bénéficiaires vivantes, telles que les Lactobacilles ou Bifidobactéries [301], qui, après avoir colonisé le tractus digestif du patient, lui confèrent des effets bénéfiques [161], [245] comme, par exemple, la restauration de l'intégrité de la barrière intestinale [62], [109]. Il a été prouvé expérimentalement que les probiotiques diminuent l'endotoxémie, le stress oxydatif, la perméabilité intestinale et la sévérité des lésions hépatiques des maladies discutées [124], [169], [271], [284], [310]. Bien que ces effets restent à être confirmés chez l'Homme [301], ces résultats sont très prometteurs. Les prébiotiques, des hydrates de carbone non-digestibles servant de fertilisant pour les bactéries bénéficiaires [133], [245], ainsi que les synbiotiques, un mélange de pro- et de prébiotiques, agissent de la même manière et présentent, eux aussi, des résultats encourageants [67], [69], [181], [216], [232], [326].

D’autres stratégies visant à restaurer l’intégrité de la barrière muqueuse incluent des molécules telles que le glucagon-like-peptide-2 (GLP-2) [69] et l’epidermal growth factor (EGF) [339] et font également l’objet d’intenses recherches. De même, les approches permettant la prévention de l’inflammation et du stress oxydatif, notamment par l'utilisation d’anticorps anti-TNF-α [3], [300], [328], [349], [366] ou d’antioxydants [398], sont au centre des investigations. Ces stratégies thérapeutiques rehaussent l'espoir de développer un traitement pour les hépatopathies alcoolique et non-alcoolique.

Des progrès importants ont été réalisés dans la compréhension de ces maladies hépatiques chroniques. Le fait que des altérations de la flore microbienne intestinale puissent aboutir à des lésions du foie ébranle les théories pré-existantes et ouvre la voie à de nouvelles possibilités thérapeutiques. Néanmoins, de plus amples investigations sont nécessaires à l'éclaircissement de certains mécanismes pathogéniques. Ce défi mènera certainement, dans un futur proche, à des découvertes fascinantes et, possiblement, à l’avènement d’un traitement efficace pour ces pathologies débilitantes.
INTRODUCTION

Alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD), conditions both causing important morbidity and mortality, remain without proven effective therapies because of incomplete understanding of their pathogenesis. Indeed, many obscure aspects are under investigation. In this framework of intense experimental and clinical research, a concept that has been evolving for more than a hundred years has slowly gained interest and, only recently, led to a revolutionary approach of the problem.

The hypothesis that the intestinal microbiota may influence liver disorders has only recently brought a radical turn in the direction of the investigations but already shed light on certain features of ALD and NAFLD. However, if this theory brings the hope of unraveling the physiopathology of these liver diseases, it also brings unanswered questions. The present work reviews the findings that corroborate the assumption that alcohol- and high-fat diet-mediated alterations in the composition of the gut microflora play a role in the development of liver steatosis, steatohepatitis and, eventually, cirrhosis.

In the second chapter, current knowledge of the intriguing and complex gut intestinal microbiota is examined. This ecosystem of microorganisms has established a symbiotic relationship with the human host and, while it is acknowledged that the beneficial interactions are continuously evolving, it is gradually being recognized that alterations in the gut microbiota composition (or dysbiosis), may be responsible for various non-infectious diseases that include the metabolic syndrome and hepatic disorders. Nevertheless, dysbiosis could not affect internal organs such as the liver without a second alteration: a defect in the intestinal mucosal barrier.

The gastrointestinal tract, due to its elaborate structure, forms a selective barrier that prevents the uptake of noxious substances and microorganisms while allowing the absorption of nutrients and other essential elements. The third chapter describes the sophisticated constitution of the intestinal barrier and discusses the important concept of intestinal permeability. Indeed, intestinal leakiness, which can be assessed clinically by different methods that are exposed in this section, plays a crucial role in the development of some models of hepatic diseases by facilitating the access of microflora-derived harmful molecules to the liver.

After briefly addressing certain aspects of ALD and NAFLD, chapter 4 explains the pathogenic mechanisms by which altered intestinal microbiota and gut barrier dysfunction lead to the development of these liver diseases. The experimental and clinical evidence is reviewed. The gut microbiota-derived endotoxin crosses a dysfunctional intestinal barrier to reach the portal bloodstream. Once in the liver, endotoxin appears to activate certain types of macrophages which consequently release inflammatory and oxidative compounds and thus trigger a necro-inflammatory cascade. Although this allegation appears straightforward, the key mediators of this newly accepted pathogenesis weave a complex and not yet fully understood network that comprises plastic interactions and modulation by various factors. This pathological system, which may be generated either by alcohol or high-fat diet, seems to be common to both ALD and NAFLD and it is conceivable that these two entities will, in the future, be considered as one disorder with different etiologies. Figure 1 illustrates the pathogeneses of these diseases and how they may be confounded into one novel pathogenic scheme.

Further research is required in order to elucidate certain mechanisms and define specific associations. Nonetheless, these recent advances in the comprehension of the discussed liver diseases stimulate the development of new therapeutic approaches. The latter are examined in chapter 5 and include the use of pro-, pre- and symbiotics which modify the microbiota composition and restore the intestinal mucosal barrier. Indeed, such approaches raise many hopes in the quest for an effective treatment for ALD and NAFLD.
Figure 1. Representation of the classical pathogenic schemes of ALD and NAFLD that may be confounded into one novel physiopathological approach. FA = Fatty Acids; FFA = Free Fatty Acids; ROS = Reactive Oxygen species.
2 GUT INTESTINAL MICROBIOTA

The human gut is home to a diverse and complex collection of microorganisms, referred to as the “gut intestinal microbiota”, which has not yet been fully analyzed. This so-called “microbial organ”, mostly contained in the colon, counts around $10^{13}$ to $10^{14}$ bacterial cells, outnumbering by ten fold the number of human body cells and representing more than a hundred times the human genome [408], [409].

2.1 A varying composition

According to a recent report, the number of species of microorganisms that compose the intestinal microbiota lies between 15,000 and 36,000 [126], although these figures seem to change with each author. Three domains are represented: Bacteria, Eukaryota and Archaea, the latter being prokaryotes which lack a DNA-containing nucleus. Bacteria predominate and 90% of their phylotypes are represented by Bacteroidetes and Firmicutes [94]. Other predominant species are, among other, Bacteroides, Eubacterium, Bilidobacterium, Fusobacterium and Peptostreptococcus. Moreover, at least 90% of the gut microbiota consists of obligate anaerobes [371].

Data suggest that the composition of the indigenous intestinal microbiota is established during the first year of life, most of the microorganism species being acquired during birth [94] or just after [158], [371]. Multiple modifying factors such as host genotype, diet, antibiotic and NSAID use, or lifestyle then participate in transforming the bacteria collection into an “adult-type microbiota”. Hence, there is a great diversity in the gut microbiotas of different persons, especially between lean and obese people [94], [208], as illustrated in Figure 2, thus demonstrating the influence of dietary factors. The composition of the gut microbiota also varies according to its anatomical location in the gastrointestinal tract, the species staying the same along the gut but their proportions changing [94].

![Figure 2. Comparison of the relative proportions of Firmicutes and Bacteroidetes between lean and obese mice [371].](image-url)
Over adult life, the microbiota composition of a person has been considered constant [94]; yet, transient and permanent changes have been shown to occur [208]. In addition, according to a small number of studies, ageing is suggested to have an important influence on the composition of the intestinal microbiota [107], [160], [228], the enterobacteria population increasing and the anaerobes decreasing in numbers in elderly [160]. This is just one of many contradictions found in the literature which illustrates how incomplete our understanding of the composition of this microbiota still is [94]. Thus, gut intestinal microbiota appears at least individual-, location- and possibly age-specific [425].

The fact that our knowledge of the intestinal microbiota is limited can be explained by the difficult accessibility of the different sections of the gastrointestinal tract and the complicated constitution of the commensal bacteria ecosystem [426]. However, technologies using small subunit ribosomal RNA (SSU rRNA) and its corresponding gene as well as phylogenetic DNA microarray analysis have been developed and recently improved. The applications of the latter include monitoring gene expression, detecting DNA sequence polymorphisms or DNA genomic mutations. These novel approaches have brought and still promise important progress in analyzing the gut microbiota diversity. Nevertheless, these results are qualitative and do not give any information about the role nor the function of the organisms. The answer to this problem resides in function-driven metagenomic studies which will certainly help unraveling mechanisms of host-microbe interactions [425]. A summary of the existing approaches is represented in Figure 3.

![Figure 3. Schematic representation of metagenomics and other approaches [425].](image-url)
2.2 A symbiotic relationship

The human host and the gut microbiota have established a symbiotic relationship [63]. Indeed, the intestine supplies the commensal bacteria with a nutrient rich, safe microenvironment while bacteria participate in digesting food, building up stocks of essential nutrients and preventing colonization by pathogenic microorganisms [23], [223]. A key issue in this “ecological mutualism” [63] lies in the fact that the microbiota is implicated in many host digestive and defensive functions including gastrointestinal motility, epithelial turnover, immune modulation and drug metabolism [94]. In addition, critical metabolic functions such as synthesizing micronutrients, breaking down carcinogens and dietary toxins, taking part in the absorption of certain electrolytes and trace minerals, fermenting indigestible substances and affecting the development and differentiation of enterocytes and colonocytes by producing short-chain fatty acids [159], [246], [317] also contribute to the wellbeing of the human host.

Most interestingly, this beneficial relationship would not be possible without a very delicate balance of the intestinal immune system. The latter has to stay hypo-responsive to commensal bacteria through inhibitory mechanisms while fighting invasive pathogenic bacteria [158]. The intestinal mucosal immune system consequently has very special and complex characteristics compared with those of other tissues.

The crucial symbiosis between host and commensal bacteria has evolved over millions of years and will further change over time [63]. The consequences of the alterations of the intestinal microbiota on human health are slowly being recognized. Poor intestinal health or dysbiosis is indeed being more and more associated with various non-infectious disorders [119], [139] including liver diseases. An example of this phenomena is small bacterial overgrowth (SIBO) which will be examined in further chapters and which is related to gut motility problems, intestinal malabsorption, diarrhea and other consequences [164], [275]. For this reason, evaluating the complex interactions of the so called “ecological pact” and learning more about the gut microbiota now seem essential to understand many of these pathologies. As will be discussed in chapter 5, treating and preventing these diseases by manipulating the commensal bacterial community is a revolutionary idea that could well become a reality.

2.3 Gut intestinal microbiota in obesity and metabolic syndrome

Recent findings, namely by the american group of J. Gordon, emphasized the fact that the intestinal microbiota composition is implicated in host nutrient acquisition along with regulation of energy homeostasis [67]. This concept is well illustrated by Backhed, et al. [22] who showed that germ free mice gained around 40% less total body fat than mice which hosted a conventional gut microbiota. In addition, when conventionalized (i.e. transplanted with a “normal” intestinal microbiota taken from the distal gut of a conventional mouse), these germ free mice increased their total body fat by 60% within two weeks in spite of being fed less. Insulin resistance and other metabolic abnormalities were associated with the increase in body fat.

Moreover, Ley et al. [208] found that gut microbiota of obese persons contain less Bacteroidetes and more Firmicutes than lean controls. After weight loss by diet therapy, the relative proportions of Bacteroidetes and Firmicutes changed, Bacteroidetes increasing and Firmicutes decreasing, resembling those of the lean participants.

These data, along with those of other groups [94], [207], [374], nurture a growing body of evidence that the commensal bacteria play a certain role in regulating weight and that the composition of the intestinal microbiota may be involved in the physiopathology of obesity and the metabolic syndrome [94], [341].
Different hypotheses have been put forward to explain the gut microbiota-obesity association and some of them are depicted in Figure 4. Firstly, commensal bacteria enhance energy extraction from indigestible dietary polysaccharides. A second mechanism could be the triggering of a low-grade chronic inflammation via the modulation of plasma LPS levels by the intestinal microbiota. This inflammation tone participates in the development of obesity and the metabolic syndrome. A third possibility suggests a role for the gut microbiota in the regulation of host genes that modulate the way energy is spent and stored [94], [371]. Yet, supplementary work is required to elucidate the causal links between obesity and the intestinal bacteria ecosystem [94].

Intestinal microbiota appears to have a role in obesity, metabolic syndrome and liver disorders through similar physiopathological mechanisms. These will be reexamined in chapter 4 in perspective with alcoholic and non-alcoholic liver diseases.
3 INTESTINAL PERMEABILITY

3.1 Intestinal mucosal barrier

The intestinal surface is the body’s largest external surface and comes in direct contact with very high concentrations of bacteria, bacterial and food antigens as well as toxic compounds. Therefore, the intestinal tract plays a crucial role by forming a barrier that prevents the uptake of luminal noxious substances into the bloodstream [39], [156]. Its ability to limit the assimilation of certain molecules while maintaining a pathway for the absorption of nutrients demonstrates how selective this specific barrier is. This capacity is clearly enabled by an elaborate structure as well as a tight and complex regulation.

3.1.1 Structure of the intestinal barrier

In simple terms, the intestinal epithelial barrier is made up of two components. The first is the cell plasma membrane itself, a phospholipid bilayer membrane that does not allow the passage of water-soluble substances [156]. Indeed, these molecules must be transported via transmembrane channels or transporters, or they must pass through the paracellular pathway between adjacent epithelial cells [362]. The second key component is this paracellular space which is controlled by dynamic intercellular junctions along the lateral margins of the epithelial cells. Closest to the luminal surface lie the tight junctions and, basolaterally, are the adherens junctions [16], [156].

Tight junctions

The tight junction (or zonula occludens) is a highly dynamic specialized intercellular junction [11], [306]. As depicted in Figure 5 and Figure 6, a diverse range of more than 50 specific proteins that include transmembrane proteins (such as occluding, tricellulin, claudin) and cytoplasmic proteins (for example ZO proteins, polarity complex proteins, cingulins) participate in the assembling of the tight junction [130], [380]. The transmembrane proteins form the paracellular channels that selectively control permeability to ions, whereas cytoplasmic proteins compose a scaffold for membrane proteins, and are implicated in a variety of signaling pathways [140], [380]. Yet, the structure of the zonula occludens is complex and the functional relationships of its constituents remain under investigation [362].

These structures close the paracellular space by forming a continuous seal around the apical poles of the cells [39], [225]. By separating the apical from the basolateral domain and preventing the free diffusion of lipids and proteins between the cells, they contribute to the establishment and maintenance of apico-basal polarity [186].
Tight junctions play a key role in the permeability of the intestinal mucosa [263]. Indeed, the actomyosin cytoskeleton is linked to the tight junctions and regulates the barrier function of the gut mucosa [238], [262], [306], [352], by opening and closing intermittently to allow fluids, nutrients and even certain microorganisms to cross from the lumen into the lamina propria via the paracellular pathway [115], [238]. On the other hand, specific permeability to ions is controlled by the size- and charge-selective gate function of claudins [380], which are thus responsible for the semi-permeable property of this diffusion barrier [238].

Interestingly, tight junctions are distributed in a particular manner. They are smaller but more numerous in the upper third of the intestinal villi which comes in contact with most of the luminal contents. In contrast, the ones in the crypts, at the base of the villi where they are less likely to come in contact with luminal compounds, are much larger in size but less numerous [156].

![Figure 5](image.png)

**Figure 5.** Two adjacent epithelial cells held together by the tight junction complex just under the brush border membrane. Within the tight junction, several classes of proteins (claudins, occludins, tricellulin and JAM) span the plasma membrane four times. Proteins in a second group, including zona occludens-1 (ZO-1) and ZO-2, attach to the proteins in the first group and link them to the cytoskeleton [362].

**Adherens junctions**

Another type of intercellular junction is represented by adherens junctions (zonula adhaerens) which are positioned basolaterally, below the tight junctions, and are illustrated in Figure 6. They are formed by transmembrane proteins (cadherins and nectins) that are connected to the actin cytoskeleton through cytoplasmic protein complexes containing beta-catenin/alpha-catenin and afadin, respectively [250].

Adherens junctions do not prevent macromolecular diffusion but control the integrity of tight junctions in an indirect, yet essential manner. For instance, the absence of E-cadherin leads to abnormal localization of important tight junction proteins resulting in permeable tight junctions [373]. Consequently, the integrity of adherens junctions clearly contributes to the regulation of the barrier function of the intestinal epithelium [306]. Tight and adherens junctions, depicted together in Figure 7, are both targets and effectors of a variety of signaling pathways linked to cytoskeletal organization and control of gene expression, cell differentiation and proliferation [140].
Figure 6. Schematic representation of the tight junction (TJ) and the adherens junction (AJ). The TJ is situated near the brush border and comprises a variety of more than 50 specific proteins, including transmembrane proteins (occludins, JAMs, claudins) and cytoplasmic proteins such as ZO proteins. Below the TJ, the AJ is composed by transmembrane proteins (cadherins and nectins) which are linked to the actin cytoskeleton through cytoplasmic protein complexes containing beta-catenin/alpha-catenin and afadin [250].
3.1.2 Intestinal barrier permeability

Before intestinal permeability is further discussed, a clear definition of the term seems essential. Intestinal permeability can simply mean the degree of leakiness of the intestinal mucosa. In addition, according to some authors, it is synonymous with the barrier function [47], [156].

However, a more precise definition is proposed by D. Hollander’s description of permeability as being the passive penetration of the intestinal epithelium by medium and large sized water soluble non-charged molecules greater than 0.4 nm in cross-sectional diameter. The monosaccharide rhamnose, the disaccharide lactulose, polymers and macromolecules such as albumin or inulin are various examples of such molecules. An increase in the absorption rate of the latter thus implies the concept of altered permeability which will be widely exploited throughout this review [155].

As mentioned above, the main determinant of the rate of intestinal permeability is the opening or closure of the tight junctions in the paracellular space [156]. Yet, new means by which the paracellular route can be activated and thus gut leakiness increased are gradually being understood. This is the case, for instance, of the zonulin pathway. This physiological route, also used by bacteria, is triggered by binding to an apical membrane receptor on the enterocyte. Consequently, an intracellular pathway is activated and leads to actomyosin contraction and increased paracellular permeability [16]. Also, sites of enterocyte apoptosis or mucosal erosions may also allow for focal leaks and contribute to a disturbed permeability [334].

Mediator of intestinal permeability, the tight junction is amazingly dynamic and responsive to various stimuli [16] which will be examined in the coming section.
3.2 Modifying factors of intestinal permeability

Studies based on animal models and human epithelial cell cultures have revealed that many endogenous and exogenous factors are able to modulate intestinal epithelial permeability across tight junctions [39], [156]. These stimuli include dietary state of the host, humoral or neuronal signals, inflammatory mediators and a variety of cellular pathways that may be exploited by bacteria or viruses [16]. A few of these factors will now be briefly discussed.

3.2.1 Physiological stimuli

The function of the gut epithelial barrier is supposed to be regulated by physiological stimuli [301]. The response of the tight junction to luminal glucose is particularly well studied [82]. Via modulation of the actomyosin cytoskeleton, the transport of Na⁺-coupled nutrient such as glucose, alanine or leucine, leads to a structural reorganization of the tight junctions, as indicated by the redistribution of ZO-1, consequently raising the tight junction permeability and increasing the uptake of nutrients [225], [362].

Additional work demonstrated that the tight junction permeability regulation triggered by the Na⁺-nutrient cotransport depends on the phosphorylation of myosin light-chain (MLC), catalyzed by myosin light-chain kinase (MLCK). Also, the pharmacological inhibition of MLCK hinders the phosphorylation of MLC along with the regulation of the epithelial barrier function [375].

3.2.2 Inflammatory mediators

Certain inflammatory mediators act as regulators of the mucosal barrier function. For instance, IL-1β manages to decrease tight junction ion selectivity in vitro, possibly through the activation of nuclear factor κB [85], [279]. Also, TNF-α and IFN-γ are able to induce a redistribution of many junctional proteins such as ZO-1, junctional adhesion molecule 1, occludin, claudin-1, and claudin-4 [4], [57], thereby increasing paracellular permeability [82] via the myosin light chain kinase-driven pathway described just above [82], [427]. Indeed, TNF-α, in association with IFN-γ, play an important role in epithelial damage in Crohn's disease [334]. Moreover, in patients suffering this disease, TNF-α neutralizing antibodies not only reduce the intestinal inflammation but also restore the small bowel mucosal barrier [357]. Hence, a growing body of evidence suggests that the immune system plays an important role in modulating intestinal permeability [82].

3.2.3 Bacterial toxins and infections

Various bacteria alter tight junction state through multiple mechanisms, most probably to enhance their own growth. For example, one of the toxins produced by Vibrio cholerae, the zonula occludens toxin, appears to increase paracellular permeability via the physiological previously mentioned zonulin pathway, by binding to an apical membrane receptor on enterocytes [16], [156], [389].

Furthermore, an in vitro model of an enteropathogenic E. coli (EPEC) infection demonstrates that EPEC cause a large increase in tight junction permeability by several mechanisms including reorganization of the actin cytoskeleton, redistribution of tight junction proteins and enhanced myosin light chain phosphorylation [420], [427]. This last mechanism is also used by Giardia to disregulate tight junction permeability [335]. It will be further examined, in section 4.5.3, how the composition of the gut microbiota strongly influences gut leakiness.
3.2.4 NSAIDs

The role of non-steroidal anti-inflammatory drugs (NSAIDS) in intestinal damage is well known [43]. By damaging the brush border through an interaction with surface membrane phospholipids or by uncoupling mitochondrial oxidative phosphorylation via intracellular signaling, NSAIDs upset the integrity of the intercellular junctions [346]. Indeed, as shown by Montalto et al. [263] in vitro, aspirin inhibits ZO-1 expression in human cell cultures. NSAIDs increase intestinal permeability in humans within 12 hours of administration [42].

3.2.5 Alcohol

Ethanol, along with its metabolite acetaldehyde, affects intracellular signal-transduction pathways leading to increased paracellular permeability. It is mainly acetaldehyde, an important metabolite of alcohol, which causes the disruption of tight junctions and adherent junctions through a mechanism that may involve phosphorylation, [307]. In addition, ethanol and acetaldehyde cause mucosal damage in the upper small intestine by inducing the loss of epithelium at the apex of the villi as well as hemorrhagic erosions which dig through to the lamina propria [56]. These mechanisms will be discussed more in detail in section 4.3.2.

3.2.6 Other factors

Many other stimuli may well influence the leakiness of the intestinal mucosa. Age [39], [72], degree of portal hypertension [72], [98], stress [329], trauma, burns [156], growth factors [131], [294], [297], spices and tobacco [344] are just a few examples of a growing list. Hereditary factors also have a clear impact on intestinal permeability [344]. Besides, more potential factors such as glucagon-like-peptide 2 (GLP-2) are under investigation [39], [69], [157]. This review will specially focus on bacteria, inflammation and alcohol as intestinal modulators which play a crucial role in the recent approach of the physiopathology of alcoholic liver disease and non-alcoholic liver disease.

3.3 Intestinal permeability in intestinal diseases

There are numerous human pathologies in which abnormal permeability has been suggested to be important. Type 1 diabetes, metabolic syndrome, Crohn’s disease, coeliac disease, infectious diarrhea and irritable bowel syndrome are some of them [16], [59], [292].

Many of these pathological states result from intestinal epithelial damage that is related to the disease but not involved in its physiopathology [16], as in enteric bacterial and parasitic infections [82]. Yet, in some conditions, it seems that increased leakiness is critical to the development of the pathology. This is the case in type 1 diabetes, Crohn’s disease or coeliac disease, for instance [16], [82]. Moreover, it will be later demonstrated that this is also true for certain liver disorders.

For these particular diseases, the assessment of intestinal permeability may help to understand cryptogenic etiologies or pathogenesis, as well as their genetic predisposition [156], and certainly promises great advances in clinical research.
3.4 Assessment of intestinal permeability

In humans, noninvasive assessment of intestinal permeability has evolved over the last 20 years and has led to the principle of differential urinary excretion of test substances. Indeed, the latter offers an index of intestinal permeability [42], [251], [252].

In order to be used as a probe molecule, the test substance must present a number of properties. For instance, it has to be small, water soluble, nontoxic, non-charged, non-degradable and non-metabolized. Also, it should not be present naturally in the urine [75], [76]. Because the probes, which are orally ingested after an overnight fast, are not metabolized, they are excreted in the urine in proportion to the amount that has been absorbed. Hence, timed collection of urine provides an assessment of the permeability rate of specific probes [156]. Figure 8 sketches out the path of a permeability probe during a permeability assessment.

![Figure 8. Illustration of the clinical principles of the permeability probes. Since the latter are not metabolized by humans, they go through the intestinal barrier. They then leave the bloodstream and are excreted into the urine in direct proportion to the amount that penetrated the intestinal barrier [156].](image)

3.4.1 Sugar probes permeability tests

Measuring the urinary excretion of orally administered, inert, non-metabolized sugars such as mannitol, lactulose, sucrose or sucralose is the most widely recognized method for the clinical assessment of intestinal permeability [29], [113]. This effective and noninvasive approach is based on the evaluation of the absorption of two or three sugars of different sizes [113], [292].

The most commonly used sugars are mannitol and lactulose which are monosaccharide and disaccharide, respectively. Both have lipophobic and hydrophilic properties which explain their poor affinity for the glycoside transport system of the intestinal mucosa. Hence, they are absorbed passively [123], [292].
Mannitol is absorbed through transcellular pathways, supposedly across the hydrophilic part of the cell membrane. Conversely, the absorption of lactulose uses paracellular pathways, i.e. the tight junctions. Therefore, lactulose represents a marker for small bowel permeability and mucosal barrier integrity [112, 123] while mannitol is correlated with the amount of mucosal absorptive surface [123]. In consequence, the lactulose/mannitol ratio from urine samples is a sensitive index of small intestine permeability [112], [292].

Dual sugar tests, as described above, only allow the assessment of small intestine permeability. Methods using three sugars have been developed to enable the evaluation of the gastric and colonic permeability concurrently to that of the small intestine. Sucrose or its derived sweetener sucralse are now frequently used in addition to mannitol and lactulose [10], [113], [114].

As mannitol, rhamnose, or lactulose can be degraded by bacterial enzymes, these inert sugar probes are not useful in assessing permeability in the large intestine, home to an abundant bacterial flora. Moreover, under conditions of small intestinal bacterial overgrowth (SIBO), loss of lactulose and mannitol is impossible to quantify and renders the permeability assessment unachievable. In opposition, sucralse is not metabolized by colonic bacteria, which allows it to be present throughout the gut. For this reason, assessment of sucralse in a 24 hour collection is an indicator of total gut permeability [49], [112], [114], [247]. Besides, newly developed partially synthetic probes such as polysucrose resist bacterial degradation and can be employed for evaluating the permeability of both small and large intestine [49]. Sucrose appears to be a valuable probe for determining the permeability of the gastroduodenal region [248]. Hence, by carefully choosing the method used, it is possible to assess the permeability of various sites within the gastrointestinal tract [16]. Figure 9 proposes a simple illustration of these regions-specific permeability measurements.

Modified highperformance liquid chromatography with pulsed amperometric detection (HPLC-PAD) as well as other chromatographic analysis are current techniques which allow the identification of sugar molecules in the urine samples. Both are precise, highly sensitive methods [29], [113], [123], [292] and are continuously being improved. For instance, Farhadi, et al. [114] developed a capillary column gas chromatography (C CGC) method which seems more sensitive than the packed column gas chromatography (PCGC) technique for what concerns sucralse detection in the urine.

The full amounts of each sugar in the urine samples are then expressed as percentages of the doses initially ingested [112], [257], [403], and the particular sugar ratios calculated.

### 3.4.2 Non-degraded radiolabeled chelates

$^{51}$Cr-ethylene diamine tetraacetate ($^{51}$Cr-EDTA) and $^{99m}$Tc-diethylenetriamine pentaacetate ($^{99m}$Tc-DTPA) are non-degraded radio-labeled chelates which share various physical properties with oligosaccharides. Yet, they have the benefit of ease of measurement and the disadvantage of being radioactive. Extensively used for assessing glomerular filtration rates, their urinary excretion can also be exploited as an index of intestinal permeability. The choice between $^{51}$Cr-EDTA and $^{99m}$Tc-DTPA is a matter of convenience [42].

Only 1-3% of the orally ingested quantity of $^{51}$Cr-EDTA permeates through the mucosal barrier. Nonetheless, if intestinal permeability is increased for any reason, a significantly higher portion of the initial dose reaches the bloodstream, where it will be cleared by glomerular filtration. Since $^{51}$Cr-EDTA is absorbed by the gastrointestinal tract through the paracellular route, its augmented presence in the urine indicates disruption of the tight junctions [91], [257].
Similarly to sucralose, $^{51}$Cr-EDTA remains stable in an environment prone to bacterial overgrowth. Hence, it is a good tracer for evaluating colonic permeability properties [247]. Evidence shows that $^{51}$Cr-EDTA excretion is a worthy marker of the severity of intestinal mucosal inflammation and damage of tight junctions [17]. Indeed, for these reasons, and also because of its small size and lipid insolubility, $^{51}$Cr-EDTA has been broadly used to assess paracellular permeability in patients with intestinal disorders [43] such as inflammatory bowel diseases [17].

The use of $^{51}$Cr-EDTA in combination with a monosaccharide appears to increase the specificity of the test [45], [46]. Also, this radio-labeled chelate can be combined with a disaccharide [42], but it should be kept in mind that some of these saccharides may be metabolized by luminal bacteria.

In the urine samples, $^{51}$Cr-EDTA is easily quantified by determining the gamma emission of radiochromium [173].

### 3.4.3 Ethylene Glycol Polymers (PEGs)

PEGs are widely employed, for example, as solvents, food additives and as a purgative in preparation for colonoscopy or in suppositories. The most commonly exploited PEG in human studies is PEG 400, which encompasses a range of polymers of different molecular mass. As for the sugar and chelate probes, it is ingested orally after fasting and quantified in urine samples. PEG polymer detection in urine is time-consuming but is eventually accomplished by gas or high-pressure liquid chromatography [42].

While PEG 400 is a theoretically ideal probe [42], it also presents a few disadvantages such as an unpleasant taste, a variable urine excretion when administered intravenously [239] and a noticeable interspecies variation in their toxicity [405]. Moreover, it appears that there is a lack of consensus on how to express PEG results. Although there are many reasons to criticize PEG 400 as a permeability test substance, this polymer still provides intriguing findings [42].

![Figure 9](image_url)

**Figure 9.** Region specific permeability measurements. By carefully choosing probes that have only a limited exposure to the gastrointestinal epithelium site, selective permeability determinations can be achieved. Since sucrose is broken down once it leaves the stomach, it is used for assessing gastroduodenal permeability. Likewise, lactulose and mannitol being destroyed in the caecum, they enable the measurement of small intestinal permeability. Probes such as sucralose and Cr-EDTA are stable throughout the gastrointestinal tract and thus, they appear to give information regarding the colonic epithelium [16].
3.4.4 Immunohistochemical analysis of ZO-1 expression

Zonula occludens-1 (ZO-1) is one of the proteins that take part in the structure of tight junctions [352] and, therefore, in the regulation of paracellular permeability [224]. ZO-1 is now clearly believed to be a good indicator of tight junction integrity [262], [263]. Indeed, in 1996, evidence brought by Gottardi, et al. [136] showed that ZO-1 not only accumulates in cell to cell contact locations but also inside the cell nucleus, where its presence might be inversely related to the degree and/or the maturity of intercellular contact. This implies that tight junction constituents such as ZO-1 take part in the regulation of nuclear processes including cellular proliferation and differentiation. More importantly, it also means that the nuclear expression of ZO-1 may well be employed as a marker of tight junction integrity [24], [257].

This method determines the expression and distribution of ZO-1 in duodenal biopsy specimens by means of immunohistochemical analysis. An anti-ZO-1 antibody is used to label ZO-1 in the mucosa and villous cell nuclei. The locations and intensity of the staining are analyzed by two different pathologists [257], low staining being associated with increased intercellular leakiness [262]. However, since the role of nuclear ZO-1 remains unclear because not all researchers have located it in the nucleus [25], this method should be considered with precaution until further investigation regarding nuclear ZO-1 is accomplished.

3.4.5 Aspirin challenge test

As mentioned above, intermittent gut leakiness can occur. Its span comprises intermittent mucosal barrier dysfunction, appearing only when the mucosa is challenged, to severe barrier impairment, exhibiting itself constantly [112]. A challenge test using acetylsalicylic acid can reveal this so-called susceptibility to intestinal leakiness [112], [153].

Aspirin is an intestinal permeability stressor which can trigger an elevation of small bowel leakiness in healthy people [112] via mechanisms discussed in section 3.2.4. Indeed, aspirin and possibly other NSAIDs can improve the detection of permeability abnormalities in relatives of patients with Crohn’s disease and may well play a role in the genesis of the disease itself [153].

Gastrointestinal permeability is first measured by means of sugar probes. The aspirin provocation test is then employed, before a second sugar permeability test is used and the results of the two sugar probes are compared. There is susceptibility to intestinal barrier leakiness if a significant difference is found between both probes, the challenged one obviously exhibiting a greater permeability [112], [153].

3.4.6 Clinical applications

Intestinal permeability tests have been broadly employed in a variety of disorders. They proved to be clinically useful as screening methods for small intestinal diseases such as celiac disease and inflammatory bowel disease [98], assessing response to treatment, confirming diagnoses and, sometimes, forecasting the clinical course of the illness [17], [42], [156].

With future research and better understanding of the mucosal barrier function, rapid, simplified and more specific permeability analysis may be accessible for detection of some of these disorders in their early subclinical stages or for following the progress of the disease under treatment [42], [156].
4 GUT INTESTINAL MICROBIOTA, INTESTINAL PERMEABILITY AND ENDOTOXEMIA IN LIVER DISEASE

4.1 Introduction

The concept that increased intestinal permeability and gut intestinal microflora may contribute to the development of certain disorders has been evolving since 1890 (Llewellyn Jones: “Theory of auto-intoxication from gut bacteria”) [47], [257]. The possibility of an interaction between the gastrointestinal tract and the liver is a fascinating hypothesis that could explain many obscure aspects of the pathogenesis of certain liver diseases. Indeed, over the past decade, mounting evidence sustaining a significant role for the gut-liver axis in the pathogenesis of ALD and NAFLD has been accumulating [257].

Substances derived from the gut microbiota cross the intestinal mucosal barrier to reach the liver where they are responsible for diverse noxious effects. While this acknowledged assertion seems straightforward, the mediators of this pathogenic mechanism weave a complex network of plastic interactions which is slowly being understood. The composition of the gut bacterial flora, along with the permeability of the intestinal barrier and the susceptibility to liver injury are varying entities influenced by many factors. This chapter offers the occasion to explore this pathogenic system with its recent developments, implications and areas for further investigation.

4.2 Alcoholic liver disease (ALD) and non-alcoholic liver disease (NAFLD)

4.2.1 Definitions

ALD

Alcohol causes a spectrum of histological lesions which may be seen as a dynamic process. The earliest and initial lesion associated with alcohol abuse is alcoholic steatosis which may completely disappear within weeks of abstinence. Alcoholic steatohepatitis represents the next histological lesion to develop, yet in only a minority of excessive drinkers with steatosis, and is a fibrogenic process which may ultimately induce cirrhosis [95]. Alcoholic steatosis, steatohepatitis (ASH) and cirrhosis are thus the three main constellations of alcohol-induced liver lesions and they generally coexist in the same patient [348].

Alcoholic steatosis consists of hepatocyte intracytoplasmic micro- and macrovesicular accumulation of triglycerides. The latter most probably results from increased fatty acid synthesis and fat mobilization or faulty fat export or breakdown in the liver [214]. This condition is the first step to steatohepatitis [348]. Alcoholic steatohepatitis (ASH) develops in only 30% of heavy drinkers [137]. It is characterized by the coexistence of three histological criteria: 1) ballooning degeneration and necrosis of hepatocytes, 2) micro- and/or macrosteatosis and 3) lobular inflammation [13]. Alcoholic cirrhosis is considered the final phase of alcohol liver disease [237].
and has long been acknowledged as a non-reversible condition which predisposes to hepatocellular carcinoma. It also arises in a minority of alcohol abusers [348].

**NAFLD**

NAFLD is defined by a fat infiltration in more than 5% of hepatocytes (as assessed by liver biopsy or magnetic resonance spectroscopy) in the absence of excessive alcohol intake. The latter is characterized by two daily standard drinks (20g of ethanol) for men and one standard drink (10g of ethanol) per day for women [132]. Comparable to ALD, NAFLD comprises a wide range of liver pathologies ranging from non-alcoholic steatosis alone, through to the necroinflammatory disorder of non-alcoholic steatohepatitis (NASH) to cirrhosis [117], [257].

Non-alcoholic steatosis is the most usual form of NAFLD and appears to be a benign condition resulting from abnormal lipid metabolism [237]. However, once present, it may cause hepatic insulin resistance [117]. On the other hand, NASH, which only 15-25% of patients with steatosis develop, is far less common and is not seen as benign [237], [393]. There is increasing evidence that NASH is the hepatic manifestation of the metabolic syndrome. Indeed, more than 85% of patients with NASH exhibit insulin resistance or metabolic syndrome [117], [210], [231], [341].

Regarding histological features, alcoholic steatosis is similar to non-alcoholic steatosis [348]. Likewise, ASH and NASH can barely be distinguished on histological bases [61]. Moreover, these two entities share risk factors and pathological mechanisms [96], [168]. Because of the shortage of clinical studies in ASH, as well as pharmacological studies in NASH, these two entities remain distinct. Yet, alcohol intake may well be one of the few aspects that differentiate alcoholic from non-alcoholic steatohepatitis [348].

### 4.2.2 Epidemiology

**ALD**

ALD represents an important cause of morbidity and mortality with over 75,000 annual deaths worldwide and an increasing incidence in the last decade [137]. Of course, the exact prevalence of ALD remains indefinite because liver biopsy is not available in large population-based studies [348]; however, this figure facilitates the appreciation of the outcome of ALD.

The sole most significant factor that foretells progression or improvement of liver function in patients with ALD is abstinence. Other than net alcohol intake and consumption patterns, gender, ethnicity, body mass index, insulin resistance and other co-morbidities, iron load and oxidant status also contribute to the risk of ALD in a given individual [137], [348]. Of course, heredity and its polymorphism of genes encoding for ethanol- and acetaldehyde-metabolizing enzymes also represent an important promoting factor for ALD [137]. According to follow-up studies, only 15-20% of heavy drinkers will develop cirrhosis over time [95].

**NAFLD**

Because of the increasing prevalence of obesity [281], NAFLD has become one of the most widespread forms of chronic liver disease throughout the world [81], [170]. Indeed, its prevalence, in many populations, ranges from 16% in lean to 76% in obese individuals and increases with age [117]. The prevalence of steatosis in obese patients is especially high and reaches 85-98%, whereas that of NASH is very variable, with an overall figure of 37% [222].
The most prevailing determinants of NAFLD severity are type 2 diabetes mellitus, evidence of metabolic syndrome, obesity and age [80], [117]. Visceral (rather than overall) obesity, type 2 diabetes mellitus and hypertriglyceridaemia appear to be the best known risk factors [117]. Besides, there are indisputable genetic factors that predispose to NAFLD/NASH [354]. In NAFLD, the estimated rate of cirrhosis development is 20%, while liver-related death is 12% over 10 years [110], [163], [242].

### 4.2.3 Pathogenesis

Although this review focuses on certain main features of the pathogenesis of ALD and NAFLD, the detailed and exhaustive mechanisms that lead to these diseases will not be examined here but may be found in the review articles by Gramenzi and colleagues [137] as well as by Farrell et al. [118] dealing with ALD and NAFLD, respectively.

The etiology of ALD is very clear. However, its complex pathogenesis is not completely understood [137]. Grossly, altered metabolic pathways, oxidative stress, elevated endotoxin levels, inflammatory mediators, apoptosis and immune reactions are well known to participate in the pathogenesis of ASH [348].

If some mechanisms of the pathogenesis of NASH/NAFLD are established, other aspects of it remain undefined. Abnormal lipid metabolism, insulin resistance and genetic factors cooperate to enlarge hepatic lipid storage and cause inflammation-induced hepatocellular injury via lipotoxicity, oxidative stress and production of proinflammatory mediators [12], [117], [170]. Yet, the means by which oxidative stress and cytokines instigate and maintain this pathological state are still poorly understood [117].

Mounting evidence suggests that the development of ALD and NAFLD depends on factors such as increased intestinal permeability, small bacterial overgrowth (SIBO), endotoxemia, inflammation and oxidative stress, which will be widely examined in the coming sections. ASH and NASH not only share a number of risk factors and histological lesions, but their pathogenic mechanisms exhibit an increasing number of similarities, especially regarding the central role of the intestinal microenvironment and its derived endotoxin, as will be revealed later on.

A long known and accepted concept of NAFLD pathogenesis is that it is a two-step process [90], illustrated in Figure 10. It is hypothesized that steatosis sensitizes hepatocytes to subsequent stress, providing the setting (or “first hit”) for NASH, while a second injury-producing process (or “second hit”), such as drugs, hypoxia, cytokines, oxidative stress, endotoxin or TNF-\(\alpha\) is needed for the progression to necroinflammatory changes, steatohepatitis and, eventually, fibrogenesis [117], [422]. The fact that this second insult may only occur in a certain number of patients with steatosis would explain why only 15-25% of such subjects develop NASH.
Figure 10. The “two hit theory”. This model considers the development of simple hepatic steatosis to represent the “first hit”, increasing the vulnerability of the liver to the “second hit” leading to hepatocyte damage (CYP2E1: cytochrome P450 2E1). Candidates for the second hit include endotoxin, TNF-α and oxidative stress [268].

It is not surprising that ALD, following a similar pattern with only 30% of alcoholics progressing to ASH, also fits this two-hit theory [124]. Whilst heavy alcohol drinking accounts for alcoholic steatosis, a second factor is necessary for triggering the appearance of ASH. Interestingly, on the basis of experimental observations, gut-derived endotoxin has been proposed as a second hit mechanism [90], [124], in both ALD [41], [272] and NAFLD [59], [416]. Hence, the way that this common factor may lead through mucosal barrier dysfunction to these liver disorders will now be analyzed.

4.3 Intestinal mucosal barrier (IMB) dysfunction

4.3.1 IMB dysfunction in liver disease

Intestinal mucosal changes in cirrhotic patients are well documented and comprise mucosal inflammatory-like abnormalities such as edema, erythema, granularity and friability, along with vascular lesions encompassing telangiectasias, angiodysplasia-like lesions or varices [92], [277]. On the microscopic level, increased intercellular space between gut epithelial cells in such patients was first observed in the 1960s [20]. However, the existence of epithelial ultrastructural defects in intestinal biopsy specimens of patients with cirrhosis has only been reported a few years ago [72], [356].

Permeability measurements confirmed that the gastrointestinal barrier could be dysfunctional in patients with cirrhosis [307], [428]. Furthermore, Cariello et al. [72] showed that the degree of intestinal leakiness (assessed by a lactulose/mannitol test) varies according to the etiology and severity of liver injury, as it is underlined by Figure 11. They also demonstrated that age, alcohol consumption and portal hypertension were indeed modifying factors of intestinal permeability. Portal hypertension, a key feature in chronic liver disease, is clearly linked to intestinal permeability alteration [277], [410].
Figure 11. Classification of the percentage of patients with modified intestinal permeability according to diagnosis and extent of liver damage. The star (*) implies a significant difference from control subjects ($p < 0.05$). Likewise, patients with chronic hepatitis (CH or ∆) considerably differ from cirrhotic (C) patients ($p < 0.01$) [72].

4.3.2 IMB dysfunction in ALD

Several studies have demonstrated how alcohol ingestion can increase intestinal permeability to macromolecules in alcoholics as well as in non alcoholics [54], [307], [180], [181], [293]. Furthermore, intestinal barrier alterations in patients with ALD are clearly recognized [307]. The increased intestinal permeability of alcoholics was first thought to be a result of the liver impairment. This was consistent with the assumption that the portal hypertension associated with ALD was responsible for an acute venous congestion, edema and ischemia which all lead to intestinal mucosal injury [149]. Yet, mounting evidence sustains a new theory which suggests that intestinal mucosal damage is a precondition to the development of liver injury and ALD [122], [182], [183].

Ethanol and acetaldehyde

Although direct intestinal mucosal damage such as loss of epithelium at the top of the villi or hemorrhagic erosions induced by acute alcohol ingestion is well documented [56], the molecular mechanisms through which ethanol may have a deleterious effect on the intestinal epithelium remain unclear [122]. There are actually two hypotheses which could explain how alcohol may lead to altered intestinal permeability.

On the one hand, ethanol may act directly on epithelial cells. Indeed, intestinal epithelial cell lines treated with ethanol exhibit barrier dysfunction [26], [220]. Nevertheless, since the ethanol concentrations need to be very high (exceeding those found in vivo after alcohol consumption) and the effect obtained on permeability is small, a direct effect of ethanol on the intestinal epithelium most probably does not account for the observed change in permeability in vivo [122].
On the other hand, the role of alcohol could be mediated by acetaldehyde, the most toxic metabolite of ethanol metabolism [301]. Acetaldehyde is produced by oxidation of ethanol to water and carbon dioxide [137] and, in the colon, is generated through the cytochrome P450 2E1 (CYP2E1) present in the epithelial cytosol [122], [137], [319], or by certain species of the resident gut microflora that bare an alcohol dehydrogenase activity [175]. A growing number of data suggest that acetaldehyde participates in the disruption of the intestinal mucosal barrier [72], [122] and thus increases permeability to endotoxin [122], [305], [307], a consequence which will be discussed later on.

Gut intestinal microbiota

Before exposing the mechanisms whereby acetaldehyde alters the mucosal barrier, it is important to point out the role of gut intestinal microbiota in alcohol-induced barrier dysfunction. Commensal bacteria may account for half of the whole ethanol metabolic activity in the gut [122], [306]. Hence, the efficiency of bacterial aldehyde dehydrogenase to metabolize acetaldehyde may influence the accumulation of acetaldehyde in the colonic lumen [278] and thereby represent a crucial cofactor in acetaldehyde-associated intestinal barrier damage.

Moreover, ethanol modifies the composition of the intestinal flora [72], reducing the number of bifidobacteria and lactobacilli [190]. Bifidobacteria are known to diminish the amounts of intestinal endotoxin in rodents and ameliorate intestinal barrier function [138], [391], [392]. Indeed, these bacteria promote a healthy microvillus environment because they do not metabolize intestinal mucous glycoproteins, to the contrary of other pathogenic microorganisms, thus avoiding permeability changes and bacterial translocation [66], [71]. This alteration could be responsible for increasing intestinal permeability, thus providing an additional mechanism whereby alcohol elicits intestinal barrier dysfunction and stressing a supplementary role for gut microbiota in alcohol-induced endotoxemia.

Mechanisms of acetaldehyde-induced barrier dysfunction

Acetaldehyde-induced mucosal barrier disruption has been observed in human colonic mucosal biopsies [306] and is caused by a redistribution of tight junction proteins (occludin and ZO-1) and adherens junction proteins (E-cadherin and β-catenin) from the intercellular junctions into the intracellular compartments, [21], [301], [336], [339]. Moreover, acetaldehyde dissociates occludin and ZO-1 from the cytoskeleton [358]. It is likely that the integrity of adherens junctions is altered first, and this may act as a signal leading to the disruption of tight junctions [338].

The redistribution of these intercellular junction proteins is most probably caused by an acetaldehyde-induced, increased phosphorylation of ZO-1, E-cadherin and β-catenin [21], [122]. Indeed, a mechanism involving a loss of phosphatase activity and a consequently enhanced tyrosine kinase activity [21], [306] upsets the regulation of the phosphorylation-dephosphorylation balance of tight junction proteins, [122], [301]. Additional information is needed to clarify certain aspects of this mechanism which is simplified in Figure 12. For instance, dephosphorylation of occludin also appears to play a role but its function remains uncertain [306].

Interestingly, data suggest that acetaldehyde, at low concentration, is a powerful mast cell degranulator in vitro [194]. Immunocytes closely linked to epithelia, mast cells receive signals from their host tissue, other immunocytes and nerves. When activated, they degranulate and liberate various mediators that include histamine, eicosanoids and cytokines [122], [255] which appear to directly increase intestinal permeability locally [31], [122], [243], [325] or activate other immune cells which, in turn, release mediators impairing the gut barrier [122]. Ferrier et al. [122] support this finding by reporting that in vitro basal permeability to dextran in rat tissues treated with the mast cell stabilizer doxantrazole is appreciably lower than in controls. In vivo, the same experiment exhibited a smaller decrease in permeability, thereby implying that mast cells may play a minor role only, in comparison with the direct effect of acetaldehyde on inter-epithelial junctions, in permeability changes.
**Role of Acetaldehyde in Increasing Intestinal Permeability to Endotoxin: A Proposed Mechanism**

Intestinal alcohol

↑ Intestinal acetaldehyde

↓ Epithelial protein tyrosine phosphatase activity

↑ Tyrosine phosphorylation of tight junction and adherens junction proteins

Redistribution of junctional proteins from intercellular junctions into intracellular compartments

↑ Disruption of barrier function

↓ Intestinal permeability to endotoxin

**Figure 12.** Role of acetaldehyde in enhancing intestinal permeability: a suggested mechanism. Acetaldehyde may increase intestinal leakiness by reducing the activity of protein tyrosine phosphatase in the intestinal epithelial paracellular space. In consequence, tyrosine phosphorylation of tight junction proteins (occluding and ZO-1) and adherens junction proteins (E-cadherin and β-catenin) is intensified and leads to the redistribution of these proteins from intercellular junctions to intracellular compartments. This reorganization possibly leads to increased intestinal permeability to endotoxin [301].

**Oxidative injury to the mucosal barrier**

One more mechanism whereby alcohol increases intestinal permeability in vivo may be local oxidative stress [124]. *In vitro* studies reported that alcohol activates oxidative pathways by inducing the CYP2E1 form of cytochrome P450 enzymes [268]. For instance, the upregulation of inducible nitric oxide synthase (iNOS) [26], [27] is achieved via the activation of the key transcription factor NFκB [27], [28]. This specific upregulation triggers an overproduction of nitric oxide (NO) and superoxide anion which react together to form peroxynitrite [301]. Ultimately, peroxynitrite and superoxide anion cause nitration and carbonylation of key proteins responsible for the integrity of the mucosal barrier, subsequently disrupting the barrier function and resulting in gut leakiness [26], [182]. This reactive cascade is depicted in Figure 13. Indeed, antioxidants have been proven to normalize intestinal permeability in alcoholic patients [382]. Yet, there is no doubt that oxidative injury to the cytoskeleton needs to be further investigated *in vivo*. 
Figure 13. Role of nitric oxide in increasing intestinal permeability: a proposed mechanism. Alcohol may increase intestinal permeability by enhancing the production of nitric oxide, through the upregulation of inducible nitric oxide synthase (iNOS) activity, and superoxide. These free radicals can react with each other to create peroxynitrite, which, in turn, can react with tubulin. This results in the damage to microtubule cytoskeleton, disruption of barrier function and increased intestinal leakiness [301].

Unanswered issues

If the mechanisms explaining how alcohol ingestion impairs the barrier function are progressively understood, some issues remain to be elucidated. For example, it is not clear whether it is gastroduodenal or intestinal barrier disruption which is responsible for the alcohol-related increase in permeability [306]. Keshavarzian et al. [183] propose that acute ethanol could raise gastroduodenal permeability alone, while chronic ethanol may be responsible for increasing intestinal permeability only.

Another significant concern is whether this alcohol-induced intestinal leakiness is transitory or permanent [306]. Based on studies by Bjarnason et al. [44], it looks likely that the effect of ethanol on gut barrier function is transient in non-alcoholics and in alcoholics without cirrhosis, whereas it may last much longer in alcoholics with the liver disorder [306].

4.3.3 IMB dysfunction in NAFLD

As discussed just above, increased intestinal leakiness has been proposed to be a main factor in alcohol-induced liver disease. Less is known about intestinal barrier dysfunction in NAFLD but recent data from animal and human studies brought up interesting results.
Evidence from animal studies

With the aim of exploring the modifications of intestinal barrier function in the evolution of NAFLD, Sheng et al. [337] created an animal model of NASH. They showed that, although no alteration of the barrier was observed at the early stage of NAFLD, the intestinal mucosa suffered critical injury during the progression from simple liver steatosis to NASH. Also, the team of P. Brun [59] demonstrated intestinal barrier disruption in two strains of genetically obese mice, which led to significant portal endotoxemia. These investigators hence revealed an association between the metabolic syndrome, with which NAFLD is clearly related, and an aberrant distribution of junctional proteins (occluding and ZO-1) within the ileal mucosa that may account for the increase in paracellular permeability.

Such evidence that intestinal barrier malfunction exists in rodents suffering NASH or metabolic syndrome is recent and the underlying mechanism is still uncertain. The actual hypotheses comprise a potential role for lipid peroxidation reaction and oxidative stress in intestinal tissue or endotoxin in intestinal tissue damage [253], [337]. Hyperinsulinemia and high circulating levels of inflammatory cytokines also represent possible factors that may impair intestinal barrier function [59], [283], [416]. Indeed, the fundamental mechanisms that could explain barrier dysfunction in NAFLD need to be further investigated.

Evidence from clinical studies

Data provided by intestinal permeability measurements in humans with NAFLD are less homogeneous. For instance, Cariello et al. [306] reported no difference in mean values of intestinal permeability, assessed by the lactulose/mannitol test, between patients suffering from NASH and controls. Yet, 25% of the sick patients exhibited a discrete increase in permeability. Likewise, the permeability assessment by a lactulose/rhamnose test performed by Wigg et al. [403] did not significantly discriminate NASH patients from controls. Interestingly, these researchers found a higher intestinal permeability in NASH patients with small bacterial overgrowth (SIBO) than in NASH patients without SIBO.

An attractive explanation for these ambiguous findings lies in the suggestion, by Farhadi’s team [112], that there may be a susceptibility to gut leakiness rather than evident gut leakiness in patients with NASH. Using aspirin which, as mentioned in chapter 3, is able to reveal susceptibility to (or intermittent) gut leakiness, Farhadi and colleagues significantly enhanced intestinal permeability in these patients. Thus, it could well be that subjects with NASH do not exhibit abnormal gut permeability at all times and that intestinal barrier stressors such as aspirin, NSAIDs [153], [156], physiological or physical stress [325] may trigger increased gut leakiness from time to time. Moreover, this could be the reason why only a minority of subjects with NAFLD develops NASH, cirrhosis and liver failure [112].

Miele et al. [257] performed the most recent study regarding barrier function in NAFLD and it is the first to clearly indicate a considerable increase in gut permeability in these patients, as elicited by Figure 14. Moreover, they show that this increase is positively correlated with the prevalence of metabolic syndrome and the severity of steatosis (Figure 14) but unrelated to scores of hepatic inflammation or fibrosis. Interestingly, Miele’s team used urinary excretion of 51Cr-EDTA to assess gut leakiness, whereas sugar probes were employed in the studies mentioned above. Also, it is noteworthy that, as reported by the group of A.J. Wigg [403], NAFLD was found to be strongly associated with SIBO which will be examined shortly.
Figure 14. (A) Intestinal permeability in the three groups studied by Miele et al.: healthy volunteers, patients with NAFLD and patients with celiac disease (which represents an excellent model of intestinal barrier dysfunction). Bars correspond to the mean (± 2 standard errors of the mean) of $^{51}$Cr-EDTA recovery dose as expression of gut leakiness. (B) Intestinal permeability increase seems to be correlated to the extent of histological steatosis [257].

Although the understanding of the role of intestinal barrier dysfunction in NAFLD is still in its infancy, especially when compared to that of ALD, these findings are very promising. Some researchers even start considering increased intestinal permeability as the condition *sine qua non* of the involvement of gut–liver axis in the development of NAFLD [257]. Indeed, intestinal mucosal barrier dysfunction plays a crucial role in allowing the crossing of bacterial compounds into the bloodstream, a concept which will be discussed in the coming sections.
4.4 Small intestine bowel overgrowth

4.4.1 Definition, pathogenesis and predisposing factors

The growth of the resident gut microflora is restrained by various physiological mechanisms which include epithelial mucus production, gastric acidity, bacterial competitive interactions and gastrointestinal motility [30], [102], [324], [396]. The latter does so via the expulsion of excessive bacteria from the intestinal lumen. On the other hand, these commensal bacteria contribute to the development and preservation of gut sensory and motor functions such as the intestinal propulsive activity [30]. The main consequence of the disruption of this motility-bacteria equilibrium is small intestinal bacterial overgrowth (SIBO) [30], [275].

SIBO is a common condition in which the small bowel contains excess bacteria which have proliferated from the distal gut, proximally, into the small bowel [185], [285]. It may promote abdominal pain, diarrhea or protein and fat malabsorption along with vitamin deficiencies and weight loss [100], [188]. SIBO is believed to occur in patients with anatomical or functional intestinal motility problems. Interestingly, toxic metabolites and lipopolysaccharides (endotoxins) released by bacteria may further upset intestinal motor function by affecting the enteric nervous system and related transmitters [111], thus generating a self-perpetuating vicious circle [112], [165].

Diverse predisposing factors of SIBO have been identified and comprise achlorhydia and thus the use of proton pump inhibitors [404], old age [166] and diabetes mellitus [384]. Bacterial overgrowth has been shown to occur in a large variety of diseases such as acute pancreatitis, acute bacterial gastroenteritis, tropical sprue, irritable bowel syndrome, celiac disease, intestinal pseudo-obstruction, autonomic neuropathy, scleroderma, rheumatoid arthritis, and after surgical interventions like jejunoileal bypass [176], [228], [229], [285], [290], [302], [303], [308], [342].

4.4.2 Diagnosis

Aspiration of small intestinal fluid for culture and bacterial counts of the aspirate is the actual gold standard in assessing the presence of SIBO [303]. The diagnosis can be made in presence of a total growth of \(>10^5\) colony-forming units/ml of intestinal fluid [322] and/or in presence of a colonic-type microbiota [206]. Because intestinal fluid aspiration is invasive and time-consuming [303], breath tests represent the most commonly used method. Simple and non-invasive, these tests are based on the preferential metabolism of substrates such as glucose, lactulose or D-xylose by bacterial enzymes, which are then detected in the expired air and indirectly measured to identify small-intestine bacterial activity [185], [303]. The glucose hydrogen breath test is the most extensively used test, especially because of its accuracy and low cost [322], although it unsuccessfully identifies the presence of SIBO when used in patients with cirrhosis [35].

An exhaustive list of all the diagnostic methods for SIBO is provided by Rana et al. [303]. However, according to Koshini et al. [185], the gold standard, and the existing tests are disappointing with varying sensibility and sensitivity [206] and superior diagnostic techniques are clearly required. Indeed, approaches such as quantitative bacterial polymerase chain reaction (PCR) may be a potential alternative in the future.
4.4.3 SIBO and bacterial translocation

An important systemic consequence of SIBO is the passage of viable, typically Gram-negative and aerobic bacteria (typically *Escherichia*, *Proteus*, *Enterobacter* and *Klebsiella*) [351] from the gut lumen to the liver, spleen, kidneys, mesenteric lymphnodes and other sites. This process, known as bacterial translocation [40], is associated with increased intestinal transit time [320] and absence of migrating motor complex (MMC) activity [275], both of which constitute the underlining mechanism of SIBO. Indeed, Sánchez et al. [324] showed that, in rodents, bacterial translocation of a given bacterium was almost always associated with intestinal overgrowth of that microorganism.

In addition to impaired intestinal motility and therefore SIBO, factors such as mucosal barrier disruption, increased amounts of bacterial endotoxins (which are, as it will be discussed shortly, facilitated by mucosal barrier dysfunction) and weaken immune defenses [40], [395], [226], [397], also enhance bacterial translocation. Thus, it is of interest that small-intestinal permeability is increased in patients with SIBO [312]. The fact that the major promoters of bacterial translocation facilitate each other or, at least, are linked to one another unveils a complex pathogenic system. The latter will of course be examined in the coming section that concerns bacterial translocation and endotoxemia.

4.4.4 Treatment

Sporadic or periodic antibiotic therapy [298] with broadspectrum antibiotics effective against Gram-negative aerobic and anaerobic bacteria [303] are used to eradicate bacterial overgrowth. Also, several studies have shown that prokinetic drugs cause a decrease in bowel overgrowth and bacterial translocation [324]. Of course, any predisposing factor for SIBO, along with any associated nutritional deficiency should be corrected [303].

4.4.5 SIBO in liver cirrhosis

In rodents, experimental SIBO promotes considerable hepatic inflammation which, in certain rat strains, progresses to fibrosis [213]. The mechanisms of liver injuries produced by bacterial overgrowth have been examined in these animal models and it appears that the peptidoglycan-polysaccharide, a bacterial cell wall polymer with powerful inflammatory and immunoregulatory properties, may participate in the development of liver damage [212].

Bacterial overgrowth was identified in 60% of patients with cirrhosis [36]. This may be explained by small intestinal dysmotility, defense system impairment [141], [421], increased adrenergic activity and portal hypertension [36], [323] which occur in the course of this disease. Furthermore, SIBO is more prevalent in rats with cirrhosis and bacterial translocation (93%) than in rats with cirrhosis but without bacterial translocation (33%) [324]. Bacterial overgrowth and its enhanced bacterial translocation, in combination with the breakdown of immune defense systems, predispose to an important risk of bacterial infection in cirrhotics [402]. For that reason, SIBO is thought to be a predictor of spontaneous bacterial peritonitis [36], [266].

According to recent reports, gut microflora may take part in the pro-inflammatory state of cirrhosis even in the absence of evident infection [313]. Indeed, in cirrhosis, SIBO promotes bacterial translocation and is clearly associated with systemic endotoxemia [415].
Hence, given the fact that intestinal permeability is increased in SIBO, that SIBO is associated with endotoxemia and that endotoxemia is facilitated by increased permeability, the link between SIBO, and endotoxemia appears to be intestinal barrier disruption (or increased intestinal permeability). Also, it could be possible that SIBO itself elicits barrier dysfunction. Once more, these interesting issues will be further discussed in the following sections.

### 4.4.6 SIBO in ALD

There is very little information, in the literature, regarding the affiliation between alcohol consumption and bacterial growth in the small intestine [301]. While duodenal bacterial overgrowth was reported in alcoholics [150], Bode et al. [54] described an increased number of microorganisms (counting Gram-negative coliform bacteria) in the jejunum of chronic drinkers. Yet, no link could be found between the quantities and types of bacteria in the lumen and the extent of liver disease in these subjects. In another study, this same team demonstrated that the incidence of bacterial overgrowth in alcoholics with ALD was increased by three fold in comparison with that of controls [52].

These findings imply that chronic alcohol consumption may promote intestinal bacterial overgrowth. Indeed, ethanol has been proven to slow down gastrointestinal motility [51]. It is thus likely that impaired gut motor function is to blame for bacterial overgrowth in alcoholics [306]. However, additional research is needed to elucidate the exact underlying mechanism and determine if alcohol has a dose-dependant influence on bacterial growth [301].

### 4.4.7 SIBO in NAFLD

If the occurrence of SIBO in ALD needs more investigation, there is no doubt that SIBO has a considerably high prevalence in patients with NAFLD. In patients with non-alcoholic steatosis, Wigg et al. [403] reported SIBO in 50% of cases. Another study revealed a higher prevalence of SIBO in NASH patients in comparison with age- and sex-matched controls [403]. Finally, Miele et al. [257] observed that the prevalence of SIBO in patients with NAFLD was more than twice that of control subjects. A possible explanation for the increased presence of SIBO in NAFLD patients lies in the fact that diabetes, linked to NAFLD by the metabolic syndrome, may facilitate SIBO by promoting intestinal dysmotility [403]. Delayed intestinal transit has in fact been reported in NAFLD [347].

In the study of Miele and coworkers [257], the prevalence of SIBO was correlated with the severity of steatosis. This interesting association is further supported by a complete or partial reversal of steatosis after metronidazole treatment in patients with intestinal bypass [101], as well as in animal models of SIBO [211], [213]. These findings suggest that SIBO plays a role in the pathogenesis of NAFLD. Evidence supporting this hypothesis is also given by Wigg et al. [403].

It appears that the presence of SIBO is considerably higher in NAFLD patients with increased intestinal permeability than that of patients with normal permeability (prevalence of 88.8% versus 29.4%) and subjects with SIBO seem to have enhanced gut leakiness [257], as already mentioned above. However, it remains to be discovered which of both, SIBO and increased intestinal permeability, comes first in NAFLD. It has been proposed that SIBO may take part in the pathogenesis of NASH by modifying small intestinal permeability and thus promoting the absorption of endotoxin [403], further supporting the hypothesis postulated earlier that SIBO may elicit barrier dysfunction. Moreover, as it will be discussed in the coming sections, several authors suggest that the consequential endotoxemia results in an inflammation-mediated extrahepatic insulin resistance with an ensuing increased amount of fatty acid to the liver which finally leads to steatosis [59], [65], [208], [221], [246], [257], [374].
The relationship between SIBO, increased intestinal permeability and endotoxemia in NAFLD is evidently an area for further research. SIBO illustrates how human gut microflora can have an important impact on health and disease. Indeed, a number of pathologies may be explained by the host response to SIBO [285]. It is now time to examine endotoxemia, the main consequence of gut leakiness and SIBO, which surely plays a key role in this newly discovered pathogenic network of ALD and NAFLD.

4.5 Endotoxemia

While endotoxemia has not yet been properly introduced, it was already mentioned throughout the first three chapters. Indeed, in sections 4.4.3 and 4.4.7, endotoxemia was presented as an important consequence of SIBO, probably through intestinal barrier disruption elicited by SIBO itself. Furthermore, gut-derived endotoxin was displayed as a potential second hit mechanism in the pathogenesis of both ALD and NAFLD (section 4.2.3). It is thus noteworthy that endotoxin and endotoxemia should be defined.

4.5.1 Definition

Endotoxins are lipopolysaccharides (LPS) that originate from the cell wall of Gram-negative bacteria that normally reside in the intestine. They comprise the LPS discarded by live microorganisms as well as dead bacteria. Hence, the gut, with its native microflora, represents the main source of endotoxins.

The intestinal mucosal barrier represents an important obstacle for the translocation of endotoxins. However, as it has been demonstrated earlier, this barrier can be disrupted by various means and a leaky gut is now clearly believed to facilitate endotoxemia [59], [69], [277], [306], [334]. The bacterial products, along with viable bacteria (i.e. bacterial translocation), may leave the gut lumen to reach the portal circulation by different means that include direct transmucosal journey across the paracellular space and thus the tight junctions, the zonulin pathway mentioned in chapter 3 and the drift through vascular channels [289]. The mechanisms that allow enteric endotoxin absorption are imprecise and therefore need to be further investigated.

Once endotoxins have crossed the barrier and spilt into the portal bloodstream, they reach the liver where Kupffer cells dispose of these molecules by phagocytosis. Yet, when the amount of endotoxins overpowers the phagocytic activity of these cells, the LPS leak into the systemic circulation, a condition known as endotoxemia. More precisely, the plasmatic concentration of endotoxins should be above 2.5 endotoxin units per milliliter of blood before one can talk of endotoxemia [306].
The standard method used to detect LPS in plasma is the in vitro Limulus amoebocyte lysate test [343], [403]. However, the detection of endotoxin in the blood is difficult and, because plasma proteins can interfere with this assay and because the limulus coagulation cascade can be triggered by agents such as fungal compounds, this test exhibits variable results and, thus, is not reliable. Even though novel assays such as the Endotoxin activity assay (a technique based on chemiluminescence) are being evaluated [233], data regarding endotoxemia values should be considered with circumspection until a reliable and precise method has been successfully developed.

4.5.2 From Kupffer cells to liver damage

![Diagram showing the flow of events from changes in gut microbiota composition to hepato-cellular damage.]

Kupffer cells, which have just been introduced as endotoxin phagocytes, are immune cells that reside in the liver and come from the macrophage lineage [122]. Their main function is to remove bacteria and unknown proteins from the blood. These cells are thus essential to the liver’s foremost role of cleaning the blood of foreign and toxic substances. In the absence of these materials, Kupffer cells remain in a resting state but may be activated by various means. Endotoxins, for instance, are potent activators of Kupffer cells [398].

Activation of Kupffer cells

Once the endotoxin is attached to the Kupffer cell by the LPS-binding protein (LBP), it activates the immune cell through two different types of receptors: cluster of differentiation-14 (CD-14) and toll-like receptor-4 (TLR-4). CD-14 is a surface receptor which lacks a cytoplasmic domain whereas TLR-4 is a transmembrane protein and owes its cytoplasmic domain the ability to transduce LPS-induced cytoplasmic signal across the cell membrane [301]. TLRs play an important role in triggering innate immunity to microbial pathogens by recognizing molecular patterns [8].

CD-14 and LBP form a structural receptor for endotoxin while the latter’s interaction with TLR-4 generates a signal that is transmitted to the cell’s interior and that initiates different signaling cascades. One of the latter involves a molecule known as interleukin–1 receptor-associated kinase (IRAK–1) [398]. IRAK–1 sends off a succession of signals which result in the activation of nuclear factor kappa B (NFκB), a transcription factor which may well control the cellular response to endotoxin.

Endotoxin is not the only substance that activates innate immune cells such as Kupffer cells. The gut microbiota discharges many other TLR ligands that include peptidoglycan, flagellin [301] and lipoteichoic acid [8] which bind to TLR-2 or TLR-5. While peptidoglycan exists in all species of intestinal bacteria, its thickness varies and is more important in Gram-positive microorganisms than in Gram-negative bacteria [301], meaning that Gram-positive bacteria also play a role in immune cell activation.
Liver damage

The activation of Kupffer cells leads to the generation of free radicals and the production of inflammatory mediators that include cytokines (such as TNF-α, IFN-γ, IL-1, IL-6, IL-8), chemokines, eicosanoids and adhesion molecules [268], [269], [288], [311], [341], [398]. The produced reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals are involved in the development of liver damage through oxidative stress [398]. Moreover, TNF-α and IFN-γ, produced by activated Kupffer cells, not only damage hepatocytes directly but also induce the transcription of iNOS, thus participating in oxidative stress. Importantly, these cytokines initiate a hepatic necroinflammatory cascade which encompasses the migration of leucocytes such as neutrophils into the liver [219]. These leucocytes produce toxic substances, especially oxidants resulting from nitric oxide [26], which can cause liver cell necrosis [182]. Eicosanoids such as PGE₂ play an important role in stimulating hepatic metabolism [73], [74], [311].

The subsequent inflammatory response eventually results in necroinflammatory changes and fibrosis in the liver [72], [301], [398]. Figure 15 summarizes the steps leading from endotoxemia to liver damage via the activation of Kupffer cells. A recent study handling mice models of NASH demonstrated that TLR-4 deficient mice suffered a lower liver injury in comparison with that of controls [314]. Similarly, CD-14 knock-out mice lack the innate immune response to bacterial LPS and, when stimulated by LPS, CD14-deficient macrophages do not produce pro-inflammatory cytokines [265]. These findings highlight the injurious consequences of Kupffer cell activation and the essential roles of TLR-4 and CD-14 in activating these cells and inducing inflammation and liver injury.

Figure 15. Schematic model of the mechanism whereby endotoxin release leads, via Kupffer cell activation, to liver injury. Endotoxins shed from certain intestinal bacteria cross from the gut lumen into the bloodstream and into the liver. There, endotoxins activate Kupffer cells through their interaction with CD-14 located on the surface of those cells. This interaction promotes the production of the regulatory nuclear factor kappa B (NFκB) which, in turn, leads to the generation of significant amounts of cytotoxic factors, namely superoxide radicals and various cytokines. TNF-α, for instance, has been shown to be a crucial factor in hepatocyte injury [398].
In the context of endotoxemia, liver damage results mainly from Kupffer cell activation. However, it appears that translocation of bacterial compounds into the mesenteric circulation and the lymphatic system activates NO synthetase in the splanchnic area, hence participating in the onset of portal hypertension and the production of pro-inflammatory mediators which both play a role in the progression of liver impairment [50], [118], [122], [403].

**TNF-α-induced hepatocyte injury**

TNF-α is considered one of the main cytokines involved in hepatocellular damage [236] and its production appears to be among the earliest events in liver injury [365]. Indeed, in animals with deficient TNF-α receptors, liver steatosis and fibrosis are markedly reduced [367]. Cytokines such as TNF-α indirectly amplify and spread the inflammatory process by recruiting and activating additional immune cells, thus leading to fibrosis [422]. They also have pro-apoptotic effects [97], [202]. For instance, Zhang et al. [422] showed that in vitro steatotic hepatocytes, characteristic of NAFLD and ALD, are sensitive to TNF-α-induced apoptosis. Although the mechanism of steatotic hepatocyte apoptosis has not been elucidated, it may involve the ASK1-JNK signaling pathway.

The excessive lipid accumulation in steatotic cells leads to the generation of intracellular reactive oxygen species (ROS) [87], [172], and low concentrations of TNF-α appreciably and more rapidly stimulate the production of additional ROS by mitochondria in these cells, in comparison to control cells under the same conditions. This induction by TNF-α appears to be dose and time-dependent. Finally, ROS may also be generated as a result of TNF-α-induced apoptosis [422].

Hence, TNF-α could represent a “second hit” in the two-stepped pathogenetic model by enhancing an already increased intracellular ROS production [422] or by inducing apoptosis, after the “first hit” of lipid accumulation, in fatty hepatocytes. Yet, further research is needed to provide a mechanistic insight into this “second hit” on steatotic hepatocytes. Even though TNF-α plays a major role in cytokine-induced liver injury, other inflammatory mediators participate in this process, as depicted in Figure 16. For example, IL-18, in addition to contributing to the inflammation, causes liver damage by induction of Fas-dependant hepatocyte apoptosis [372]. Moreover, the ultimate effect of TNF-α on liver cells is very much influenced by other cytokines present in the hepatic tissue [365].

Interestingly, while cytokines mediate hepatic inflammation, apoptosis and necrosis of hepatocytes, cholestase and fibrosis [259], certain also appear to have a protective function. Indeed, data indicate that, after partial hepatectomy, TNF-α initiates the regeneration of hepatic tissue via the activation of type I TNF receptors [5], [412] and, possibly, of the transcription factor NFκb [37]. Therefore, when exposed to TNF-α, cell death is not the natural outcome for hepatocytes, thereby implying that during liver damage, the normal response of liver cells to TNF-α is incapacitated [365]. Another example of the protective effects of some cytokines is the anti-inflammatory function of IL-6. Mice lacking the gene coding for IL-6 that are exposed to endotoxin generate threefold more TNF-α than controls [88]. Moreover, IL-6 has been shown to induce hypo-responsiveness to LPS and to inhibit TNF-α emission in rodents [379]. Hence, there is a delicate balance between pro- and anti-inflammatory mediators in the liver and it is understandable that this equilibrium is lost in disorders such as steatohepatitis. In this context, Tilg et al. [365] support a slightly different two-hit theory for liver injury that includes a first hit that increases hepatocyte exposure to TNF-α and a second hit which interferes with the liver cell’s normal ability to protect itself from TNF-α-induced apoptosis.
Figure 16. Physiopathological role of cytokines in chronic liver disease. Most types of cells in the liver, including Kupffer cells, hepatocytes and stellate cells, either produce or respond to cytokines. In the early stage of chronic liver disease, specific factors (for example viruses, ethanol and toxins) may stimulate the synthesis of cytokines. In later stages, endotoxin could be the key agent stimulating cytokine production. While pro-inflammatory cytokines such as TNF-α and IL-6 typically participate in cholestasis and acute-phase proteins synthesis, TGF-β emitted by activated Kupffer cells and hepatocytes may be one of the critical cytokines involved in fibrosis. In patients with progressive liver disease, the equilibrium between pro-inflammatory and anti-inflammatory cytokines may be shifted towards the pro-inflammatory axis. Therefore, the counteracting anti-inflammatory cytokines are incapable of controlling inflammation and fibrosis [365].

Hepatic stellate cells

In the liver, other than Kupffer cells, the cellular targets of LPS comprise hepatocytes, neutrophils [154], sinusoidal endothelial cells [106] and hepatic stellate cells (HSC), the latter being the most important type of fibrogenic cell in the wounded liver [301]. HSC activation is typically consequent to liver injury and due to the disruption of the normal extracellular matrix and to the liberation of pro-inflammatory cytokines from Kupffer cells, hepatocytes, and infiltrating inflammatory cells [59]. According to different studies in rodents and culture-activated human HSCs, endotoxin appears to activate HSCs through the same mechanism than that of Kupffer cells, which includes the CD-14 and TLR-4 receptors, NFκB activation and the upregulation of gene expression of inflammatory mediators [60], [286], [364]. Thus, in vivo, LPS might well activate HSCs which contribute to the hepatic inflammatory network and liver fibrosis [59], [301].
Interesting data from Brun et al. [59] show that HSCs from the livers of obese mice are more sensitive to endotoxin than HSCs from lean control mice. This finding may explain, at least in part, the fact that liver susceptibility to LPS-mediated injury is increased in these animals [416]. Moreover, HSCs of obese mice appear to lack the tolerance to endotoxin that arises in different cell types after LPS stimulation. This tolerance mechanism prevents the synthesis of inflammatory cytokines to avoid autotoxic effects of excessive amounts of these mediators [286]. These issues remain to be clarified and proven in humans, but they certainly bring up new hypotheses regarding the important incidence of NAFLD in patients with obesity.

### 4.5.3 Modulating factors of endotoxemia

So far, several factors promoting endotoxemia have been mentioned in this review. Indeed, SIBO, weakened immune defenses and increased intestinal permeability have been shown to enhance bacterial translocation and hence endotoxemia. Some of these factors will now be examined more thoroughly.

**Increased intestinal permeability**

If it has been acknowledged that increased intestinal permeability facilitates endotoxemia, less is known about endotoxin-induced mucosal barrier dysfunction. Animal studies have shown that endotoxemia causes intestinal injury. Also, the overproduction of NO by iNOS which are upregulated after endotoxin challenge has been established in many models. Hence, the hypothesis that LPS-induced emission of NO by enterocytes results, in an autocrine fashion, in mucosal injury, loss of barrier function and bacterial translocation has slowly emerged. This vicious circle whereby barrier dysfunction facilitates endotoxemia which, itself, increases intestinal permeability is supported by a mounting body of evidence [125], [258], [411]. Moreover, it appears that hyperglycemia enhances endotoxin-induced gut barrier dysfunction and thus bacterial translocation [411].

Another vicious-circle worth mentioning is the fact that inflammatory cytokines such as TNF-α are able to induce a redistribution of several junctional proteins [4], [57], [240], as discussed in section 3.2.2. Hence, endotoxemia leads to the production of TNF-α which, in turn, alters the gut barrier, facilitates bacterial translocation and increases endotoxemia [59], [369].

**High-fat diet**

One of the mechanisms whereby LPS crosses the mucosal barrier may be related to fat absorption [48], the endotoxins being transported in the blood by lipoproteins [387]. Indeed, Cani et al. [65] performed a study on LPS-infused mice and demonstrated that high-fat food augments the plasmatic concentration of LPS sufficiently to increase body weight, fasted glycemia and inflammation. These findings are of special interest since, as will be discussed shortly, metabolic syndrome, which is characterized by high-fat intake, is clearly associated with increased levels of endotoxemia. Moreover, this diet-induced gut leakiness appears to be mediated by commensal bacteria.

**Gut intestinal microbiota**

Fat-rich diet not only has an influence on endotoxin concentrations but has also been shown to alter the composition of the intestinal microflora (section 2.1), similarly to alcohol. In animal studies, high-fat regime decreases the number of bifidobacteria [65], [68]. However, other bacteria of the dominant Gram-positive group (namely the Eu. rectale-Cl. coccoides group) are also lessened after fat-rich diet which, overall, appears to increase the Gram-negative-to-Gram positive-ratio [65].
As discussed earlier, this alteration of gut bacteria composition in response to high-fat feeding may lead to a robust increase in intestinal permeability, possibly by decreasing the expression of genes coding for the tight junction proteins ZO-1 and occluding [66], a mechanism known to be used, for instance, by enteropathogenic E. coli (section 3.2.3). Furthermore, Cani and coworkers [66] showed that commensal bacteria are undoubtedly involved in this mechanism since antibiotic treated mice displayed normal intestinal integrity, even though the rodents were still on a fat-rich diet.

Therefore, these data imply that changes in intestinal microbial composition in response to a high-fat diet or to motility problems (SIBO) may account for increased permeability and thus elevated endotoxemia, which in turn would trigger the development of metabolic disorders and liver diseases [65], [66], [68], as shown in Figure 17.

![Figure 17. Hypothesis for bacteria-induced metabolic and liver diseases. On a high-fat diet, the composition of intestinal microflora is altered. This modification is associated with an increased intestinal permeability. In consequence, endotoxemia intensifies and triggers inflammation, metabolic and hepatic disorders [66].](image)

### 4.5.4 Endotoxemia and inflammation

**In the metabolic syndrome**

In chapter 2, the hypothesis that intestinal microbiota could trigger the low-grade inflammation that characterizes insulin resistance, diabetes and obesity via the modulation of plasma LPS levels has been put forward and appears to be true. Indeed, Cani et al. [65] demonstrated that endotoxemia is responsible for the onset of metabolic diseases. For instance, in humans, endotoxemia leads to a hypermetabolic response which results in insulin resistance [254]. Moreover, some authors suggest that gut microbiota, by controlling intestinal permeability and thus endotoxemia levels, determines the threshold at which LPS-induced metabolic disorders occur [65]. Nonetheless, a causal link between intestinal bacteria, endotoxemia, and metabolic disease has not yet been proven [66].

It has already been discussed how metabolic endotoxemia, mediated by high-fat diet and changes in the microbiota composition, triggers the expression of inflammatory factors by a CD-14/TLR-4-dependent mechanism. Furthermore, recent data revealed that free fatty acids can bind TLR-4 to activate cells from the innate immune system [340], thus leading to the emission of cytokines [65]. Hence, it is worth mentioning, once again, the crucial role played by the LPS-CD-14-4 system in endotoxemia-induced inflammation.
LPS-treated mice develop inflammation and the expression of genes coding for cytokines, namely IL-6, TNF-α and IL-1, is increased in adipose depots, muscle and liver. The latter is most probably the first organ affected by endotoxin treatment [65]. The coming sections of this chapter will now focus on the hepatic consequences of endotoxemia.

**In liver disease**

Several studies have demonstrated how pro-inflammatory cytokines significantly contribute to the development and exacerbation of liver damage. As discussed above, the endotoxin-mediated activation of Kupffer cells and other macrophages is certainly responsible for the overproduction of these inflammatory mediators [108], especially in conditions of increased LPS translocation and decreased hepatic clearance [272], [315].

However, so far, no significant correlation between circulating levels of endotoxin and those of cytokines has been proven [77], [184], [403]. According to some authors [8], this indicates that the presence of endotoxin alone is not sufficient to significantly raise the amounts of TNF-α and other cytokines, implying that additional stimuli may be needed.

In this regard, TLRs may provide a possible answer. A few lines above, it has been described how TLR-4 and TLR-2, in response to endotoxin and Gram-positive microbial stimuli, respectively, elicit signal transduction resulting in TNF-α production. It is of interest that Riordan et al. [313] revealed an increased expression of TLR-2, but not TLR-4, on peripheral blood mononuclear cells (PBMCs) in cirrhotics without evident infection. Moreover, they exhibited a correlation between PBMC expression of TLR-2, but not of TLR-4, and TNF-α. Indeed, these data suggest that TLR-2 signaling may substantially contribute to the important levels of circulating TNF-α, namely in cirrhosis, and thereby underline the role of Gram-positive microorganisms [8].

As elicited above, hepatic steatosis seems to be an early consequence of endotoxemia because only four weeks of LPS infusion are needed to promote it in mice. Also, this condition is closely related to inflammation since it is totally blunted in CD-14-deficient mice infused with endotoxin [65]. In humans, increased circulating levels of both endotoxin and pro-inflammatory cytokines have been observed in patients with chronic liver disease, even in the absence of overt infection [8], [77], [129], [144], [385]. Furthermore, a growing body of evidence shows that endotoxemia is strongly associated with the occurrence and severity of cirrhosis [291], [307]. It is now time to consider endotoxemia in perspective to ALD and NAFLD.

### 4.5.5 Endotoxemia in ALD

Accumulating data indicate that endotoxin plays an important role in the induction and possibly progression of ALD [301]. It has been established that the levels of circulating endotoxins in patients affected with different stages of ALD are notably higher than those in healthy controls [129], [289], [331] or patients with non-alcoholic cirrhosis [129]. Indeed, endotoxin concentrations in the plasma of patients with ALD span from 8.5 to 206 (pg/mL), while the values in healthy subjects range from 0.3 to 10.4 (pg/mL) [306]. Even though these figures exhibit an important variability and should be considered carefully given the difficulty of assessing endotoxemia, the plasma endotoxin levels in patients with ALD have always been found to be 5 to 20 times greater than those in normal subjects [306].

Interestingly, a gender-dependent difference has been recorded in alcoholic endotoxemia [273], [332]. Kono et al. [197] reported significantly higher levels of plasma endotoxin in female rats after chronic ethanol intake. This could be explained by the fact that females are more susceptible than males to alcohol-mediated increases in intestinal permeability to endotoxin [398]. Indeed, gut leakiness is enhanced in animals treated with the female hormones estradiol and progesterone [195]. Furthermore, alcohol-induced elevated endotoxemia after ethanol activates more Kupffer cells in females than in males as there is an enhanced CD-14 expression in females [197].
Evidently, more information is required to understand whether these mechanisms explain the acknowledged observation that women are more prone to alcohol-induced liver damage than men [398].

**Mechanisms of alcohol-induced endotoxemia**

Animal as well as human studies suggest that alcohol mediates the transfer of endotoxin from intestinal lumen to the liver that leads to the elevation of plasma endotoxin concentration [301]. Figure 18 illustrates this association in regard to the plasma concentration of both factors after acute alcohol ingestion. Different mechanisms have been proposed to underlie this causal link.

![Figure 18](image-url)

**Figure 18.** Time course of blood ethanol and endotoxin concentrations after acute ethanol administration. (A) Rats were given 5 g/kg ethanol intra-gastrically and the concentration of ethanol was determined by gas chromatography at 30-min intervals for 3 h. Blood ethanol levels were determined from the concentration in breath. In separate experiments, blood samples were collected directly from the portal vein before and 30, 60, 90, 120, or 180 min after ethanol or saline. (B) Plasma endotoxin concentration, measured with the limulus amebocyte lysate assay [315].

As discussed previously, alcohol not only directly damages the intestinal mucosa but also induces intestinal barrier disruption leading to gut leakiness by redistribution of intercellular junction proteins, by local oxidative stress and by altering the intestinal microbiota composition (section 4.3.2). This ensuing gut leakiness is regarded as the main cause of endotoxemia in ALD [306]. Keshavarzian and coworkers [181] elegantly illustrate this assertion by showing that restoring a normal permeability by dietary means reduces endotoxemia and hepatic injury in alcohol-fed rats.
Earlier, it has been described how alcohol, by impairing intestinal motility, promotes SIBO, an important contributor to endotoxemia (section 4.4.6). Although additional research is required to clarify this mechanism, SIBO almost certainly accounts for alcohol-induced endotoxemia via the proliferation of Gram-negative bacteria and mucosal barrier disruption.

Another way by which alcohol increases plasma LPS levels is by preventing Kupffer cells from effectively clearing these molecules from the bloodstream [315]. In vitro, phagocytosis by Kupffer cells is impeded by the addition of ethanol directly to the culture medium [180]. Rivera et al. [315] came to the same conclusion in vivo, in rodents, and propose that ethanol may inhibit the scavenger receptor function of the immune cells.

Hence, until now, three major mechanisms of alcohol-induced endotoxemia have been identified [306]. Increased intestinal permeability, ethanol-induced bacterial overgrowth and delayed endotoxin clearance from the circulation may not be the only contributors to increased endotoxemia in ALD and others remain to be discovered. For instance, liver disease itself may elevate plasma endotoxin levels since they are significantly high in non-alcoholic cirrhotic patients [53], [129], [301]. Finally, understanding these underlying mechanisms may help to design strategies for the prevention or treatment of alcohol/endotoxin-associated disorders [301].

Alcohol and endotoxin-induced liver damage

Many animal studies support the hypothesis that endotoxin plays a critical role in alcohol-induced liver damage. For example, administration of endotoxin in rats fed with chronic alcohol led to the progression of liver injury from fatty liver to necroinflammatory changes [15], [41], [293]. In another rat model of alcoholic liver injury, considerable attenuation of liver damage was achieved by reducing plasma levels of endotoxin via sterilization of the gut with antibiotics [2].

While acute [235], [270] and chronic [174], [235], [273], [307] ethanol-induced increases in endotoxin plasma concentration appear to coincide with the development of liver damage [306], the demonstration of a correlation between the degree of endotoxemia and the severity of the disease is only in its infancy [272]. However, evidence such as the fact that endotoxemia was observed in alcoholics exhibiting minimal symptoms of ALD [331] suggests that endotoxemia occurs during the early stage of liver injury [306]. Data from the time course study by Keshavarzian et al. [182] show that fatty liver occurs early and before significant endotoxemia and that endotoxemia occurs prior to development of steatohepatitis, thereby confirming that it is improbable that endotoxemia is the consequence of liver inflammation. Contrarily to what has been proposed above, simple steatosis may be primarily due to the direct effects of alcohol on hepatic lipid metabolism, rather than a consequence of endotoxemia, and is not dependant on endotoxin [146]. In opposition, the onset of steatohepatitis seems to depend on endotoxin, the triggering factor for necroinflammatory cascade [182].

It is now acknowledged that alcohol exerts its damaging effects on the liver in synergy with endotoxin [182], [306]. Indeed, in an animal study conducted by Keshavarzian and coworkers [182], neither alcohol alone nor endotoxin alone (at least not low endotoxin levels) led to severe liver injury in rats. Yet, the combination of the two mediators effectively resulted in significant hepatocellular damage. Alcohol sensitzes hepatocytes to LPS-induced cellular injury [152], [363], [386], and exacerbates Kupffer cell production of cytokines such as TNF-α, IL-6 and IL-8 in the liver [398], in response to endotoxin.

Hence, Kupffer cells play an important role in the detrimental effect of alcohol [398]. Indeed, the destruction of Kupffer cells in an animal model of chronic ethanol exposure reduces liver injury [1]. Moreover, hepatocellular damage is prevented in both CD-14 [417] and TLR-4 deficient mice [377], once again emphasizing the essential role of these receptors. It is of interest that, in rodents, CD-14 levels in the liver increase in many inflammatory hepatic diseases, including alcoholic and non-alcoholic liver injury [398]. Furthermore, the expression of CD-14 in macrophages varies with different stages of alcohol-induced damage [191], [197].
Although the meaning of these variations is not entirely understood, these changes in CD-14 levels and thus IRAK-1 activity may determine how the liver reacts to the noxious effects of endotoxin [413]. Indeed, alcohol modifies the degree to which Kupffer cells, hepatocytes and other cells of the liver respond to LPS by promoting both tolerance and sensitization to endotoxin [398]. While acute alcohol administration classically induces tolerance (i.e. hypo-responsiveness), chronic alcohol sensitizes (i.e. renders hyper-responsive) the liver to the toxic consequences of endotoxin [235]. For instance, in contrast to normal hepatocytes which resist TNF-α-induced apoptosis, liver cells from rodents with alcohol-mediated fatty livers die rapidly following in vitro exposure to TNF-α [120]. Several mechanisms put forward to explain this phenomenon of sensitization involve hepatocyte mitochondria which play a role in TNF-α-induced apoptosis [199]. Interestingly, mitochondria of alcohol-induced fatty livers exhibit ultrastructural abnormalities [58]. Further research will certainly clarify these interesting findings which may shed light on an important aspect of the pathogenesis of ALD.

This alcohol-endotoxin synergy, which corresponds to the two-hit model postulated earlier, along with other direct metabolic effects of alcohol on the liver, not only initiates liver injury by means involving Kupffer cells described in section 4.5.2, but also, via sensitization of liver cells, creates a vicious circle that maintains a chronic necroinflammatory process and accelerates the onset of liver failure [182].

### Oxidative stress and alcohol-induced liver damage

Oxidative stress is accepted as a major mechanism of alcohol-induced organ injury [182], as shown in Figure 19. Inhibiting superoxide production by impeding NADPH oxidase activity reduces alcohol-induced liver injury in rats chronically fed with alcohol [196]. Antioxidants confer considerable protection against ALD in rodents [18], [244]. Furthermore, Kang et al. [177] postulated that, in acute alcohol intoxication, oxidative stress may mediate endotoxin-induced hepatic production of TNF-α, described earlier as responsible for major hepatocellular damage.

In section 4.3.2, it was explained how alcohol, in the intestine, activates oxidative pathways in a CYP2E1-dependant manner. It appears that the same mechanism takes place in the liver. However, a number of studies reports that the hepatic oxidative stress related to chronic alcohol ingestion is mostly accredited to LPS-induced Kupffer cell activation [197], [398], [418], once again underscoring the central role of these cells.
Figure 19. CYP2E1-dependent oxidant stress and toxicity in alcoholic liver injury. Alcohol raises CYP2E1 protein and activity by stabilizing the enzyme against proteasome-mediated degradation. CYP2E1 generates ROS (superoxide and hydrogen peroxide) and, in the presence of iron which is increased after ethanol intake, more powerful oxidants such as hydroxyl radicals. Lipid peroxide formations are proposed to be key mediators of the CYP2E1 toxicity and mitochondrial injury. Hepatic stellate cells are activated by superoxide and induce hepatic fibrosis. ATP = adenosine triphosphate; GSH = Glutathione; MMP = mitochondrial membrane potential; ROS = reactive oxygen species [268].
Deductions

In accordance with the scheme in Figure 20, which summarizes the interactions of the main protagonists of ALD pathogenesis, endotoxin appears to mediate many effects of alcohol on the liver. The fact that treatments such as antibiotics and lactobacillus, which prevent endotoxin production by the gut microbiota, significantly impede the development of steatohepatitis in alcohol-fed animals [2], [167], [271] raises both the possibility that the main triggering factor of ALD has been identified and the hopes of elaborating new therapies for this disease, but also of other disorders. Indeed, endotoxin may participate in the development of other alcohol-mediated tissue or organ damage [301] such as pancreatitis [390], acute respiratory distress syndrome [321] and brain injury [104].

Even though gut microbiota and its derived endotoxin play a major role in ALD pathogenesis, mucosal barrier dysfunction, Kupffer cells, TNF-α and other cytokines along with oxidative stress have an equally important responsibility in this complex pathogenic network and should be considered in the development of novel therapeutic strategies, as will be elaborated in the next chapter.

![Figure 20. Summary of the interactions between alcohol, intestinal bacteria and intestinal permeability to endotoxin, and Organ Injury: A Summary.](image)

**Figure 20.** Summary of the interactions between alcohol, intestinal bacteria and intestinal permeability to endotoxin which lead to liver injury. Alcohol consumption can promote the growth of Gram-negative bacteria in the intestine which results in the accumulation of endotoxins. Additionally, alcohol metabolism by Gram-negative bacteria and intestinal epithelial cells can lead to the accumulation of acetaldehyde, which in turn can increase intestinal permeability to endotoxin. Alcohol-induced generation of nitric oxide may also be involved in this increased gut leakiness. The latter allows the passage of endotoxins from the gut lumen to the portal vein which brings LPS to the liver where it binds to Kupffer cells to initiate a cascade of events leading to TNF-α production, oxidative stress (not represented here) and liver injury. Also, endotoxins that escape to general circulation may induce injury to other organs. A part of TNF-α produced in the liver may reach the intestine via bile duct or general circulation and further elevate intestinal permeability to endotoxin. Nitric oxide may mediate the endotoxin-induced liberation of TNF-α in the liver, although the arrow here is missing [301].
4.5.6 Endotoxemia in NAFLD

Mounting evidence from experimental studies shows that endotoxin considerably contributes to the development of obesity-related inflammatory liver diseases such as NAFLD and NASH [2], [59], [211], [213]. For instance, genetically obese rats and mice rapidly develop steatohepatitis after exposure to low amounts of LPS [59]. Also, specific elimination of Gram-negative microorganisms and thus lessening of endotoxin levels by polymyxin B treatment reduces hepatic steatosis [287].

In humans, the likely role of endotoxin in hepatic damage was supported by indirect evidence only [59], [309], [403] until, recently, Farhadi and colleagues [112] observed significantly elevated endotoxemia in patients with NASH. Although Wigg et al. [403] failed to detect this correlation in the same kind of subjects, they proposed reasonable explanations for the negative results of their study. Indeed, they assessed endotoxin retrospectively and did not measure “hidden” endotoxin that is bound to plasma proteins.

Mechanisms for increased endotoxemia in NAFLD

Miele and coworkers [257] recently demonstrated that patients with NAFLD exhibit a considerable increase in gut permeability (section 4.3.3) and the latter is very likely the cause for the observed elevated plasma LPS. Also, SIBO, which is significantly prevalent in subjects with fatty liver disease, may well increase gut permeability to endotoxin [59], [318], [403] as elicited in section 4.4.7. Moreover, mucosal barrier dysfunction and SIBO have been shown to be associated with the severity of hepatic steatosis in NAFLD [257].

These two features might reflect a modification in intestinal microflora composition. Yet, the role of the intestinal flora in NAFLD is poorly understood. Evidence that surgical procedures which provoke intestinal stasis and secondary bacterial overgrowth, such as jejuno-ileal bypass, hasten the progression of fatty liver disease in obese patients [143] indicates that increased exposure to intestinal bacterial products may take part in the pathogenesis of NAFLD [210]. Also, hepatic steatosis has been identified in other settings that lead to intestinal bacterial overgrowth in humans [403]. Thus, changes in gut intestinal microbiota may influence the development of steatosis [257], but more data are needed to prove such an association.

It is noteworthy that the two factors demonstrated to mediate endotoxemia in NAFLD, i.e. intestinal barrier disruption and SIBO, have also been put forward as factors of alcohol-induced endotoxemia (section 4.5.5). Hence, ALD and NAFLD might share important pathogenic mechanisms.

Endotoxin- and TNF-α-induced liver injury in fatty liver disease

Yang et al. [416] revealed that, in genetically obese rats, systemic endotoxemia leads to steatohepatitis via the release of TNF-α. This allegation is further supported by several studies. For example, neutralizing anti-TNF antibodies considerably ameliorate histological evidence of hepatocellular lipid accumulation as well as the hepatic content of fatty acids and clearly lower hepatic inflammation [210]. Additionally, as already mentioned, NASH model mice with deficient TNF-α receptors have reduced liver steatosis and fibrosis [367]. Finally, TNF-α concentration in rat models of NASH was significantly higher than in controls [406].

If clinical studies are, again, less abundant than experimental data, they also show higher levels of TNF-α among patients with NASH and NAFLD in comparison with controls [162], [403]. Hence, patients with NAFLD exhibit high amounts of plasma endotoxin and of TNF-α. However, no study was yet able to demonstrate a statistically significant positive correlation between TNF-α and endotoxin plasmatic concentrations in NAFLD patients [403].
These data sustain the concept that TNF-α plays a significant role in the development of NASH as a “second hit” subsequent to the development of steatosis [171], [416]. Moreover, they provide indirect evidence for the central role of endotoxin, also candidate for the “second hit”, in the pathogenesis of NASH via Kupffer cell activation and TNF-α production [403], as TNF-α is the proven effector of endotoxin-mediated liver injury [19], [260].

Earlier in this section, the process of TNF-α-induced hepatocellular damage, especially in a fatty liver, has been discussed. Further findings from some authors suggest that TNF-α is an important contributor to increased activity of stearoyl-CoA desaturase-1 (SCD-1), a lipogenic enzyme involved in fatty acid metabolism, fatty acid synthesis and excessive triglyceride accumulation in hepatocytes of obese mice [210]. Indeed, anti-TNF antibodies decrease the activity of SCD-1 and mediate the reduction in total hepatic fatty acid content [83]. Therefore, these data support the idea that endotoxemia results in steatosis via an inflammation-mediated extrahepatic insulin resistance and increased supply of fatty acid to the liver (sections 4.4.7 and 4.5.4), by proposing TNF-α as a possible causal link. Yet, it is not yet understood if TNF-α-induced steatosis is dependant or not on endotoxin, since these findings are different from the results of Keshavarzian et al. [182] which report steatosis prior to endotoxemia. It could be that steatosis is a consequence of the metabolic syndrome (or alcohol in ALD) and that endotoxin further potentiates this steatosis. Obviously, supplementary investigation is needed.

Hence, in addition to providing the “second hit” by acting on sensitized and vulnerable steatotic hepatocytes, TNF-α might, by enhancing SCD-1 activity, also contribute to the sensitization of hepatic cells to injurious agents such as endotoxin and TNF-α, i.e. the “first hit”. Nevertheless, while CD-14 levels in the liver have been shown to be increased in ALD and NAFLD [398] and as alcohol has been postulated to induce this up-regulation and thus promote hepatocellular sensitization to endotoxin in ALD (section 4.5.5), there is a lack of information regarding this same potential role for TNF-α in NAFLD. Interesting data show that patients with NASH have noticeably more soluble TNF-α receptor 2 (sTNFR2) than patients with simple steatosis and controls [162]. Moreover, sTNFR2 expression in steatotic hepatocytes is around 2-fold higher than that in control hepatocytes and the expression of sTNFR1 and of sTNFR2, particularly, is up-regulated by TNF-α in steatotic liver cells. Thus, TNF-α, which was expected to sensitize steatotic hepatocytes to endotoxin as alcohol does in ALD, renders these cells hyper-responsive to itself, thereby creating a vicious circle. Furthermore, Zhang and colleagues [422] suggest that sTNFR2 stimulation is involved in the evolution from reversible steatosis to progressive steatohepatitis. Therefore, TNF-α may mediate the early stages of fatty liver disease as well as the transition to more advanced stages of liver injury [365].

**Oxidative stress-mediated liver damage**

As in ALD, oxidative stress plays an important role in hepatocyte injury in NAFLD. Indeed, NASH was reported to be tightly linked to lipid peroxidation and oxidative stress [33], [90], [198], which results from both overproduction of free radicals and poor hepatic reserve of antioxidants [234]. In NAFLD, as in ALD, ROS are generated by Kupffer cells, by intracellular fat accumulation, by TNF-α-induced apoptosis of hepatocytes and by TNF-α-stimulated mitochondria. Interestingly, as in alcohol-induced fatty livers, ultrastructural and functional alterations have been observed in hepatic mitochondria of obese mice [79], [179]. ROS participate in the hepatic necroinflammatory cascade. The latter, itself, is also responsible for the accumulation of oxidants as it includes the hepatic migration of leucocytes which produce, among other injurious compounds, molecules resulting from NO [182]. Finally, as mentioned at the beginning of this section, endotoxins arriving into the mesenteric circulation and the lymphatic system activate NO synthetases in the splanchnic area, thereby participating in the onset of portal hypertension and inducing regional and systemic liberation of pro-inflammatory cytokines that contribute to the progression of liver impairment.
Inferences

Altogether, the discussed findings support a strong relationship between intestinal microflora, gut permeability, systemic and hepatic inflammation (prominently mediated by Kupffer cells and TNF-α) and oxidative stress in the pathogenesis of NAFLD. It is noteworthy that these factors are the same as the ones that mediate ALD, thereby further linking the two disorders. It appears increasingly obvious that the gut microbiota, via endotoxin, has a potent influence on the occurrence of these liver diseases, even though some points in this newly discovered pathogenic network still need to be clarified. However, since ALD and NAFLD have many physiopathological features in common, unraveling mechanisms for one of them certainly benefits the understanding of the other. Figure 21 represents a scheme of the recent pathogenic mechanisms which have been discussed throughout this review and which appear to be common to both ALD and NAFLD. With advances in research, it may be possible that these two diseases are shown to be one entity with two different etiologies. Moreover, the revelation of these pathological interactions offers many possibilities for the development of therapeutic strategies for ALD and NAFLD which will be examined in the coming chapter.
Figure 21. Summary of hypothetical interactions between gut microbiota, intestinal permeability, endotoxemia, TNF-α- and oxidative stress-mediated liver injury. This scheme includes the most important modifying factors that influence the main protagonists of this complex pathogenic cascade. HSC: hepatic stellate cells, ROS: reactive oxygen species, SIBO: small bowel overgrowth.
5 NOVEL THERAPEUTIC APPROACHES IN ALD AND NAFLD

5.1 Introduction

Current therapies for liver diseases related to alcohol consumption or obesity are based on abstinence and weight loss, respectively [365]. Many treatments that include corticosteroids [348], supplemental amino acid infusion [256] and colchicine [6], [368] have been tested in patients with ASH but none have been consistently proven to have a clear beneficial outcome nor has reached a consensus among hepatologists. Likewise, no pharmacological treatment has been reliably shown to be efficient in NASH, for which the current therapeutic recommendations comprise weight loss and reversal of other components of metabolic syndrome with, for instance, insulin sensitizers, lipid lowering agents or cytoprotective agents. Thus, despite more than a decade of research and clinical trials, there is no presently established treatment for ALD or NAFLD, most probably due to the lack of comprehension of their physiopathology [89], [203].

Given the recent progress in the understanding of the pathogenesis of these liver disorders, new molecular mechanisms to target are gradually emerging [267]. In particular, since abnormal gut intestinal microflora profile plays an initial and central role in this context, manipulation of the commensal bacteria ecosystem may represent a novel and revolutionary approach [94]. Other important potential targets are, of course, intestinal permeability, endotoxemia, inflammation and oxidative stress. This chapter discusses the latest advances in the development of therapeutic strategies for ALD and NAFLD, in perspective with the novel pathogenic findings contemplated in this review.

5.2 Manipulation of the intestinal microbiota

So far, the therapeutic strategies used to limit bacterial translocation have been either very aggressive, such as colectomy in experimental models [64], or conservative, with the use of antibiotics [276], [381] despite their side effects. Recently, the emergence of probiotics, prebiotics and synbiotics has brought a new challenge: the reconstitution of an intestinal microflora that would restore well-being and prevent the over activity of a dysbiotic commensal microbiota generated by an excess of alcohol or food and/or a fat-rich diet [63].

5.2.1 Antibiotics

As already mentioned in previous chapters, the suppression of Gram-negative bacteria intestinal growth reduces the amount of plasma LPS, thereby protecting the liver (and other organs) from endotoxin-associated damage [2], [209], [301]. This has been demonstrated in antibiotic-treated animal models of ALD [2] and NAFLD.

By changing the composition of the bowel microbiota, treatment with antibiotics such as gentamycin significantly lowers intestinal permeability, oxidative stress, macrophage infiltration as well as the inflammatory tone [66], [209]. Indeed, norfloxacin appears to normalize LPB, CD-14 and cytokine levels in cirrhotic patients [7]. Antibiotics also block the effects of alcohol on intestinal permeability, especially by reducing the intracolonic formation of acetaldehyde [122].

Information regarding the influence of antibiotics on the severity of liver damage in humans is difficult to find. Also, these substances are well known for their secondary effects that include, in addition to their chronic toxicity [105], the emergence of multidrug-resistant strains [178], [280], [359] or E. coli overgrowth and translocation [40]. Thus, other means to eliminate certain species of commensal bacteria in order to alleviate hepatic impairment have been explored.
5.2.2 Probiotics

Probiotics are live microbial food supplements which are given orally, in adequate amounts that allow the colonization of the colon [161], [245]. Because they have favorable effects on the host [109], they are also known as beneficial bacteria. Lactobacilli species that reside in the small intestine and Bifidobacteria, which are found in the colon and have been mentioned in chapter 4 to improve mucosal barrier function, are examples of endogenous probiotics [301] and appear to be the most used probiotic strains [63]. Other commonly used probiotics are listed in Table 1.

<table>
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<tr>
<th>Table 1. Bacteria and yeasts used as probiotics [245]</th>
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<tr>
<td><em>Bifidobacterium</em> longum</td>
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<tr>
<td><em>B. breve</em></td>
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<tr>
<td><em>B. infantis</em></td>
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<tr>
<td><em>B. bifidum</em></td>
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<tr>
<td><em>B. adolescentis</em></td>
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<td><em>Lactococcus</em> cremonis</td>
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<td><em>L. lactis</em></td>
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<td><em>Enterococcus faecium</em></td>
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<td><em>Lactobacillus rhamnosus</em></td>
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<td><em>L. acidophilus</em></td>
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<td><em>L. casei</em></td>
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<td><em>L. bulgaricus</em></td>
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<td><em>L. gasseri</em></td>
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<td><em>Saccharomyces boulardii</em></td>
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<td><em>S. cerevisiae</em></td>
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Beneficial bacteria stimulate intestinal development and mucosal immunity (for example by increasing the number of IgA secreting cells), synthesize antioxidant and antibacterial substances [249], [360], [414] (thus lessening intestinal oxidative stress) [360], modulate the intestinal inflammatory response along with the levels of TNF-α, IFN-γ, IL-10, IL-6, and decrease intestinal permeability [124], [169], thereby improving the intestinal barrier function [62], [109], [124], [209], [282], [310], [326], [383] and lowering endotoxin levels [138], [301], [391], [392]. Indeed, animals supplemented with oral *Bifidobacterium* spp. have a lower incidence of bacterial translocation [209], [391] and endotoxemia has been shown to correlate negatively with the presence of these bacteria [68]. Moreover, *L. acidophilus* may be responsible for an increase in the number of Kupffer cells [274]. A very interesting finding is that the ingestion of probiotics appears to alter the gut microbiota composition, restoring the numbers of *Bifidobacteria* and *Lactobacillus* species [124], [189], [423] which are reduced in alcoholics, for example. Probiotics have also been shown to attenuate the intestinal proliferation of Gram-negative bacteria [295], [301], [370], [419]. The detailed mechanisms of the mentioned positive effects of probiotics are well reviewed by Forsyth and coworkers [124]. They include, for instance, the downregulation of the NFkB family genes, and may be mediated by additional factors such as epidermal growth factor (EGF), as will be examined shortly.

These data suggest that, since probiotics ameliorate intestinal barrier function and lessen endotoxemia, they may reduce the extent of hepatocellular injury. Indeed, some authors have demonstrated that probiotics can prevent liver damage [407]. This has been shown to occur in animal models of ALD fed with *Lactobacillus rhamnosus* Gorbach-Goldin (LGG), the first used probiotic which received most clinical attention to date [135]. LGG lowers endotoxin levels, attenuates alcohol-induced intestinal and hepatic oxidative stress, strongly reduces gut leakiness...
and subsequently decreases severity of liver injury [124], [169], [271]. Other probiotics have been tested in ALD animal models and researchers have come to the same conclusions [232]. In patients with alcohol-induced liver injury, short-term oral supplementation with beneficial bacteria has been reported to restore the bowel flora and greatly diminish hepatic damage in comparison with standard therapy [190]. However, further investigation is required to confirm whether probiotics can attenuate ALD in humans [301].

Similarly to experimental ALD, gavage with Lactobacillus sp. prevents non-alcoholic endotoxin-induced hepatic injury in rats [227], [284], [333]. In addition, a decrease in liver steatosis and hepatic inflammation markers was achieved in obese mice treated four weeks long with a mixture of viable, lyophilized bifidobacteria, Lactobacilli, and Streptococcus thermophilus [210]. In patients with NAFLD, only two small studies have been conducted with probiotics and suggest that the latter are well tolerated, may improve liver function and reduce markers of lipid peroxidation [215]. Moreover, Loguerchio and coworkers [217] demonstrated that the use of the probiotic VSL#3 (a mixture containing 450 billion bacteria of different strains) in patients with NAFLD improves various serum markers of hepatic damage. However, no biopsies were performed. Hence, the lack of randomized clinical trials makes it difficult, so far, to support or refute probiotics as a treatment for patients with NAFLD [215].

Probiotics have been proven to be efficient in various clinical disorders, ranging from infantile- or antibiotic-associated diarrhea, necrotizing enterocolitis, Helicobacter pylori infections, inflammatory bowel disease to cancer, female uro-genital and surgical infections [309]. While it is obvious that additional research regarding the benefits of probiotics in humans with different stages of ALD and NAFLD is necessary, accumulating experimental data exhibit probiotics as a promising potential treatment for these liver diseases.

5.2.3 Prebiotics

Prebiotics consist of non-digestible carbohydrates that act as “fertilizers” of the gut microbiota and promote the growth of the whole indigenous population of bifidobacteria or lactobacilli (or both) [133], [245], [316]. They include oligosaccharides such as inulin, fructo- and galacto-oligosaccharides [245]. Other examples of prebiotics are listed in Table 2. Prebiotic dietary fibers escape digestion in the upper gastrointestinal tract and hydrolysis by host enzymes because of their chemical structure [134] and thus provide a substrate to a restricted group of microorganisms which have clearly identified health-promoting properties [245]. Consequently, they induce specific changes both in the composition and activity of the gastrointestinal microbiota [134]. This alteration of the gut ecology leads to enhanced villus height and crypt depth and hence a thicker mucosal layer in the jejunum and colon [192]. Indeed, these effects are mediated by the bacterial fermentation of the prebiotics. The products of fermentation are mainly short-chain fatty acids (SCFAs) with butyrate, for example, acting as an energetic substrate for the enterocytes and possibly exerting a trophic effect on the mucosa [32], [361].

<table>
<thead>
<tr>
<th>Table 2. Examples of Prebiotics [245]</th>
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<tr>
<td>Fructo-oligosaccharides</td>
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<td>Inulin</td>
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<td>Pyrodextrins</td>
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<tr>
<td>Transgalactosylated oligosaccharides</td>
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<tr>
<td>Galacto-oligosaccharides</td>
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<tr>
<td>Soy oligosaccharides</td>
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<tr>
<td>Xylo-oligosaccharides</td>
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<tr>
<td>Isomalto-oligosaccharides</td>
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<tr>
<td>Lactulose</td>
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Therefore, prebiotics and probiotics, via two slightly different ways, both increase the gut Bifidobacteria and Lactobacilli content. This increase has been shown to attenuate the important endotoxin levels in mice with metabolic syndrome [67], most probably via the ability of these bacteria to enhance intestinal barrier function by various mechanisms described just above. Besides the assumed role of the SCFAs and specific bacterial strains such as Bifidobacteria, there may be additional mechanisms, yet to be discovered, by which prebiotic-induced changes in gut microflora improve intestinal barrier function [69]. Indeed, as will be mentioned shortly, glucagon-like-peptide-2 may mediate the beneficial effects of prebiotics.

Fermentable fibers have been shown to attenuate liver damage in experimental ALD and NAFLD. In a rat model of ALD, oat supplementation markedly lessened gut leakiness, endotoxemia and steatohepatitis [181]. Furthermore, in a recent study by Cani and colleagues [69], prebiotic-treated obese and diabetic mice exhibited lower plasma endotoxin and cytokine levels along with a decreased hepatic expression of inflammatory and oxidative stress markers. The decreased inflammatory tone was associated with a lower intestinal permeability and improved tight-junction integrity, in comparison with controls.

Liu and coworkers [216] demonstrated an improvement of 29% in the Child-Turcotte-Pugh class of cirrhotic patients treated with prebiotics. This progress occurred in association with a significantly reduced hepatic necro-inflammatory activity and an improved hepatic function. However, there are no available data regarding the use of these dietary fibers in patients with ALD and NAFLD, thereby stressing the need for further investigation in this challenging field.

### 5.2.4 Synbiotics

A synbiotic is a mixture of probiotics and prebiotics. In order to maintain colonization, probiotics must be taken regularly [193] because, without a prebiotic substrate, they do not survive well in the gastrointestinal tract where factors such as oxygen, temperature, pH and competing microbial species impede their development. Therefore, this combination appears to be more stable and thus more efficient than probiotics or prebiotics alone. Indeed, this approach enables the stimulation of the growth and implantation of both exogenous and endogenous bacteria in the colon [134].

So far, there is hardly any information regarding the effect of synbiotics on the outcome of liver impairment. The above mentioned clinical study of Liu et al. [216] has addressed this question and showed an amelioration of 50% in the Child-Turcotte-Pugh class along with reduced hepatic necroinflammatory activity and enhanced liver function in patients with cirrhosis. As expected, synbiotics had a greater beneficial effect on the liver than prebiotics alone (50% versus 29% of improvement in Child-Turcotte-Pugh class). Of course, additional investigation is required to assess the ability of synbiotics to effectively attenuate hepatocellular injury as well as the underlying mechanisms.
5.3 Restoration of intestinal barrier integrity

As discussed in section 4.3, intestinal barrier dysfunction plays a crucial role in the pathogenesis of ALD and NAFLD. It may even be considered as a rate-limiting step in the development of these liver diseases. Therefore, restoring the mucosal barrier function in order to impede the elevation of endotoxemia and thus its noxious consequences represents a very attractive and conceivable challenge.

5.3.1 Glucagon-like-peptide-2 (GLP-2)

GLP-2 is a potent intestinotrophic factor. In addition to promoting intestinal mucosal growth, this peptide enhances intestinal barrier function by affecting both paracellular and transcellular pathways. The mechanisms that grant GLP-2 these properties remain unclear but may include tight junction modulation. Moreover, according to research currently in progress, several pathways may be involved [39].

Cani and coworkers [69] demonstrated that chronic GLP-2 pharmacological treatment lowers endotoxaemia (by about 50%) and improves gut permeability markers in obese mice. Also, it attenuates systemic and hepatic inflammation, oxidative stress and macrophage infiltration markers. Furthermore, ZO-1 and occluding staining immunohistochemical score analysis confirmed the important impact of GLP-2 on tight-junctions proteins.

Most interestingly, the use of a GLP-2 antagonist in these same mice totally blunts the prebiotic-induced reduction in endotoxaemia. Thus, most of the prebiotic-induced changes in hepatic inflammation and oxidative stress markers may also be mediated by a GLP-2-dependent mechanism. These findings led the authors to stipulate that gut microbiota modulation could control and increase the endogenous production of GLP-2 and subsequently improve gut barrier function in a GLP-2-dependent manner. Such data stress the utility of developing specific therapeutic strategies using GLP-2 in order to prevent metabolic endotoxaemia and its derived disorders [69].

5.3.2 Epidermal growth factor (EGF)

EGF promotes growth and differentiation of gastrointestinal mucosa and also protects the latter from injurious agents [301]. Sheth and coworkers [339] demonstrated that, experimentally, EGF can preserve intestinal paracellular permeability by attenuating acetaldehyde- or E. coli-induced disruption of tight junctions and adherens junctions (by impeding the redistribution of the actin cytoskeleton and its interaction with occluding, ZO-1, E-cadherin, and β-catenin). In the human colonic mucosa, an equivalent protective effect of EGF on acetaldehyde-induced disruption of intercellular junctions was reported [26], [27], [34], [310].

Interestingly, researchers found that Lactobacillus spp. is able to modulate EGF receptor (EGFR)-mediated intracellular signaling [310], thereby providing a new mechanism whereby probiotics may improve intestinal barrier function.
5.3.3 L-glutamine

Glutamine is a nonessential amino acid which has been shown to improve intestinal barrier function experimentally [93], [401]. Moreover, it has been proven to attenuate acetaldehyde-induced mucosal barrier disruption in vitro [34], [336]. Indeed, glutamine reduces occluding, ZO-1, E-cadherin, and β-catenin redistribution to the intracellular compartments and their dissociation from the actin cytoskeleton. Interestingly, it appears that L-glutamine protects the gut barrier function by an EGF receptor-dependent mechanism [336].

Therefore, EGFR and its activators (such as EGF, L-glutamine and possibly certain bacterial strains) emerge as a very interesting target for mucosal barrier restoration and, thus, therapeutic strategies for the contemplated hepatic disorders. These promising findings justify supplementary research about this EGFR-mediated pathway.

5.3.4 Insulin-like growth factor 1 (IGF)-1

An interesting novel hypothesis focuses on the role of the anabolic hormone IGF-1. The latter is markedly reduced in cirrhosis, since the major source of the circulating hormone is the liver [121]. IGF-1 is most probably mandatory for normal gastrointestinal tract structure and function [277]. In cirrhotic rats, researchers have shown that treatment with IGF-1 enhances the intestinal barrier function and reduces bacterial translocation and endotoxemia. Moreover, these changes were accompanied by a diminished TNF-α expression in the intestine [218]. Evidence from a small clinical randomized trial showed that IGF-1 therapy is safe and results in a decrease in Child-Pugh class. Yet, gut barrier function was not assessed [84].

5.3.5 Inhibition of myosin light chain kinase (MLCK)

As discussed earlier (sections 3.2.1 and 4.3.2), myosin light chain kinase-mediated phosphorylation of myosin light chain is a central event in one pathway of tight junction regulation. Indeed, MLCK activation, for instance by glucose, acetaldehyde, IFN-γ or TNF-α, leads to intestinal barrier dysfunction [82]. Therefore, the inhibition of MLCK should restore the barrier function. Although MLCK inhibitors have only been proven to do so experimentally [335], [388], [427], their success in re-establishing intestinal barrier integrity suggests that these molecules may be used as a future therapy for ALD and NAFLD.

5.3.6 Zinc supplementation

Zinc is an essential trace element involved in various physiological functions and its major binding protein, metallothioneinein, is found in the liver [177]. Alcohol consumption seems to cause zinc depletion by impairing its intestinal absorption and interfering with its excretion [14], [204], [99], [378]. Zinc depletion was reported in animal models of alcohol-induced hepatic injury, in alcoholic patients with hepatitis or cirrhosis [55], [187], [241] as well as in patients with increased intestinal leakiness [151], [345]. Moreover, the reduction in zinc levels has been proposed to play a role in alcohol-induced liver injury [241]. Researchers using a mouse model of acute alcohol toxicity demonstrated that zinc supplementation attenuates ethanol-induced increases in endotoxemia, serum alanine aminotransferase activity and the amounts of hepatic TNF-α. Importantly, this supplementation prevents ethanol-induced liver injury in this animal model [205]. Also, the intake of complementary zinc appears to preserve intestinal permeability in patients with Crohn's disease [355]. Therefore, it may be possible that zinc achieves these changes, at least in part, by enhancing mucosal barrier function and thus inhibiting ethanol-mediated endotoxemia. However, the mechanism whereby zinc preserves intestinal permeability is still under investigation [301].
It may be possible that zinc may protect the liver from alcohol-mediated damage through other ways than solely avoiding endotoxemia. For instance, zinc inhibits alcohol-induced ROS accumulation and LPS-induced hepatic TNF-α production (both via abrogation of the NFκB pathway in Kupffer cells) [424] and hepatocyte apoptosis. Hence, zinc may also benefit NAFLD since its targets also appear to mediate the pathogenesis of this disorder. However, the effect of zinc in animals or patients with NAFLD has not yet been evaluated. Even though further research is needed in order to define mechanistic insights and to confirm the clinical benefits of zinc, the latter may indeed have an important therapeutic potential in the prevention and treatment of ALD [177] and NAFLD, at least by impeding the elevation of endotoxemia and inhibiting the production of TNF-α and oxidative compounds, thus lessening hepatic injury.

5.4 Prevention of inflammation and oxidative stress

The release of inflammatory mediators and reactive oxygen species that injure the liver corresponds to the last stage of the gut-liver pathological axis. Similarly to intestinal dysbiosis and gut barrier dysfunction, this part of the pathogenesis also represents a target for potential therapeutic strategies.

5.4.1 Anti-TNF antibodies

Since it is acknowledged that TNF-α is critically involved in hepatocellular damage, it is justified to hypothesize a therapeutic role for TNF-α blockage in chronic liver disorder. Li and colleagues [210] demonstrated that treatment of obese mice exhibiting fatty livers with anti-TNF antibodies results in a complete resolution of steatosis and a significant reduction in liver inflammation. Serum ALT levels were also decreased and, interestingly, hepatic mitochondrial abnormalities tended to normalize. In addition, this treatment led to a reduction in the activity of NFκB, a transcription factor introduced in section 4.5.2 and thought to control the cellular response to endotoxin. Anti-TNF antibodies also proved their efficiency in animal models of ALD [167], [299].

Pentoxifylline, a xanthine derivative that inhibits TNF-α [103], has been studied in NASH patients. Improvement in transaminases levels, liver steatosis, inflammation and fibrosis was observed [3], [327], [300], [328]. Similar findings were noted in several studies with the anti-TNF antibody infliximab (Remicade) in patients with ASH [264], [349], [366]. These data, among others, support a potential role for anti-TNF antibodies in the treatment of ALD and NASH, but there is no doubt that further research with larger clinical trials is necessary.

5.4.2 IL-6

The endogenous cytokine IL-6 exhibits both pro- and anti-inflammatory properties and appears, like zinc, to prevent excessive TNF-α and ROS production. Indeed, Nandi and colleagues [269] showed that treatment with IL-6 modulates the response to LPS (i.e. inducing hyporesponsiveness to endotoxin) and strongly reduces its lethal toxicity in mice. Also, this cytokine was found to inhibit TNF-α and IFN-γ production. Also, this study suggests that IL-6 treatment prevents excessive LPS-induced ROS release. These recent and convincing findings, corroborated by additional studies [88], [379], should hopefully lead to clinical trials and raise the possibility that other endogenous cytokines such as IL-10 [230], [269], [350] could also be candidates for LPS-induced liver disorder therapy.
5.4.3 Antioxidants

As discussed in the previous chapter, oxidative stress plays an important role in inducing intestinal hyperpermeability and hepatocellular damage in both ALD and NAFLD. Therefore, antioxidants represent a logical potential therapeutic strategy for these liver diseases. In a mouse model of acute alcohol-mediated hepatotoxicity, antioxidant treatment with N-acetylcysteine or dimethylsulfoxide did not succeed in attenuating plasma endotoxin levels but significantly inhibited alcohol-induced hepatic lipid peroxidation, TNF-α production, steatosis and necrotic hepatocellular death [424]. This beneficial outcome has been shown to result from the down-regulation of TNF-α release from LPS-activated Kupffer cells along with the inhibition of NFκB activation [38], [70], [78], [142].

If this approach has been successful in experimental models [398], the results of clinical trials are less promising. For instance, improvement in transaminase levels and histological findings in NASH patients treated with vitamin E has been observed [147], [148] but another study showed no benefit with this therapy [201]. Other antioxidants such as β-carotene, vitamins C and E, selenium, methionine, allopurinol, desferrioxamine and N-acetylcysteine have been studied in patients with ASH and did not prove their efficiency [296], [353]. Indeed, the available data are of mixed quality; the study sizes are small and the results range from heterogeneous to conflicting. A large randomized, multicenter, double-blinded, placebo-controlled trial including vitamin E is currently in progress [203].

5.4.4 Genetically engineered viruses

The use of genetically engineered viruses that produce excessive amounts of specific enzymes or signaling molecules has been successful, experimentally. For instance, the viral over-expression of an enzyme that converts superoxide into harmless products almost completely prevents alcohol-induced liver injury and lessens alcohol-induced TNF-α production in animals [400]. A similar outcome has been achieved with a genetically engineered virus that produces a molecule able to suppress NFκB activation [376]. However, it has yet to be determined whether this virus-mediated gene transfer may be of clinical use [398].

5.5 Conclusion

These novel therapeutic approaches based on the recently discovered pathogenic mechanisms are summarized in Figure 22. They raise many hopes in the quest for an effective treatment for ALD and NAFLD. At present, the most promising strategy lies in the use of the three “biotics”, a simple, safe and revolutionary approach. However, the next few years could yield fascinating advances in liver disease research and thus treatment. The discussed potential therapies will certainly be investigated, tested experimentally and clinically and ameliorated. While additional targets such as the TLR signaling pathway will be examined, new ones will be identified. Therefore, it is essential that the obscure pathogenic aspects of these liver diseases should be recognized and further investigated.
Figure 22. Schematic representation of the targets of the discussed potential therapeutic strategies for ALD and NAFLD.
6 CONCLUSION

The aim of this thesis was to review the established and assumed mechanisms of the novel and revolutionary pathogenic concept linking the gut microbiota to liver disease. Mounting experimental and clinical evidence corroborates the theory that alcohol- and high-fat diet-mediated modifications in the intestinal microflora composition participate in the development of ALD and NAFLD.

The microbial community that resides in the human gut outnumbers by ten fold the number of human body cells, represents more than a hundred times the human genome and thus remains poorly understood. It is now known to have a plastic composition. Indeed, there is a great diversity in the gut microfloras of different persons, especially between lean and obese people but also between young and old subjects. Various factors that include alcohol and fat-rich regime have been shown to render the microflora dysbiotic, i.e. with reduced populations of beneficial bacteria, such as Bifidobacteria and Lactobacilli, and an overgrowth of noxious strains. One can consider the intestinal microflora as an individual organ which can dysfunction. Furthermore, an altered bacterial community has the ability to induce, through the toxins that it sheds, various pathological conditions. The fact that this indistinct and varying population of microorganisms may influence and even trigger the onset of certain diseases such as liver impairment represents an unexpected but fascinating hypothesis.

The intestinal surface, the body’s largest external surface, comes in direct contact with the gut microflora. It forms an effective barrier against the high concentrations of bacteria, multiple antigens and toxic compounds. Unfortunately, it is now clear that this intestinal barrier can dysfunction, thereby representing the condition sine qua non of the involvement of the so-called gut-liver axis. Although there are numerous human pathologies in which abnormal permeability has been suggested to be important, hepatic diseases were not counted among them until recently. Indeed, increased intestinal permeability which can be elicited by alcohol, a fatty diet, certain bacterial strains or other agents, has been reported in ALD as well as in NAFLD. It is even considered as a rate-limiting-step of their pathogenesis. Interestingly, while many pathologies comprise intestinal epithelial damage, namely inflammatory bowel diseases, they are not complicated by NAFLD.

Although the mechanisms whereby alcohol ingestion and a fatty diet impair the barrier function are becoming progressively understood, many issues remain to be elucidated. For instance, it is not yet clear how the dysbiotic microbiota itself increases gut permeability, although the hypothesis that the microorganisms decrease the expression of genes coding for the tight junction proteins is very attractive. It has yet to be shown which part of the gut barrier (gastro-duodenal versus intestinal) is responsible for the alcohol- or diet-related increase in permeability. Another intriguing question is whether gut leakiness is transitory or permanent, and if it really is gender-dependent. Moreover, the concept of individual susceptibility to intestinal barrier leakiness must be kept in mind and will probably explain, with future investigation, the tendency of certain subjects to develop hepatic disease.

Intestinal permeability assessment tests, broadly employed in a variety of disorders such as celiac disease and inflammatory bowel disease, have proved to be clinically useful in assessing response to treatment, confirming certain diagnoses and, sometimes, forecasting the clinical course of the illness. Yet, the available methods seem unequal, as illustrated by the variability in the results obtained by different research groups investigating the same phenomenon. For instance, it needs to be clarified which part of the intestine is assessed by the particular test substance and the factors interfering with the probe should be identified. With future research and better understanding of the mucosal barrier function, rapid, simplified and more specific permeability analysis may be accessible.
SIBO, a common condition in which the small bowel contains excess bacteria which have proliferated from the large intestine into the small bowel, is a model of dysbiotic microbiota. It is an important contributor to endotoxemia, being a source of LPS but also very likely by eliciting barrier dysfunction and thus promoting the absorption of endotoxin. Thus, the fact that SIBO has a significantly high prevalence in patients with ALD as well as NAFLD is certainly not a coincidence. Although intestinal motility impairment is a feature of both hepatic diseases and may explain the high occurrence of SIBO, accumulating data support the theory that the latter is a cause rather than a consequence of ALD and NAFLD. The relationship between SIBO, increased intestinal permeability and endotoxemia in patients with ALD and NAFLD is evidently an area for further clinical research and may provide fascinating information.

A leaky gut allows endotoxins to reach the liver where they mediate hepatic injury. They do so mainly by activating Kupffer cells via receptors such as CD-14 and TLR-4, thus leading to a signaling cascade in which the molecules IRAK-1 and NFκB appear to play a key role. The subsequent necro-inflammatory process encompasses cytokines, such as TNF-α, and oxidative stress, both of which are thought to cause most of the hepatocellular damage. This pathogenic scenario conveniently fits the popular two-hit theory in which steatosis, the accepted first hit, sensitizes hepatocytes to a consequent stress for which endotoxin and TNF-α are the preferred candidates. Indeed, the onset of steatohepatitis seems to depend on endotoxin (or TNF-α), the triggering factor for necroinflammatory cascade.

Endotoxins, lipopolysaccharides discarded by live microorganisms as well as dead bacteria, mainly originate from the gut microbiota. Once they are present in the bloodstream one speaks of endotoxemia. Yet, the terms “endotoxin” and “endotoxemia” should be considered with circumspection since the exact nature of this collection of bacterial compounds still lacks precision. Furthermore, the methods of detection of endotoxins in blood samples do not seem reliable, namely because some LPS appear to be bound to plasma proteins and thus more difficult to identify. Because endotoxins play a central role in the new pathogenic scheme discussed in this review, the need for these clarifications is doubtless one of the most urgent appointments.

TNF-α is considered one of the main cytokines involved in hepatocellular damage, amplifying and spreading the inflammatory process, mediating apoptosis and necrosis of hepatocytes and thus leading to cholestasis and fibrosis. Surprisingly, so far, no significant correlation between circulating levels of endotoxin and those of cytokines has been proven. Is the presence of endotoxin alone not sufficient to significantly raise the amounts of TNF-α and other cytokines, implying that additional stimuli and pathways (namely including certain TLRs) may be needed? Or may this be explained by the sensitization, via alcohol or a fat-rich regime, of the liver cells to endotoxin, the latter thus capable of triggering the production of a disproportionate amount of cytokines? Although the various assumptions are appealing, they require further confirmation. Also, investigating the sensitization phenomena, which involves hepatocyte mitochondria and represents a capital lead, should certainly bring about interesting results.

It is noteworthy that the pathogenic network described in this thesis involves numerous amplification loops. For instance, increased intestinal permeability facilitates endotoxemia while the latter probably induces mucosal injury and thus loss of barrier function. Also, endotoxemia leads to the production of TNF-α which, in turn, alters the gut barrier, facilitates bacterial translocation and increases endotoxemia. Therefore, the identification and prevention of these vicious circles may help to contain the extent of the liver damage.

A key concept conveyed by this review is that the discussed novel pathogenic mechanisms appear to be common to both ALD and NAFLD, which already share histological features and certain risk factors. Thus, unraveling mechanisms for one of them indisputably benefits the understanding of the other. This is particularly convenient since, with respect to gut microbiota, scientific data concerning ALD is more abundant than that regarding NAFLD. Indeed, for a certain reason, there is a lack of clinical work with NAFLD patients, which will hopefully be completed
soon, as this intriguing topic is becoming increasingly popular. It is likely that, one day, these two diseases may be shown to be one entity with two different etiologies.

The fact that the incidence of these liver disorders has been rapidly growing in the last decade is of concern, especially given the important morbidity and mortality of these conditions and the need for an effective therapy. Therefore, the discovery of pathogenic mechanisms of ALD and NAFLD and thus potential targets for novel therapeutic strategies is essential. Since abnormal gut intestinal microflora profile plays an initial and central role in this context, manipulation of the commensal bacteria ecosystem may represent a novel and revolutionary approach. The latter is embodied by the “biotics” (pro-, pre- and synbiotics) which have been shown to improve liver health experimentally. While probiotics seem to be clinically efficient, there are at this time no available data regarding the use of pre- and synbiotics in patients with ALD and NAFLD, thereby stressing the need for further investigation.

Other important potential therapeutic targets are, of course, intestinal permeability, endotoxemia, inflammation and oxidative stress. While additional targets such as the TLR signaling pathway will be examined, new ones will be identified. The discussed potential therapies will hopefully be developed, tested experimentally and clinically, and improved.

Altogether, the discussed findings support a strong relationship between intestinal microflora, gut permeability, endotoxemia, systemic and hepatic inflammation (prominently mediated by Kupffer cells and TNF-α) and oxidative stress in the pathogenesis of these chronic hepatic disorders. Nonetheless, this review also demonstrates the extent of our ignorance of certain important mechanisms or features. Unraveling the missing threads of the complex pathogenic network weaved by the numerous mediators of ALD and NAFLD represents a challenge that will certainly yield fascinating advances in liver disease research and treatment in the near future.
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