UNIVERSITÉ CATHOLIQUE DE LOUVAIN
INSTITUTE OF NEUROSCIENCE

New targets in the management of alcohol dependence: which role for gut microbiota, gut barrier and immunity?

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To my Syrian sister Manar,

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En écrivant ces quelques lignes, qui sont en fait quelques pages, je me rends compte qu'une thèse est loin d'être un travail solitaire. Les nombreuses personnes citées ci-dessous, de par leur aide scientifique et technique, leur bonne humeur et leurs encouragements ont toutes contribué à l'élaboration de ce travail.

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FOREWORD

While it may seem that the rise in drug-related deaths has become a recent phenomenon, the loss of some of the most celebrated men and women in history due to alcohol has been a surprisingly constant and tragic reality over time. Plenty of actors, writers and singers have experienced the descent into alcoholism. Some recovered and some did not win the battle. Here are various examples.

The most recent loss is the depression-related suicide of Robin Williams whose genius comic impressions touched a generation of moviegoers. He experienced two decades of sobriety before his three-year alcohol relapse occurred. Sir Anthony Hopkins, renowned for his portrayal of Hannibal Lecter in *The Silence of the Lambs*, has been sober for 30 years and claims "the compulsive side of my nature is still present but now it does not control my life. I feel like a survivor". Other actors came to alcoholism such as Mickey Rourke, whose erratic career has perfectly matched his state of sobriety, and Mel Gibson, whose neighbors repeatedly filed complaints with police during very pronounced crises.

Crafters of the written word have not been immune to this disease, among them such influential writers as Edgar Allan Poe, one of the pioneers of Science Fiction; F. Scott Fitzgerald, author of The Great Gatsby as well as lauded poets Verlaine, Rimbaud, Baudelaire. All have fallen victim not only to their success, but also to the excessive consumption of alcohol.

Many singers have struggled with alcoholism like the legendary guitarist Eric Clapton, who has known many tragedies in his life and wrote in his autobiography: "in the lowest moments of my life, the only reason I didn't commit suicide was that I knew I wouldn't be able to drink any more if I was dead". Recently, the British singer Amy Winehouse joined Club 27 because of her alcohol and drug addiction. Also, David Bowie, in addition to cocaine addiction, spiraled down into alcoholism. In 1977 Berlin, he said "I've been a real idiot some of my life and I put myself in ridiculously dangerous situations. Now I seem to have been getting through it". And that became part of that wonderful song "Heroes", reflecting that you can overcome some incredible odds.

"*And I'll drink all the time*

'Cause we're lovers

We can beat them, forever and ever

We can be heroes, just for one day"

SUMMARY

Alcohol dependence has traditionally been considered a brain disorder. Neurobiological studies have demonstrated the impact of chronic ethanol consumption on the concentration of several neurotransmitters involved in the brain reward circuit. Therefore, pharmacological approaches targeting these neurotransmitters have been developed to treat alcohol addiction. Most of the clinical studies clearly showed that these pharmacological agents are far from being totally convincing at improving clinical outcomes, and the relapse rate in this disease is still very high. Alcohol-dependent subjects frequently develop emotional and psychological disturbances such as depression, anxiety and craving that are known to play an important role in the drinking behavior and influence the probability of relapse after a period of sobriety.

In addition to the deleterious effect of ethanol on the brain, many other peripheral organs are damaged. In this PhD work, we have focused on the effect of heavy and chronic alcohol consumption on the gut and more particularly on the gut barrier and on the gut microbiota which is now considered an "exteriorized" organ placed within the body. We tested whether alterations in the gut barrier and in the gut microbiota composition and functions occur in alcohol-dependent subjects and whether these changes could be related to psychological symptoms of addiction. We also analyzed the systemic inflammatory response which could be seen as a mediator in the gut-brain interactions.

In the first study, we found that alcohol-dependent subjects presented with increased intestinal permeability (also called leaky gut), which permit the translocation of endotoxin LPS from the gut lumen to the systemic circulation. LPS comes from the cell wall of Gram negative bacteria inhabiting the gut and is a potent pro-inflammatory agent. Alcohol-dependent subjects also presented with a low-grade systemic inflammation which persisted after 3 weeks of alcohol abstinence. Finally, we showed an important relationship between inflammatory markers and the severity of depression and alcohol craving.

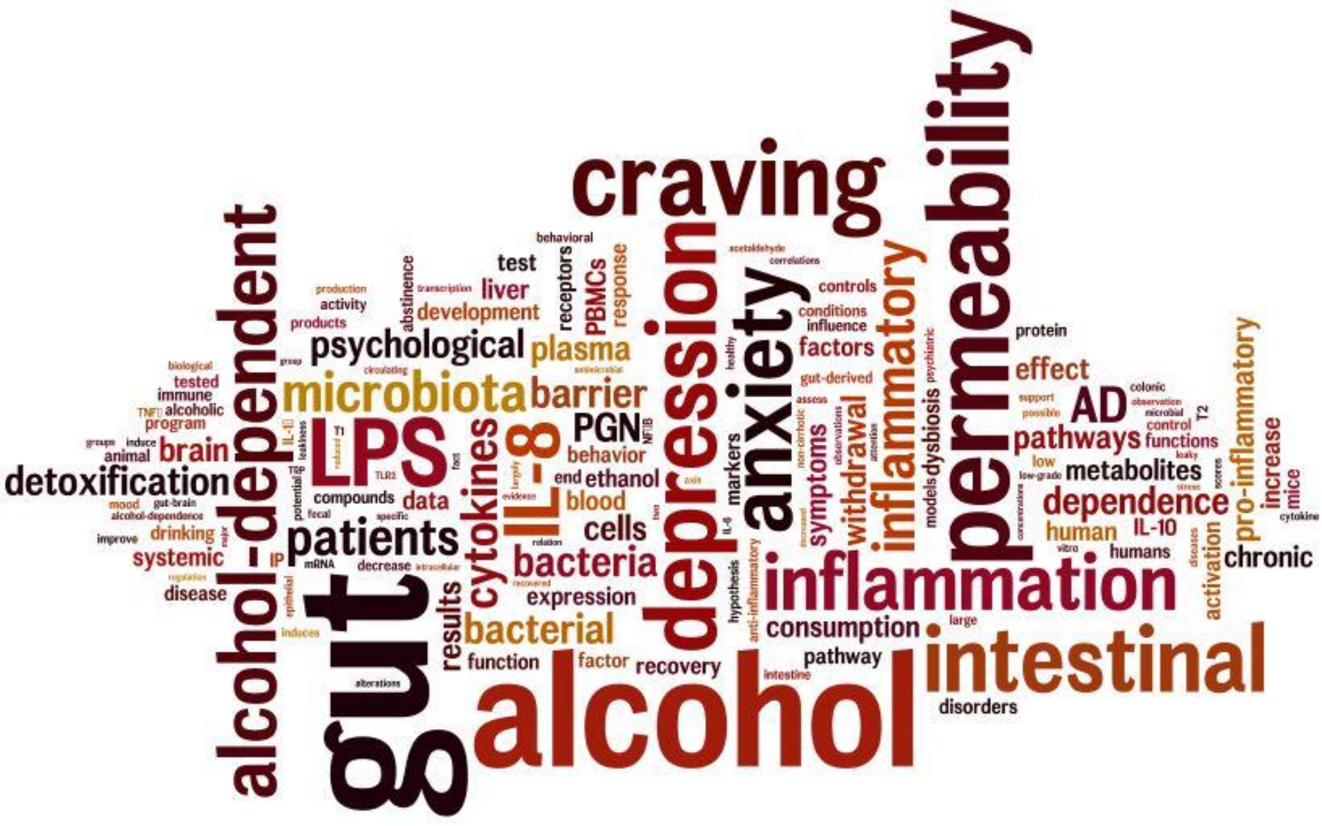
In the second study, we found that in addition to LPS, peptidoglycan which derived from Gram positive bacteria could also cross the leaky gut barrier and both could activate the peripheral blood mononuclear cells (PBMCs) to induce the release of pro-inflammatory

cytokines IL-1β and IL-8. The improvement of alcohol craving score during the alcohol withdrawal was associated with the decrease of specific inflammatory markers in PBMCs, and IL-8 was found to be the best predictor of the psychological outcome.

In the last study, we observed that only a subset of alcohol-dependent subjects presented gut leakiness, independently of the amount of alcohol consumed. Deep analysis of the gut microbiota composition and functionality revealed that subjects with leaky gut had also altered gut microbiota (called dysbiois) that persisted after 3 weeks of alcohol abstinence. Specific bacteria or metabolites produced by the bacteria could therefore be involved in the gut barrier regulation. More importantly, subjects with leaky gut and altered gut microbiota presented with a more severe form of dependence including higher levels of depression, anxiety and alcohol craving compared to subjects without dysbiosis, and this could influence the probability of relapse after detoxification. The results of this third study showed, for the first time in human alcohol-dependent subjects, an association between the gut and the psychological symptoms of addiction.

All these data are in favor of the existence of gut-brain axis in alcohol-dependent subjects, where inflammation could play a role in the communication between a peripheral organ that was initially "simply" devoted to absorption and fermentation of nutrients, and the central nervous system. However, other mechanisms underlying gut-brain interactions remain to be investigated, in particular the role of the vagus nerve, of tryptophan metabolism or the possibility that bacterial metabolites would enter the bloodstream and exert neuroactive properties.

The prevalence of alcohol use disorders reaches 7.5% of the population in Europe. All social classes are concerned. The high fraction of patients who resume alcohol drinking few months, few weeks or even directly after a detoxification program is a reality difficult to accept for the medical community, for the patients and their family. The persistence of inflammation and dysbiosis observed at the end of detoxification supports the idea that new strategies, targeting the gut and not the brain, might be beneficial in the treatment of alcohol dependence. More particularly, the use of nutritional factors called prebiotics appears to be the most promising strategy to improve, naturally and in a safe way, gut and psychological health in alcohol-dependent subjects.



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depression
anxiety
inflammation
intestinal
alcohol-dependent
LPS
IL-8
microbiota
inflammatory
patients
detoxification
psychological
cytokines
bacterial
bacteria
barrier
plasma
dependence
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metabolites
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consumption
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INTRODUCTION

The introduction has been divided into three sections:

In the first part, we describe the health, social and economic consequences of the harmful use of alcohol. Then, we expose the brain neurochemical mechanisms involved in the development of alcohol dependence and of alcohol withdrawal syndrome and the current treatments proposed to alcohol-dependent patients. As emotional and psychological symptoms are frequently developed in alcohol-dependent subjects, the psychological models of alcohol dependence are also briefly introduced.

Alcohol consumption has many deleterious consequences on the brain, but other peripheral organs are also damaged. The second part of the introduction focuses on the effect of heavy and chronic alcohol consumption on the gut, and more particularly on the intestinal barrier function as well as on the gut microbiota composition and functionality. In order to understand the potential impact of gut dysfunction on the psychological symptoms of alcohol dependence, the mechanisms underlying the communication between the gut and the brain are described.

In the last section, we develop the concept of sickness behavior which suggests that pro-inflammatory cytokines, produced following peripheral infection, act on the brain to induce mood and behavioral changes. When inflammation persists and becomes chronic, mood changes can lead to the development of depression thought the activation of a specific enzyme described in this third section.

1 ALCOHOL USES AND MISUSES

Alcohol consumption has been described in society since early history. It is frequently associated to important social events and takes an important place in many cultures. However, excessive drinking is also an important public health problem as recently defined by the World Health Organization (WHO):

"Besides the numerous **chronic and acute health effects**, alcohol consumption is also associated with widespread **psychosocial consequences**, including violence, child neglect and abuse, absenteeism in the workplace, and many other impacts. Considering the significance of alcohol consumption compared to other health risks, **the harmful use of alcohol is not given proper attention in public policy, particularly since other lesser health risks have higher priority".**

Global status report on alcohol and health, WHO, 2011 [1].

In the first section of this chapter, we will describe the health, social and economic consequences of harmful alcohol-drinking. In the second section, we will elaborate on different characteristics of excessive drinking when it becomes a psychiatric disorder.

1.1 Harmful use of alcohol: health, social and economic consequences

Alcohol is a psychoactive substance with dependence-producing properties. Consumption of alcohol and problems related to alcohol vary widely around the world, but the burden of disease and death remains significant in most countries. The harmful use[1] of alcohol is the third leading risk factors for disease and disability and is a causal factor in more than 200 disease and injury conditions. The harmful use of alcohol is responsible for approximately 3.3 million deaths each year. In addition to its deleterious effect on health, the harmful use of alcohol can also have serious social and economic consequences for individuals other than the drinker and for society at large [2].

1.1.1 Alcohol consumption

Alcohol is consumed almost worldwide but there is wide variation in total alcohol consumption among regions (figure 1-1) due to socio-demographic factors, prevalence rates of abstention, culture such as Islam religion and level of economic development. Globally, individuals above 15 years of age drink on average 6.2 liters of pure ethanol per year (or 13.5 grams of pure alcohol per day). The highest consumption levels are found in the developed countries, in particular the WHO European Region (which includes the Russian Federation). In Belgium, the average consumption is 11 liters of pure ethanol per person per year.

[1] The harmful use is defined as a pattern of alcohol use that is causing damage to health, and the damage may be physical (e.g. liver cirrhosis) or mental (e.g. depressive episodes secondary to heavy alcohol consumption).

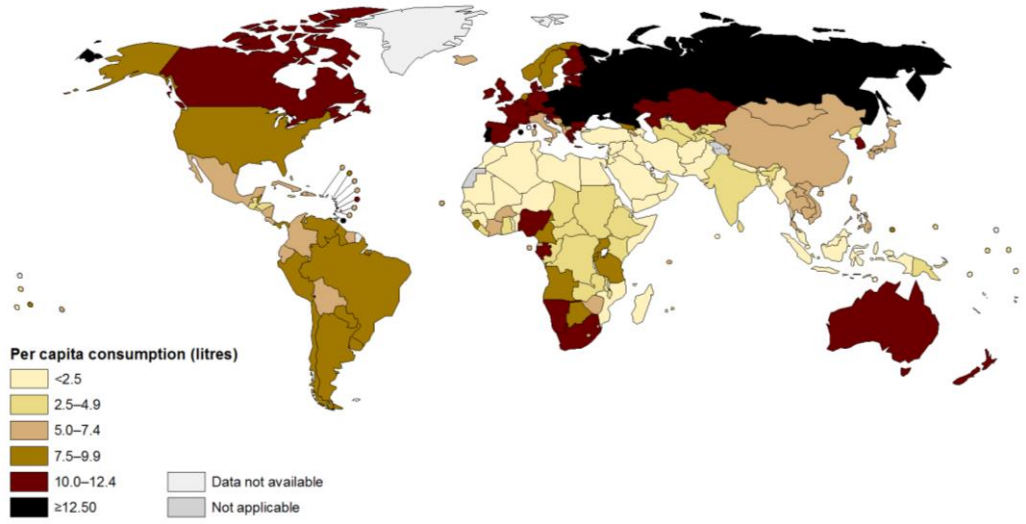

Figure 1-1: Total alcohol per capita (aged 15 years and older) consumption, in liters of pure alcohol, WHO 2010 [1].

1.1.2 Health consequences for drinkers

The harmful use of alcohol is the third leading risk factor for disease and disability (figure 1-2). In 2012, 5.1% of the global burden of disease and injury, as measured in DALYs[2], were attributable to alcohol. Because alcohol starts killing or disabling people at relatively young age, the harmful use of alcohol is the first leading risk factor for mortality and the overall burden of disease in the 15-59 age group. Alcohol consumption has been identified as a component cause for more than 200 disease and injury conditions and more than 30 conditions include alcohol in their name or definition (e.g. alcohol dependence, alcoholic liver disease, fetal alcohol syndrome). This indicates that these disease conditions would not exist at all in the absence of alcohol consumption.

[2] Disability-adjusted life years (DALYs) represent a time-based measure of overall burden of disease for a given population. DALYs are the sum of years of life lost due to premature mortality as well as years of life lost due to time lived in less than full health.

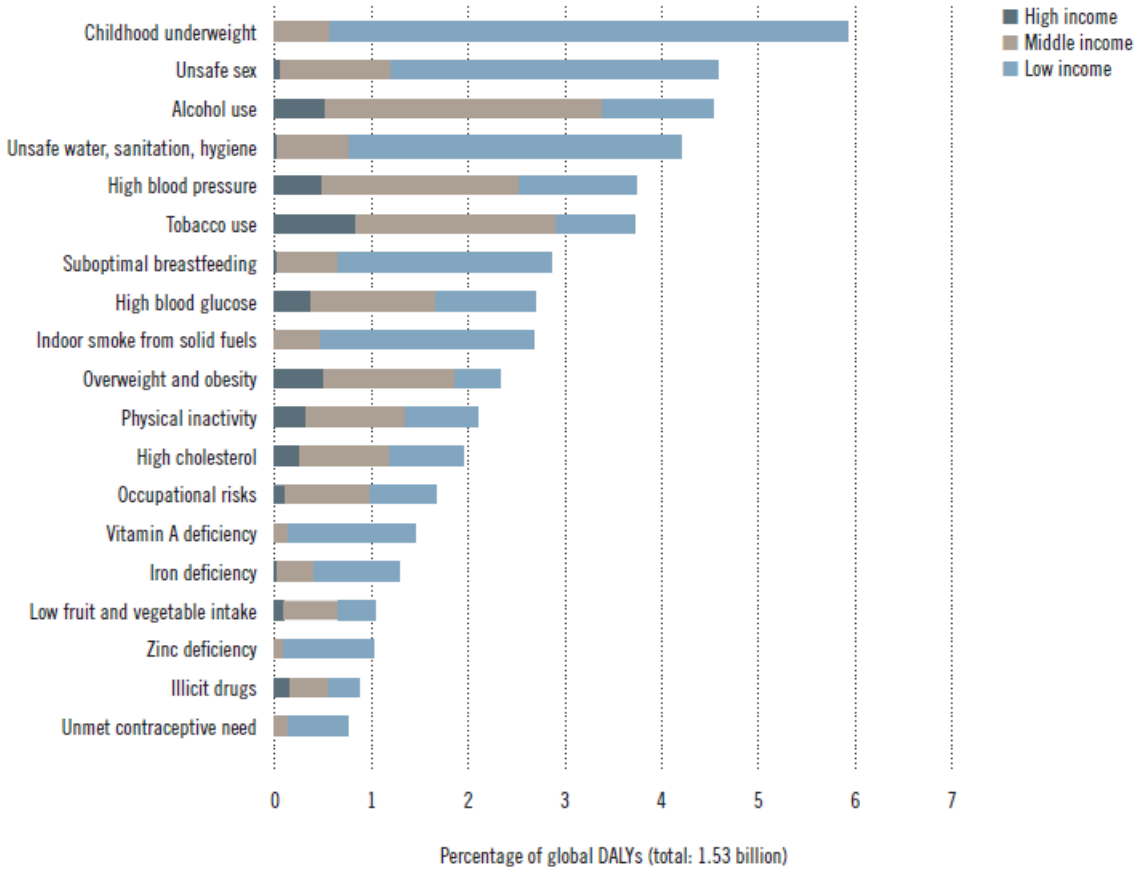

Figure 1-2: Global percentages of DALYs attributes to 19 leading risk factors by income group, WHO 2004 [1]**.**

In 2012, 5.9% of all deaths worldwide (7.6% for men, 4.0% for women) were caused by alcohol consumption. It means that one death out of twenty is due to alcohol. Figure 1-3 shows the distribution of these 5.9% alcohol-related global deaths by broad disease category. The highest numbers of deaths are from cardiovascular diseases, followed by unintentional injuries, gastrointestinal diseases (mainly liver cirrhosis) and cancers. Compared to other diseases, neuropsychiatric disorders cause more disability than mortality. Almost 25% of alcohol-attributable DALYs were due to neuropsychiatric disorders whereas only 4% for all alcohol-attributable deaths would have a neuropsychiatric origin.

The degree of risk for harm due to alcohol use varies with the drinker's age, sex, familial factors, socioeconomic status as well as drinker's behavior and alcohol exposure. Indeed, alcohol-related harm is also determined by three related dimensions of drinking: the volume of alcohol consumed, the pattern of drinking and also the quality of alcohol consumed.

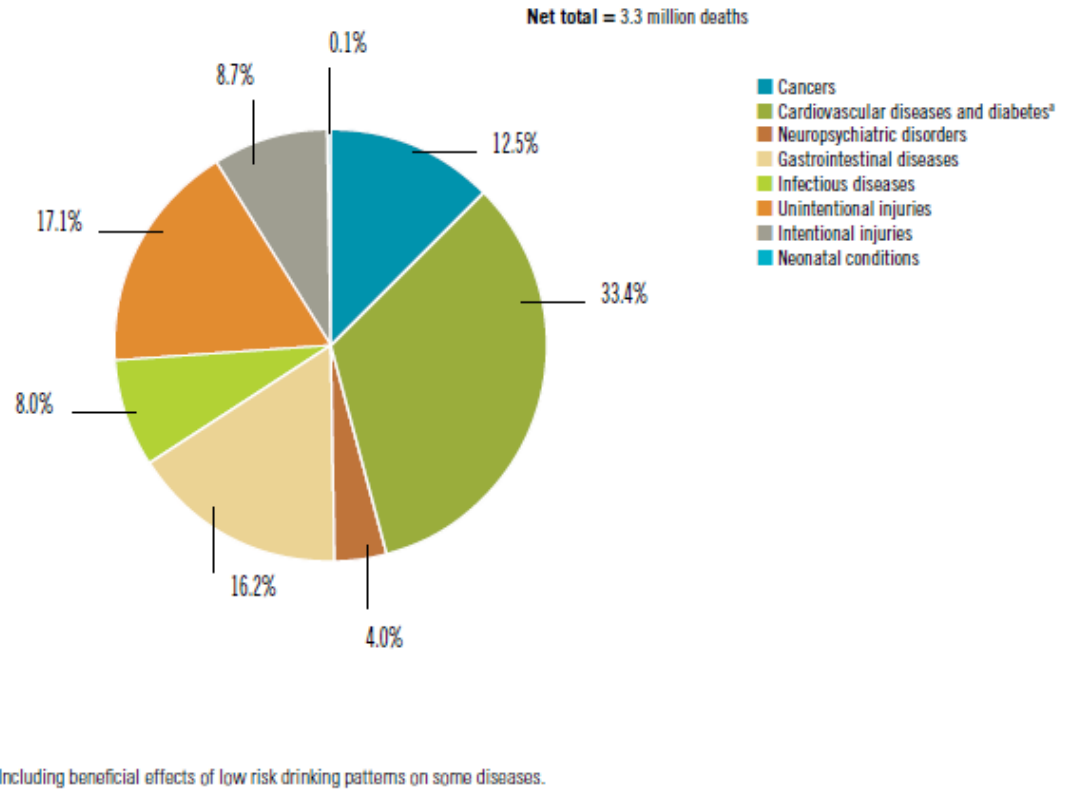

Figure 1-3: Distribution of alcohol-attributable deaths, as a percentage of all alcohol-attributable deaths by broad disease category, WHO, 2012 [2]

1.1.3 Socioeconomic consequences for drinkers

In addition to harm to physical and mental health, alcohol consumption is often associated with socioeconomic consequences such as loss of earnings, unemployment, family problems, stigma and barriers to accessing health care.

Alcohol is a valued commodity. When earnings are low, heavy drinking may further impoverish the drinker and the drinker's family [3, 4]. Alcohol intoxication, dependence or withdrawal states results in poor performance, loss of productivity or absenteeism at the work place, break-up and dysfunction in family life [3]. Also, there is a clear tendency to marginalize and exclude from society habitually intoxicated persons and their families and it has been shown that heavy alcohol users receive less priority in health care [5, 6].

1.1.4 Harms to other individuals

The harmful use of alcohol can have health and social consequences for the drinker but can also result in harm to other individuals. The harms done by people's drinking to others involve both socioeconomic consequences and health problems such as intentional (e.g. violence, homicide) or unintentional (e.g. traffic crash, workplace accident) injuries, child neglect or abuse, fetal alcohol syndrome, as well as mental health impact for relatives who may be frightened by the actions of the drinkers [7].

A recent study assessed the harms caused by the misuse of twenty drugs, which were scored according the harms that the drug produces to the individuals (harms to users) and the harms that the drug produces to others (harms to others) [8]. As shown in figure 1-4, the study revealed that the most harmful drugs to users were heroin, crack cocaine and metamphetamine, whereas the most harmful drug to others was alcohol. When the two scores were combined, **alcohol was the most harmful drug**, with heroin and crack cocaine in second and third positions.

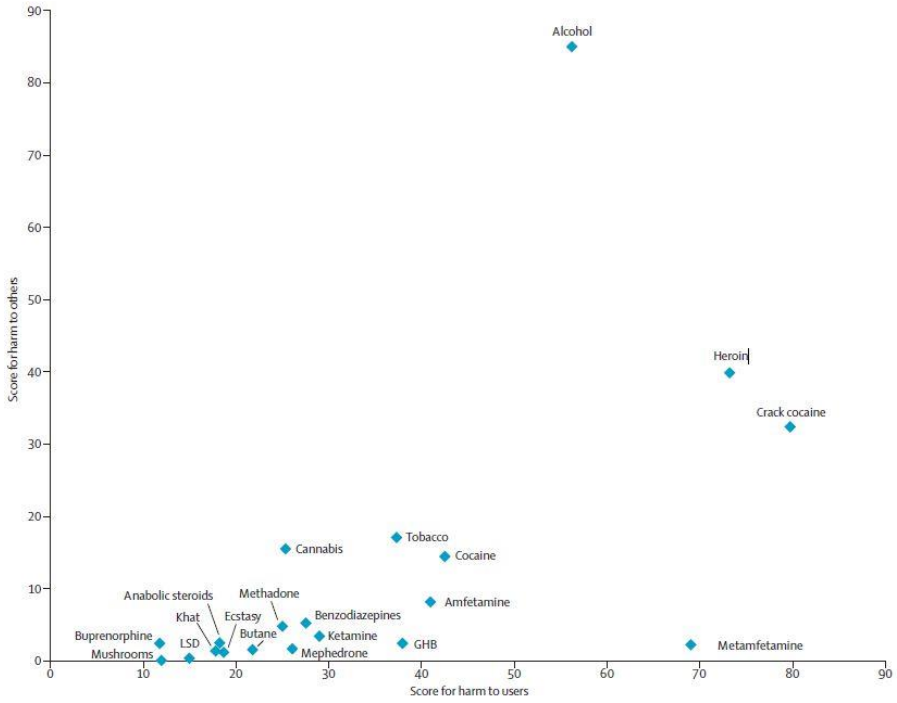

Figure 1-4: Scatter plot displaying harm to users and harm to others of different drugs, Nutt *et al.*, The Lancet, 2010 [8].

1.1.5 Harms to society at large

The harmful use of alcohol results in a significant health, social and economic burden on society. There are three major categories of alcohol-attributable social and economic costs. The first category is the direct economic costs of alcohol consumption which include health care services (e.g. hospitalizations, ambulatory care, nursing home care, prescription medicines) and the police and criminal justice system [9]. The second category is indirect costs due to absenteeism, loss of productivity, unemployment, premature pension [10]. A third category is intangible costs which are costs assigned to pain and suffering, and more generally to a reduced quality of life for the drinkers and their families [10]. Alcohol-attributable costs have been estimated at about 125 billion euros in the European Union in 2003 [10].

Despite the large health, social and economic burden associated with harmful use of alcohol, it remained a relatively low priority in public health policy [2].

1.2 Alcoholism as a psychiatric disease

"*All things are poison and nothing is without poison, only the dose permits something not to be poisonous*."

Paracelsus (1496-1541)

1.2.1 Definition, diagnosis and epidemiology

Alcohol use in itself may not be considered a pathology. The British Medical Association published the following guidelines in order to minimize the risk of health harms associated with drinking [11]:

- Men should drink no more than 21 units of alcohol per week, no more than 4 units in any given day, and have a least 2 alcohol-free days a week.
- Women should drink no more than 14 units of alcohol per week, no more than 3 units in any given day, and have a least 2 alcohol-free days a week.
- Pregnant women should not drink alcohol at all.

World-wide recommendations on alcohol consumption show wide disparity among countries, also because one standard alcohol unit varies greatly. In most European countries, one unit contains 10 grams (g) of pure alcohol. The amount of liquid in the glass is therefore adapted according to the strength of alcoholic beverage (figure 1-5). In the United Kingdom, one unit is equal to 8g of ethanol, in the USA 14g and in Japan 19.75g. In Belgium, there is no official guideline on alcohol consumption.

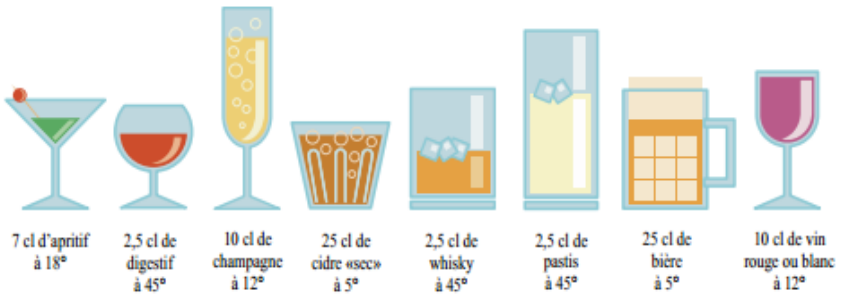

Figure 1-5 : One standard drink (one alcohol unit) contains 10g of pure alcohol, Bertin A., Addica, 2004 [12].

In psychiatry, several definitions have been proposed to describe the misuse of alcohol. The distinction between different pathologies related to alcohol use in psychiatry depends on the presence or absence of different psychiatric symptoms. The diagnosis of alcohol dependence is based on criteria described in the Diagnostic and Statistical Manual of Mental Disorders (DSM). In 1952, the American Psychiatric Association published the first edition (DSM-I). In all the studies described in this manuscript, patients were selected upon criteria of the fourth edition (DSM-IV) published in 1994.

Alcohol dependence is defined as a cluster of behavioral, cognitive and physiological phenomena that develop after repeated alcohol use and that typically include a strong desire to consume alcohol, difficulties in controlling its use, persisting in its use despite harmful medical and social consequences, a higher priority given to alcohol use than to other activities and obligations, increased tolerance, and sometimes a physiological withdrawal state [2]. The exact criteria are depicted in table 1-1.

Since May 2013, the fifth edition (DSM-5) is available, in which a general evolution can be observed compared to DSM-IV: substance abuses are described as a continuous progression rather than a categorical perspective. Although there is considerable overlap between DSM-5 and DSM-IV, there are several important differences. For instance, DSM-IV distinguishes alcohol-dependence from alcohol abuse, which is a less severe psychiatric disease and which essentially insists on the consequences of drinking rather than on a specific symptomatology, while DSM-5 integrates the two disorders into a single disorder called “alcohol use disorder” (AUD) where a progression in the severity is described by a sub-classification in mild, moderate and severe forms of AUD. Moreover, DSM-5 adds **alcohol craving** as a criterion for AUD diagnosis, which was not included in the prior edition [13].

DSM–IV			DSM–5	
Any 1 = ALCOHOL ABUSE	Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home (e.g., repeated absences or poor work performance related to alcohol use; alcohol-related absences, suspensions, or expulsions from school; neglect of children or household.	1	Alcohol is often taken in larger amounts or over a longer period than was intended. (See DSM–IV, criterion 7.)	The presence of at least 2 of these symptoms indicates an **Alcohol Use Disorder (AUD).** The severity of the AUD is defined as: **Mild:** The presence of 2 to 3 symptoms **Moderate:** The presence of 4 to 5 symptoms **Severe:** The presence of 6 or more symptoms
	Recurrent alcohol use in situations in which it is physically hazardous (e.g., driving an automobile or operating a machine when impaired by alcohol abuse).	2	There is a persistent desire or unsuccessful efforts to cut down or control alcohol use. (See DSM–IV, criterion 8.)	
	Recurrent alcohol-related legal problems (e.g., arrests for alcohol-related disorderly conduct). ****This is not included in DSM–5****	3	A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects. (See DSM–IV, criterion 9.)	
	Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the alcohol (e.g., arguments with spouse about the consequences of intoxication, physical fights).	4	Craving, or a strong desire or urge to use alcohol. ****This is new to DSM–5****	
Any 3 = ALCOHOL DEPENDENCE	Tolerance, as defined by either of the following: a) A need for markedly increased amounts of alcohol to achieve intoxication or desired effect b) Markedly diminished effect with continued use of the same amount of alcohol	5	Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home. (See DSM–IV, criterion 1.)	
	Withdrawal, as manifested by either of the following: a) The characteristic withdrawal syndrome for alcohol b) Alcohol is taken to relieve or avoid withdrawal symptoms	6	Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol. (See DSM–IV, criterion 4.)	
	Alcohol is often taken in larger amounts or over a longer period than was intended.	7	Important social, occupational, or recreational activities are given up or reduced because of alcohol use. (See DSM–IV, criterion 10.)	
	There is a persistent desire or unsuccessful efforts to cut down or control alcohol use.	8	Recurrent alcohol use in situations in which it is physically hazardous. (See DSM–IV, criterion 2.)	
	A great deal of time is spent in activities necessary to obtain alcohol (e.g., driving long distances), use alcohol, or recover from its effects.	9	Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol. (See DSM–IV, criterion 11.)	
	Important social, occupational, or recreational activities are given up or reduced because of alcohol use.	10	Tolerance, as defined by either of the following: a) A need for markedly increased amounts of alcohol to achieve intoxication or desired effect b) A markedly diminished effect with continued use of the same amount of alcohol (See DSM–IV, criterion 5.)	
	Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance (e.g., continued drinking despite recognition that an ulcer was made worse by alcohol consumption).	11	Withdrawal, as manifested by either of the following: a) The characteristic withdrawal syndrome for alcohol (refer to criteria A and B of the criteria set for alcohol withdrawal) b) Alcohol (or a closely related substance, such as a benzodiazepine) is taken to relieve or avoid withdrawal symptoms. (See DSM–IV, criterion 6.)	

Table 1-1 : A comparison between DSM-IV and DSM-5, National Institute on Alcohol Abuse and Alcoholism, 2013 [13]**.** Under DSM-IV, two distincts disorders were described: alcohol abuse and alcohol dependence. Anyone meeting one or more out of 4 of the "abuse"criteria (items 1→4) within a 12-month period would be diagnosed as "alcohol abuser". Anyone meeting three or more out of 7 of the "dependence" criteria (items 5→11) within the same 12-month period would be diagnosed as "alcohol-dependent". Under DSM-5, anyone meeting any two of the 11 criteria during the same 12-month period would receive a diagnosis of "alcohol use disorder" (AUD). The severity of AUD is defined as mild if 2 to 3 symptoms are present, moderate if 4 to 5 symptoms are present, severe if 6 or more symptoms are present. Overall, the DSM-5 alcohol use disorder criteria are nearly identical to the DSM-IV alcohol abuse and dependence criteria combined, with two exceptions: DM-5 eliminates legal problem as a criterion but adds craving as a new criterion for AUD diagnosis.

The most recent statistics related to AUDs date back to 2010 and include alcohol-dependence as well as harmful use of alcohol [2]. Worldwide, AUDs are significantly more prevalent among males compared to females. Table 1-2 shows the prevalence of AUDs in some countries of the WHO European Region. In Belgium, 5.8% of the population suffers from AUDs whereas the disorder is present in 30% of males in some countries of Eastern Europe.

	Alcohol use disorders		
	Total	Male	Female
WHO European Region	7.5	12.6	2.9
Italy	1.0	1.3	0.8
Netherlands	1.2	1.7	0.8
Spain	1.3	2.3	0.4
France	5.5	8.8	2.5
Belgium	5.8	8.8	3.0
Switzerland	8.0	13.5	2.6
United Kingdom of Great Britain and Northern Ireland	11.1	16.3	6.0
Slovenia	11.6	19.7	3.8
Belarus	16.6	29.8	5.5
Russian Federation	17.4	31.0	6.2
Hungary	17.7	31.0	6.0

Table 1-2: This table shows the extent of alcohol use disorders (AUDs) prevalence (%) in selected countries of the WHO European Region in 2010. Based on 12-month prevalence estimate including people aged 15 years or more. WHO, 2012 [2].

1.2.2 Biological and psychological models of alcohol dependence

1.2.2.1 Biological models

The person's initial decision to use alcohol is influenced by genetic, psychosocial and environmental factors. Once it has entered the body, alcohol promotes continued alcohol-seeking behaviors by acting directly on the brain. A major goal of basic research is to understand the neural underpinnings associated with the transition from alcohol use to alcohol abuse and dependence. Reinforcements (positive or negative) and **neuroadaptation** contribute to the development of addiction.

Reinforcement is a process in which a response or behavior is strengthened based on previous experiences and it appears to be regulated by multiple neurotransmitter systems described below [14]. **Positive reinforcement** describes a situation in which a rewarding stimulus, such as euphoria or pleasure induced by alcohol ingestion, increases the probability to drink alcohol. This positive motivating factor is frequent in the early stages of alcohol use and abuse. **Negative reinforcement** occurs when the individual drinks alcohol to avoid or alleviate aversive stimulus such as withdrawal symptoms (anxiety, irritability, tremors …) or negative affects related to drinking. It is involved in a later stage of addiction, when dependence has developed, and is commonly accepted as a major factor involved in the persistence of the drinking habits in alcohol-dependent subjects.

Neurobiological hypothesis currently ascribes these positive and negative reinforcement processes to changes in brain neurotransmitters. Indeed, alcohol interacts with several **neurotransmitters systems** in the **brain's reward circuit**, a functional area that plays an important role in the emergence of the addiction and that is constituted by neurons located in the mesolimbic system (ventral tegmental area, nucleus accumbens, and prefrontal cortex), amygdala, striatum and hippocampus (figure 1-6). Neurotransmitters involved in this circuit are dopamine, opioids, serotonin, gamma-aminobutyric acid (GABA) and glutamate [15].

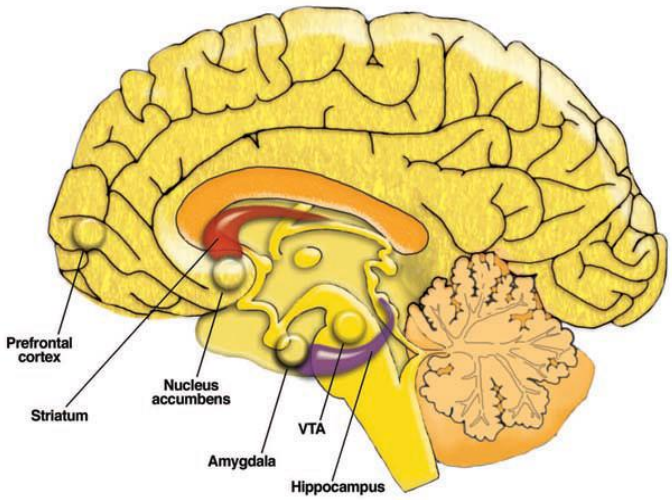

Figure 1-6: Location of human brain regions that constitute the brain reward circuit and are affected by alcohol drinking, Clapp P. *et al*., Alcohol Research and Health, 2008 [15].

Dopamine and endogenous opioids are released in the brain after alcohol ingestion and are both involved in the positive reinforcement. Serotonin (or 5-hydroxytryptamine, 5-HT) is involved in the regulation of emotional states and evidence indicates that it is also important in the regulation of alcohol consumption [16]. For example, increasing the level of serotonin in synapses can decrease alcohol intake [17]. However, serotonin would not play a role in positive or negative reinforcement but rather in the ability to control impulsions or compulsions. GABA is the major inhibitory neurotransmitter in the brain and acts via two receptors called $GABA_A$ and $GABA_{B.}$ Conversely, glutamate is the major excitatory neurotransmitter in the brain and exerts its effects via several receptor subtypes, including the N-methyl-D-aspartate receptor (NMDA).

A short term alcohol exposure increases inhibitory neurotransmission and inhibits excitatory neurotransmission

A short term alcohol exposure increases GABA activity in the brain through two mechanisms: it acts on the pre-synaptic neuron to increase GABA release, and acts on the postsynaptic neuron to facilitate the $GABA_A$ receptor activity. In contrast to its effects on GABA, alcohol inhibits glutamate activity in the brain. A short-term alcohol exposure leads therefore to a state of sedation and decreased anxiety [18].

Long-term alcohol exposure leads to compensatory neuroadaptation that induces decrease in inhibitory neurotransmission and increase in excitatory neurotransmission.

Following chronic exposure, evidence suggests that the brain restores equilibrium by decreasing $GABA_A$ function and increasing excitatory activity of glutamate. This compensatory changes participate in the development of sensitization (i.e. an increase in the reinforcement value of alcohol following repeated exposures), tolerance (i.e. a decrease in the reinforcing efficacy of alcohol following repeated exposures, which means that a person must drink progressively more alcohol to obtain a given effect on brain function), alcohol withdrawal syndrome (see section 1.2.3) and dependence [14].

Alcohol addiction also involves negative reinforcement driven by activation of the stress system

The transition to dependence involves the dysregulation not only of the brain reward circuit (mainly involved in the positive reinforcement) but also of circuit that mediates behavioral response to stressors. The **brain stress system** involves the corticotropin-releasing factor (CRF) which is produced by the hypothalamus (and by extrahypothalamic brain areas) and activates the hypothalamic-pituitary-adrenal (HPA) axis that results in the release of stress hormone cortisol. CRF release is activated under conditions of stress as well as during chronic administration of alcohol and during acute withdrawal. The dysregulation of the brain CRF system could contribute to the negative emotional state (e.g. anxiety) and the alcohol-seeking behavior that fosters increased alcohol consumption and could also participate to relapse. The development of aversive emotional states that drive negative reinforcement has been defined as the "dark side" of addiction by Koob G. and Le Moal M. [19]. This negative reinforcement process also involves the dysregulation of other systems such as dynorphin, norepinephrine and neuropeptide Y.

1.2.2.2 Psychological models

Neurobiological models of addiction developed in animals cannot be directly transposed to the humans. The behavioral expression of addiction measured in animals cannot exactly mimic the human behavior and we need to rely on psychological models to develop appropriate testing of hypothesis.

Alcohol-dependent subjects are characterized by an **intense desire to drink alcohol referred as craving** [20]. Craving may occur in situations where the dependent subject cannot obtain a drink, or when he is exposed to situations that evoke drinking (cues). The latter dimension may be related to positive reinforcement. Craving is also frequently linked to negative emotional states including anxiety and depression and correlations have frequently been observed between these dimensions in alcohol-dependent subjects [21, 22]. This may be related to the negative reinforcement which is a major process involved in drinking tendency in chronic alcoholism.

In psychology, the dual process model of addiction recently developed by Wiers and colleagues in a cognitive and neuroscience perspective proposes that addictive behaviors develop as a result of an imbalance between two systems in the brain: an impulsive system which includes **automatic responses** to emotional and motivational stimuli, and a regulatory **executive system** which includes controlled processes [23]. Briefly, the executive system regulates the ability to inhibit a motivational process (e.g. alcohol drinking). Evidence indicates that the long-term effects of heavy alcohol consumption sensitize the appetitive motivational processes and negatively affect the controlled regulatory processes, with impairment of cognitive and executive abilities. In other words, chronic alcohol consumption strengthens the motivational and emotional processes and weakens the controlled executive system. This results in an increased alcohol use. The **loss of control** of alcohol drinking behavior, that may result from this imbalance, is an important criterion for alcohol dependence diagnosis and is referred as “failure of efforts to cut down alcohol drinking due to the inability to control alcohol use” in the DSM-IV.

In order to evaluate both automatic and controlled processes, psychological questionnaires that test mood, emotions and motivation and neuropsychological tests that evaluate control processes were used in our studies:

- Emotional states: depression, anxiety
- Motivational state: alcohol craving
- Cognitive functions: selective attention, working memory
- Executive functions: inhibition, flexibility and decision making

A previous study [22] has analyzed the effect of a 3-week alcohol withdrawal on these functions and has shown that emotional state and alcohol craving decreased significantly during withdrawal but remained higher than in a control group at the end of withdrawal. This study also showed that executive functions were impaired in alcoholic subjects at the onset of withdrawal and no improvement was observed at the end of withdrawal. These remaining deficits likely contribute to the weak ability of alcohol-dependent subject to resist drinking, and thus increase the probability of relapse. However, another study [24] showed that executive functions improve significantly after 6 months of abstinence.

One aim of this thesis is to investigate whether the psychological variables described above are related to biological parameters (inflammation, intestinal permeability and gut microbiota) and whether they progress in parallel during alcohol withdrawal.

1.2.3 Alcohol withdrawal syndrome

1.2.3.1 Clinical symptoms of alcohol withdrawal

Alcohol withdrawal syndrome consists of the physiological and psychological effects that occur if a chronic drinker suddenly abstains from alcohol use [20]. It results in hyperactivity of the glutamate system and varies significantly among alcoholics in both its clinical manifestations and its severity. Alcohol withdrawal symptoms generally appear within hours after the last drink and are the following: hand tremors, agitation, higher heart rate, elevated blood pressure, headache, sweating, nausea, vomiting, loss of appetite, craving for alcohol, anxiety, irritability, insomnia, and also most severe manifestations such as hallucinations (auditory or visual), seizures and delirium tremens. Even without treatment, most of these manifestations will usually resolve several hours to several days after their appearance [25]. Also, it has been shown that repeated cycles of chronic alcohol exposure and

withdrawal may have progressively important consequences on the brain, exacerbate the psychological and physiological symptoms of withdrawal and increase the incidence and severity of seizures, and finally render the dependent subjects more vulnerable to relapse. This process referred as "kindling" is due to increased neuronal hyperexcitability and excitotoxicity following repeated withdrawal experiences [26, 27].

1.2.3.2 Neurochemical mechanisms underlying alcohol withdrawal

The first neurochemical explanations for withdrawal from alcohol were proposed by Himmelsbach in 1941 and involved mainly the major inhibitory neurotransmitter GABA, the major excitatory neurotransmitter glutamate and their receptors $GABA_A$ and NMDA, respectively [28]. The Himmelsbach's concept can be expressed as a seesaw (figure 1-7). Before alcohol exposure, the brain is in a balanced state. When a person consumes alcohol, its acute effect is to unbalance the neurochemical equilibrium of the brain leading to inhibition (e.g. sedation and incoordination). This inhibition is induced by the potentiation of GABA actions and by the inhibition of NMDA receptors. If the alcohol exposure continues over a long period of time, an opposing chemical adaptation (neuroadaptation) occurs within the brain, that tends to restore the equilibrium. This neuroadaptation consists in reducing the number of $GABA_A$ receptors on neurons' surface (down regulation) and producing more NMDA receptors (up regulation). In other words, the brain develops tolerance and dependence for alcohol. When alcohol exposure ceases, the brain neurochemistry is now unbalanced thereby tilting the seesaw in the opposite direction, with an increased action of the excitatory neurotransmitter glutamate. The result is the development of a withdrawal syndrome that includes signs and symptoms of hyperactivation that were described above. Moreover, it has been shown that during alcohol withdrawal, dopamine and serotonin release is reduced in the nucleus accumbens [29]. This could result in the development of depression, anxiety and emotional discomfort which are important psychological aspects of the withdrawal syndrome.

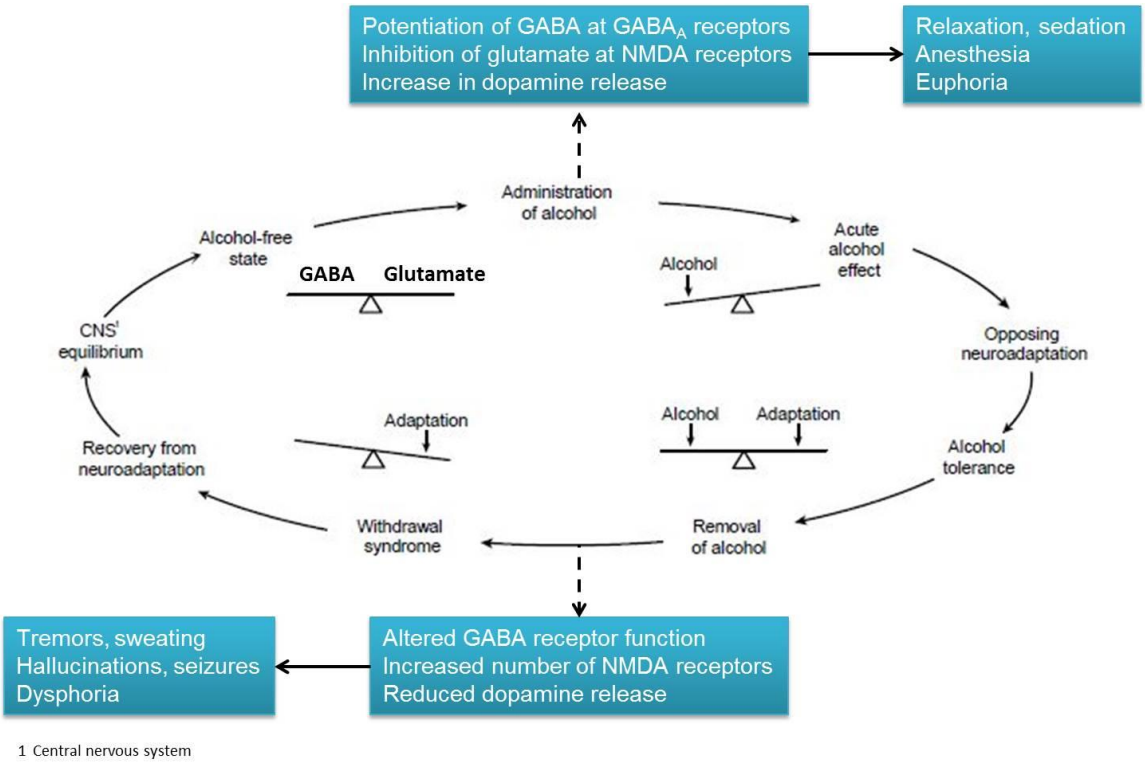

Figure 1-7: The Himmelsbach model for dependence and withdrawal, adapted from Littleton J., Alcohol Health & Research World, 1998 [28].

1.2.3.3 Treatment of withdrawal symptoms

The most common pharmacological treatment given during detoxification is a benzodiazepine that acts, like alcohol, to enhance activation of $GABA_A$ receptors [30, 31]. The main goal of this treatment is to restore the balance between excitation and inhibition in neurons in order to prevent the life-threatening onset of major withdrawal signs such as seizures and delirium tremens. However, benzodiazepines should be carefully used as there is a risk of dependence. Also, it is possible that other harmful sequelae of withdrawal (e.g. nerve cell damage [32]) exist that are not controlled by current treatments.

Alcoholics are often deficient in thiamine (vitamin B1) due to insufficient nutritional intake and reduced intestinal absorption [33]. Thiamine is an essential cofactor required for the functioning of several enzymes involved in carbohydrate metabolism that leads to the production of various molecules such as nicotinamine adenine dinucleotide phosphate (NADH), neurotransmitters (glutamate, GABA), nucleic acids, antioxidant (glutathione), myelin, and adenosine triphosphate (ATP) that provides energy to the cells [34]. Cells of the nervous central system are particularly sensitive to thiamine deficiency. Decrease in thiamine-

using enzymes activity leads to brain lesions due to oxidative stress, cell damage and cell death (i.e necrosis) induced by lack of ATP. These brain lesions in alcoholics are associated with a neurological disorder known as Wernicke-Korsakoff syndrome that consists in mental confusion, impaired ability to coordinate movements and cognitive deficits. As described earlier, alcohol withdrawal is associated with a hyperexcitability of neurones which therefore require more energy. Accordingly, thiamine is administrated to patients who start a detoxification program in order to limit the development of neurological disorders.

1.2.4 Treatment of alcohol dependence

Alcohol dependence is a chronic relapsing disease. Without pharmacological adjunct to psychosocial therapy, up to 70% of patients resume drinking within one year [35]. Although abstinence remains the ultimate goal in treating alcohol dependent subjects, pharmacological agents have been developed in order to reduce the frequency of heavy drinking, the craving and improve abstinence rate and quality of life. Most of these agents target the neurotransmitters that mediate alcohol reinforcement effects and to date, two medications have been approved by the Food and Drug Administration (FDA): naltrexone and acamprosate. A third FDA approved agent, disulfiram (Antabuse®) targets alcohol metabolism.

Naltrexone is a μ-opioids receptor antagonist. In two meta-analytic studies [36, 37], naltrexone has been demonstrated to be efficacious at reducing the risk of relapse among recently abstinent alcohol-dependent individuals. However, the naltrexone's effect size is small, with a corresponding number needed to treat[3] of 7. Due to the small effect size and the bad pill-taking compliance (due to adverse effects such as nausea), some studies have failed to demonstrated naltrexone's efficacy in treating alcohol-dependence [38]. A recent study including 1383 patients have shown that naltrexone associated with medication management to enhance compliance reduced the risk of a heavy drinking day compared with placebo [39].

[3] Here, the number needed to treat is the number of individuals who need to be treated to prevent relapse in a single individual.

However, to date, there is insufficient data to ascertain naltrexone's efficacy over more prolonged periods [36]. Nalmefen (Selincro®), another drug targeting opioid receptors has recently been recognized in Europe as a treatment for alcohol-dependence allowing a reduction of alcohol consumption rather than increased abstinence rates [40]. It also appears as a new strategy for the treatment of alcohol-dependence.

Acamprosate is an NMDA glutamate receptor antagonist. European studies have clearly demonstrated efficacy for acamprosate as a treatment for alcohol dependence although its therapeutic effect size is small, with a number needed to treat of 8 [41]. In contrast, to date, studies in the U.S. have been unable to find any therapeutic benefit of acamprosate compared to placebo among a heterogeneous group of alcohol-dependent individuals [39]. The reason for this discrepancy between the results of U.S. and European studies has not been established. However, in 2013, a new double-blind, placebo-controlled randomized trial conducted in Germany in 426 patients found that neither acamprosate nor naltrexone treatment for 3 months supplied any additional benefit compared with placebo [42].

Serotonin reuptake inhibitors (SSRIs) have been shown to decrease ethanol consumption in animals [43]. Although the preclinical results were promising, there is, at present, little support for the proposal that SSRIs are efficacious in the treatment of alcohol dependent individuals [44]. Moreover, there is no current evidence to recommend SSRIs over placebo for the treatment of depressed alcoholics [45]. Regarding dopamine system, neither dopamine receptor antagonists nor agonists have been demonstrated to be efficacious in the treatment of alcohol dependence [44].

Baclofen is a $GABA_B$ receptor agonist. Evidence for the efficacy of baclofen for alcohol dependence is largely based on three placebo-controlled, double-blind trials [46–48]. In two studies conducted in Italy [46, 47], compared to placebo, baclofen was shown to promote abstinence and reduce alcohol-associated craving and anxiety. However, the third American study [48] did not find evidence that baclofen is superior to placebo in the treatment of alcohol dependence. Moreover, questions still remain about optimal dosing and duration. To date, there is not enough evidence to support the use of baclofen as first line medication but it may be considered in patients with liver cirrhosis when other medications are contraindicated [49].

Disulfiram has been used for treating alcohol dependence since the 1940s. It is an aversive agent which inhibits the enzyme aldehyde dehydrogenase and prevents the

metabolism of acetaldehyde, the primary alcohol metabolite. The accumulation of acetaldehyde in blood causes unpleasant effects such as headache, sweating, nausea, palpitation, lowered blood pressure. These symptoms discourage further alcohol consumption. However, serious side effects have also been reported, including hepatotoxicity and psychotic reactions. Disulfiram has no effect on craving for alcohol. Therefore the efficacy is limited to patients who are highly compliant and receive their medication under supervision [44].

Given the complex neuroadaptive changes that occur during chronic alcohol exposure and withdrawal, it is no surprising that no single pharmacological agent has proven to be fully successful in the treatment of alcoholism. Most of the studies clearly showed that agents targeting neurotransmitters have no effect or small effect in the improvement of clinical outcomes. Moreover, among mental disorders, alcohol abuse and dependence had the largest **treatment gap**[4], with a mean of 92% in Europe, indicating that less than 10% of the patients are actually treated [50] (figure 1-8). This treatment gap could be explained by the denial associated to the disease, the social stigma but also by the lack of efficiency of current pharmacological treatments.

Altogether, these observations support the idea that up to now, all medications targeting the brain failed to treat in a convincing manner this important population of patients. The aim of the thesis is to displace the focus from the brain to peripheral organs, such as the gut, in order to find new potential targets in the treatment of alcohol dependence.

[4] The treatment gap represents the absolute difference between the true prevalence of a disorder and the treated proportion of individuals affected by the disorder. Alternatively, the treatment gap may be expressed as the percentage of individuals who require care but do not receive treatment.

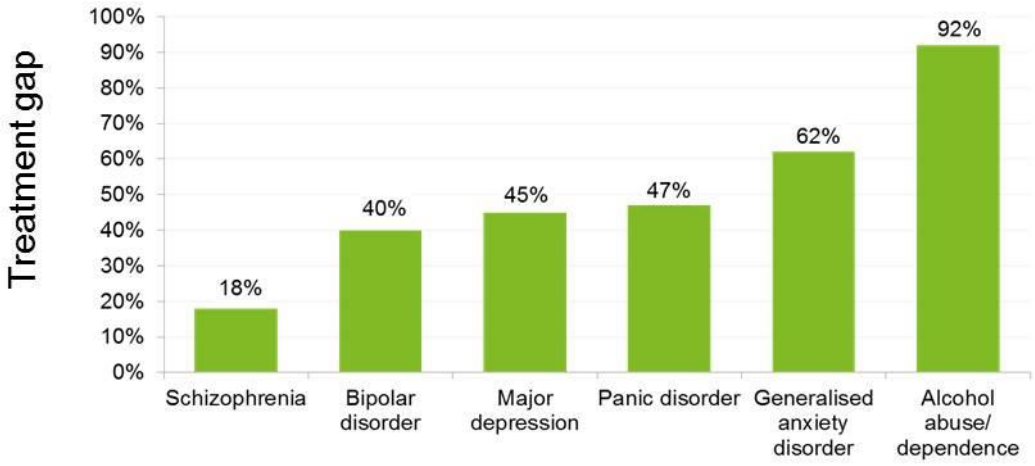

Figure 1-8: Treatment gap in mental disorders, adapted from Kohn R. *et al*., Bulletin of the WHO, 2004 [50].

2 ROLE OF THE GUT IN ALCOHOL DEPENDENCE

"*All diseases begin in the gut*"

Hippocrates (460-370 B.C.)

2.1 Gut barrier

2.1.1 Physiological, morphological and immunological characteristics of the gut barrier

The intestinal mucosal surface is unique among tissues in that it is constantly in contact with a vast, diverse and dynamic microbial community. Therefore, the intestinal cells must sense and respond appropriately to this potential immunological challenge of the luminal content. In humans, the intestinal epithelium encompasses 200 m^2 of surface area and the most abundant cell is the **enterocyte** [51]. Enterocytes are linked with their neighboring cells thanks to apical intercellular junctional proteins, known as **tight junctions** (TJs) as well as **adherens junctions** (AJs). TJs are composed by several proteins such as claudins, occludin and zonula occludens and regulate the paracellular pathway. Enterocytes, together with the junctional complexes, constitute a physical barrier that prevents bacterial penetration while allowing nutrient absorption.

Also, enterocytes produce a variety of **antimicrobial substances** that kill or inactivate microorganisms and maintain homeostasis [52]. These natural "antibiotic" molecules are members of diverse families: α- and β-defensins, cathelicidins, lysozyme, phospholipase A2, RNAses and C-type lectins such as RegIIIγ in mice (called HIP/HAP in humans) [53].

Gut surfaces harbor other less-abundant epithelial cells that limit the penetration of bacteria into host tissues: the **Goblet cells** and the **Paneth cells** [51] (figure 2-1). Goblet cells express proteins (like MUC2) responsible for the secretion of large quantities of mucins

which form a protective layer of gel-like mucus over the luminal site of the epithelium [54]. In the colon, the **mucus** is composed by two distinct layers. The outer layer is colonized with bacteria whereas the inner layer is resistant to bacterial penetration – likely due to the fact that antimicrobial factors secreted by epithelial cells are retained in this inner layer. Paneth cells are situated at the base of small intestinal crypts and harbor secretory granules containing antimicrobial proteins that are discharged in the gut lumen following bacterial stimuli [52]. This type of cell is absent in the colon [55].

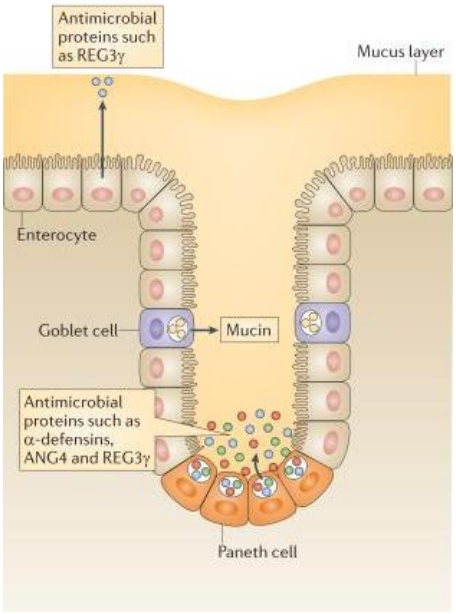

Figure 2-1: Epithelial barrier of the intestine, Gallo R. and Hooper L., Nature Review Immunology, 2012 [53].

Also, subepithelial **immune cells** play an essential role in defending the intestinal mucosa against invading bacteria [56] (figure 2-2) . With the help of **dendritic cells**, **B Lymphocytes** (plasma cells) present in the lamina propria secrete bacteria-specific **immunoglobulin A** (IgA), which are translocated across the epithelium and deposited on the apical surface of epithelial cells. IgA are essential in maintaining luminal compartmentalization of intestinal bacteria. Other **T lymphocytes** intercalated between intestinal epithelial cells contribute to the restoration of homeostasis following mucosal injury by secreting growth factors and pro-inflammatory cytokines. Also, **macrophages** located in the lamina propria can quickly phagocyte and kill invading bacteria that breach the mucosal surface. Finally, **M cells**, which are present in the lymphoid tissue of the small intestine, and **enteroendocrine cells** function as important sensors of intestinal luminal contents [57].

The intestinal immune response towards gut bacteria is regulated by pattern recognition receptors (PRRs) which recognize specific molecular pattern of bacteria and other microorganisms (called PAMPs). These receptors include Toll-like receptors (TLRs) which are transmembrane proteins highly expressed by intestinal epithelial cells and immune cells, and nucleotide oligomerization domain-like receptors (NLRs such as NOD1 and NOD2) which are expressed in the intracellular cytoplasmic compartment. The stimulation of these receptors by bacteria activates a signaling cascade that leads to the synthesis of pro-inflammatory cytokines, chemokines, and antimicrobial proteins [58, 59].

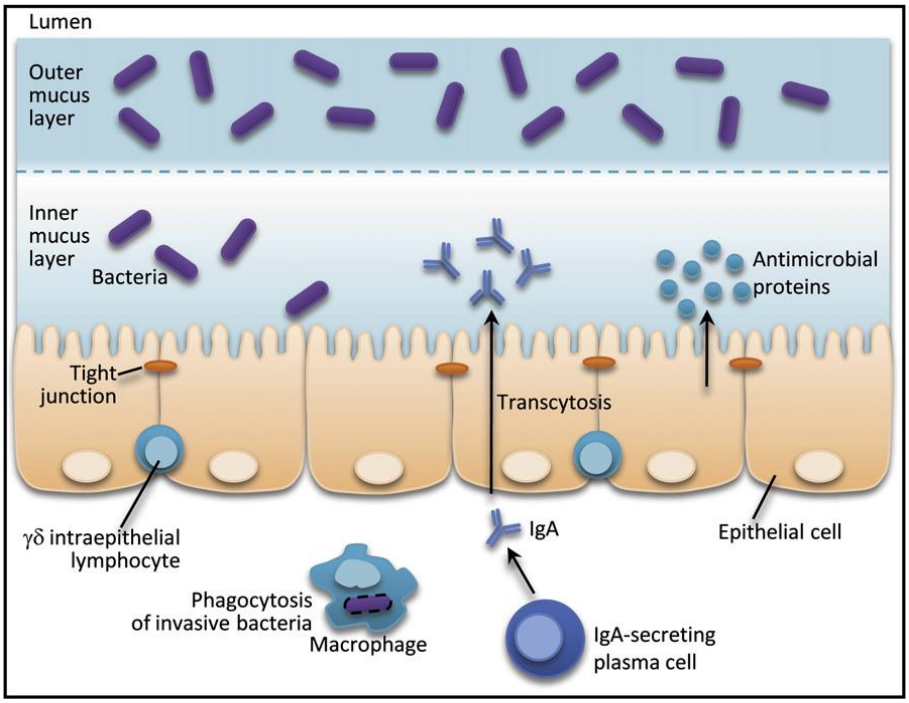

Figure 2-2: Cells and molecules forming a physical barrier against bacterial invasion, Duerkop BA *et al*., Cell, 2009 [51].

Several studies [60, 61] suggest that defects leading to reduced antimicrobial proteins and/or mucus production are associated with bacterial invasion of the intestinal barrier and subsequent inflammation that characterizes inflammatory bowel diseases. Also, the alteration of enterocytes and the disruption of TJs contribute to the increase in intestinal paracellular permeability, also referred as “leaky gut” which enables the translocation of bacterial toxins from the lumen to the systemic circulation.

2.1.2 Methods to assess intestinal permeability

The evaluation of gut barrier alterations includes histological analysis of TJs structures from intestinal biopsy (invasive method) or the assessment of blood endotoxins. However, noninvasive intestinal permeability test *in vivo* has been widely used to assess the intestinal barrier function in health and diseases conditions.

The principle of the intestinal permeability test is to measure urinary excretion of orally administrated tests substances. The "ideal" test substance must have specific physicochemical properties such as water solubility, non-toxic, not metabolized before, during and after permeating the intestine, nondegradable by intestinal bacteria, not naturally present in urines [62]. The most common test probes are the following:

- Polyethylene glycol-400 (PEG-400)
- Monosaccharides: mannitol, L-rhamnose
- Disaccharides: lactulose, sucrose
- Radioactive probes: ^{51}Cr-EDTA and ^{99m}Tc-DTPA

Each probe has its own advantages and disadvantages. Despite its radioactivity, ^{51}Cr-EDTA presents the closest characteristic of an ideal probe. Indeed, very low ^{51}Cr-EDTA permeability has been reported through intact intestinal mucosa [63]. However, the absorption of the probe is increased following damage of intercellular junctions, which regulate the paracellular pathway. Moreover, ^{51}Cr-EDTA has been proved to be useful for the assessment of colonic permeability as this probe is not degraded by enteric bacteria [64]. The other tests probes have characteristics that prevented their use in our study. For instance, PEG-400 demonstrates a considerable physiologic absorption, probably due to its lipophilic properties which allows transcellular permeation. Sugars could be produced endogenously, have osmotic action, and more importantly, can be degraded by colonic bacteria [62, 63].

In our study, a Nutridrink[5] solution containing 50 µCi of ^{51}Cr-EDTA was drunk by the subjects after an overnight fast and bladder emptying. Urines were collected during 24 hours. Normal food and fluid intake was allowed during the test day. Radioactivity was detected in

[5] The permeation rate of the probe can be influenced by the osmolarity of the solution. The study by Peeters *et al*. [65] reported the lowest interindividual variations with the Nutridrink, compared with other solutions.

urines by using a gamma-counter and results were expressed as the percentage of ingested dose found in urines.

Bjarnason *et al*. reported an increased in intestinal permeability in alcoholics using ^{51}Cr-EDTA, which returns to normal after at least 2 weeks of abstinence [66]. They also demonstrated increased gut permeability in inflammatory bowel diseases [67] and coeliac disease [68]. Interestingly, in 1987, Wood *et al.* [69] reported abnormal gut permeability in psychiatric patients, which could not be attributed to established bowel disease.

2.1.3 Effect of ethanol and its metabolites on the gut barrier function

Few studies have investigated the effect of ethanol intake on intestinal barrier function in humans. Some studies have shown that acute alcohol intake induces an increase in intestinal permeability in non-alcoholic healthy subjects [70–72], while the majority of data focuses on the effect of chronic alcohol abuse and report an increased in intestinal permeability in alcoholic subjects [66, 71–74]. The subjects taken into consideration have been often diagnosed with hepatic damage. However, these studies have mainly investigated the effect of ethanol on small intestinal permeability. Data on the effects of ethanol on colonic barrier function are lacking. Only one animal study has shown that oral administration of ethanol in rats resulted in increased colon permeability [75]. Yet, although ethanol is mainly absorbed in the small intestine, ethanol in excess and its metabolites can also reach the colon and their effect on the colonic permeability deserve to be tested.

Mechanisms by which ethanol and its metabolites alter the intestinal barrier

Several direct and indirect mechanisms underlying the ethanol-induced barrier dysfunction have been proposed.

Ethanol and its metabolite acetaldehyde can induce direct epithelial cell damages, with ultrastructural and histological changes in intestinal mucosa including enterocyte alterations, decrease in mucosal surface area, inflammatory cell infiltration, decreased number of mucin-secreting goblet cells [76–78]. In vitro studies using Caco-2 cell monolayer (human epithelial colorectal adenocarcinoma cells) have highlighted several mechanisms by which ethanol and

acetaldehyde disrupt TJs, destabilize cytoskeleton and subsequently form large gaps between adjacent cells. These include myosin-light chain kinase (MLCK) activation [79], NF-κB activation [80], up-regulation of intestinal circadian clock gene expression [81], decrease ZO-1 protein expression through ethanol-induced miR-212 overexpression [82]. Oxidative stress has also been involved in gut barrier alteration. Indeed, ethanol induces increased paracellular permeability of Caco-2 cells via inducible nitric oxide synthase (iNOS)-mediated generation of reactive oxygen species (ROS), resulting in oxidation of microtubule cytoskeleton and TJs disassembly [83, 84]. The involvement of iNOS has been confirmed in vivo where the inhibition of this enzyme attenuated the ethanol-induced gut leakiness and the associated endotoxemia in rats [85]. Evidence revealed that acetaldehyde has higher potency than ethanol to induce intestinal barrier dysfunction [86]. Acetaldehyde reduces the activity of protein tyrosine phosphatase that results in increased phosphorylation of TJs and AJs proteins and loss of interaction between these junctional complexes [87, 88]. It also induces hyperacetylation of microtubular protein [89].

Also, a recent study in mice [90] has shown that chronic alcohol exposure suppresses gene and protein expression of RegIIIg, an antimicrobial peptide which might contribute to alteration of gut barrier integrity and microbial changes. Pro-inflammatory cytokines, especially TNFα and IFNγ, has been demonstrated to regulate TJs and cause disruption of the gut barrier [91]. Finally, the intestinal microbiota can also modulate the gut barrier integrity. Indeed, animal studies have shown that amelioration of ethanol-induced barrier dysfunction can be achieved using antibiotics [75], dietary fibers [92] or live bacteria (probiotics) [93]. Therefore, ethanol-induced gut microbiota alterations [94] could be considered an indirect mechanism by which ethanol can alter the gut barrier. This hypothesis has never been tested in human alcoholics and is one of the main topics of the Chapter 3 of this PhD work, in which we have measured in parallel the intestinal permeability and the gut microbiota of alcohol-dependent subjects.

Overall, this alcohol-induced increased intestinal permeability enables the translocation of luminal antigens, mainly bacterial endotoxins such as lipopolysaccharides (LPS) into the portal circulation. This can have drastic effect on the liver as bacterial toxins can activate hepatocytes and Kupffer's cells (liver macrophages), which subsequently release pro-inflammatory cytokines, that results in hepatocellular damage, and consequently, alcoholic liver disease (ALD) [95].

2.2 Gut microbiota

2.2.1 Gut microbiota composition

The human gastrointestinal tract is the natural habitat for 10^{14} microorganisms [96], which represent 10 times the number of human cells in the body. This large and dynamic bacterial community is called the "gut microbiota" and has an approximate mass of 2 kg. The microbiome, that refers to the collective genomes of all members of the gut microbiota, contains 150 times more genes than the human genome [97]. The gut microbiota can be considered an organ within an organ and maintain a symbiotic relationship with the host. The human host provides a nutrient-rich environment and the microbiota provides indispensable functions that humans cannot exert themselves. Indeed, intestinal bacteria have long been appreciated for the benefits they provide to the host: they supply essential nutrients such as vitamins, metabolize indigestible compounds, defend against pathogen colonization. Moreover, they contribute to the development of intestinal architecture and to the functioning of immune system. Evidences now support that certain aspects of health and disease may depend on the status of the microbiota [98].

The gut microbial communities of an individual can be represented as a phylogenetic tree with several hierarchical levels (figure 2-3). The higher taxonomic level is the "phylum". Firmicutes is the major Gram positive phylum and Bacteroidetes is the major Gram negative phylum. Together they constitute over 90% of the total gut bacteria. The remaining bacteria, accounting for less than 10% of the total population, belong to the Proteobacteria, Fusobacteria, Actinobacteria, Verrucomicrobia, Spirochaetes, and Cyanobacteria [51]. Then, each phylum is divided successively into class, order, family, genus, species, and strains. It is generally accepted that, in adult, the microbiota consists up to 500-1000 different species [99]. Arumugam et al. [100] has identified 3 clusters called "enterotypes" (enriched in *Bacteroides*, *Prevotella*, or *Ruminococcus*) in the human gut microbiome that vary in species and functional composition. They suggest that enterotypes are driven by groups of species that together contribute to the preferred community compositions. However, this notion of "enterotypes" is currently under debate [101, 102], and diet appears to be an important factor that mediates variations in bacterial compositions [103].

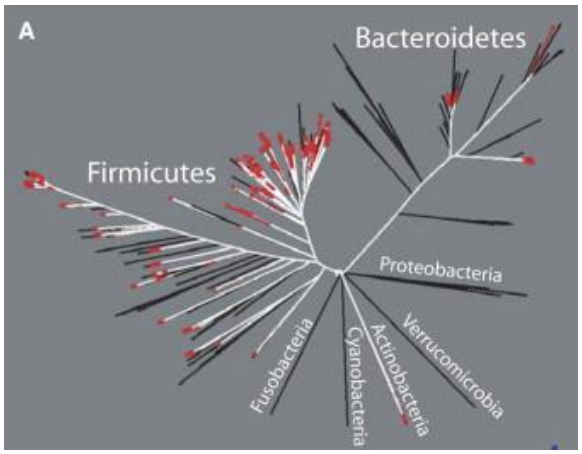

Figure 2-3: Phylogenetic tree based on the 16S rRNA bacterial sequences, representing colonic bacterial diversity of an individual, adapted from Ley RE *et al.*, Cell 2006 [96].

The scarcity of bacteria in the upper tract seems to be due to the composition of the luminal content (acids, bile, and pancreatic secretions) which kills most ingested microorganisms, and because of the phasic propulsive motor activity toward the ileal extremity. By contrast, the large intestine contains the core of the microbial ecosystem with high densities of living bacteria (figure 2-4) [104].

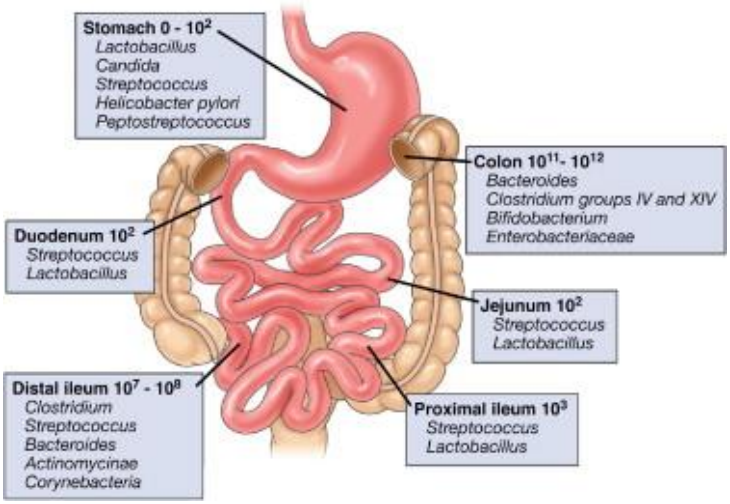

Figure 2-4: Composition and luminal concentrations of dominant microbial species in various regions of the gastrointestinal tract, Sartor RB., Gastroenterology, 2008 [105].

A large number of factors can influence the gut microbiota composition, from birth to old age (figure 2-5). At birth, the method of delivery (vaginal vs. Cesarian section) already shapes the microbial communities of babies [106, 107]. Then, other environmental factors such as diet, life-style, antibiotic use, stress, injury can modulate the microbiota. Among

dietary factors, probiotics and prebiotics are interesting nutritional tools that can positively modified the microbial composition and/or activity.

Probiotics are live microorganisms, which when administrated in adequate amounts confer a health benefit on the host (definition of the World Health Organization / Food and Agricultural Organization of the United Nation, 2001). Most of probiotics are bacteria (mainly *Lactobacillus*, *Bifidobacterium*) or yeasts (*Saccharomyces*). The probiotics can introduce missing bacteria or supplement present bacteria with known beneficial functions for the host.

The concept of prebiotics was introduced for the first time by Gibson and Roberfroid in 1995 [108]. A **prebiotic** is a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health [109]. Unlike probiotics, prebiotics target the microbiota already present within the ecosystem. Human enzymes are not able to degrade most of the complex carbohydrates. These ingredients can be prebiotics that reach the colon and are fermented by commensal bacteria. However, not all undigested carbohydrates are prebiotics. Three criteria are required to have prebiotic effect: 1) resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; 2) fermentation by intestinal microbes; 3) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing [110]. The most widely accepted prebiotics are inulin-type fructans such as fructo-oligosaccharide (FOS) and galactooligosaccharide (GOS). Although promise does exist with several other dietary carbohydrates, well conducted human trials are required to confirm their prebiotic effects.

Disruption of the gut microbiota, known as dysbiosis, can lead to a variety of diseases including inflammatory bowel diseases, colon cancer, irritable bowel disease, obesity and metabolic syndrome, nonalcoholic fatty liver diseases, auto-immune diseases and finally mood and behavior changes [98]. The relationship between gut microbiota alteration and psychological changes in alcohol-dependent subjects is one of the main topics of the Chapter 3 of this PhD work.

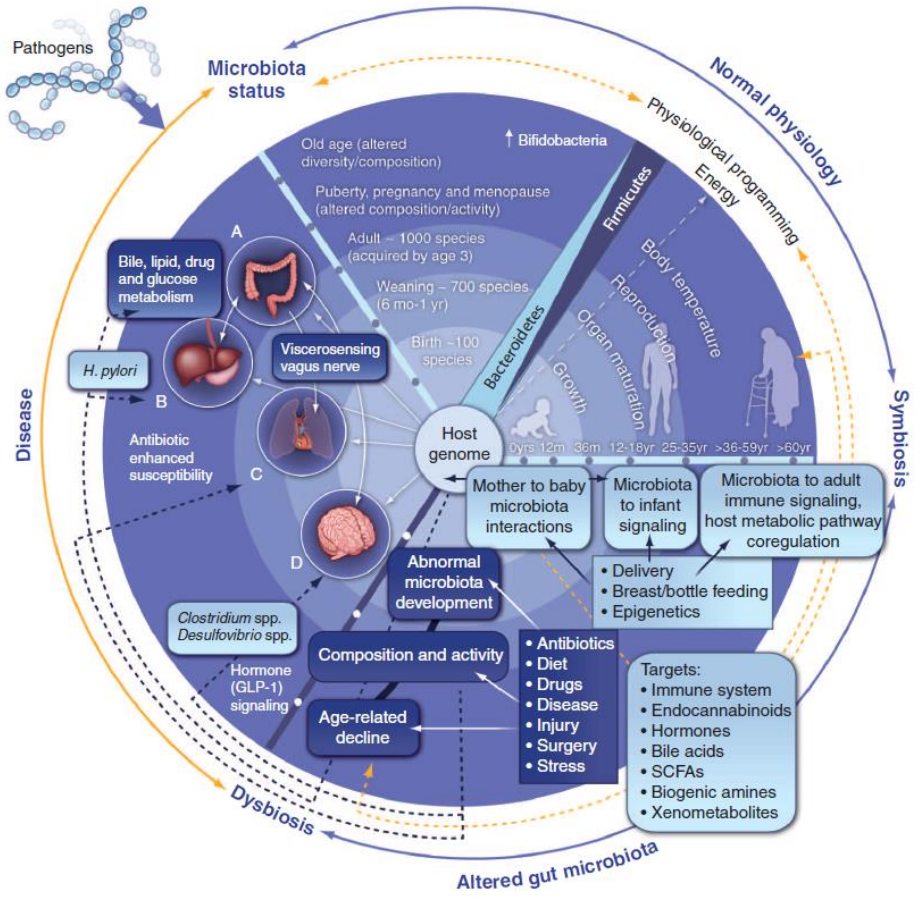

Figure 2-5: The gut microbiota in development and disease, Nicholson *et al*., Sciences, 2012 [98].

2.2.2 Gut microbiota activity and functionality

The intestinal microbiota has a huge metabolic capacity which complements our physiology with functions that are indispensable for human life. For example, the intestinal microbiota is responsible for host metabolism regulation and energy harvest from non-digested nutrients. It is involved in the synthesis of vitamin K and in the metabolism of bile acids and polyphenols. Moreover, the gut microbiota provides colonization resistance toward potential pathogens, provides trophic effects on epithelial cells and stimulates the immune function of the host [111]. The human microbiota is characterized by a significant degree of functional redundancy, meaning that different bacteria can perform similar functions and metabolize the same substrates [96]. This functional redundancy confers stability. Therefore, not only the composition but also the functional capacity of the intestinal microbiota is highly important regarding clinical endpoints. Metagenomic studies have applied next generation pyrosequencing techniques to assess the functional capacity of the gut microbiota by detecting

the bacterial genes [112]. However, it is important to note that the detection of genes does not mean that they are functionally relevant. Therefore, to gain better insight into the activity and functionality of the intestinal microbiota, other approaches must be considered such as metabolomics [113]. Metabolomics can be applied to different type of samples such as feces, breath, urines and plasma. Certain breath, blood and urine metabolites result from co-metabolism by the host and the intestinal microbiota, whereas large amount of fecal metabolites are primarily derived from the action of the intestinal bacteria. In addition, fecal metabolic profiling appears to be a potential useful approach to understand microbiota-gut barrier interactions.

In summary, the main functions of the gut microbiota are the following: metabolic, trophic, immune and protective.

2.2.2.1 Metabolic function

The colonic microbial fermentation produces various metabolites which type and amount depend on the composition of the gut microbiota, on the transit time and on substrates available for fermentation. The colonic bacteria can ferment endogenous host-derived substrates such as mucus and pancreatic enzymes, as well as dietary components that escape digestion in the upper part of the gastrointestinal tract. Basically, two types of bacterial fermentation occur in the colon (figure 2-6) [113]:

- **The carbohydrate fermentation** which occurs mainly in the proximal part of the colon. The metabolic endpoint is the generation of short-chain fatty acids.
- **The protein fermentation** of dietary and endogenous peptides (from mucus and pancreatic enzymes), which takes place in the distal colon, can generate compounds with toxic properties.

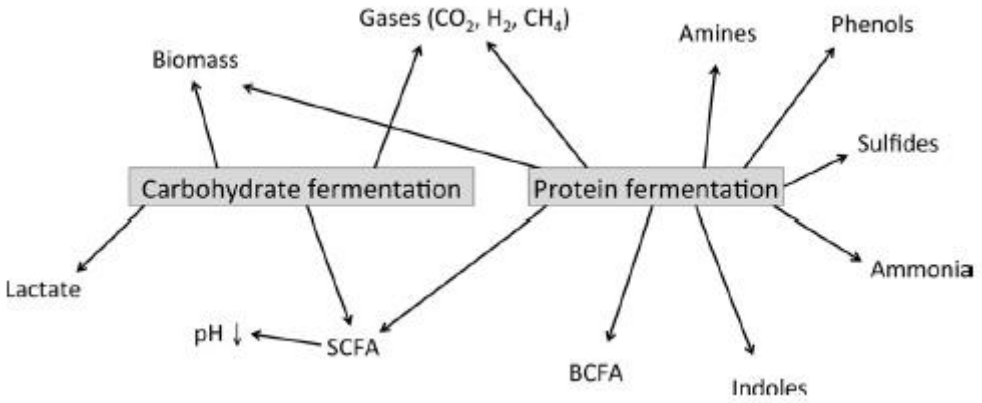

Figure 2-6: Different metabolites produced from colonic fermentation of carbohydrates and proteins, Hamer HM *et al*., AJP-Gastrointestinal and liver physiology, 2012 [113].

Although they received less attention in the literature, the gut microbiota is also able to transform lipid compounds from the diet, such as cholesterol and polyunsaturated fatty acids (PUFAs). The PUFA-derived metabolites like conjugated linoleic acids or conjugated linolenic acids could have beneficial effects on the host and should be taken into account in the next future [114, 115].

2.2.2.1.1 Saccharolytic microbial fermentation

The majority of intestinal bacteria use the glycolytic pathway to derive energy from carbohydrates that have not been digested in the upper part of the intestine [116] such as inulin, oligofructose, oat bran, resistant starches, cellulose, hemicellulose, pectins, gums and other unabsorbed sugars. These carbohydrates are initially converted to pyruvate and acetyl-CoA (figure 2.7). Pyruvate is then transformed into lactate, succinate and propionate while acetyl-CoA is converted into acetate, butyrate and ethanol. Acetate, propionate and butyrate are the main short-chain fatty acids (SCFAs) found in fecal samples. SCFAs, particularly butyrate, have been shown to have beneficial impacts for health: they provide energy for colonocytes, decrease luminal pH to inhibit pathogen growth, improve ions absorption (calcium, magnesium, iron), reinforce various components of the colonic defense barrier, have anti-inflammatory properties, improve insulin sensitivity and promote satiety [117, 118].

After carbohydrate fermentation, some intestinal bacteria which possess the enzymes aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) can catalyze the reduction of acetate to acetaldehyde and then to ethanol [116] (figure 2-7). These bacteria are

called ethanol-producing bacteria. Ethanol found in the gut can therefore have two origins: exogenous (alcohol consumption) and endogenous (produced by bacterial enzymes).

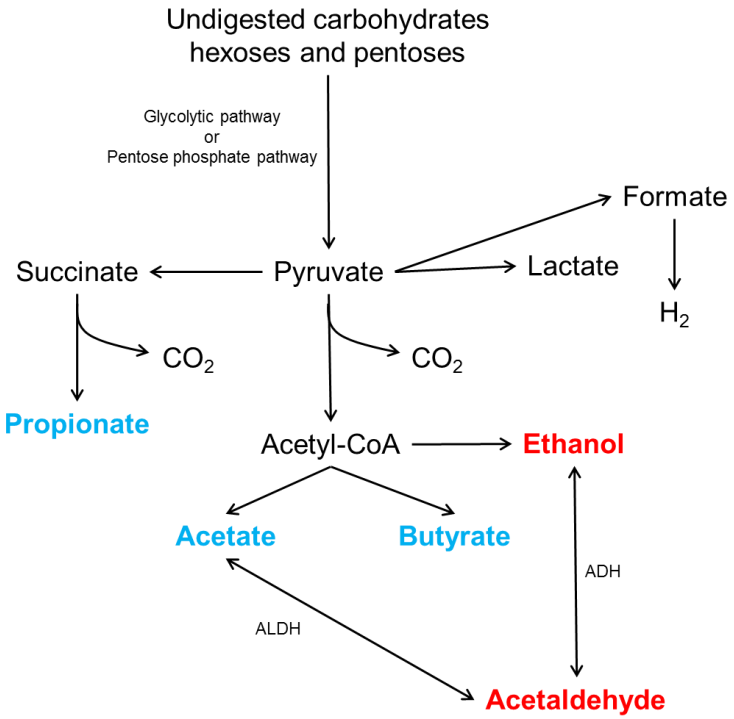

Figure 2-7 : Simplified diagram of carbohydrate fermentation in the large intestine, adapted from Macfarlane S. and Macfarlane GT, 2003 [116]. The SCFAs predominantly found in colon appear in blue.

2.2.2.1.2 Proteolytic microbial fermentation

Although the carbohydrate fermentation has shown to be beneficial for the host, the proteolytic fermentation results in the production of metabolites that could be detrimental for the host's health and that have been implicated in the etiology of colorectal cancer and ulcerative colitis [119]. Proteins and peptides that enter the large intestinal can have exogenous (diet) or endogenous (pancreatic enzymes, host tissues) origin and are the target of various proteases and peptidases [120]. The resulting short peptides and amino acids then become available for fermentation by the intestinal bacteria. The fermentation of branched-chain amino acids (BCAAs – valine, isoleucine and leucine) leads to the production of branched-chain fatty acids (BCFAs) such as 2-methyl propanoic acid, 2-methyl butanoic acid and 3-methyl butanoic acid. The bacterial degradation of aromatic amino acids tyrosine and tryptophan results in the production of phenolic and indole compounds, respectively (figure 2-

8). The end-products of tyrosine metabolism include mainly phenol and 4-methyl phenol (also called p-cresol) while tryptophan degradation generates indole and 3-methyl indole (also called skatole). Fermentation of sulfur amino acids (methionine, cysteine, taurine) by sulfate-reducing bacteria results in the production of hydrogen sulfide (H_2S). SCFAs can also arise from protein fermentation but in small amount.

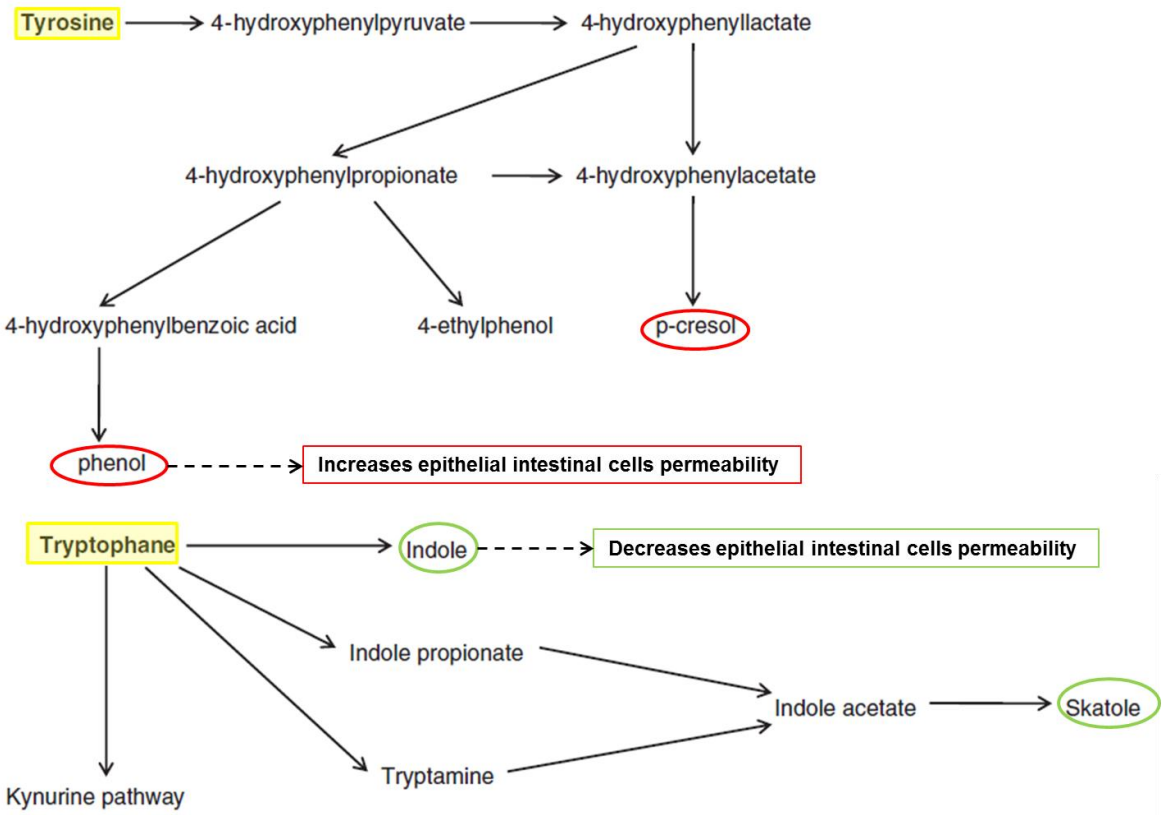

Figure 2-8: Degradation pathway of the aromatic amino acids tyrosine and tryptophane, adapted from Windey K. *et al.*, 2012 [119]. Metabolites detected in the study described in chapter 3 are circled.

Phenolic and sulfur-containing compounds are potentially toxic. For instance, phenols are believed to act as co-carcinogens [121]. Moreover, two independent studies [122, 123] have shown that phenol exposure decreases the transepithelial electric resistance in a dose-dependent manner, in parallel with an increase in the paracellular flux of mannitol or dextran-FITC in intestinal epithelial cells culture, reflecting a general increase in epithelial permeability. The change in paracellular permeability observed after phenol treatment correlate with the delocalization of TJs claudin-1 and ZO-1 from intercellular junction to cytosol [122]. By contrast, indole has been shown to reinforce the epithelial intestinal cell barrier in vitro [124]. H_2S produced by sulfate-reducing bacteria is an extremely toxic agent that has been shown to induce genomic DNA damage in colonocytes [125].

It is important to note that BCFAs, phenolic and indoles compounds are not produced by human enzymes and are therefore unique colonic bacterial metabolites. Any changes observed in these metabolites may therefore result from a change in the gut microbiota composition or a change in dietary protein intake.

2.2.2.2 Trophic function

It has been reported that modulation of gut microbiota by using prebiotics are associated with an increased endogenous production of glucagon-like-peptide 2 (GLP-2), whose production may improve mucosal barrier function by increasing the rate of crypt cell proliferation and villus elongation [126]. Also, SCFAs stimulate epithelial cells proliferation and differentiation in the small and large intestine in vivo through mechanisms involving the autonomic nervous system [127], the release of growth factors or gastrointestinal peptides like gastrin or cholecystokinin [128, 129], or by acting directly on genes that regulate proliferation and cell growth [130].

2.2.2.3 Immune function

The gastrointestinal tract is the primary site of interaction between the host immune system and microorganisms. The intestinal immune system is able to distinguish commensal from pathogenic bacteria. On the other hand, intestinal bacteria can also shape and modulate the immune responses and these complex interactions play an important role in health and diseases [131]. Many examples can illustrate the gut microbiota – immune system interactions. For instance, germ-free animals show defects in the development of gut-associated lymphoid tissues, decreased IgA and antimicrobials production, thinner and less cellular lamina propria and are therefore more susceptible to infection [111]. Deficiencies of specific immune genes such bacterial flagellin receptor TLR5, inflammasome component NLRP3 and NLRP6, or intracellular receptor NOD2 for peptidoglycan recognition are associated with gut dysbiosis [132–135]. The bacterium *Faecalibacterium prausnitzii* exhibits anti-inflammatory properties by increasing the production of IL-10 by peripheral mononuclear cells, reducing TNFα production in the colon and improving intestinal disease in mice [136]. Also, it has recently been shown that Crohn's disease and ulcerative colitis

patients have a specific reduction in this gut bacterium [136, 137]. *F. prausnitzii* can also exhibit anti-inflammatory action via the production of butyrate which, apart from being an important energy source for the colonocytes, can exert direct immune-modulatory effects through inhibition of NF-κB, inhibition of the IFN-γ production and signaling and up-regulation of the peroxisome proliferator-activated receptor-γ (PPARγ) [117]. Several clinical studies suggest that the luminal administration of butyrate improve inflammation and symptoms in active ulcerative colitis patients [138–140]. Finally, butyrate as well as other SCFAs, are the ligands of G-protein-coupled receptors, GPR41 and GPR43 expressed in immune cells and in colonic mucosa. Activation of these receptors is required to induce an appropriate immune response during immunological challenges [141].

2.2.2.4 Involvement in the barrier function

The bacteria inhabiting the gut participated to the intestinal "barrier effect" through several mechanisms [104]. Bacteria compete for attachment sites in the brush border of intestinal epithelial cells and non-pathogenic adherent bacteria therefore prevent the attachment of pathogenic and enteroinvasive bacteria. Bacteria also compete for nutrient availability and produce antimicrobial substances which inhibit the growth of their competitors. SCFAs also play a role in the regulation of gut barrier function. Indeed, in vitro studies have shown that SCFAs up-regulate the expression of antimicrobial peptides in different colon epithelial cell lines [142]. Moreover, propionate and butyrate have been shown to increase the expression of MUC2 gene which encodes the most important mucin of the intestinal mucus layer [143]. The protective effect of butyrate on intestinal permeability seems to occur at low concentration (up to 2mM) [144, 145] while at higher concentrations (8mM), butyrate increased the permeability of Caco-2 cell line [146].

2.2.3 Techniques used to characterize the gut microbiota composition and functionality

2.2.3.1 Taxonomic analysis

Until the 1990s, knowledge of the gut microbiota was limited to culture-based technique, an approach that has been used since the early 20th century. However, this approach left substantial gaps in the catalog of intestinal bacterial species because anaerobic (uncultivable) bacteria outnumber aerobic bacteria by a factor of 100-1000 [104]. The development of culture-independent techniques has revolutionized our understanding and identifying of microbial communities and revealed their complexity. Most of these new techniques are based on sequence analysis of the 16S ribosomal RNA gene (16S rRNA gene also called 16S rDNA). Basically, these techniques bring information about 1) the microbial diversity of the gut microbiota, 2) the qualitative and quantitative measure of bacterial species, 3) the changes in the gut microbiota in relation to disease [147].

The ribosome is a complex molecular machine indispensable for messenger RNA (mRNA) reading and protein synthesis (translation). Ribosome consists of two major components, a small subunit and a large subunit. Each subunit is composed of one or more ribosomal RNA (rRNA) molecules as well as proteins. Prokaryotes have 70S ribosomes[6] that consist of a small (30S) subunit and a large (50S) subunit. The 30S subunit contains one 16S RNA molecule and the 50S subunit contains two RNA molecules, 5S and 23S (figure 2-9). 16S rRNA genes are highly conserved between bacterial species and are therefore used for phylogenetic (bacterial) identification. However, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature.

[6] The unit of ribosome measurement is the Svedberg unit, a measure of the rate of sedimentation in centrifugation.

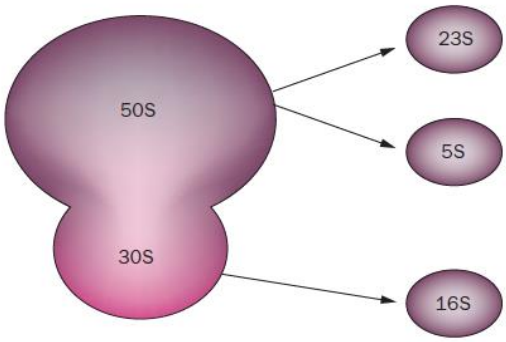

Figure 2-9: Representation of 70S bacterial ribosome, Fraher MH *et al*., Nature Review Gastroenterology and Hepatology, 2012 [147].

Culture-independent techniques used to characterize the intestinal microbiota are based on the extraction of DNA from a sample containing microbial communities (stool sample, colon biopsy) followed by PCR amplification of 16S rRNA gene (figure 2-10). These techniques include denaturating gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), fluorescence in situ hybridization (FISH), DNA microarray, quantitative polymerase chain reaction (qPCR) and next-generation sequencing of the 16S rRNA gene. The two latter techniques have been used in this thesis for the characterization of gut microbiota of alcohol-dependent subjects and are therefore described below.

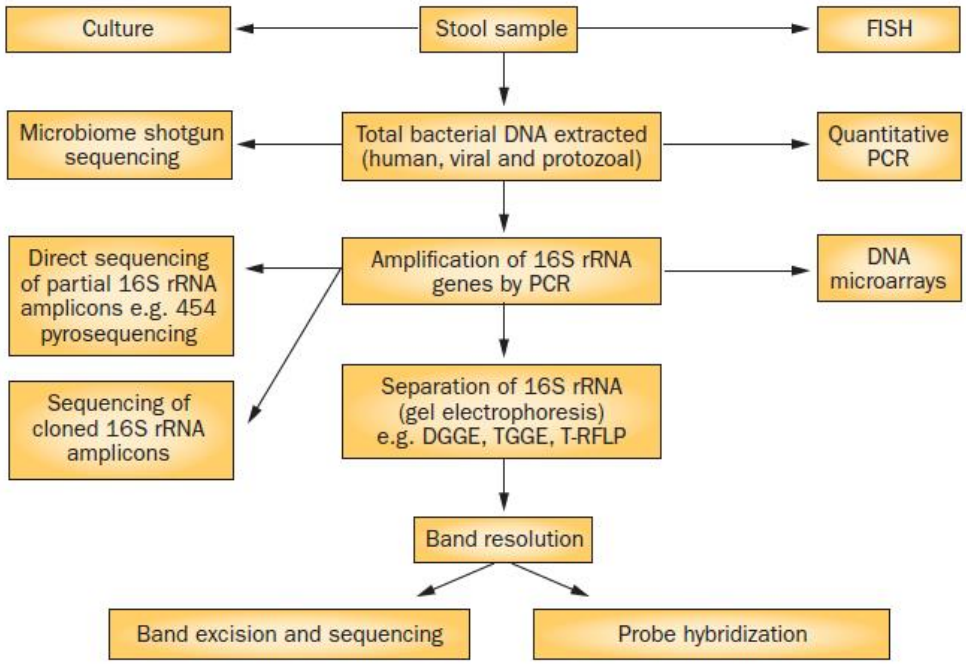

Figure 2-10: Overview of the techniques used to characterize the gut microbiota, Fraher MH *et al*., Nature Review Gastroenterology and Hepatology, 2012 [147].

Next-generation sequencing describes sequencing technologies that deliver sequence data at fast rate (and quite low cost). "454 Pyrosequencing" was used in this thesis and consists of a massive parallel sequencing of 16S rRNA gene amplicons. Briefly, following DNA extraction from stool samples, 16S rDNA was amplified by PCR using universal oligonucleotides primers[7] that target highly conserved regions of 16S rDNA. This amplification results in the formation of new double-stranded 16S rRNA gene amplicons. Amplicons are then fragmented into single-strand and then immobilized on beads. Pyrosequencing differs from Sanger sequencing in that pyrophosphate is released when a nucleotide is incorporated by the polymerase rather than chain termination with dideoxynucleotide. This technique allows phylogenetic identification and identification of new unknown bacterial species. However, it requires complex bioinformatics and biostatistics analyses. The kind of bacteria present in the sample can be identified by comparison of their 16S rRNA gene sequences to previously analyzed sequences found in the Ribosomal Database Project [148]. By convention, all the 16S rRNA gene sequences are assembled into operational taxonomic units (OTUs) based on the percentage of sequences identity. Therefore, the percentage of sequence identity defines the taxonomic level. For instance, OTUs containing sequences with ≥ 99% identity are considered to be from the same strain; OTUs with ≥ 97% identity are considered to be from the same species, while different species from the same genus have ≥ 95% identity [149].

In our study, the pyrosequencing was used in combination with qPCR, also known as real-time PCR. qPCR is useful to quantify specific bacteria or a specific group of bacteria using appropriate primers. Indeed, primers can be designed to target all bacterial phyla (to known the total bacterial load) as well as single species. This technique therefore reliably quantifies the amount of DNA present in the sample by reference to a standard curve derived from parallel amplification of known target bacteria number. The disadvantage of qPCR is the inability to identify novel and unknown species.

One limitation of all these new technologies is that they map the content of the microbes present in the gut without giving any indication on their activity. Therefore, in this thesis, metabolomics analyses of stool samples were performed in order to assess the

[7] In this study, 27F and 338R primers were used to amplified 16S rDNA

functionality of the gut microbiota. In reality, knowing "who is there" is not sufficient. Essential question is "what are these microbes really doing?" [112].

2.2.3.2 Functional analysis

Metabolomics aims at the comprehensive and quantitative analysis of wide arrays of metabolites in biological samples. The "metabolome" is the complete set of metabolites that exists in a biological sample. There is a strong possibility that changes in metabolite levels reflect the functional status of a biological system because alterations of their levels occur downstream of DNA, RNA and proteins [150]. Therefore, metabolomics is the endpoint of the "omics cascade" and is the closest to phenotype. Moreover, it involves the study of fewer molecules than genomics, transcriptomics and proteomics (figure 2-11).

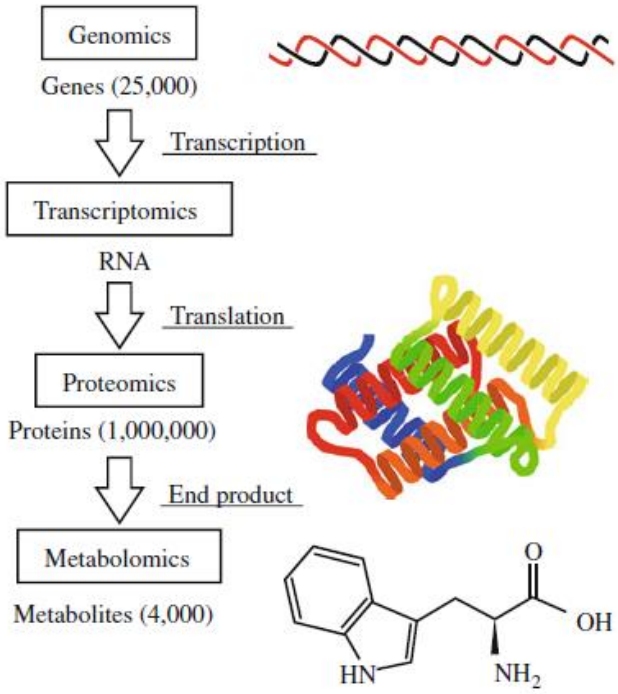

Figure 2-11: The "omics" cascade, from Yoshida M. *et al*., J Gastroenterol (2012) [150].

Large-scale metabolome analysis commonly used analytical platforms such as ^{1}H-nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) [113]. Each of these methods has its own merits and demerits and there is no single-instrument platform that currently can analyze all metabolites. MS technique requires a prior separation of the metabolites present in the sample by using gas chromatography (GC) or liquid chromatography (LC). This separation reduced the complexity of the mass spectra due to

metabolite separation in a time dimension and delivers additional information on the physico-chemical properties of the metabolites. MS-based methods are considerably more sensitive than NMR spectroscopy. NMR has the potential for high-throughput fingerprint, minimal requirement for sample preparation but only metabolites present in high abundance will be detected.

GC-MS method was used in this thesis to characterize the volatile organic compounds (VOCs), which are important component of the metabolome, in alcohol-dependent subjects. Briefly, the metabolites present in the sample pass through a capillary column in the GC along with a carrier gas (e.g. helium gas). As they travel the length of the column, the metabolites are separated according to their chemical properties and are eluted from the column at different times, called the retention time. Then the metabolites are directed to the MS. A conventional MS consists of an ionization source, mass analyzer, and detector. The first step is to ionize the molecules which results in the creation of fragments with specific mass and charge. Then mass analyzer separates the molecule based on their mass to charge value (m/z ratio). The information is then converted into an electric current by electron multiplier (detector) for quantification and data analysis [151].

Therefore, each metabolite is defined by:

- Its retention time (RT), measured by GC
- Its *m/z* value, which is its molecular mass divided by its charge, measured by MS

Metabolite identification is achieved by use of existing libraries of chromatographic retention times and *m/z* values such as Automated Mass Spectral Deconvolution and Identification System (AMDIS) provided by the US National Institute of Standard and Technology (NIST).

Due to the overwhelming amount of metabolites found in samples, multivariate analysis is commonly used in metabolomics studies. The multivariate methods should be selected according the aim of the study. However, it is generally appropriate to use several methods to maximize the extraction of information. If the aim of the study is sample classification and prior information about the sample identity is not known, unsupervised method such as principal component analysis (PCA) can be used. PCA attempts to reduce multi-dimensional data on a 2D or 3D plot, which can be comprehended by the human mind. The plot is a

Cartesian coordinate system in which the axes (principal components) represent the greatest variation of the data. PCA is a good data visualization tool. On the other hand, if the sample identity is known and the aim of the study is to discover characteristic biomarkers (e.g. comparing samples from healthy and diseased subjects), supervised method such as principal least square-discriminant analysis (PLS-DA) can be used. Loading plots derived from PCA or PLS-DA are used to determine the metabolites that contribute to the separation of subjects into different groups. If the concentration of a particular metabolite is found to be altered through multivariate analysis, univariate analysis can be used to test the statistical significance of the change. This typically involves the use of Student's t-test or one-way analysis of variance (ANOVA) depending on the number of study groups.

2.2.4 Effect of ethanol on gut microbiota composition and functionality

The idea that the gut microbiota could be altered by ethanol consumption came from experimental and clinical data showing that the bacterial endotoxin lipopolysaccharide (LPS), which derives from the cell wall of Gram negative bacteria inhabiting the gut, is a key factor in the development of alcoholic liver disease. Indeed, elevated endotoxin levels and liver injury has been reported in rats fed with ethanol [152] and in human alcoholics [74, 153, 154]. Lowering serum endotoxins levels by giving antibiotics [155], oats [92] or probiotics *Lactobacillus* [93, 156] attenuates ethanol-induced liver damage in rats. Thus, endotoxin and possibly other gut-derived bacterial products are involved in the development of liver disease in alcoholics. Three possible mechanisms have been proposed to explain the elevation of blood endotoxins in alcoholics:

- Increased production of endotoxins by abnormal gut microbiota composition or bacterial overgrowth [90, 157–159].
- Increased permeation of endotoxins through the gut due to gut leakiness.
- Decreased function of liver Kupffer cells.

It was therefore pertinent to characterize the composition of gut microbiota and identify the alterations that occur in response to chronic alcohol consumption. The first experimental study that investigated whether chronic ethanol consumption affects gut bacterial composition

was conducted by Mutlu *et al*. in 2009 [160]. The authors showed that chronic ethanol treatment induced alterations of the mucosa-associated colonic bacterial microbiota in rats. The authors hypothesized that dysbiosis could contribute to endotoxemia and liver disease both by increasing endotoxins production and by chronic deleterious consequences on gut barrier function. Then, another study investigated in more details the change in enteric microbiome by using pyrosequencing technology and found reduced abundance of Firmicutes and *Lactobacillus* as well as increased Bacteroidetes in mice continuously fed with ethanol [90]. The third and most recent study [161] examining the gut microbiota of alcohol fed mice showed that chronic alcohol consumption induced a reduction in Firmicutes (unclassified Lachnospiraceae and Ruminococcaceae*)*, Bacteroidetes, and an increase in Proteobacteria, Actinobacteria and in *Lactobacillus*. The authors suggested that alcohol-induced microbial shifts could be driven by change in luminal pH. The discrepancies seen across the different animal studies could be due to the different time periods of alcohol feeding and methods used. However, in animals, the improvement of the alcohol-induced gut microbiota alterations have been reported after supplementation with prebiotics [90, 160] and probiotics [160, 161].

In human, only very few studies tried to relate changes in the gut microbiota with alcohol consumption. A study by Kirpich *et al*. [162] showed, by culturing stool samples, that alcoholic patients had decreased numbers of *Bifidobacterium*, *Lactobacillus* and *Enterococcus* compared to healthy controls. After 5 days of probiotic supplementation and alcohol abstinence, these bacteria returned to numbers seen in controls and improvement of liver enzymes was reported. In 2012, Mutlu *et al*. [94] characterized the gut microbiota composition in alcoholics using, non-cultured next generation sequencing technologies and showed that only a subgroup of alcoholics had altered colonic microbiota composition. Indeed, 31% of subjects were defined as “dysbiotic” and included alcoholics with and without liver disease, as well as actively drinking and sober alcoholics. The dysbiosis of colonic mucosa consisted mainly in lower abundances of Bacteroidetes and higher ones of Proteobacteria. These results suggest that chronic alcohol consumption rather than liver disease is the most important event that alters the gut microbiota. Moreover, the effects of alcohol abuse were not temporary but rather long-lasting since sober (> 1month) alcoholics were also dysbiotic. The authors also suggested that dysbiosis could contribute to gut leakiness but this remained speculative since the intestinal permeability has not been measured. Finally, although they characterized the microbial composition, the authors did not

assess the gut bacterial metabolites which may better differentiate alcoholics from healthy subjects and alcoholics with or without liver disease.

Metabolomics analysis of fecal samples has already been used to study numerous digestive tract disorders including colorectal cancers, irritable bowel syndrome and inflammatory bowel diseases. However, metabolome analysis of alcohol-dependent subjects has never been reported. In 2013, a study by Xie *et al* [163] characterized the metabolic alterations of the whole gastrointestinal tract contents in rats following 8 weeks of chronic ethanol consumption. Elevated acetic acid levels were observed in the upper part of intestine in ethanol-fed rats, which is presumably due to the oxidation of ethanol to acetaldehyde and subsequent oxidation by mucosal or bacterial aldehyde dehydrogenase to acetic acid. However, in colon contents, ethanol consumption induced a significant decrease in SCFAs, BCAAs and BCFAs. No significant alteration of phenol and derivatives was observed. The authors suggested that the reduced abundance of some metabolites in ethanol-treated rats could results from alterations of the gut microbiota. Unfortunately, the composition of gut microbiota was not analyzed in this study.

2.3 Gut-brain axis

2.3.1 Mechanisms underlying gut to brain interactions

Recent years have witnessed the rise of the gut microbiota as a major topic in health and disease physiology. Very quickly, it has become evident that gut microbiota can influence many aspects of physiology and studies revealed how changes in the composition of the gut microbiota could contribute to somatic diseases such as obesity, diabetes and inflammatory bowel diseases. Accumulating experimental data now indicate that the gut microbiota also communicates with the central nervous system (CNS) and thereby influence brain functions and behavior [164]. Indeed, four independent groups have shown that germ-free mice exhibited reduced anxiety-like behavior [165–167], as well as deficit in cognitive function like working memory [168]. Another study showed that antibiotic treatment administrated orally induces behavioral changes in mice while intraperitoneal (i.p.) injection did not influence behavior [169]. In these experimental studies, change in behavior was accompanied by alterations in brain chemistry.

This chapter highlights the potential mechanisms underlying the communication between the gut and the brain (figure 2-12). The experimental approaches used to elucidate these mechanisms mainly include the use of germ-free animals (animals microbiologically sterile and maintained in sterile environment and thus lacking intestinal microbiota), animals with pathogenic bacterial infections and animals exposed to antibiotics or to probiotics agents. Importantly, there is growing evidence that this communication is bidirectional: microbiota influences CNS function, and the CNS influences gut microbiota composition.

2.3.1.1 Involvement of the hypothalamus-pituitary-adrenal (HPA) axis

It has been a long time since we know that stress and the associated activation of HPA axis can influence the gut microbiota composition [170]. Indeed, animal studies have shown that early-life stress [171] as well as chronic stress in adult mice [172] induce changes in gut microbiota composition which was associated with increased circulating inflammatory cytokines. On the other hand, the microbial content of the gastrointestinal tract can influence

the development of the HPA axis, as shown in a study of Sudo *et al.* where exposure to stress induce an exaggerated activation of the HPA axis in germ-free mice, which was fully reversed by reconstitution with the probiotic *Bifidobacterium infantis* [173].

It is also important to note that chronic stress disrupts the intestinal barrier [174] and increased the circulating levels of bacterial cell wall components [175]. Moreover, the high co-morbidity between stress-related psychiatric diseases, such as anxiety and depression, and gastrointestinal disorders is further evidence of the importance of this gut-brain interaction in pathophysiology [176].

2.3.1.2 Involvement of the immune system

Gut microbiota and probiotics agents can have direct effects on the immune system [177] and are involved in the maintenance of gut homeostasis [51]. In addition, indirect effects of gut bacteria on the immune system can result in alterations of circulating levels of pro-inflammatory cytokines that affect the brain functions (see third part of the introduction). A study showed, in a rat model of maternal separation, mood disorders associated with enhanced peripheral IL-6. Behavioral and immune alterations were normalized by the administration of probiotic *B. infantis* [178].

2.3.1.3 Involvement of the vagal tone

The vagus nerve is the major nerve of the parasympathetic division of the autonomic nervous system and regulates several organ functions such as heart rate and gut motility. Approximately 80% of nerve fibers are sensory, conveying information about the state of the body's organs to the CNS [179]. Some studies [180–183] have shown that the vagus nerve is involved in the gut-brain communications while others have demonstrated vagus-independent mechanisms [169, 184]. For instance, ingestion of *Lactobacillus rhamnosus* has been shown to decrease anxiety and depression-like behavior in healthy mice which was associated with alterations of brain GABA receptor expression. The behavioral and neurochemical effects of this bacterium were not found in vagotomized mice [182]. Similarly, the ability of *B. longum* to attenuate anxiety induced by chemical colitis was abolished by vagotomy [183]. However, how the gut microorganisms activate vagal afferents are currently unclear.

2.3.1.4 Involvement of tryptophan metabolism

Tryptophan is an essential amino acid and the precursor of neurotransmitter serotonin. It can be converted into kynurenine by either indoleamine-2,3-dioxygenase (IDO) or hepatic tryptophan-2,3-dioxygenase (TDO). These enzymes are activated by inflammatory mediators and by corticosteroids. Kynurenine can then be converted into other metabolites which may have neuroactive properties (see chapter 3 of the introduction). A study has shown that the probiotic bacterium *B. infantis* can alter the tryptophan/kynurenine pathway which was associated with an antidepressant effect [185]. Interestingly, germ-free animal exhibit elevated plasma tryptophan levels and hippocampal serotonin concentrations compared with control animals [167].

2.3.1.5 Involvement of microbial metabolites

Several molecules with neuroactive functions have been reported to be microbially-derived and many of which have been isolated from bacteria within the human gut. For instance, *Lactobacillus* spp. and *Bifidobacterium* spp. produce the neurotransmitter GABA [186] through the same biosynthetic pathway as in neuronal tissue [187]. Serotonin and dopamine are produced by *Lactobacillus* spp., *Escherichia* spp., *Streptococcus* spp. [188] and metabolomics study showed that plasma serotonin level was strongly reduced in GF mice compared to conventional mice [189]. The levels of serotonin in the cortex and hippocampus were also significantly reduced in GF mice [165]. A recent study demonstrates that bacteria which constitute the normal microbiome in mice are able to produce large quantities of catecholamines [190]. These secreted neurotransmitters from bacteria may stimulate intestinal epithelial cells to release molecules that in turn modulate neural signaling to influence brain functions and behavior.

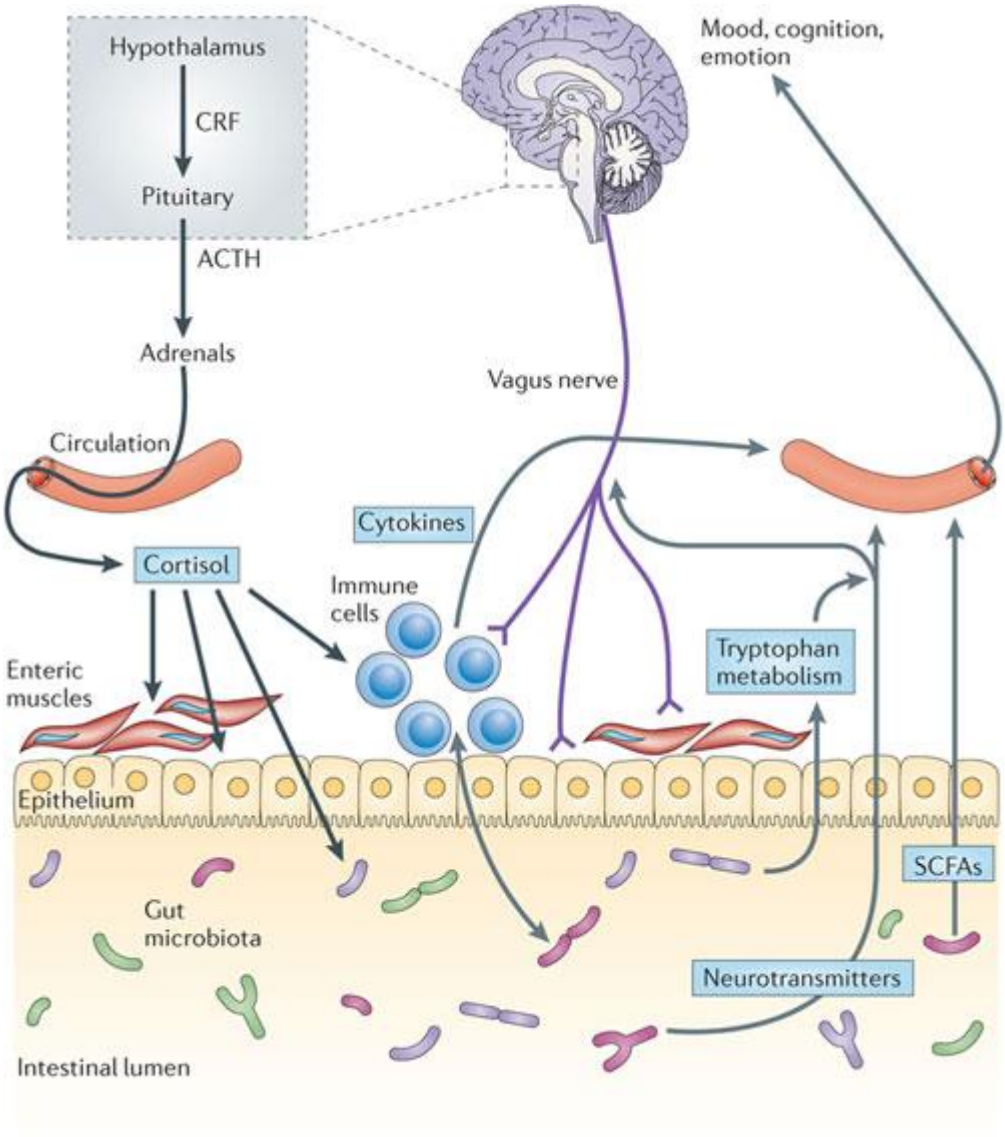

Figure 2-12: Pathways involved in the bidirectional communication between the gut microbiota and the brain, Cryan JF and Dinan TG, Nature Reviews, 2012 [164].

Bercik *et al*. recently published an interesting study which brought evidence that the gut microbiota could signal to the brain through multiple pathways [184]. The authors infected mice with a parasite to induce chronic gut inflammation and observed behavioral alterations consisting in increased anxiety-like behavior. These infected mice presented with decreased hippocampal level of brain-derived neurotrophic factor (BDNF) mRNA, increased plasma levels of kynurenine and pro-inflammatory cytokines TNFα and IFNγ. Several strategies were tested to prevent the behavioral alterations induced by the intestinal inflammation. First, they showed that vagotomy before infection did not prevent anxiety-like behavior, indicating that vagus nerve did not mediate the behavioral effects of the infection.

Secondly, treatment with anti-inflammatory agents normalized behavior of infected mice, reduced circulating cytokines and kynurenine levels, but did not influence BDNF expression. Thirdly, administration of probiotic *Bifidobacterium longum* also normalized behavior of infected mice, restored brain BDNF expression but did not affect cytokines and kynurenine levels. These results clearly showed that multiple routes are involved in the gut-brain communication.

2.3.2 Modulation of the gut microbiota improves the psychological symptoms in clinical populations

Individuals with chronic fatigue syndrome have altered gut microbiota composition and emotional disturbance like anxiety. There is now clinical evidence of a role of probiotic intervention in reducing anxiety and neurocognitive functions in these patients [191, 192]. A recent double-blind, placebo controlled, randomized study demonstrated that the oral intake of a probiotics formulation (*Lactobacillus helveticus* and *Bifidobacterium longum*) had beneficial psychological effects in healthy human volunteers [193]. Also, four-week intake of a fermented milk product containing mixture of probiotics by healthy women affected activity of brain regions that control central processing of emotion and sensation [194].

Gut microbial alterations are now well-established in somatic diseases such obesity, diabetes, and inflammatory bowel diseases. Recent clinical studies have shown variation of gut microbiota composition in psychiatric diseases such as autism [195, 196] and only one paper, published during the course of this PhD work, suggest that such changes occur upon alcohol-dependence [94].

3 ROLE OF THE INFLAMMATORY RESPONSE IN ALCOHOL DEPENDENCE

3.1 Sickness behavior theory

Anyone who has experienced a bacterial or viral infection knows what it means to feel sick. When they are sick, the behavior of people changes dramatically and consists in lassitude, fatigue, inability to concentrate, loss of appetite, irritability, altered sleep pattern and withdrawal from normal social activities [197]. These uncomfortable symptoms are frequently ignored by physician or considered rather banal. However, these symptoms contribute to a highly organized strategy of the organism to fight infection and facilitate the recovery following pathogen exposure. This strategy is called "sickness behavior" and consists in three components: fever, neuroendocrine changes (i.e., activation of the hypothalamic-pituitary-adrenal (HPA) axis) and behavioral effects of sickness described above [198]. The "sickness behavior" is triggered by pro-inflammatory cytokines produced by immune cells in contact with the invading pathogens. Indeed, infectious pathogens encounter dendritic cells, blood monocytes and tissue macrophages which express pattern-recognition receptors such as Toll-like receptors (TLRs). In humans, 10 TLRs have been identified that recognize mainly viral and bacterial structures [199, 200]. For instance, lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is recognized by TLR4 whereas peptidoglycan (PGN) originating mainly from Gram-positive bacteria is recognized by TLR2 [201, 202]. Upon TLR activation, a signal transduction cascade leads to transcription factor activation and, in the end, production of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 [203]. It has been shown that physiological concentrations of pro-inflammatory cytokines that occur after a peripheral infection act in the brain to induce the three components of sickness behavior (figure 3-1).

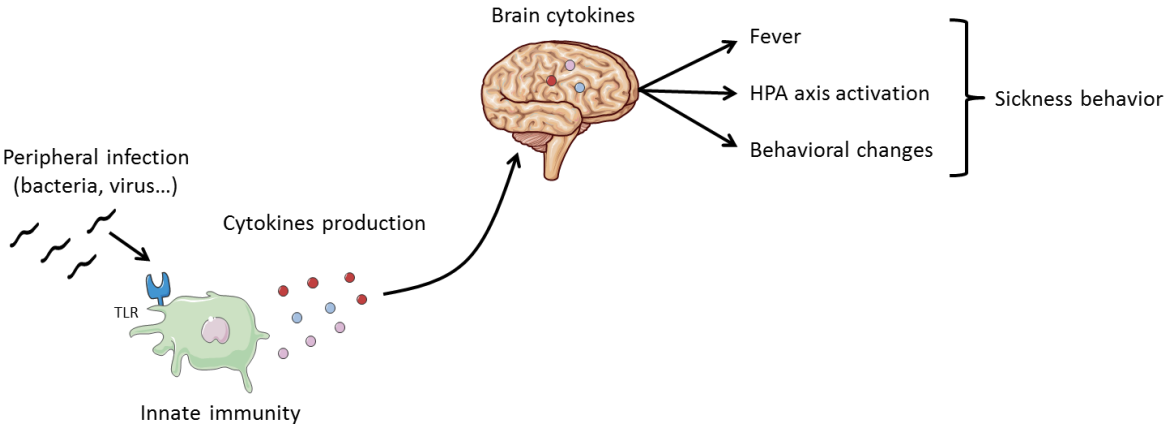

Figure 3-1: Cytokines-induced sickness behavior following peripheral infection.

This communication between the peripheral cytokines and the brain raised an important question: what happens when the sickness response is prolonged? This condition actually occurs during a variety of chronic inflammatory diseases. In the 90's, scientists suggested that there is an analogy between sickness behavior and depression and thereafter a role for cytokines in the development of depression was proposed [204–206] (figure 3-2).

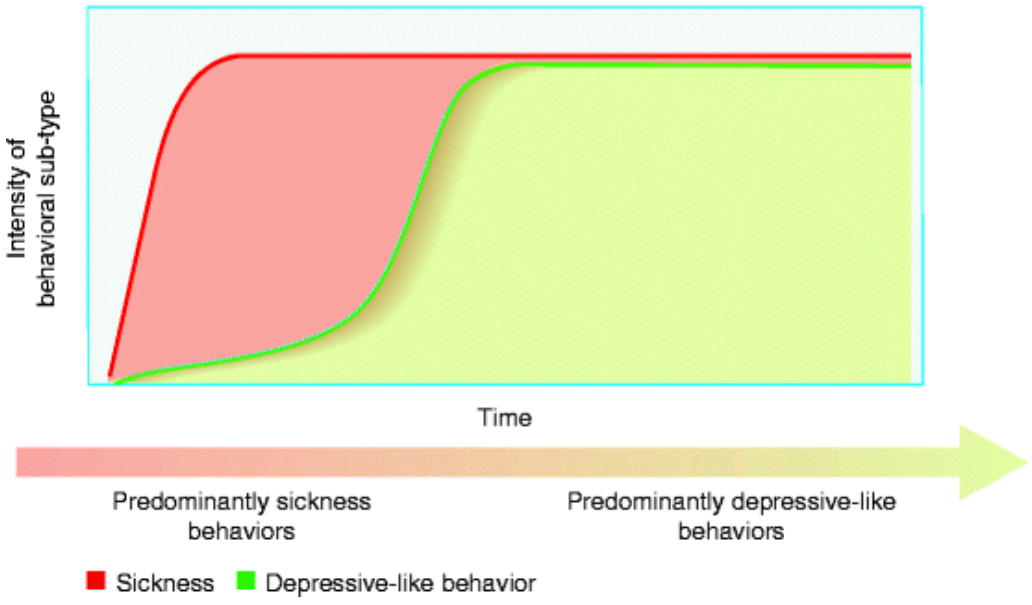

Figure 3-2: The relative onset, duration, and intensity of sickness behaviors and depressive-like behaviors following inflammation, Mood disorders and immunity, Walker A. *et al.*, 2012. [207]

This concept was supported by several clinical indirect evidences. For instance, it was reported that depressed individuals have increased circulating levels of pro-inflammatory

cytokines [208]. Furthermore, the prevalence of depressive disorders in patients afflicted with chronic inflammatory diseases is higher than normal. More direct clinical evidence comes from the potent neuropsychiatric side effects of immunotherapy. Indeed, 45% of cancer patients treated with IFN-α and IL-2 developed symptoms of depression [209–211]. However, this hypothesis of "cytokines-induced depression" illustrated by clinical observations failed to attract the interest of the psychiatrists' community because the direct demonstration of a mechanistic link between inflammation and depression was missing. Therefore, behavioral tests that mimic human depression as well as pharmacological and genetic approaches were developed in animal models that allowed to prove a causative role for cytokines in depression [212]. More recently, clinical models in which healthy humans were injected intravenously with endotoxins to induce an inflammatory response, were used to establish the causality [213]. For instance, injection of *Salmonella abortus* endotoxin induced an increase in serum TNFα, IL-6 and cortisol together with the development of a depressed mood, increased anxiety and a decrease in memory performance [214]. Similar results were obtained after vaccination of healthy volunteers with *Salmonella typhi* who reported negative changes in mood [215]. Taken together, animal and human studies have demonstrated that inducing a pro-inflammatory state greatly increases the incidence of mood symptoms.

Animal models have also enabled researchers to identify the mechanisms that mediate the behavioral effects of peripherally released cytokines on the brain. Several communication pathways were described: a fast neural route and slower humoral pathways (figure 3-3).

3.1.1 Neural transmission of the cytokine message

The vagus nerve innervates organs of the abdominal cavity. Its afferent nerves, which convey sensory information about the state of body's organs to the central nervous system, are the target of pro-inflammatory cytokines.

Peripheral administration of LPS induces the expression of cytokines in the brain [216]. The first demonstrations of the role of the vagus nerve in the transmission of information from the periphery to the brain have been published in the 90's. It was found that intraperitoneal (i.p.) injection of LPS caused a rapid increase in *c-fos* immunoreactivity within

the *nucleus tractus solitarius*. This marker of neuron activation localized to areas of projection of the vagus [217]. Other studies showed that subdiaphragmatic vagotomy abrogates the induction of synthesis of IL-1β in the brain in response to i.p. injection of LPS [218] and IL-1β [219] while leaving unaffected the pool of peripheral cytokines. Finally, vagotomy drastically reduced the sickness response to i.p. LPS in rats [220]. Also, it has been proposed that activation of vagal afferents by peripheral cytokines is then transduced back into immune message in the brain. Locally produced cytokines would then alter brain functions by acting directly or indirectly in neurons [221].

In contrast to the previous findings, vagotomy does not block the pyrogenic action of i.p. LPS [222] or IL-1β [223] and does not block the induction of sickness behavior when cytokines are administrated intravenously or subcutaneously [224]. The latter findings suggest that additional humoral pathways are also required to mediate the brain effects of peripheral infection.

3.1.2 Humoral transmission of the cytokine message

Since cytokines are relatively large hydrophilic molecules that are unable to cross the blood-brain barrier, it was obvious that their action on the brain could not be direct. Several pathways of transmission from the immune system to the brain have been identified and are often represented by the production of intermediates at the level of blood-brain interface in response to circulating cytokines or microorganism components.

For instance, the macrophage-like cells residing in the circumventricular organs and the choroid plexus express TLRs and can respond to pathogens by producing pro-inflammatory cytokines. As these brain structures are devoid of a functional blood-brain barrier, the cytokines can enter the brain by volume diffusion [225]. Another way of communication involves IL-1 receptors that are located on perivascular macrophages and on endothelial cells of brain venules [226, 227]. Activation of these IL-1 receptors by circulating cytokines results in the local production of prostaglandin E2 (PGE2), which are the main mediators of cytokines-induced fever [226] and HPA axis activation [228]. Finally, studies have also shown the existence of cytokine transporters at the blood-brain barrier. The pro-

inflammatory cytokines overflowing in the systemic circulation can gain access the brain though these saturable transport systems [229].

Engagement of these immune-to-brain communication pathways ultimately leads to the production of pro-inflammatory cytokines by microglial cells. In this way, the brain forms a “mirror” of the peripheral immune response.

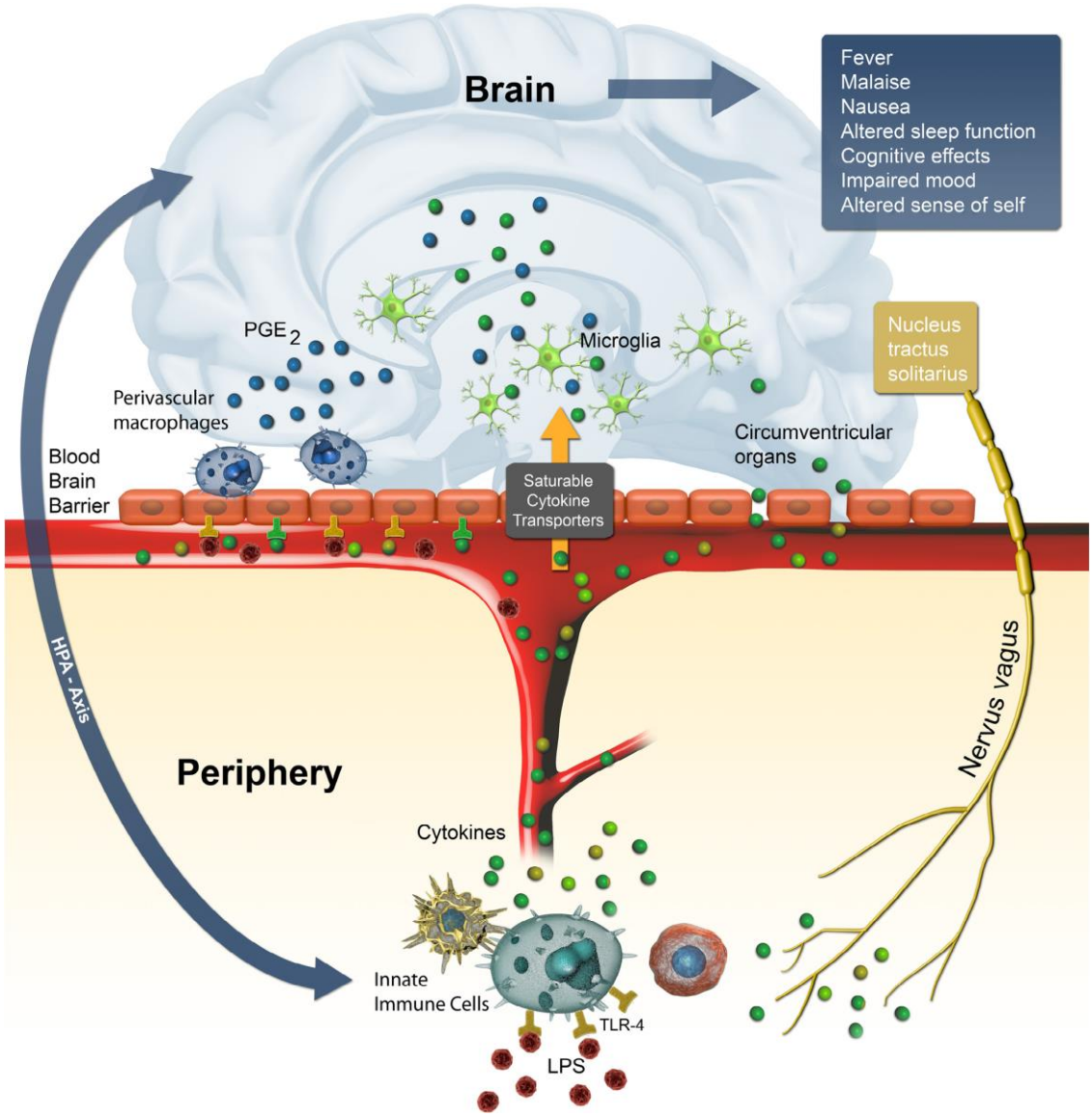

Figure 3-3: Afferent communication pathways from the periphery to the brain. Schedlowski M. *et al*., Brain, Behavior, and Immunity, 2014 [213].

3.2 Role of indoleamine-2,3-dioxygenase in depression

One key player of the action of cytokines in the brain is an enzyme known as indoleamine-2,3-dioxygenase (IDO) [230]. This enzyme is expressed in all tissues mostly in macrophages and microglia and is activated by pro-inflammatory cytokines, particularly IFN-γ and TNFα [231]. IDO degrades the essential amino acid tryptophan, which is usually actively transported into the brain for the synthesis of serotonin. Degradation of tryptophan by IDO generates kynurenine which is biologically inactive but can be further metabolized into biologically active metabolites (figure 3-4). For instance, astrocytes, neurons and brain endothelial cells metabolize kynurenine into kynurenic acid which antagonizes NMDA receptors and shows neuroprotective effects [232]. On the other hand, macrophages and microglial cells metabolize kynurenine into downstream neurotoxic metabolites such as quinolinic acid which easily crosses the blood-brain barrier, generates oxidative radicals and acts as an agonist of NMDA receptors [232, 233]. This results in alteration of glutamatergic neurotransmission that could trigger the necessary conditions for the development of depression.

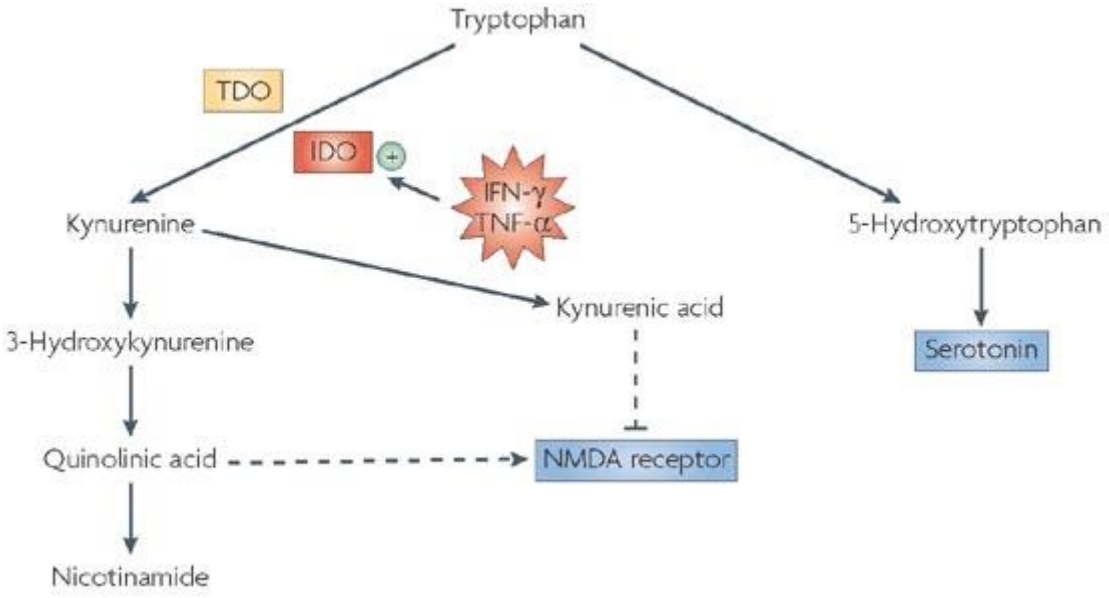

Figure 3-4: IDO degrades tryptophan through the kynurenine pathway, Dantzer R.*et al.*, Nature Reviews Neuroscience, 2008 [198].

In order to demonstrate the link between tryptophan metabolism and symptoms of depression, mice were treated with a selective competitive inhibitor of IDO prior to an intraperitoneal injection of bacille Calmette-Guérin (BCG) [234]. This treatment did not affect the expression of cytokines in the brain but reversed the rise in the kynurenine/tryptophan ratio in both blood and brain and finally prevented the BCG-induced depressive-like behavior. In this animal model, results indicate that BCG-induced pro-inflammatory cytokines caused the activation of IDO which subsequently caused depressive-like behavior by changing tryptophan metabolism. Similar results were obtained using genetic approach, with IDO knockout mice, which responded to bacterial infection by producing pro-inflammatory cytokines but did not develop depressive-like behavior [234]. In cancer patients treated with cytokines, a reduction in serum tryptophan, without a change in other neutral amino acids, was observed after 1 month of therapy. The lower the circulating levels of tryptophan, the higher the severity of depressive symptoms [235, 236].

Animal studies have shown that brain pro-inflammatory cytokines are the cause of sickness behavior. These cytokines induce the downstream expression of IDO that causes depressive-like behavior. Therefore, IDO is suggested to be a master regulator responsible for the switch from sickness to depressive-like behavior [237, 238].

Also, a recent study [239] has shown that the enzyme tryptophan 2,3-dioxygenase (TDO) previously known to be expressed in the liver is also present in the brain (mainly in neurons and astrocytes). TDO can be activated by cortisol and its substrate tryptophan. Wu et al. found high expression of TDO and quinolinic acid in the hippocampus of Alzheimer's disease patients. The authors suggested that elevated brain TDO expression and excessive formation of kynurenine-derived metabolites could be involved in the neurodegenerative processes and influence brain functions.

3.3 Chronic inflammation in alcohol dependence

During the past few years, a new player has emerged in the field of alcohol abuse. As discussed above, this player is the immune system represented by peripheral and central inflammation. A substantial body of data now indicates that chronic alcohol abuse causes an increase in systemic and brain cytokines [240–242]. As previously described, brain cytokines could be responsible for behavioral changes and for psychological and cognitive symptoms associated with alcohol abuse.

TLR4 seems to be required to induce neuroinflammation following alcohol consumption. Indeed, a specific mouse strain C3H/HeJ was identified to have a spontaneous mutation in the TLR4 gene making them endotoxins resistant as well as resistant to LPS-induced sickness behavior [243, 244]. Moreover, alcohol-induced increase in cortical cytokines did not occur in TLR4-deficient mice [245].

However, alcohol-dependent subjects are not permanently infected by environmental bacterial or viral components that could trigger the production of inflammation. Therefore, the inflammatory response is likely induced by microorganisms that inhabit naturally the human body. One of the aims of this thesis is to analyze the activation of inflammatory pathways in response to bacterial components originating from the gut.

AIMS AND GENERAL DESIGN OF THE WORK

Alcohol dependence has traditionally been considered a brain disease. Pharmacological treatments that target neurotransmitters involved in the brain reward circuit has been widely tested but has proven only very partial efficacy, if any. Heavy and chronic alcohol consumption is known to have toxic impact on the gastrointestinal tract and organs that participate to the digestive process (liver, pancreas) and alcoholics are at risk of malnutrition.

The aim of this PhD work is to analyze gut alterations in alcohol dependence and to investigate whether what happens on the gut is related to alterations in emotional, motivational and cognitive functions that have been related to the development of alcohol dependence. We also investigated which factors may underlie the potential gut-brain interactions. Finally, we tested the effect of a short-term alcohol withdrawal on gut functions and psychological dimensions.

To follow that general aim, several hypotheses were developed, step by step, during the course of the 3 main studies of the thesis. Each study raised new questions that we tried to answer in the following study (figure 1).

1. In vitro and animal studies, in the field of hepatology, have shown that alcohol exposure induced an increase in intestinal permeability as well as bacterial toxins translocation that may play a key role in the development of hepatic inflammation and damage leading to alcoholic liver disease. In the first chapter of this thesis, we developed a method to assess the small bowel and the colonic permeability of alcohol-dependent subjects versus matched healthy controls. We tested whether LPS translocation occurred and if it was related to the systemic inflammatory response. We also investigated whether inflammatory markers were correlated with the psychological and cognitive symptoms of alcohol dependence.

   The results of the first chapter revealed that circulating cytokines were related to depression and anxiety symptoms as well as craving for alcohol. However, the source of inflammation in alcohol dependence remained unclear.

2. The second chapter of this thesis focused on peripheral blood mononuclear cells (PBMCs) and their potential contribution to the systemic inflammatory response. We tested whether bacterial toxins LPS and peptidoglycans, which originated from the gut and entered the bloodstream due to leaky gut, activated PBMCs and which intracellular pathways were

involved. Furthermore, we investigated whether the activation of inflammatory pathways were correlated with alcohol consumption and craving.

3. Since leaky gut and gut-derived bacterial products seem to play an important role in the release of circulating pro-inflammatory cytokines that may influence emotions and alcohol craving, we analyzed, in the third chapter, the effect of alcohol dependence on the gut microbiota which has previously been described as a key factor in the regulation of gut barrier function and immune response. On the one hand, we performed a deep analysis of the gut microbiota composition by using two complementary approaches: the pyrosequencing of the 16S rRNA gene and the quantitative PCR. On the other hand, we performed metabolomics analysis in order to assess the functionality of the gut microbiota. Finally, we looked for the possible associations between gut microbiota, gut permeability and psychological symptoms of alcohol dependence.

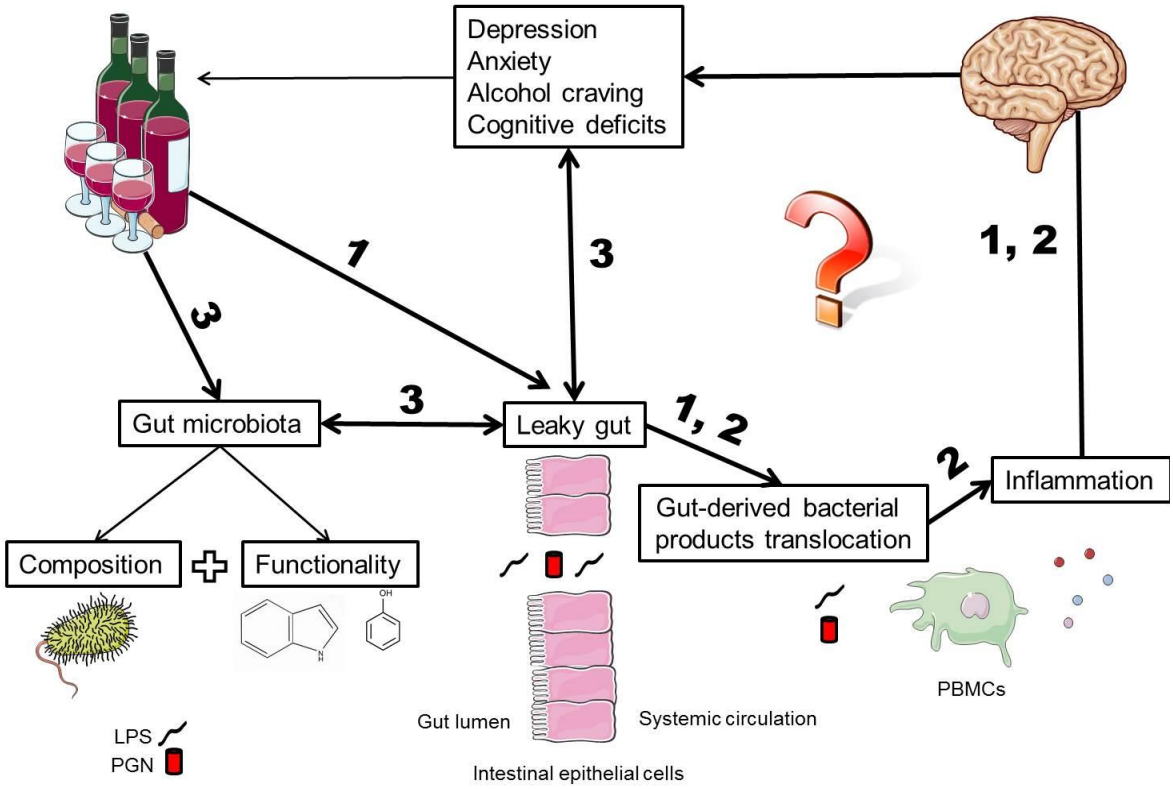

Figure 1: PhD work hypothesis. The number indicated on the line refers to the chapter in which the hypothesis was tested.

Although raising different questions, the three studies reported in the thesis were conducted according to a similar design which is directly dependent on the alcohol-withdrawal procedure of the patients during their stay in the medico-psychological unit dedicated to the treatment of alcohol dependence in our academic hospital. The patients included in our study were diagnosed as alcohol-dependent according the DSM-IV criteria and were hospitalized for a detoxification program at the Unité Intégrée d'Hépatologie, Cliniques Universitaires Saint-Luc, Brussels. This unit was created in a collaborative project of the department of Gastroenterology and Psychiatry to allow treatment of alcohol dependence and of the medical consequences of chronic alcohol consumption, and to provide psychiatric and psychological support to alcohol-dependent subjects in order to facilitate the possibility of developing a prolonged abstinence. An important aspect of the rehabilitation process is the alcohol withdrawal. The alcohol withdrawal program consists in 2 weeks at the hospital separated by one week where the patients go back home (figure 2).

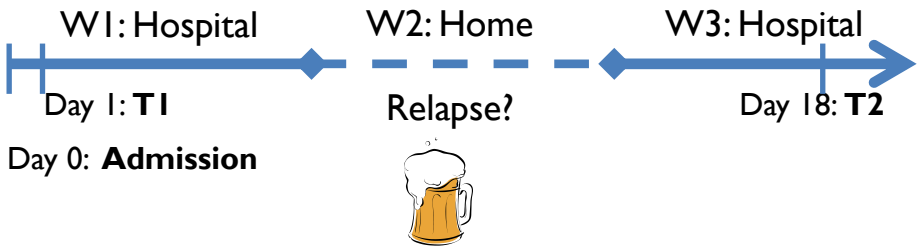

Figure 2: Time line of the 3-week detoxification program.

When the alcohol detoxification unit was created, in 2003, it was organized in a very well standardized manner, in a way to facilitate patients' treatment but also the development of research activities. All patients arrived on Monday morning at the hospital (Day 0). They received vitamins B and most of them received benzodiazepines for which the dose is adapted according withdrawal symptoms. We selected, for the studies, patients that had consumed alcohol until their admission in the unit or the day before (on Sunday, or the night between Sunday and Monday). They were informed about the study and signed an informed consent form if they agreed to participate on Monday noon. Neuropsychological tests were performed Monday afternoon before they received the first dose of valium, if possible. All biological tests started Tuesday morning (Day 1), after an overnight fast. The patients were tested twice, at the beginning of the detoxification program (T1) as explained above, and at the end of the

third week of the detoxification program, after 18 days of abstinence (T2). The patients who resumed alcohol consumption during the second week (the week at home) were excluded from the study at T2.

Patients were compared to 16 healthy subjects who consumed socially low amount of alcohol (<20g/day). The healthy controls were matched for age, sex and BMI.

The patients presenting the following characteristics were excluded from the study:

- diabetes, BMI > 30 kg/m2 and metabolic disorders
- inflammatory bowel diseases : Crohn's disease, ulcerative colitis
- bypass gastric
- cirrhosis and liver fibroscan > F1
- viral hepatitis
- auto-immune diseases such as rheumatoid arthritis or lupus erythematosus
- other dependence excepted tobacco dependence

The patients who had used the following drugs within 2 months preceding the study were also excluded:

- anti-inflammatory drugs
- antibiotics
- probiotics

RESULTS AND DISCUSSION

1 CHAPTER 1

ROLE OF INTESTINAL PERMEABILITY AND INFLAMMATION IN THE BIOLOGICAL AND BEHAVIORAL CONTROL OF ALCOHOL-DEPENDENT SUBJECTS

Increased intestinal permeability and elevated blood lipopolysaccharides (LPS) have been described in animal models of chronic alcohol consumption and in patients that have already developed an alcoholic liver disease (ALD). Bacterial LPS are potent pro-inflammatory agents and have been shown to be involved in the development of hepatic inflammation and liver damage. The first aim of this study was to measure intestinal permeability, blood LPS levels and systemic inflammation in alcohol-dependent patients without ALD, to test the effect of alcohol-dependence itself and avoid bias linked to hepatic injury. In parallel to the biological measurements, we assessed the negative affects (depression, anxiety), alcohol craving and cognitive function (selective attention). The second aim of this study was to evaluate the effect of a short-term alcohol withdrawal on these biological and psychological parameters. Finally, in order to collect the first elements of a possible interaction between the gut and the brain in AD subjects, we tested correlations between the biological parameters and the behavioral ones.

The results of this first chapter have been published in *Brain, Behavior and Immunity 26 (2012), 911-918.*

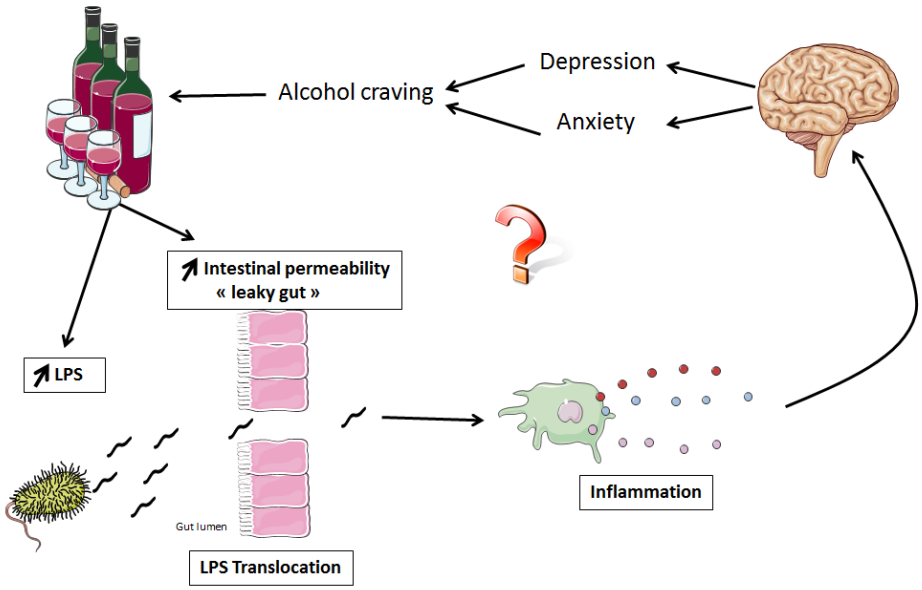

Figure 1-1: Hypothesis scheme of Chapter 1. The aims of the study were to assess intestinal permeability, LPS translocation and systemic inflammation in alcohol-dependent subjects. We also tested whether inflammation was related to psychological symptoms such as depression, anxiety and alcohol craving.

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1.1 Abstract

Background and aims: Mood and cognition alterations play a role in the motivation for alcohol-drinking. Lipopolysaccharides (LPS) are known to stimulate inflammation that was shown to induce mood and cognitive changes in rodents and humans. Enhanced intestinal permeability and elevated blood LPS characterize alcohol-dependent mice. However, no data have been published in non-cirrhotic humans. Our first goal was to test whether intestinal permeability, blood LPS and cytokines are increased in non-cirrhotic alcohol-dependent subjects before withdrawal and if they recover after withdrawal. Our second goal was to test correlations between these biochemical and the behavioral variables to explore the possibility of a role for a gut-brain interaction in the development of alcohol dependence.

Methods: 40 alcohol-dependent (AD) subjects hospitalized for a 3-week detoxification program were tested at onset (T1) and end (T2) of withdrawal and compared for biological and behavioral markers with 16 healthy subjects. Participants were assessed for gut permeability, systemic inflammation (LPS, TNFα, IL-6, IL-10, hsCRP) and for depression, anxiety, alcohol craving and selective attention.

Results: Intestinal permeability and LPS were largely increased in alcohol-dependent subjects at T1 but recovered completely at T2. A low-grade inflammation was observed at T1 that partially decreased during withdrawal. At T1, pro-inflammatory cytokines were positively correlated with craving. At T2 however, the anti-inflammatory cytokine IL-10 was negatively correlated with depression, anxiety and craving.

Conclusion: Leaky gut and inflammation were observed in non-cirrhotic alcohol-dependent subjects and inflammation was correlated to depression and alcohol craving. This suggests that the gut-brain axis may play a role in the pathogenesis of alcohol dependence.

1.2 Introduction

Alcohol-dependence is a disorder that is present in 5 to 7% of the population of developed countries (Anderson and Baumberg, 2006; Grant et al., 2004). In addition to its familial, social, and professional consequences, it is also a major risk factor for several pathologies including liver or pancreatic diseases, cardiac diseases, cancers, and neurological or psychiatric disorders. This addiction is also the first cause of malnutrition in developed countries. Indeed, both human and animal studies have shown that heavy chronic alcohol consumption leads to mucosal damages that will interfere with the absorption of micro and macronutrients and with mucosal enzyme activities contributing to malnutrition (Bode and Bode, 2003). Alcohol-fed animals are characterized by an intestinal hyperpermeability and possibly a Gram-negative bacterial overgrowth in the upper small intestine that contribute to increased leakage of lipopolysaccharides (LPS) into the circulation (Adachi et al., 1995; Yan et al., 2010). LPS are potent pro-inflammatory agents that through a CD14-TLR4 receptor mechanism (Akira and Hemmi, 2003) activate the transcriptional factor NFκB to induce the production of inflammatory mediators such as pro-inflammatory cytokines, chemokines, nitric oxide and reactive oxygen species (Wheeler, 2003). This LPS-activated inflammatory process has been thought to play an important role in the development of alcoholic liver disease (Wheeler, 2003). These mechanisms have been mainly studied in rodent models of alcohol-dependence (Adachi et al., 1995; Mathurin et al., 2000; Rao et al., 2004; Wheeler, 2003; Yan et al., 2010) but data on whether this also occurs in alcohol-dependent humans are scarce.

Among the human studies, increased circulating LPS levels have been reported in subjects that have already developed liver damage and cirrhosis (Fukui et al., 1991; Parlesak et al., 2000) where the increased LPS levels have been also be attributed to dysfunctional Kupffer cells with reduced ability to detoxify endotoxins (Rao et al., 2004). Therefore, the rise in plasma LPS and subsequent inflammation in these patients can also be the consequence of the liver disease. The analysis of the effects of alcohol consumption in non-cirrhotic alcoholics would allow us to test the hypothesis that changes in intestinal permeability, LPS and low-grade inflammation may not only be a consequence but also play a role in continuing alcohol-dependence.

The influence of circulating cytokines on the human brain functions and the development of depression is supported by several studies (Dantzer and Kelley, 2007; Dantzer et al., 2008; Maes, 1999). Furthermore, an infection (Dantzer et al., 2008; Konsman et al.,

2002) or the injection of LPS (Reichenberg et al., 2001) will induce several symptoms (fever, fatigue, anorexia, sleep abnormalities, loss of interest in social activities) and cognitive disturbances (e.g. memory or attention dysfunctions) that are close to symptoms of depression. These symptoms are induced by the effect of inflammation on the brain through both humoral and autonomic pathways (Konsman et al., 2002). These observations support the idea that inflammation induces a Sickness Behavior, which may play a role in the pathophysiology of some psychiatric diseases. Consistent with this idea, depressed subjects have higher plasma pro-inflammatory cytokines concentrations than controls (Dowlati et al., 2010; Maes, 1999). On the other hand, the anti-inflammatory cytokine IL-10 seems to prevent LPS-mediated mood and cognition disturbances (Bluthe et al., 1999; Richwine et al., 2009; Van den Boogaard et al., 2010). In addition, depression plays an important role in the behavioural mechanism of addiction (Schuckit, 1994) and has been shown to be strongly and consistently correlated to alcohol craving (Andersohn and Kiefer, 2004; de Timary et al., 2013) which could be defined as the appetitive urge to drink alcohol (Anton, 1999). Also, recent psychological studies indicated that negative affects (depression, anxiety) and craving largely improve during the alcohol-withdrawal (Cordovil De Sousa Uva et al., 2010; de Timary et al., 2008). Selective attention also improved during the detoxification programme whereas other cognitive functions such as executive functions (inhibition, flexibility, decision making) remained unchanged (Cordovil De Sousa Uva et al., 2010).

Our first goal was to show that heavy chronic alcohol consumption induced an increase in intestinal permeability. Then, we tested whether this hyperpermeability was associated with a rise in plasma LPS levels (Cani et al., 2007; Cani et al., 2008) and systemic inflammatory response. Finally, we hypothesized that these biological changes would in turn influence the severity of depressive symptoms, the intensity of craving and consequently alcohol consumption. To test these hypotheses, alcohol-dependent subjects were carefully selected upon diagnosis as non-cirrhotic. They were assessed for intestinal permeability, plasma LPS levels, plasma pro- and anti-inflammatory cytokines levels, high-sensitivity C-reactive protein (hsCRP) levels and for behavioural measures both at the onset and end of alcohol-withdrawal. This alcohol-dependent group was compared to a control group consisting of individuals matched for age and body mass index (BMI) and who socially consume low amounts of alcohol.

1.3 Material and methods

Participants and procedure

The study protocol was approved by the ethical committee of the hospital and all subjects signed an informed consent form prior to the investigation (B40320096274, Commission d'éthique biomédicale hospitalo-facultaire de l'UCL). A total of 52 subjects with diagnosis of alcohol dependence according to the DSM-IV criteria (APA., 1994) were clinically evaluated by a psychiatrist (PdT) and admitted to the gastroenterology ward for a 3-week detoxification and rehabilitation programme. This programme consists of 2 weeks of hospitalization separated by 1 week where the patients return back home. All patients had kept on drinking until the day of admission to the detoxification ward or the day before. Patients were tested twice, on the day of or the day following their admission (T1) and on day 18-19 (T2) corresponding to the last days of the detoxification program. Among the 52 patients who were included, only 40 participated at both times of the testing. The subjects who abandoned their treatment or who relapsed during the week at home were excluded. Also excluded prior to the study entry were patients who suffered from diabetes and Crohn's disease or other chronic inflammatory diseases (such as rheumatoid arthritis), those who presented gastric bypass or other bariatric surgery, those who took antibiotics, probiotics or glucocorticoids and nonsteroidal anti-inflammatory drugs during the two months preceding enrollment, and subjects who presented with a diagnosis of cirrhosis. Transient liver elastrography (Fibroscan®) was applied to all patients in order to quantify liver stiffness which correlates with fibrosis grades, according to the METAVIR classification system of fibrosis (Bedossa and Poynard, 1996). Based on this evaluation, only patients without significant fibrosis (F0 and F1 scores) were included in the study (see supplementary material section 1). Alcohol-dependent patients were compared to 16 age- and BMI-matched controls who consume low amounts of alcohol (< 20g/day) (Table 1).

Table 1: Characteristics of the alcohol-dependent and the control groups

Variable	AD-T1	AD-T2	Controls
Number of participants	52	40	16
Age	47 ± 11[a]	49 ± 11[a]	50 ± 11[a]
Gender, *n* (%)			
Male	42 (81%)	32 (80%)	9 (56%)
Female	10 (19%)	8 (20%)	7 (44%)
BMI (kg/m^2)	24.7 ± 4.1[a]	25.3 ± 3.9[a]	27.4 ± 4.1[a]
Alcohol intake (g/day)	195 ± 79[a]	0	10 ± 7[a]

a data are means ± SD. AD - T1 and AD - T2 refer to alcohol-dependent subjects tested at onset and end of the detoxification program respectively

Measurement of intestinal permeability with ^{51}Cr-EDTA

We measured intestinal paracellular permeability with ^{51}Cr-EDTA. This non-toxic probe was neither degraded by gut flora nor metabolized by the body and not naturally present in the urine (Bjarnason et al., 1995; Peeters et al., 1994). After an overnight fast and emptying of the bladder, patients drank a Nutridrink® (200ml, 150kcal/100ml) (Nutricia, Brussels, Belgium) containing 50µCi (1.85 MBq) ^{51}Cr-EDTA. Urine was collected for 24 hours during two periods that were expected to reflect small (0-4h) and large (4-24h) bowel permeability, respectively. Urine creatinine was measured to ensure completeness of the collections and subjects with very low creatinine clearance rates (< 8mg/kg/24h) were excluded (De Ms et al., 1993). Complete collections at T1 and T2 were obtained in 26 alcohol-dependent subjects and 16 controls. The radioactivity was measured with a gamma counter (Cobra5003 Canberra Packard, Downers Grove, IL, USA). Results were expressed as percentages of the ingested dose normalized for creatinine.

Biochemical analyses

The LPS concentration was measured with the Limulus amebocyte lysate kinetic chromogenic methodology that measures the colour intensity directly related to the endotoxins concentration in a sample, using Endosafe-MCS (Charles River laboratories, Lyon, France). Plasma were diluted 1/10 with endotoxin-free buffer to minimize interferences in the reaction (inhibition or enhancement) and heated for 15 minutes at 70°C. Each sample was diluted 1/70 with endotoxin-free Limulus amebocyte lysate reagent water (Charles River

Laboratories, Wilmington, MA) and treated in duplicate, and 2 spikes per sample were included in the determination. All samples have been validated for recovery and coefficient of variation determination. The lower limit of detection was 0.01 EU/mL (Everard et al., 2011; Luoto et al., 2011).

Plasma cytokines (interleukin (IL)-6, IL-10 and tumor necrosis factor (TNF) α) were determined in duplicate by multiplex immunoassay (Millipore, Belgium) and measured using Luminex®xMap® technology (Biorad, Nazareth, Belgium) following the manufacturer's instructions. Plasma hsCRP was measured by an automated turbidimetry method (DxC 800, Beckman Coulter).

Psychological tests

All patients were tested for depression, anxiety, craving for alcohol and selective attention at T1 and T2 (see supplementary material section 2).

The Beck Depression Inventory (BDI) is a 21-item self-report inventory designed to measure the severity of depressive symptoms, with a maximum score of 63 (Beck et al.,1996). The validated French translation of the second version of the BDI (BDI-II) was used in this study (Bourque and Beaudette, 1982).

The state report of the State-Trait Anxiety Inventory (STAI Form YA) is a valid and reliable 20-item self-report inventory for measuring the state of anxiety (Spielberger et al., 1983). The scores range from 20 to 80 where higher scores indicate greater anxiety. A valid French version was administered (Bruchon-Schweitzer and Paulhan, 1993).

The Obsessive-Compulsive Drinking Scale (OCDS) is a questionnaire that assesses the cognitive aspects of alcohol craving during the preceding 7 days.(Anton et al., 1995) This 14-question questionnaire provides a global craving score, as well as two sub-scores: an obsessive score (6 items) and a compulsive score (8 items). A valid French version was used in this study (Ansseau et al., 2000).

Selective attention was evaluated with the validated double binary computerized task from the "Batterie d'Attention de William Lennox" (BAWL) in its version 4.0 (Leclercq, 2007). Briefly, the subject was asked to react when a specific target appeared on the screen by pressing as quickly as possible the response button on the computer. The reaction time needed to press the button is calculated for each of the 40 targets.

Statistical analyses

For each variable, normal distribution was tested with the Kolmogoroff-Smirnov test. As all biological measures exhibited non-normal distributions, a log-transformation was applied to obtain normality and statistical analyses were performed on log-transformed data. The data presented in the graphs are, however, non-transformed means ± standard error of the mean (SEM). Biological and psychological measures were analyzed using t-tests. Paired t-tests were performed to compare alcohol-dependent (AD) subjects at T1 and T2. Unpaired t-tests were performed to compare AD to controls (CT). Concerning selective attention, to detect a practice effect as a result of test-retesting, the control group was tested twice and data were analyzed with repeated-measures analysis of variance (ANOVA), with time as a within factor (T1 vs. T2) and group as a between factor (AD vs. CT). Correlations were calculated using Pearson's moment correlations. Statistical analyses were performed using SPSS version 18.0 and graphs with GraphPad Prism version 4.0. Statistical significance was defined for a p-value lower than 0.05.

1.4 Results

Increased intestinal permeability and LPS in alcohol-dependent subjects and recovery after withdrawal

Intestinal permeability was measured by calculating the quantity of ^{51}Cr-EDTA found in urines. At T1, the small bowel permeability was significantly higher in alcohol-dependent subjects than in controls, $t(40) = 2.44$, $p < .05$. It decreased significantly from T1 to T2, $t(25) = 5.63$, $p < .001$, and this decrease was observed in 92.5% of patients. At T2, it did not differ anymore from controls, $t(39) = 1.01$, $p = .32$. At T1, alcohol-dependent subjects had higher colon and total intestinal permeability than those of controls but the difference between groups did not reach significance. However, both colon and total intestinal permeability decreased significantly during withdrawal, $t(25) = 2.22$, $p < .05$ and $t(25) = 3.66$, $p < .01$ (fig 1A). These observations suggest that the effects of alcohol on gut permeability are more prominent at the level of the small bowel than the colon. Patients at T1 also exhibited higher plasma LPS concentrations than controls, $t(37) = 2.21$, $p < .05$. It significantly decreased during withdrawal, $t(23) = 2.27$, $p < .05$, and at T2, LPS levels did not differ from controls (fig 1B).

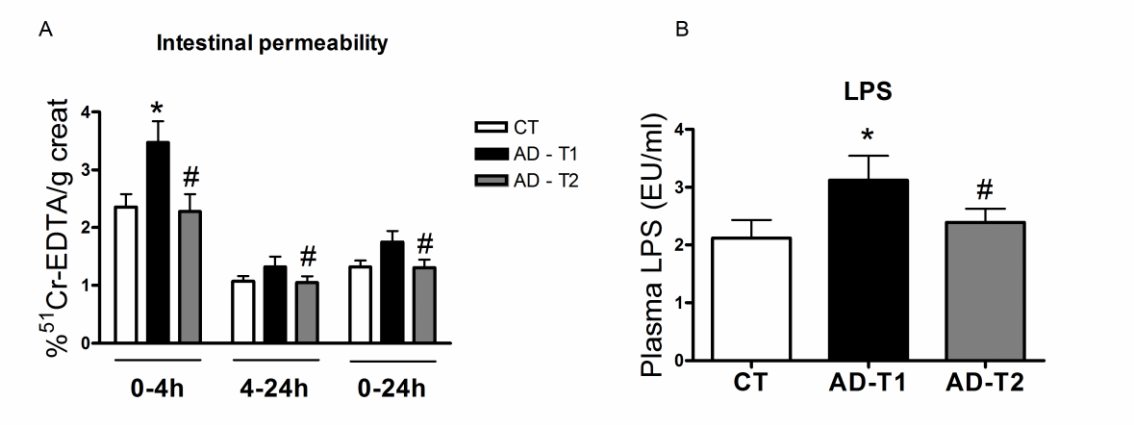

Figure 1: **A 3-week detoxification program induced a total recovery of intestinal permeability and blood LPS**. (A) Intestinal permeability was assessed by measurement of ^{51}Cr-EDTA excreted in urine (n =26). The first 4-h urinary collection reflects the small bowel permeability and the second 20-h collection large bowel permeability. Total intestinal permeability was also calculated. (B) Plasma LPS concentrations measured in AD subjects (n = 27) and in CT. Results are means ± SEM. Creat: creatinine; EU: endotoxin units; LPS: lipopolysaccharide; CT: control group; AD-T1 and AD-T2 refer respectively to the alcohol-dependent group at the onset and at the end of withdrawal.* $p < 0.05$ as determined by a two-tailed Student's unpaired t-test (AD vs CT).# $p < 0.05$ as determined by a two-tailed Student's paired t-test (AD-T1 vs AD-T2).

Induction of plasma cytokines in alcohol-dependent subjects and partial decrease after short-term withdrawal

The levels of pro-inflammatory cytokines TNFα and IL-6 were significantly higher in alcohol-dependent subjects than in controls at T1, $t(44) = 3.77$, $p < .001$ and $t(31) = 2.185$, $p < .05$, and at T2, $t(44) = 3.50$, $p < .01$ and $t(28) = 2.20$, $p < .05$ (fig 2A, B). The mean plasma cytokine concentrations decreased during withdrawal, although not significantly. The levels of plasma IL-6 decreased in only half of the patients. The TNFα concentrations decreased in 66% of patients but increased in 33% of them. HsCRP, which is one of the acute phase proteins that increases during systemic inflammation, was significantly higher in alcohol-dependent subjects than in controls at both T1, $t(47) = 2.45$, $p < .05$, and T2, $t(48) = 3.12$, $p < .01$ (fig 2D). There was no significant decrease of hsCRP levels from T1 to T2. The anti-inflammatory cytokine IL-10 was two-fold higher in alcoholics at T1 than in controls but this difference did not reach significance (fig 2C). Surprisingly, plasma IL-10 decreased significantly during withdrawal, $t(26) = 4.20$, $p < .001$, and this decrease was observed in 85% of the patients. At the end of withdrawal, IL-10 returned to the same level as controls. These results permit us to conclude that alcohol-dependent subjects indeed present with a low-grade inflammation, which is defined by a 2- to 3-fold increase in inflammatory cytokines and acute phase proteins (Petersen and Pedersen, 2005; Ross, 1999).

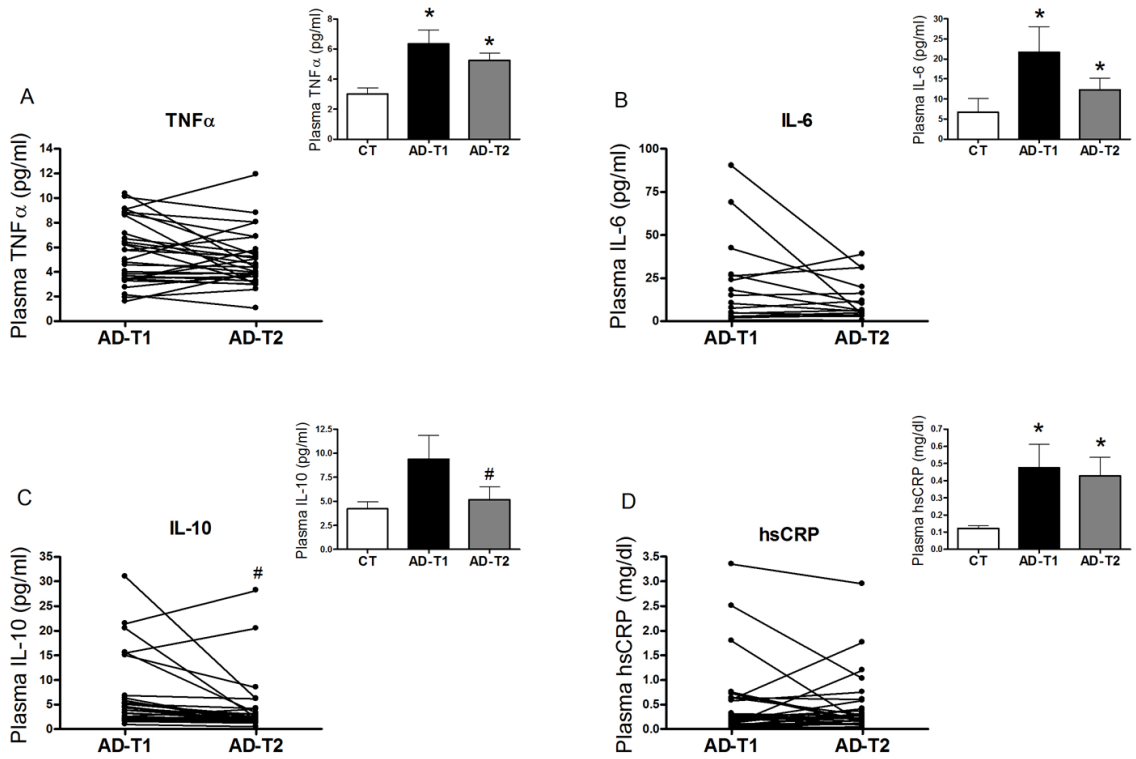

Figure 2: Change in inflammatory state during alcohol-withdrawal. Plasma (A,B) pro-inflammatory and (C) anti-inflammatory cytokines levels and (D) plasma hsCRP in AD and in CT group. Results are means ± SEM but corresponding p-values are derived from log-transformed data. TNFα: tumor necrosis factor α (n = 30); IL-6: interleukin-6 (n = 16); IL-10: interleukin-10 (n = 26); hsCRP: high-sensitive C-reactive protein (n = 31); CT: control group; AD-T1 and AD-T2 refer respectively to the alcohol-dependent group at the onset and at the end of withdrawal. * $p < 0.05$ as determined by a two-tailed Student's unpaired t-test (AD vs CT). # $p < 0.05$ as determined by a two-tailed Student's paired t-test (AD-T1 vs AD-T2).

Recovery of psychological markers during alcohol-withdrawal

Depression and anxiety scores were significantly higher in alcohol-dependent subjects at T1 than in controls, $t(51) = 9.95$, $p < .001$ and $t(45) = 6.74$, $p < .001$, and decreased significantly from T1 to T2, $t(39) = 10.11$, $p < .001$ and $t(39) = 6.83$, $p < .001$. However, at the end of withdrawal, both scores remained significantly higher than those of controls, $t(50) = 4.51$, $p < .001$ and $t(42) = 3.18$, $p < .01$ (fig 3A, B). For craving, the total OCDS score as well as the obsession and compulsion subscores were significantly higher at T1 in alcohol-dependent subjects than in controls, $t(53) = 22.61$, $p < .001$ and $t(45) = 20.94$, $p < .001$ and $t(51) = 19.86$, $p < .001$, and all three decreased significantly during withdrawal, $t(39) = 15.63$, $p < .001$ and $t(39) = 14.33$, $p < .001$ and $t(39) = 12.93$, $p < .001$, while remaining

significantly higher than those of controls at the end of withdrawal, t (50) = 5.51, p < .001 and t (45) = 6.04, p < .001 and t (53) = 3.88, p < .001 (fig 3D-F). Selective attention was evaluated using reaction times from the double binary task of BAWL. The results of the repeated-measures ANOVA show a main effect of group, F (1, 54) = 19.98, p < 0.001, η2 = 0.27, meaning that the performances of alcohol-dependent subjects were lower than in controls. As expected, there was also a main effect of time, F (1, 54) = 17.13, p < 0.001, η2 = 0.24, indicating that subjects had a quicker reaction time at T2 than at T1 (practice effect) (fig 3C). Most importantly, there was a significant Time × Group interaction, F (1, 54) = 6.93, p = 0.011, η2 = 0.11, showing that reaction times in alcohol-dependent subjects improved more than those of controls from T1 to T2.

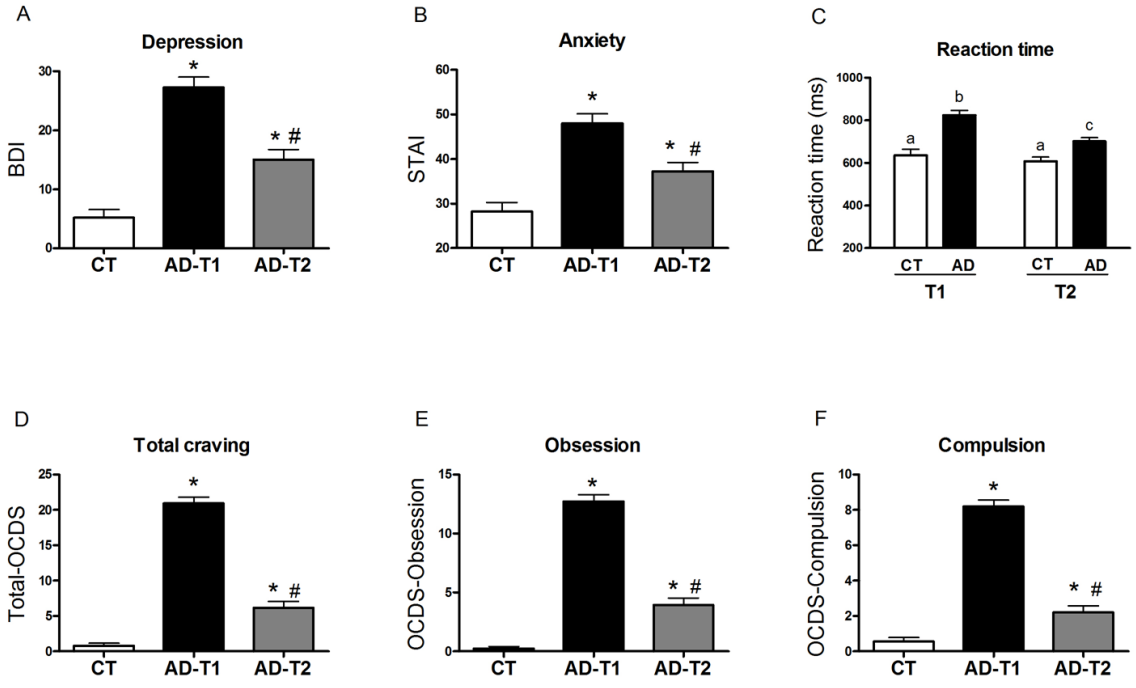

Figure 3: Decrease in emotional, motivational and cognitive disturbances during alcohol-withdrawal (n = 40). (A, B) Comparison of levels of depression and anxiety in AD subjects and in controls. (C) Selective attention, measured by reaction times, was evaluated twice on each participant to detect a possible practice effect. Analyses were performed using repeated-measures analysis of variance (ANOVA), with time as a within factor (T1 vs. T2) and group as a between factor (AD vs. CT). (D,E,F) Comparison of total craving, obsessive and compulsive components of craving in AD subjects and in CT groups. Results are means ± SEM. BDI: Beck depression inventory; STAI: state-trait anxiety inventory; OCDS: obsessive-compulsive drinking scale; ms: milliseconds; AD: alcohol-dependent group; CT: control group; AD-T1 and AD-T2 refer respectively to the alcohol-dependent group at the onset and at the end of withdrawal.* p < 0.01 as determined by a two-tailed Student's unpaired t-test (AD vs CT).# p < 0.001 as determined by a two-tailed Student's paired t-test (AD-T1 vs

AD-T2). Data with different superscript letters were significantly different according to t-tests following 2 × 2 MANOVA statistical analysis.

Correlations between biological and psychological factors

Cytokines have in the past been suggested to play a role in the development of psychiatric diseases and mostly in major depression (Dantzer et al., 2007; Dantzer et al., 2008; Konsman et al., 2002; Maes, 1999). Here, we decided to test the possibility that intestinal permeability and inflammation that were observed to be abnormal in alcohol-dependent subjects could be related to some behavioural symptoms that are specific to alcohol-dependence, particularly depression, anxiety, selective attention and alcohol craving.

We first examined correlations in patients at T1. We found that IL-6 was positively correlated with depression ($r = 0.44$, $p < .05$). Moreover, all cytokines measured in this study were positively and significantly correlated with alcohol craving (TNFα: $r = 0.44$, $p < .05$; IL-6: $r = 0.52$, $p < .05$; IL-10: $r = 0.52$, $p < .01$, hsCRP: $r = 0.46$, $p < .01$).We also observed that selective attention was significantly correlated with small bowel permeability ($r = 0.38$, $p < .05$) (fig 4).

After three weeks of abstinence, it was the anti-inflammatory cytokine IL-10 that was this time negatively correlated with all psychological factors: depression ($r = -0.45$, $p < .05$), anxiety ($r = -0.44$, $p < .05$), craving ($r = -0.48$, $p < .05$) and selective attention ($r = -0.39$, $p = .05$). This suggests a potent psychological role for anti-inflammatory cytokines. However, the pro-inflammatory cytokine TNFα was still positively correlated with craving ($r = 0.41$, $p < .05$). Finally, we also observed that selective attention was positively correlated with large bowel permeability ($r = 0.39$, $p < .05$) and with hsCRP ($r = 0.37$, $p < .05$) (fig 4).

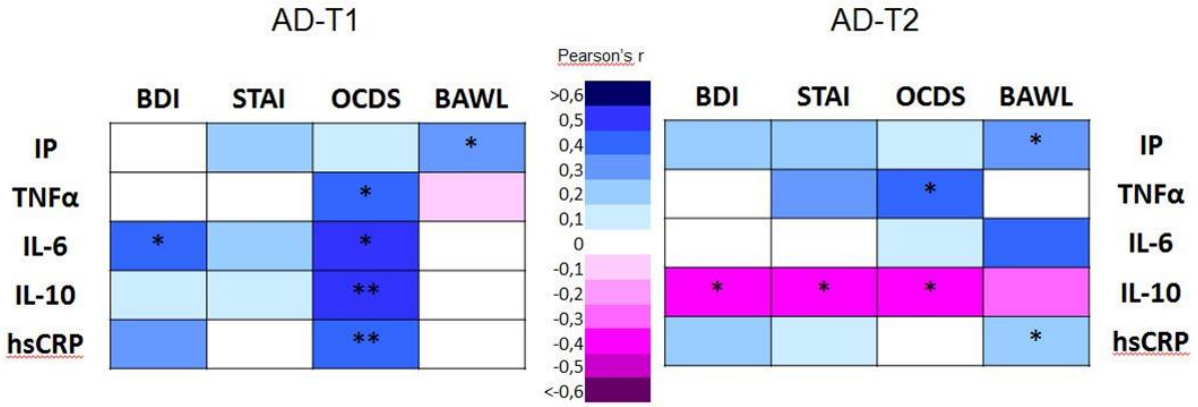

Figure 4: Correlations between biological and psychological factors at the onset (T1) and at the end (T2) of withdrawal. BDI: Beck depression inventory; STAI: state-trait anxiety inventory; OCDS: obsessive compulsive drinking scale; BAWL: batterie d'attention de William Lennox; IP: intestinal permeability.* $p < 0.05$, ** $p < 0.01$.

1.5 Discussion

Several animal and human studies support the view that alcohol-induced increase in intestinal permeability and portal LPS play a role in the development of alcoholic liver disease (Adachi et al., 1995; Bode et al., 2003; Keshavarzian et al., 2009; Mathurin et al., 2000; Mutlu et al., 2009; Parlesak et al., 2000; Ramachandran et al., 2002; Rao et al., 2004; Wheeler, 2003; Yan et al., 2010). However, these previous human studies have always been done in alcohol-dependent subjects that had already developed liver damage (Parlesak et al., 2000). The first aim of our study was to test whether intestinal permeability, circulating LPS and inflammatory response would be elevated in non-cirrhotic alcohol-dependent subjects. Our results clearly showed that these patients presented with a greater intestinal permeability than control subjects, predominantly in the small intestine. Interestingly, the permeability of all intestinal segments decreased significantly during withdrawal and reached at T2 the level observed in controls. This confirms the deleterious effect of alcohol on the small intestine, where alcohol is mainly absorbed (Inserm, 2001). The rapid recovery suggests that three weeks of abstinence are sufficient to restore a functional gut barrier, probably because of the rapid turn-over of intestinal mucosa (Creamer et al., 1961).

Concerning circulating LPS that can be elevated in the case of gut impairment (Muccioli et al., 2010), we found that plasma LPS levels were significantly higher in alcohol-dependent group at T1 than in controls but returned to normal at T2. This observation supports the view that severe alcohol intake may increase LPS levels independently of the development of cirrhosis. Our study is the first to observe intestinal hyperpermeability and increased LPS levels in non-cirrhotic alcohol-dependent humans, thereby confirming the results of a recent study in rats (Keshavarzian et al., 2009). The increase in LPS levels can probably be attributed to the increase in small intestine permeability. However, we cannot exclude that small intestinal bacterial overgrowth, that characterizes alcohol-dependent subjects (Bode et al., 2003; Bode et al., 1993), might also contribute to the increase in LPS. As mentioned above and in contrast with a previous study performed in obese animals (Cani et al., 2009) we failed to find a significant correlation between intestinal permeability and plasma LPS concentrations. This discrepancy may be explained by two reasons: firstly, in our study, blood samples were taken from the antecubital and not from the portal vein; secondly, the probe we used to assess permeability was ^{51}Cr-EDTA, that has a molecular mass of 350 Da, whereas LPS have a molecular weight of 2,000-20,000 Da (Caroff et al., 2002). Indeed, in

a previous study, LPS were found to correlate with permeability only when probes of large molecular weight were used (Mathurin et al., 2000; Parlesak et al., 2000).

These bacterial lipopolysaccharides are the major components of the outer surface of Gram-negative bacteria and are considered to be powerful pro-inflammatory agents (Caroff and Karibian, 2003). We assessed the inflammatory markers of the alcohol-dependent subjects and observed that the levels of TNFα, IL-6, IL-10, and hsCRP were significantly higher than in controls at T1. Surprisingly, the anti-inflammatory cytokine IL-10 was the only one to totally recover at T2 whereas the pro-inflammatory cytokines TNFα, IL-6 and hsCRP only partially decreased. This indicated that IL-10 was the first cytokine that responded to the decrease in intestinal permeability and circulating LPS. Although the exact source of plasma cytokines remains unknown in alcohol-dependent subjects, we may hypothesize that IL-10, which is mainly produced by leucocytes (Sabat et al., 2010) and epithelial intestinal cells (Autschbach et al., 1998) could be more related to the intestine and that TNFα and IL-6 are mainly produced by the liver, after activation of Kupffer cells by LPS (Elsharkawy and Mann, 2007). Indeed, IL-10 is commonly considered as a Th3 cytokine which is involved in immune tolerance by T cells at sites for first line of defense such as intestinal mucosa, where they have a protective role against the development of uncontrolled inflammation in the gut (Allez and Mayer, 2004). Moreover, IL-10 inhibits the production of pro-inflammatory cytokines by monocytes and macrophages (de Waal et al., 1991) and might hence play a significant role in maintaining a non-inflammatory immune status in the normal intestine (Autschbach et al., 1998). In case of inflammatory bowel disease (IBD), the immunosuppressive effect of IL-10 is probably not sufficient to control local inflammation (Autschbach et al., 1998; Kucharzik et al., 1995) since patients with IBD also have high levels of pro- and anti-inflammatory cytokines (Kucharzik et al., 1995). Our data also suggested that in alcohol-dependent subjects in whom there is systemic immune activation, the anti-inflammatory cytokines are increased to counteract the actions of pro-inflammatory cytokines. This may explain the strong correlations between IL-10 and TNFα (T1: $r = 0.50$, $p < .01$; T2: $r = 0.51$, $p < .01$) or IL-6 (T1: $r = 0.74$, $p < .001$; T2: $r = 0.45$, $p = .06$) that we found in our study. Another evidence for the role of IL-10 in the intestine immune tolerance is the observation that IL-10 knock-out mice spontaneously develop chronic enterocolitis (Kuhn et al., 1993). All of these data support the fact that the recovery of plasma IL-10 observed in alcohol-dependent subjects during withdrawal could be related to the recovery of local inflammatory state that induced

intestinal permeability. On the other hand, the explanation for the lack of recovery of the pro-inflammatory cytokines is unclear. It could be due to the persistence of liver inflammation. There is evidence of ongoing liver damage at the end of a 3-week withdrawal, as assessed by the persistence of liver stiffness on transient elastography measures after 30 days of abstinence (Gelsi et al., 2010) and the absence of total recovery of cytolytic enzymes observed in our patients (data not shown). However, we can also propose the hypothesis that the incomplete resolution of the general immune response could also contribute to the continuing low-grade inflammation observed at the end of the detoxification program.

Our study also has a second important aim: to test the possible role of these markers of permeability and inflammation on psychological factors that play a central role in the development of alcohol dependence. We therefore decided to assess psychological factors that play a role in the development of alcohol dependence but that are also known to change during withdrawal. We observed, as expected, that scores of depression, anxiety, alcohol craving and selective attention were largely altered in alcohol-dependent subjects. All the scores recovered during withdrawal, following a pattern parallel to that of biological markers. However, despite a total recovery of intestinal permeability and circulating LPS after alcohol-withdrawal, markers of inflammation and of psychological distress had only partially recovered. This suggests that they are not related only to the intestinal permeability.

To test this last hypothesis, we looked for correlations between biological and psychological factors. Previous animal or human studies have shown some emotional and cognitive disturbances when inflammation was artificially induced by the injection of LPS. For instance, Sparkman et al. (2006) reported that IL-6 could be a key mediator of the deficit in working memory in mice injected with LPS. Reichenberg et al. (2001) reported that LPS injection in humans induced memory dysfunctions and an increase in depression and anxiety. Moreover, LPS-induced depression and anxiety were correlated with cytokines and cortisol levels. Interestingly, we observed that alcohol-drinking is a condition that naturally induces changes in LPS, intestinal permeability and cytokines, and hence resembles the artificial situations described above. In accordance with these previous reports, we observed at T1, when LPS levels are elevated, a correlation between the pro-inflammatory cytokine IL-6 and depression. However, we also found at T1 that cytokines IL-6, TNFα and IL-10 are correlated with alcohol craving. It is, to our knowledge, the first observation of a relationship between inflammation and craving in alcohol-dependent subjects, where craving is expected to play a central role in drinking behavior. Selective attention correlated with intestinal permeability at

both times of withdrawal. More importantly, we found at T2 that IL-10 was negatively correlated with depression and alcohol craving. These two markers are known to be important predictors of relapse after withdrawal (Andersohn et al., 2004) suggesting therefore an important role for the anti-inflammatory cytokine in abstinent alcohol-dependent subjects. Animal studies have suggested a role for IL-10 to prevent the effects of LPS on mood and cognition. Bluthé et al. (1999) showed that IL-10 injection in mice abrogated the depressive symptoms induced by central or peripheral LPS administration. More recently, LPS-induced fatigue and deficits in psychomotor coordination were found to be exacerbated in mice deficient in IL-10 (Krzyszton et al., 2008). Richwine et al. (2009) reported that LPS injection induced cognitive disturbances in IL-10-/- mice but not in wild-type mice. However, only one recent study mentioned a positive impact of IL-10 on human cognitive performances (Van den Boogaard et al., 2010). Whether IL-10 influences behavior through an inhibition of the secretion of pro-inflammatory cytokines (Bluthe et al., 1999; Di Se et al., 1995) or through a direct effect on the brain (Mesquita et al., 2008) is still under debate.

In conclusion, our study is the first to demonstrate a relationship between heavy chronic alcohol consumption, increased intestinal permeability, increased circulating LPS and low-grade inflammation in non-cirrhotic alcohol-dependent subjects. Furthermore, the parallel changes of these biological markers and of psychological markers related to drinking behavior observed during withdrawal, suggest a role for a gut-brain interaction in alcohol-dependence. While pro-inflammatory cytokines are positively correlated with depression and craving at the onset of withdrawal, the anti-inflammatory cytokine IL-10 becomes negatively correlated with depression and craving at the end of the detoxification program, suggesting a potential protective role for this cytokine against relapse. Moreover, we can suggest that the incomplete recovery of pro-inflammatory cytokines after a short-term withdrawal could also contribute to the high number of relapses observed among these patients within several weeks or months following the detoxification program.

Because correlation does not imply causal connections, further experimental studies using agents that directly affect the levels of inflammation are definitely needed to reinforce the evidence of the important role of cytokines in the behavior of AD subjects. However, this potential gut-brain interaction opens a new field of research for pharmacological interventions in alcohol-dependent subjects, targeting the gut or inflammation. Indeed current

pharmacological approaches of alcohol-dependence that only target brain neurotransmitters have proved so far to be modestly efficient (Johnson, 2010), eventually because they fail to take into account of intestinal permeability and inflammation as a driving force for the psychological components of alcoholic pathology. An interesting perspective would be to test whether nutritional factors such as probiotics or prebiotics that are known to have positive impacts on the gut barrier (Delzenne et al., 2011) will be able to reduce inflammation and improve mood and craving, as a new treatment of alcohol-dependence.

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1.7 Supplemental information

Section1: Diagnosis of cirrhosis

Liver biopsy is still regarded as the gold standard for grading and staging the liver disease. However, this invasive procedure carries the risk of potential life-threatening complications (e.g. bleeding) and therefore, for obvious ethical reasons, liver biopsy cannot be justified in all patients for study purposes. Therefore, all of the subjects have systematically been evaluated by blood tests (biochemistry, haematology, coagulation), by a gastro-intestinal endoscopy (to check the signs of portal hypertension such as oesophageal and gastric varices), by liver Doppler-ultrasound (to evaluate the shape of the liver, splenomegaly with collateral intra-abdominal venous circulation or slowed down or inverted portal venous blood flow) and by a measurement of transient liver elastrography (Fibroscan®). This new non-invasive technique has been designed to quantify liver stiffness which has been correlated to liver fibrosis grades based on the METAVIR classification system of fibrosis. Validated cut-off has been published and studies clearly show that this technique is reliable in ruling out significant fibrosis and in confirming cirrhosis. Accordingly, we only selected F0 and F1 patients (i.e. absence of significant fibrosis) for the study. Liver biopsy has only been performed in patients in whom the non-invasive work-up suspected advanced fibrosis/cirrhosis which were by definition excluded from the study.

Section 2: Assessment of psychological variables

All patients were tested for depression, anxiety, craving for alcohol and selective attention at T1 and T2.

The Beck Depression Inventory (BDI) is a 21 question multiple-choice self-report inventory designed to measure the severity of depressive symptoms. This questionnaire is valid, reliable and thought to be a good measure of depression severity in alcohol-dependent subjects. The validated French translation of the second version of the BDI (BDI-II) was used in this study. The BDI-II consists of 21 items measuring characteristic attitudes and symptoms of depression. Each item is rated on a 4-point scale ranging from 0 to 3. The BDI-II is scored by summing the ratings for the 21 items, with a maximum score of 63.

The *State-Trait Anxiety Inventory (STAI Form YA)* is a valid and reliable self-report inventory for measuring anxiety. It evaluates how subjects are feeling at the present time

(state). The questionnaire consists in 20 items with a range of four possible responses to each (Likert scale; 1 = not at all, 2 = somewhat, 3 = moderately so, 4 = very much so). The range of scores is 20-80 and the higher score indicates the greater anxiety. A valid French version was administered.

The Obsessive-Compulsive Drinking Scale (OCDS) is a self-report questionnaire that measures the cognitive aspects of alcohol craving during the preceding 7 days. This questionnaire comprises a total of 14 items, which can be divided into two subscales, a 6-item "obsessive" subscale (*e.g. How much of your time where you are not drinking is occupied by ideas, thoughts, impulses, or images related to drinking?*) and an 8-item "compulsive" subscale (*e.g. How much of an effort do you make to resist consumption of alcoholic beverages?*). Participants responded to each OCDS item on a Likert scale ranging from 0 to 4. Four compulsive items are related to alcohol consumption (*e.g. How many drinks do you drink each day?*). Because alcohol was prohibited during withdrawal, these items were eliminated and a modified 4-item "compulsive" subscore and a modified 10-item "total" score were computed. A valid French version was administered to all participants.

Selective attention is evaluated by a computerized test that comes from the BAWL (Batterie d'Attention de William Lennox) in its version 4.0. The double binary task involves 4 kinds of visual signals that appear one after the other on a screen and that are distinct by two features: the form (cross and circle) and the color (red and blue). The subjects have to react as quickly as possible only when the targets (red cross or blue circle) appear on the screen by pressing the response button on the computer. Therefore, they have to refrain from responding when the distractors (blue cross and red circle) are presented. There are 64 items and for each target, reaction time needed to press the button is calculated. The median of reaction times is considered for statistical analyses. To help subjects during this task, a picture with the different targets is put in front of them.

1.8 Complementary discussion and conclusion

Besides the different aspects that were evoked in the discussion of the first paper published in 2012, the results obtained for the publication lead us to propose new interpretations that will be developed in this complementary discussion.

Increased intestinal permeability and blood LPS levels have been involved in the pathophysiology of alcoholic liver disease. Here, we have shown for the first time that leaky gut and systemic endotoxemia occur in alcoholic subjects without liver disease, meaning that alcohol-dependence itself, and not hepatic injury, induces these changes. We have also shown that the enhanced gut permeability and blood LPS are transient and that they recover completely after 3 weeks of alcohol abstinence (T2). However, the low-grade systemic inflammation is still present at the end of the detoxification program. The psychological symptoms that are largely expressed in the patients at the beginning of the treatment are reduced at the end, but are also still present (figure 1-1).

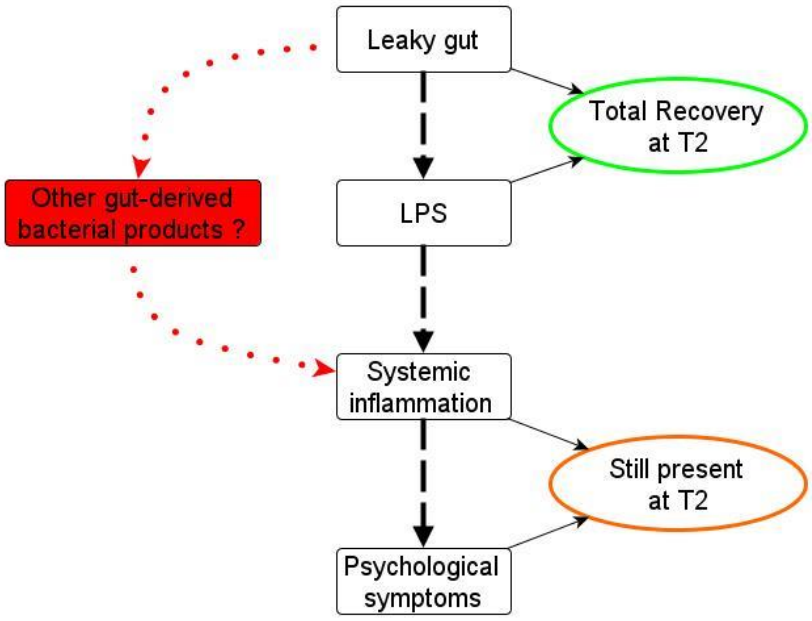

Figure 1-1: Summary of the results obtained in Chapter 1.

These observations led us to discuss several important points:

First, LPS arise from the cell wall of intestinal Gram-negative bacteria and can induce a potent inflammatory response through an interaction with their receptors TLR4/CD14. Because LPS recovered totally at the end of withdrawal while pro-inflammatory cytokines were still present, we suggest that LPS is not the only bacterial toxin inducing a systemic inflammation. Indeed, the paracellular gut permeability is not selective to LPS and the altered gut barrier could also permit the translocation of other gut-derived bacterial toxins such as peptidoglycans that originate mainly from Gram-positive bacteria. The role of peptidoglycans in the generation of inflammatory response will therefore be investigated in Chapter 2. Also, it is important to note that the LPS measurement performed in this first study reflects actually the "endotoxin activity" and we did not take into account the different types of LPS originating from different bacteria and that could induce different immune responses. Moreover, the different ways of translocation (paracellular vs. transcellular – via chylomicrons), the degradation processes as well as the binding to proteins (like LPS-binding protein (LBP) or sCD14) make the plasma LPS level difficult to correlate with other parameters, such as the gut permeability. The measurement of LPS in feces supernatants could better reflect its real production-release by bacteria.

Secondly, the comorbidity of alcoholism and depressive/anxiety disorders has been extensively documented [246, 247]. However, it remains unclear whether one of the disorders causes or predisposes to the other. Studies referred to the "tension reduction hypothesis" which proposes that alcohol is consumed for its anxiolytic effect which then promotes future alcohol intake [248]. Alcohol consumption is therefore a self-medication against anxiety or depression symptoms. Alternatively, clinical observations revealed that alcohol intake may also induce the development of anxiety symptoms and is seen as anxiogenic [249]. Although it has been shown that depressive/anxiety disorders promote alcohol consumption and vice versa, alcoholism promotes the development of depressive/anxiety disorders, the results of our study support the hypothesis that alcohol intake induces psychological symptoms. Indeed, at the beginning of withdrawal, alcohol-dependent subjects had high scores of depression and anxiety. However, after 3 weeks of alcohol abstinence, the anxiety and depressive symptoms were significantly reduced. Furthermore, in clinical practice, alcohol detoxification is always required before treating

depressive disorders. However, even though alcohol abstinence largely improved the psychological symptoms, the patients included in our study remained with higher scores of depression, anxiety and craving at the end of withdrawal, compared to a control group. As the inflammatory markers correlated with the severity of psychological symptoms, these observations highlight the possibility that, in addition to the psychological interventions by psychologists or psychiatrists, reducing inflammation could help the patients to feel better and improve their psychological well-being, that is likely related to the probability of a relapse after detoxification [21].

Finally, we assessed in vivo the intestinal permeability of patients and controls by measuring in urine the percentage of an ingested dose of ^{51}Cr-EDTA. The originality of this study resides in the fact that we make a distinction between small bowel and colon permeability that was poorly reported in the literature. It has been shown that chronic alcohol consumption induced changes in the composition of gut microbiota which consists mainly in bacteria inhabiting the colon. Furthermore, gut bacteria may be involved in the regulation of intestinal permeability. Therefore, it does make sense to separate small and large bowel permeability which was made possible by using the probe ^{51}Cr-EDTA that is not degraded by the colonic bacteria, unlike sugars (e.g. lactulose, mannitol). The results of the intestinal permeability test showed that the small bowel was mainly altered whereas the increase in colon permeability was not significant. However, one year after the publication of this study, we repeated the same test on a larger sample size and found that, on average, all intestinal segments were this time significantly altered by chronic alcohol abuse. Then, we observed carefully the value of intestinal permeability for each patient, one by one, and realized that, in a cohort of 60 alcohol-dependent patients, 43% of them had altered gut permeability whereas 57% had gut permeability similar to that of healthy controls. This difference cannot be explained by the amount of alcohol consumed and is therefore related to another factor. This concern is discussed in details in Chapter 3.

The conclusions and perspectives of Chapter 1 are the following:

- Increase in intestinal permeability and LPS translocation occurs in alcohol-dependent subjects independently of the presence of liver disease.
- The low-grade systemic inflammation is correlated with the psychological symptoms of alcohol-dependence, in particular with alcohol craving.
- After a short-term alcohol withdrawal, leaky gut and endotoxemia fully recovered whereas inflammation and psychological symptoms persist.
- The persistence of inflammation suggests that other gut-derived bacterial toxins could induce the release of inflammatory cytokines
- Strategies aiming at reducing the inflammatory state could help alcohol-dependent patients to improve their psychological distress.

2 CHAPTER 2

ROLE OF INFLAMMATORY PATHWAYS, BLOOD MONONUCLEAR CELLS AND GUT-DERIVED BACTERIAL PRODUCTS IN ALCOHOL-DEPENDENT SUBJECTS

Recent studies suggest that inflammation could play a role in the development of psychiatric disorders. However, there are currently only little hypotheses on the mechanisms by which inflammation could develop in these disorders. In chapter 1 of the results section, we have shown that alcohol-dependent subjects developed a chronic low-grade systemic inflammation but the origin of blood pro-inflammatory cytokines is unknown. As alcohol abuse is associated with increased intestinal permeability and LPS translocation from the gut lumen to the blood, it is plausible that LPS induce an inflammatory response through interaction with their receptors expressed by immune cells. However, alteration of the gut barrier may also permit the translocation of other gut-derived bacterial products, such as peptidoglycan (PGN), that originate mainly from Gram positive bacteria. The immune cells that expressed Toll-like receptors which recognized specifically LPS and PGN are located in the gut mucosa, in the liver (Kupffer cells) as well as in the systemic circulation. Peripheral blood mononuclear cells (PBMCs) represent an essential defense barrier against gut-derived bacterial products entering the bloodstream and may potentially contribute to the systemic inflammation.

The role of PGN has generally been neglected in the literature, while most studies on inflammation focused on the importance of LPS. In this Chapter 2, we tested whether gut-derived bacterial products LPS and PGN activate the PBMCs, which intracellular pathways are involved, and whether PBMCs could contribute to the systemic inflammation. We also investigated whether activation of inflammatory pathways are related to alcohol consumption and alcohol craving. Finally, we tested the recovery of pathways activation after a short-term alcohol withdrawal.

The main strengths of this study reside in the fact that, first, we obtained data on a large cohort of alcohol-dependent subjects (n = 63). Secondly, as described in Chapter 1, we selected only alcohol-dependent subjects without alcoholic liver disease in order to assess the effect of alcohol-dependence itself and not to be biased by hepatic inflammation. Furthermore, the inflammatory pathways in PMBCs were analyzed under naturalistic conditions, without culturing cells or applying pathogen stimulation. Therefore, our results likely better reflect the real and pathophysiological impact of gut-derived bacterial products on immune response.

The results related to this chapter have been published in *Biological Psychiatry* (2014) *1;76(9):725-33.*

To make the reading and the understanding of this study easier to the reader, several figures originally inserted in the supplementary information of the article published in Biological Psychiatry will be inserted in the core of the manuscript.

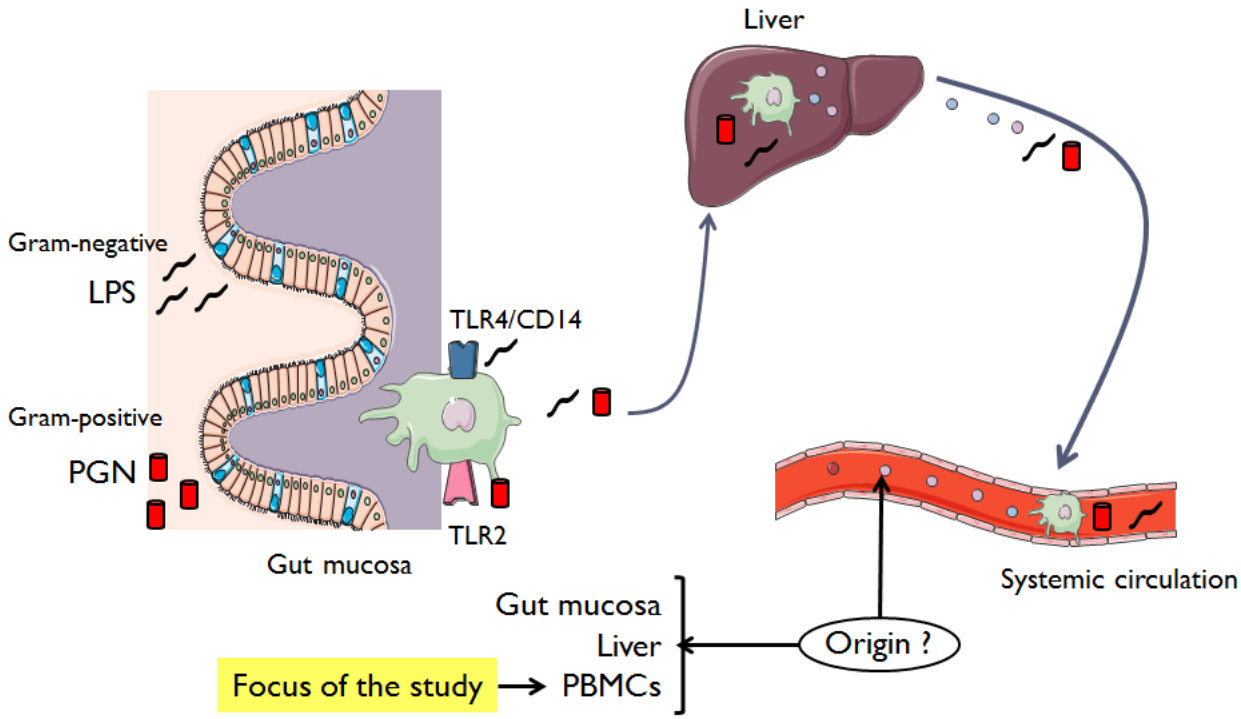

Figure 2-1: Hypothesis scheme of chapter 2. Alcohol-dependent subjects are characterized by a chronic systemic low-grade inflammation. However, the origin of inflammation is unknown. Leaky gut permits the translocation of LPS from the gut lumen to the systemic circulation. LPS induces an inflammatory response through interaction with receptors CD14/TLR4. These receptors are located in immune cells which can be found in the gut mucosa, in the liver or in the systemic circulation. The aims of this Chapter 2 were to determine whether LPS can activate peripheral blood mononuclear cells (PBMCs), which intracellular pathways are involved and whether PBMCs contribute to the systemic inflammation. Moreover, we investigated whether peptidoglycan can also cross the leaky gut barrier and whether it activates PBMCs though interaction with receptor TLR2.

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2.1 Abstract

Background: Inflammation might play a role in the development of several psychiatric diseases. However, the origins of processes that mediate inflammation remain unknown. We previously reported, in alcohol-dependent (AD) subjects, increased intestinal permeability, elevated blood lipopolysaccharides (LPS) levels, and low-grade systemic inflammation associated with psychological symptoms of alcohol dependence. In this study, we tested during detoxification peripheral blood mononuclear cells (PBMCs) inflammatory responses to gut-derived bacterial products and its relation to alcohol-craving.

Methods: Sixty-three actively-drinking non-cirrhotic AD subjects were tested at the beginning (day 2) and end (day 18) of alcohol detoxification and compared with 14 healthy subjects. Activation of various intracellular signaling pathways by gut-derived bacterial products was analyzed by qPCR, Western blotting and DNA binding assays (for transcription factors). Toll-like receptors activation was assessed by cell cultures.

Results: In addition to LPS, we showed that peptidoglycans (PGN) may also cross the gut barrier to reach the systemic circulation. Both activate their respective Toll-like receptors in PBMCs. Chronic alcohol consumption inhibited the nuclear factor kappa B pro-inflammatory cytokine pathway, but activated the mitogen-activated protein kinase/activator protein 1 pathway, together with the inflammasome complex. This resulted in increased mRNA and plasma levels of interleukin (IL)-8, IL-1β, and IL-18. Activated pro-inflammatory pathways, in particular IL-8 and IL-1β, were positively correlated with alcohol consumption and alcohol-craving scores. Short-term alcohol withdrawal was associated with the recovery of LPS- but not PGN-dependent receptors.

Conclusions: LPS and PGN from the gut microbiota stimulate specific inflammatory pathways in PBMCs that are correlated with alcohol craving.

2.2 Introduction

Recent studies suggest a role for inflammation in the development of several psychiatric diseases (1), including alcohol dependence (2), a disorder that affects 5–7% of the population in developed countries (3). Inflammation in alcohol dependence has been ascribed to a local pro-inflammatory effect of ethanol, either in the brain or in the liver (2). However, heavy chronic alcohol consumption induces gut mucosal damage, increases intestinal permeability (4-6), induces changes in the composition of the gut microbiota (7,8) and bacterial overgrowth in the small intestine (9,10). Gut bacteria are classified into one of two major groups, according the multi-layered structure of their cell envelopes. Gram-negative bacteria are surrounded by a thin peptidoglycan (PGN) cell wall, which itself is surrounded by an outer membrane containing lipopolysaccharide (LPS). Gram-positive bacteria lack the LPS-associated outer membrane but are surrounded by layers of PGN many times thicker than is found in the Gram-negatives (11). The view that systemic inflammation could play a role in alcohol dependence and be induced by increased intestinal permeability and permeation of LPS is supported by recent data in humans (6). Nevertheless, the possibility of an inflammatory effect of other bacterial components such as PGN has not been tested to date.

Gut-derived bacterial LPS and PGN interact with receptors on lymphocytes and monocytes, the membrane-bound Toll-like receptors (TLR4 and TLR2, respectively), and the cytosolic Nod-like receptors (NLRs) to elicit inflammatory responses (12,13). Upon receptor activation, a signal transduction cascade converges toward a common set of signaling molecules, leading to the activation of various transcription factors that drive the production of pro-inflammatory cytokines and type I interferons (14,15).

TLR4 interacts with co-receptors CD14 and MD2 to activate signal transduction pathways through adaptor molecules, including myeloid differentiation primary-response protein 88 (MyD88) and TIR domain-containing adapter inducing IFN-beta (TRIF) (16-18). The MyD88-dependent pathway, which is common to most TLRs, leads to the activation of two distinct intracellular pathways: the inhibitor of nuclear factor kappa-B (NFκB) kinase (IKK) pathway, culminating in the activation of the transcription factor NFκB (14), and the mitogen-activated protein kinase (MAPK) pathway inducing another transcription factor, activator protein 1 (AP-1) (19,20). TLR4 is also able to activate a second MyD88-independent

pathway, which results more specifically in the induction of interferon regulatory factor 3 (IRF3), leading to increased production of type I interferons, in particular interferon-beta (IFNβ) (21). PGN directly stimulate TLR2 receptors, whereas muramyl dipeptide (MDP), which is the minimal bioactive cytosolic structure of PGN, interacts with nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins NOD2 and NLRP3 (22,23). TLR2 and NOD2 also activate the MyD88-dependent pathway. The receptor NLRP3 forms the inflammasome, which is a multiprotein complex that also comprises the enzyme caspase-1. This converting enzyme cleaves the precursor forms of interleukin (IL)-1β and IL-18 into mature and active cytokines (24).

The aim of the present study is threefold: 1) Mechanistic: to test whether gut-derived bacterial products activate PBMCs, which intracellular pathways are involved, and whether they contribute to the systemic pro-inflammatory cytokines response under natural conditions in non-cirrhotic AD subjects; 2) Clinical: to assess whether activation of specific pathways, and especially the pro-inflammatory cytokines, are related to the amount of alcohol consumed and to alcohol craving; and 3) to investigate the recovery of pathway activation after 18 days of detoxification.

2.3 Material and methods

Patients and Study Design

Sixty-three actively drinking AD inpatients were recruited from the alcohol-detoxification unit of the departments of gastroenterology and psychiatry, Saint-Luc Academic Hospital, Brussels, Belgium. The following minimal eligibility criteria were required: alcohol dependence according to the DSM-IV (25), and alcohol drinking until the day of admission. Exclusion criteria were as follows: the use of antibiotics, probiotics, glucocorticoids, and nonsteroidal anti-inflammatory drugs currently or during the two months preceding enrollment; patients with metabolic disorders such as diabetes and obesity (BMI > 30 kg/m^2), chronic inflammatory diseases (such as inflammatory bowel disease or rheumatoid arthritis), cancer, or other severe medical conditions, including cirrhosis or significant liver fibrosis (F≥2 on transient liver elastography) (see Supplemental Material). Fasting blood was drawn from the antecubital vein on the day following admission (T1) and at the end of the detoxification program, on day 18 (T2). Among the 63 patients, 41 participated to both time points (subjects who abandoned their treatment or resumed alcohol consumption were excluded at T2). AD patients were compared with 14 age-, gender-, and body mass index-matched controls who consumed socially low amounts of alcohol (< 20 g/day). The study protocol was approved by the ethical committee of the hospital (reference B40320096274) and written informed consent was obtained from all subjects.

Alcohol Consumption

At T1, patients were asked to self-report the number of drinks that they were having each day, prior to hospitalization. In a subset of 21 patients, alcohol consumption was evaluated more carefully, with the time-line follow-back (TLFB) approach (26), as detailed in de Timary et al. (27).

Isolation of Human PBMCs and RNA

PBMCs were isolated from blood by centrifugation on a Ficoll-Paque Plus gradient (GE Healthcare, Uppsala, Sweden) (Supplemental Material). RNA was extracted using TRIzol Reagent (Invitrogen, California, USA).

Reverse Transcription and Real-Time Quantitative Polymerase Chain Reaction

cDNA was synthetized and quantitative PCR was performed with the Step One Plus device and software (Applied Biosystems, California, USA) by using the fluorogenic SYBR Green PCR Master Mix (Applied Biosystems), as previously described (28). The ΔΔ CT method was used for quantification normalized to ribosomal protein L19 RNA (internal standard). Primers (Supplemental Table S1) were designed with Primer Express design software (Applied Biosystems).

Quantification of Transcription Factor Activation

Activation of transcription factors (p65, c-Fos, phospho-c-Jun) in PBMCs was assessed, in whole cell extracts (Nuclear Extract kit, Active Motif, la Hulpe, Belgium) by using a sensitive TransAM detection kit (Active Motif) according to the manufacturer's guidelines.

Western Blotting

Western blot analysis was performed on whole cell extracts according to standard electrophoresis and transfer techniques. Membranes were revealed with the PerkinElmer Western Lightning chemiluminescent detection system (PerkinElmer, Boston, USA) prior to quantification of the blots with the Molecular Imager ChemiDoc XRS System (Biorad, Nazareth, Belgium). Membranes were stripped (Fisher Scientific, Erembodegem, Belgium) and re-probed with several antibodies (Supplemental Table S2). β-Actin was used as a loading control.

Plasma PGN and Cytokine Measurements

Plasma was diluted 1:2 and detection of PGN was performed by using the Peptidoglycan ELISA kit (Cusabio Antibodies-online, Aachen, Germany) following the manufacturer's guidelines. Plasma cytokines (tumor necrosis factor (TNF)α and IL-6, IL-1β, and IL-8 were assayed in duplicate with a multiplex immunoassay (Millipore, Molsheim, France) and Luminex xMap technology (Bio-Rad) following the manufacturer's instructions.

Short-term Cell Culture and In Vitro Stimulation

PBMCs from four AD patients and four healthy controls were cultured to test in vitro TLR activation upon LPS and PGN stimulation (Supplemental Material).

Assessment of Alcohol Craving

The Obsessive-Compulsive Drinking Scale (OCDS) questionnaire assesses the cognitive aspects of alcohol craving during the preceding 7 days (29) and provides a total craving score, as well as two subscores: an obsessive and a compulsive subscore (Supplemental Material). A validated French version was used in this study (30).

Statistical Analysis

Statistical analyses were performed with SPSS version 20.0 after log-transformation for non-normally distributed data. Independent t-tests were performed to compare AD subjects with controls (CT), and paired t-tests were performed to compare AD subjects at T1 and T2. Correlations were calculated by using the Pearson product-moment correlation coefficient and multiple regression by the stepwise method (Supplemental Material). Statistical significance was defined as a p-value of less than .05. Data presented in the graphs are non-transformed means ± standard error of the mean (SEM).

2.4 Results

Demographic Data and Alcohol Consumption

The principal demographic data are described in Table 1. The average values of alcohol consumption obtained by using the self-reporting approach (160 ± 100 g/day) and the TLFB approach (145 ± 69 g/day) were not statistically different (p = .53).

Table 1: Characteristics of the alcohol-dependent and control groups

	AD-T1	AD-T2	CT
No. Participants	63	41	14
Age (Years)	48 ± 11[a]	49 ± 11[a]	43 ± 11[a]
Gender, *n* (%)			
Male	35 (56%)	21 (51%)	7 (50%)
Female	28 (44%)	20 (49%)	7 (50%)
BMI (kg/m^2)	25.6 ± 5.3[a]	ND	25.0 ± 5.4[a]
Alcohol Intake (g/day)	155 ± 90[a]	0	<20

a Data are means ± SD. AD - T1 and AD - T2 refer to alcohol-dependent subjects tested at the beginning and end of the detoxification program respectively; CT: control group; BMI: body mass index; ND: not defined.

2.4.1 Mechanistic Analyses

Increased Expression and Activation of the TLR4 Receptor Complex in PBMCs of AD Patients

Since the TLR4 complex is principally involved in the recognition of bacterial LPS in immune cells, we first studied its expression in PBMCs. The mRNA levels of TLR4 and CD14, the co-receptor required for LPS recognition by TLR4, were significantly increased in AD subjects (Figure 1A). In order to test activation of the TLR4 complex, we designed a cell culture based-experiment in which PBMCs were treated with physiological doses of LPS. After 6 h of stimulation, TNFα mRNA levels in PBMCs and TNFα protein concentrations in culture medium were higher in AD subjects than in controls, suggesting a higher reactivity of

AD subjects to LPS, in line with up-regulated activation of the TLR4 receptor complex (Supplemental Figure S1A, B).

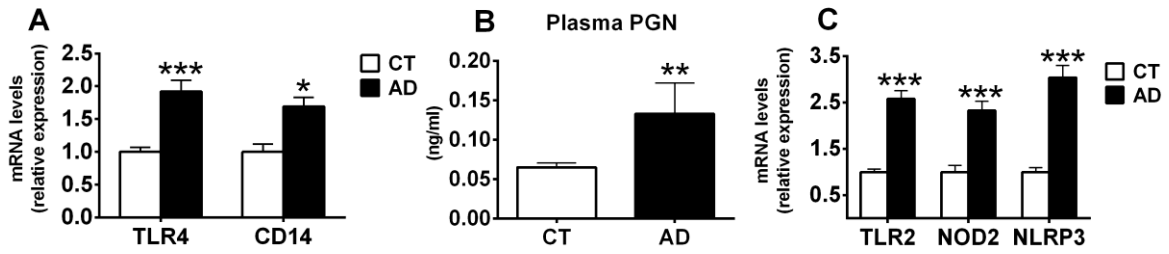
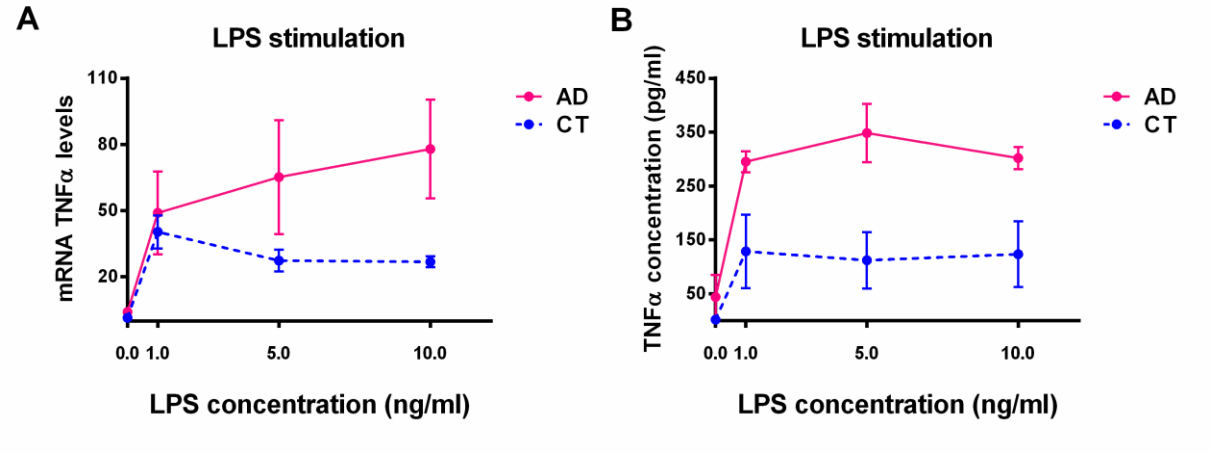


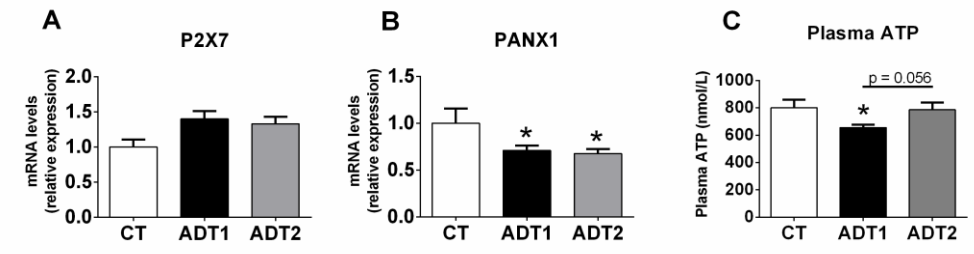
Figure 1: Increased expression of LPS and PGN-associated receptors in peripheral blood mononuclear cells (PBMCs) of alcohol-dependent (AD) patients. (A) Increased mRNA expression of TLR4 and CD14 in PBMCs of AD subjects under naturalistic conditions. (B) Increased plasma PGN concentration in AD subjects. (C) Increased mRNA levels of PGN-associated receptors (TLR2 and NOD2) and NLRP3 in PBMCs of AD subjects under naturalistic conditions. Data are means ± SEM. AD: alcohol-dependent group; CT: control group; *p < .05, **p < 0.01, ***p < .001.



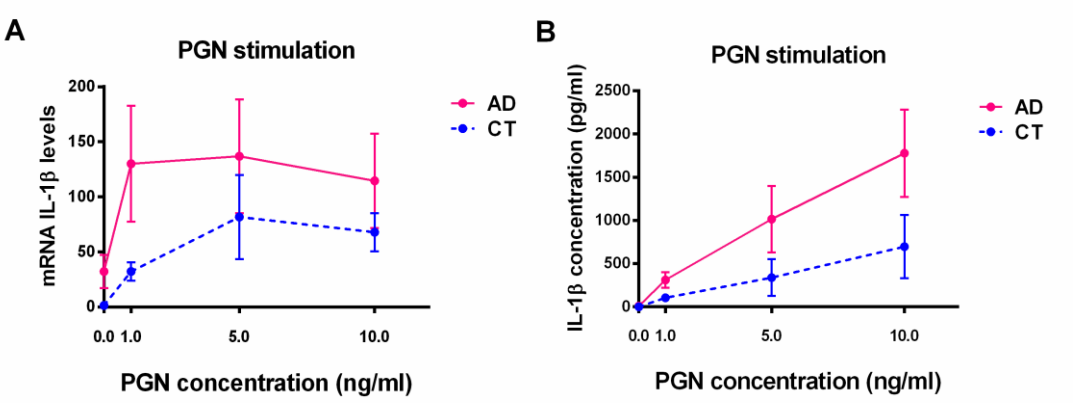
Supplemental figure 1: Increased activation of LPS-associated receptor in peripheral blood mononuclear cells (PBMCs) of alcohol-dependent (AD) patients. To test TLR4 activation, PBMCs from AD and control subjects were cultured for 3 h and stimulated with low doses of LPS (0, 1, 5, 10 ng/mL) for 6 h. Tumor necrosis factor alpha (TNFα) mRNA levels were measured in PBMCs (A) and TNFα protein levels were assayed in culture medium (B). Data are means ± SEM. AD: alcohol-dependent group; CT: control group; LPS: lipopolysaccharides.

Elevated Plasma PGN Levels and Increased Expression and Activation of PGN-Associated Receptors in PBMCs of AD Patients

We hypothesized that PGN might also cross the gut barrier. Plasma PGN levels were indeed elevated in AD subjects compared with controls (Figure 1B). We determined the expression of TLR2 and NOD2, which recognize PGN-associated components, and MDP, the minimal bioactive structure of PGN. Both mRNA levels were significantly increased in AD subjects compared with controls (Figure 1C). In addition, mRNA levels of NLRP3, a component of a multiprotein complex called inflammasome known to be activated by MDP, were also strongly increased in AD patients (Figure 1C). Interaction of ATP with the P2X7 receptor may activate the inflammasome complex. Therefore, we measured plasma ATP levels, P2X7 and pannexin1 (PANX1) mRNA expression which is known to increase upon P2X7 activation (31,32). Plasma ATP levels were lower in AD subjects and the expression of P2X7 and PANX1 remained unchanged or decreased, respectively, suggesting no involvement of the ATP/P2X7 complex in activating the inflammasome and IL-1β secretion (Supplemental Figure S2). Finally, PBMCs were cultured in the presence of physiologically relevant doses of PGN. After 6 h of stimulation, mRNA levels of IL-1β were higher in PBMCs from AD than control subjects. In particular, a very small dose of PGN (1 ng/mL) induced strong up-regulation of IL-1β mRNA levels (Supplemental Figure S3A) and increased the IL-1β concentration in the cell culture supernatant from AD subjects (Supplemental Figure S3B). These results suggest that PGN also cross the gut barrier of AD subjects and likely contribute to the activation of PGN-specific receptors in PBMCs.



Supplemental figure 2: No significant change in P2X7 receptor mRNA (**A**) and down-regulation of PANX1mRNA (**B**), a surrogate marker of receptor activation, in AD subjects at both time points (T1, T2) compared with CT. Furthermore, the plasma ATP level was lower in AD subjects at T1 compared with the CT. Taken together, these data argue against a role for ATP/P2X7 complex in activating the inflammasome and IL-1β secretion. Data are means ± SEM. AD: alcohol-dependent group; CT: control group; T1 and T2 refers to the beginning and end of a 3-week alcohol withdrawal, respectively. *$p < .05$ compared with CT.



Supplemental figure 3: **Increased activation of PGN-associated receptors in peripheral blood mononuclear cells (PBMCs) of alcohol-dependent (AD) patients**. To test TLR2 activation, PBMCs from the AD and CT subjects were cultured for 3 h and stimulated with low doses of PGN (0, 1, 5, 10 ng/mL) for 6 h. IL-1β mRNA levels were measured in PBMCs (**A**) and the active form of IL-1β was assayed in culture medium (**B**). Data are means ± SEM. AD: alcohol-dependent group; CT: control group; PGN: peptidoglycans.

Activation of TLR-Dependent Downstream Signaling in PBMCs of AD Patients

TLR activates down-stream signaling pathways through interaction with various molecules. We therefore investigated the expression and activation of key components of the different pathways. First, we showed that the expression of MyD88 and the phosphorylated form of IL-1 receptor-associated kinase (IRAK-1) were higher in AD subjects, suggesting activation of the MyD88-dependent pathway (Figure 2A-C).

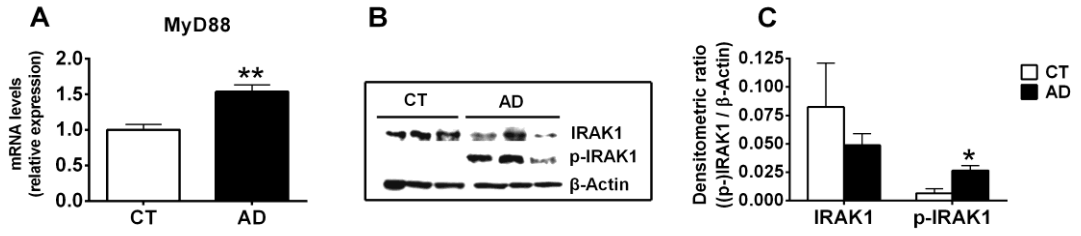


Figure 2: Activation of the MyD88-dependent pathway in peripheral blood mononuclear cells (PBMCs) of alcohol-dependent (AD) subjects. (**A**) Increased mRNA expression of MyD88 in PBMCs of AD subjects under naturalistic conditions. (**B**) Representative Western blots of total and phosphorylated forms of interleukin-1 receptor-associated kinase (IRAK1) protein expression. (**C**) Quantification of IRAK1 and p-IRAK1 expressions in Western blots by densitometry normalized to the loading control β-actin, showing that the expression of the phosphorylated and activated form of IRAK1 is higher in AD subjects compared with healthy CT. Data are means ± SEM. AD: alcohol-dependent group; CT: control group. *$p < .05$, **$p < .01$.

Inhibition of the NFκB proinflammatory cytokine pathway in PBMCs of AD patients

The NFκB proinflammatory cytokine pathway is one of the principal pathways activated upon TLR stimulation. In AD patients, mRNA levels of the NFκB subunits p65 and p105 (NFκB p50 precursor) did not differ from those of controls (Figure 3A). Moreover, the mRNA levels and protein expression of IκBα, the principal inhibitor of NFκB, were more than 3-fold increased in AD patients (Figure 3A-C). Subsequent analysis of the DNA binding activity of p65 showed that NFκB DNA binding was inhibited in AD patients (Figure 3D). To confirm inhibition of NFκB, we also assessed mRNA expression of the cytokines TNFα and IL-6 that are directly regulated by this transcription factor. TNFα levels were significantly lower in AD patients and levels of IL-6 did not differ significantly from controls (Figure 3E). All of these observations demonstrate inhibition of the NFκB proinflammatory cytokine pathway in PBMCs of AD patients.

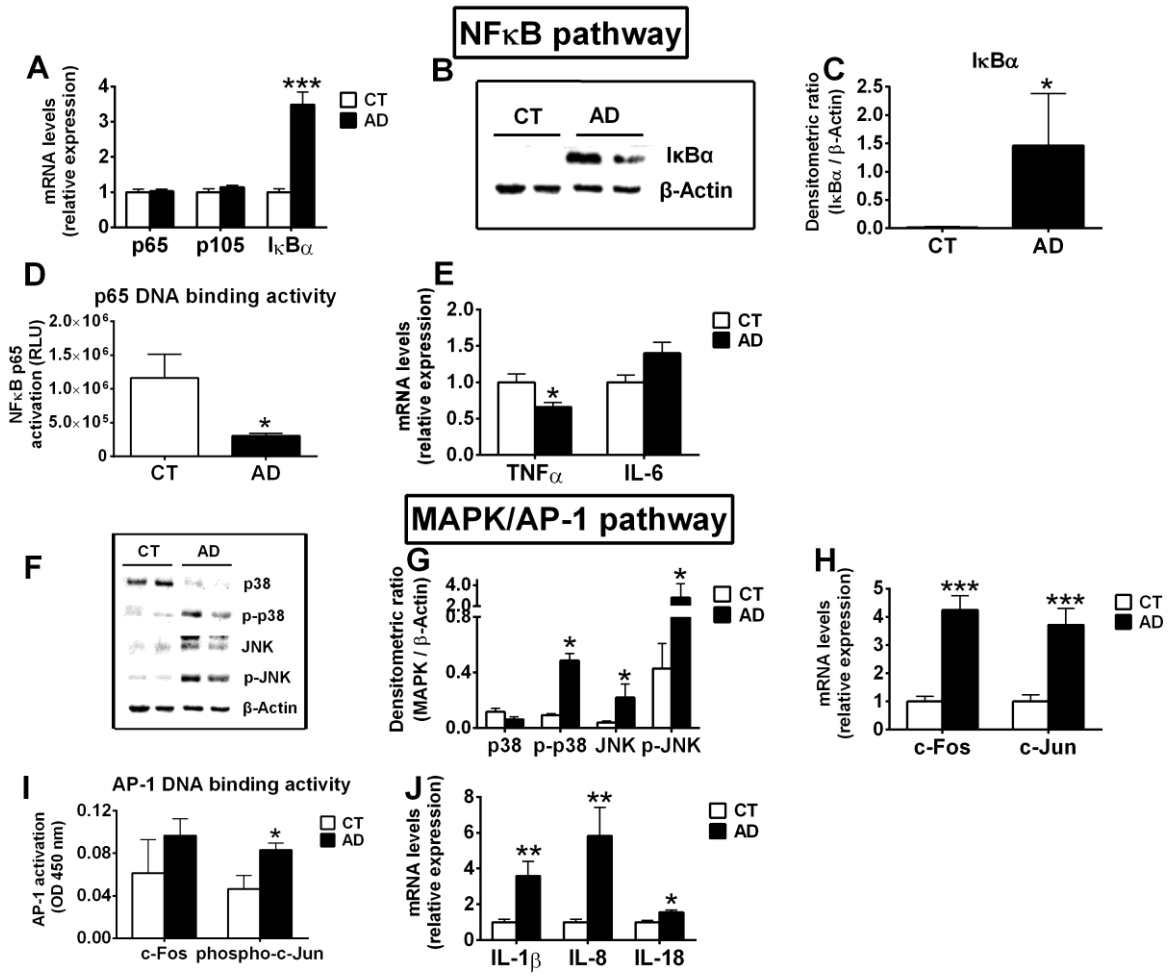


Figure 3: Inhibition of the nuclear factor kappa B (NFκB) pro-inflammatory signaling pathway and activation of the mitogen-activated protein kinase (MAPK)/ activator protein 1 (AP-1) pathway in peripheral blood mononuclear cells (PBMCs) of alcohol-dependent (AD) subjects. (**A**) No induction of mRNA p65 and p105 NFκB subunit expression but a large increase in the mRNA level of the NFκB inhibitor IκBα in PBMCs of AD subjects. (**B**) Representative Western blots of IκBα protein expression in PBMCs and quantification normalized to the loading control β-actin (**C**), showing increased IκBα expression in AD subjects compared with CT. (**D**) NFκB p65 DNA-binding assay showing decreased p65 activation in AD subjects. (**E**) Decreased expression of tumor necrosis factor alpha (TNFα) and no significant change of interleukin (IL)-6 mRNA levels, respectively, in PBMCs of AD subjects. (**F**) Representative Western blots of MAPK protein expression in PBMCs and quantification normalized to the loading control β-actin (**G**) showing an increased p38 phosphorylation (p-p38) and increased expression and phosphorylation (p-JNK) of the c-Jun amino-terminal kinase (JNK) in AD subjects compared with CT. (**H**) Increased mRNA levels of both AP-1 subunits, c-Fos and c-Jun, in PBMCs of AD subjects. (**I**) AP-1 DNA binding assay showing increased c-Fos and phospho c-Jun activation in AD subjects. (**J**) Increased mRNA levels of interleukin (IL)-1β, IL-8, and IL-18, which are, at least in part, regulated by transcription factor AP-1. Data are means ± SEM. AD: alcohol-dependent group; CT: control group; $*p < 0.05$, $**p < .01$, $***p < .001$.

Activation of MAPK and AP-1 pathway in PBMCs of AD patients

MyD88-dependent TLR signaling also activates the MAPK signaling pathway, leading to activation of the transcription factor AP-1. We first assessed protein expression of MAPK p38, and JNK, known activators of AP-1. Western blot analysis showed increased phosphorylation of both proteins in AD patients compared with those of controls, suggesting activation of the MAPK pathway (Figure 3F, G). In addition, mRNA levels of c-Fos and c-Jun, the two principal subunits of AP-1, were significantly up-regulated in AD patients (Figure 3H). Furthermore, c-Fos and phospho c-Jun DNA binding activities were also increased in AD subjects, confirming activation of AP-1 (Figure 3I). Finally, a 3.5-fold and a 6-fold up-regulated expression of the cytokines IL-1β and IL-8, respectively, which are at least in part regulated by AP-1, as well as significantly higher levels of IL-18 (Figure 3J), add further arguments in favor of AP-1 activation in PBMCs of AD patients.

Weak activation of MyD88-independent pathway in PBMCs of AD patients

The MyD88-independent pathway classically leads to activation of the interferon pathway and production of type I interferons. Although assessment of IRF3 and IRF7 mRNA levels did not show any significant difference between AD patients and controls (Figure 4A), the phosphorylated form of IRF3 was more expressed in AD subjects (Figure 4B, C). Therefore, we looked at the expression of the end product IFNβ and found no difference between groups (Figure 4A). In addition, genes directly regulated by IFNβ, such as OAS-1 and ISG6-16, were not increased or only mildly increased in AD subjects, respectively (Figure 4A). Overall, these results suggest only a weak, if any, activation of the MyD88-independent pathway in PBMCs of AD patients.

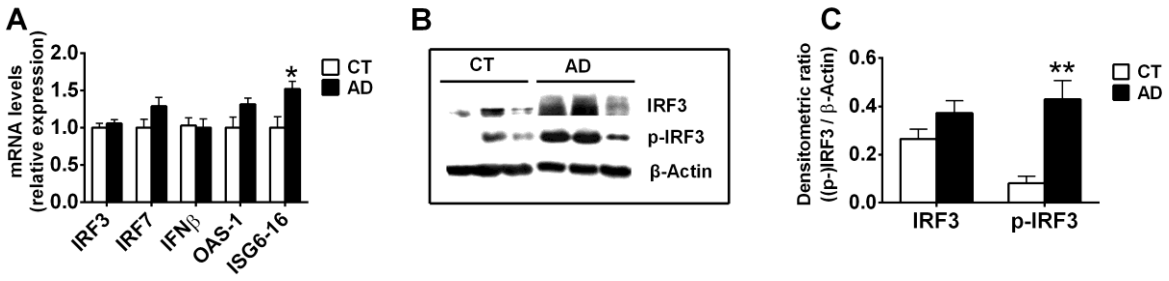
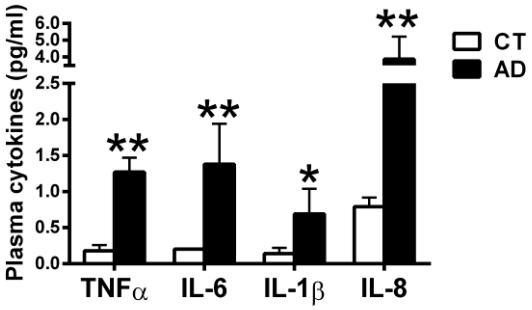


Figure 4: MyD88-independent pathway in peripheral blood mononuclear cells (PBMCs) of alcohol-dependent (AD) subjects. (**A**) No induction of IRF3, IRF7 and interferon beta (IFNβ) mRNA levels as well as no or weak increase of the IFN-induced genes OAS-1 and ISG6-16, respectively, in PBMCs of AD subjects, suggesting that the MyD88-independent interferon pathway is not activated in AD subjects. (**B**) Representative Western blots of the total and phosphorylated forms of interferon regulatory factor 3 (IRF3) protein expression in PBMCs. (**C**) Quantification of IRF3 and phosphorylated IRF3 (p-IRF3) expression in Western blots by densitometry normalized to the loading control β-actin, showing increased expression of p-IRF3 in AD subjects. Data are means ± SEM. AD: alcohol-dependent group; CT: control group. *$p < .05$, **$p < .01$.

Induction of Plasma Pro-inflammatory Cytokines in AD Patients

We measured plasma TNFα, IL-6, IL-1β, and IL-8 concentrations and showed that they were all significantly higher in AD than in control subjects (Supplemental Figure S4).



Supplemental figure 4: Increased levels of plasma pro-inflammatory cytokines in AD subjects compared with healthy CT. Cytokines were measured in duplicate with a multiplex immunoassay. Data are means ± SEM. AD: alcohol-dependent group; CT: control group; IL, interleukin; TNFα, tumor necrosis factor alpha. *$p < .05$, **$p < .01$.

2.4.2 Clinical Analyses

Correlations Between Alcohol Consumption and Expression of pro-inflammatory cytokines

In the subset of 21 patients in whom alcohol consumption was evaluated with the TLFB approach, correlations between the amount of alcohol consumed and the mRNA levels of pro-inflammatory cytokines IL-1β and IL-8 and signaling pathways contributing to their activation was observed at T1 (Table 2).

Table 2: **Activation status of signaling molecules and correlations with alcohol consumption and scores of craving**

		Correlations with Alcohol Consumption T1		Correlations with OCDS-Obs T1		Correlations with Δ OCDS-Obs	
	Activation	*r* Pearson	*p* Value	*r* Pearson	*p* Value	*r* Pearson	*p* Value
Receptors							
TLR4	Activated	.47	<.05				
CD14	Activated	.62	<.01				
TLR2	Activated	.65	<.01				
NOD2	Activated	.55	<.05	.44	<.05	.03	NS
NLRP3	Activated	.68	<.001	.53	<.01	.65	<.01
Signal transduction							
MyD88	Activated	.73	<.001				
MAPK	Activated	ND					
IκBα	Activated	.66	<.01	.52	<.01	.51	<.05
Transcription factors							
p65	Inhibited	.40					
p105	Inhibited	.31					
c-Fos	Activated	.71	<.001	.58	<.001	.59	<.01
c-Jun	Activated	.59	<.01	.62	<.001	.28	NS
IRF3	Controversial	.30					
Cytokines							
TNF-α	Inhibited	.19					
IL-6	Inhibited	.20					
IL-1β	Activated	.65	<.01	.58	<.001	.57	<.01
IL-8	Activated	.55	<.05	.65	<.001	.52	<.05
IL-18	Activated	.16					
IFN-β	Inhibited	.10					

Recap chart of signaling molecule status and correlations with alcohol consumption (expressed in g/day) at T1, with the obsessive subscore (Obs) of alcohol craving at T1 and correlations between the difference scores (Δ = T1 – T2) of inflammatory signaling molecules and the obsessive subscore of craving. OCDS: Obsessive Compulsive Drinking Scale; T1: beginning of withdrawal; T2: end of withdrawal. **p* < .05, ***p* < .01, ****p* < .001; NS: not significant; ND: not determined.

Correlations Between Activated Inflammatory Pathways and Alcohol-Craving Scores

The OCDS was administrated to 38 patients in order to assess alcohol craving. At T1, significant correlations were observed between the total score as well as the obsessive subscore of OCDS and the mRNA expression of pro-inflammatory cytokines IL-1β and IL-8, and the intracellular signaling pathways involved in their activation. As the OCDS total scores decrease during withdrawal (T1: 18 ± 6 vs. T2: 5 ± 4, p<0.001) in parallel with reduction in inflammation, we also tested correlations between the difference scores (Δ) calculated by subtracting "inflammation T1 – inflammation T2" and "OCDS T1 – OCDS T2." Significant correlations were found between Δ for IL-1β, IL-8 and Δ for total and obsessive OCDS scores (Table 2).

As several inflammatory parameters were associated with alcohol craving, we used a multiple linear regression in order to detect which parameters were the best predictors of

craving. Using the stepwise method, we found that the IL-8 mRNA level was the best predictor of the outcome over and above all other predictors and accounted for 49% of the variance in craving (Table 3).

Table 3 : Multiple regression report (stepwise method)

Model	B	SE B	β	*t* Test	Significance
Constant	8.83	.52		16.86	.000
mRNA IL-8	.16	.03	.697	4.86	.000

Multiple R = .697, R^2 = .49 (IL-8 mRNA accounted for 49% of the variance in craving with a significant association of the t-test with the β-value). B and SE B represent the unstandardized beta values and the associated- standard errors. β is the standardized coefficient with the associated t-test and significance.

2.4.3 Effect of a Short-term Alcohol Withdrawal on Inflammatory Pathways

To test the effect of alcohol withdrawal on the recovery of inflammatory pathways in PBMCs of AD patients, data at T1 and T2 for the patients that completed the entire study were compared (Table 4). Concerning the LPS activated pathway, TLR4 and CD14 mRNA levels decreased significantly with abstinence to values observed in controls. For the PGN pathway, TLR2 and NLRP3 levels decreased from T1 to T2, but remained high in AD patients at T2. High NOD2 expression also persisted and was not affected by withdrawal. The AP-1 subunits c-Fos and c-Jun decreased significantly from T1 to T2, but both remained higher than in controls at T2. Despite evidence that stimulation of the TLR2 and MAPK/AP-1 pathways was not completely abrogated after 18 days of abstinence, the mRNA levels of IL-1β, IL-8, and IL-18 decreased to control levels at T2. Alcohol withdrawal reduced MyD88, but it remained above control levels at T2. High IκBα levels persisted, and no change in the mRNA expression of NFκB subunits, TNFα, and IL-6 levels were found during withdrawal.

Plasma cytokines (TNFα, IL-6, IL-1β, and IL-8) remained higher at T2 in AD than in control subjects (not shown).

Table 4: Changes in the expression of inflammatory signaling molecule mRNA levels during alcohol withdrawal

Signaling Molecules	AD-T1	AD-T2	CT
TLR4	1.82 ± 1.42	1.31 ± .90[a]	1.00 ± .26
CD14	1.65 ± 1.07	1.28 ± .79[a]	1.00 ± .44
MyD88	1.61 ± .80	1.33 ± .60[b,c]	1.00 ± .30
c-Fos	5.13 ± 5.30	3.55 ± 2.98[b,d]	1.00 ± .69
c-Jun	3.02 ± 2.41	2.51 ± 3.14[b,c]	1.00 ± .62
IL-1β	4.00 ± 7.34	2.06 ± 2.91[b]	1.00 ± .59
IL-8	7.32 ± 13.44	1.33 ± 1.08[e]	1.00 ± .57
IL-18	1.58 ± .93	1.29 ± .80[b]	1.00 ± .42
TLR2	2.69 ± 1.53	2.25 ± 1.05[b,d]	1.00 ± .25
NOD2	2.40 ± 1.64	2.35 ± 1.52[d]	1.00 ± .49
NLRP3	3.02 ± 2.24	2.32 ± 1.39[b,d]	1.00 ± .36

Data are means ± SD normalized for controls. AD-T1 and AD-T2 refer to the alcohol-dependent group at the beginning and at the end of withdrawal, respectively; CT: control group. *a* $p < .01$ compared with AD-T1; *b* $p < .05$ compared with AD-T1; *c* $p < .05$ compared with CT subjects; *d* $p < .001$ compared with CT subjects; *e* $p < .001$ compared with AD-T1.

2.5 Discussion

Inflammation might play a role in the development of several psychiatric disorders, including major depression, schizophrenia, and autism (1, 33-36). In alcohol dependence, several preclinical and clinical studies have also suggested that inflammation plays a role in addictive behaviors (2, 37-39).

To elucidate the contribution of PBMCs to inflammation, we analyzed pro-inflammatory cytokines and their intracellular signaling pathways in PBMCs of 63 non-cirrhotic, actively drinking AD subjects. AD subjects had elevated plasma PGN, IL-1β, and IL-8 levels and increased mRNA expression of IL-1β, IL-8 and IL-18 in PBMCs. Induction of these cytokines were likely related to increased expression and activation of the TLR2 receptors, as well as activation of the transcription factor AP-1 and the NLRP3 inflammasome, but to a lesser extent to LPS stimulation of the TLR4-MyD88-dependent pathway (Figure 5). Up-regulation of IL-1β and IL-8 mRNA levels positively correlated with alcohol consumption and craving. Alcohol withdrawal was associated with recovery of LPS- but not PGN-dependent receptors.

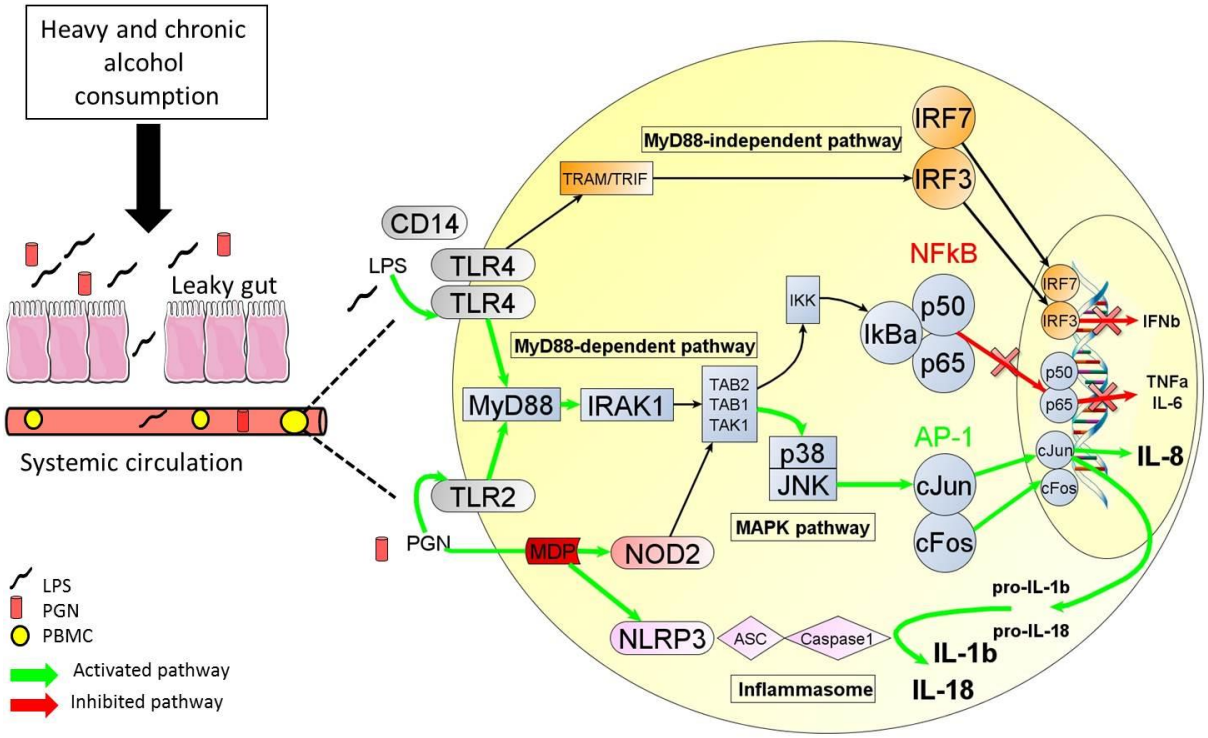


Figure 5: Proposed working model of the Toll-like receptor (TLR) and Nod-like receptor (NLR) signaling pathways in peripheral blood mononuclear cells (PBMCs) of non-cirrhotic alcohol-dependent (AD) subjects. AD subjects present with increased intestinal permeability (leaky gut) that favors the translocation of bacterial components (LPS and PGN) from the gut lumen to the systemic circulation. The gut-derived bacterial products interact with their respective receptors (TLR4 and TLR2, NOD2) located in PBMCs and activate downstream signaling. These events principally result in activation of the MAPkinase-AP1 transcription factor pathway as well as in activation of the inflammasome leading to secretion of the pro-inflammatory cytokines interleukin (IL)-1β, IL-8 and IL-18. IL-1β and IL-18 are first transcripted as precursors (pro-IL1β and pro-IL-18) and then converted into mature and bioactive cytokines by the inflammasome complex. The NFkB pathway and the MyD88-independent interferon pathway are not or only weakly activated. Green arrows represent activated pathways and red arrows represent inhibited pathways. AP-1, activator protein 1; IFN, interferon; IRAK1, IL-1 receptor-associated kinase; IRF, interferon regulatory factor; JNK, c-Jun amino-terminal kinase; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinase; MDP, muramyl dipeptide; MyD88, myeloid differentiation primary-response protein 88; NFκB, nuclear factor kappa B; PGN, peptidoglycan; TNF, tumor necrosis factor.

In AD subjects, the origin and impact of inflammation was mainly attributed to a local effect of alcohol in the brain or the liver. We recently reported increased intestinal permeability, elevated blood LPS, and low-grade systemic inflammation in AD patients, findings that were also related to psychological factors (6), suggesting indirectly that gut-derived bacterial products could play a role in the development of inflammation.

Circulating PBMCs represent an essential defense barrier against gut-derived bacterial products entering the bloodstream that may potentially contribute to systemic inflammation. Since alcohol dependence naturally increases blood LPS (6) and PGN levels, inflammatory pathways were tested in PBMCs without culturing cells or applying pathogen-associated molecular pattern stimulation. The inflammatory response in PBMCs of AD subjects has previously been analyzed only in studies that included low numbers of patients with significant liver disease (40,41). Monocytes that were extracted from these patients were cultured and artificially stimulated with high LPS dose and showed an increase in NFκB activity and TNFα production, in contradiction to our findings. We included only patients that had not developed liver damage to test the effects of alcohol dependence itself and avoid potential bias linked to liver disease. Furthermore, the inflammation was tested under natural conditions. Therefore, our results likely better reflect the physiological impact of gut-derived bacterial products on immune responses of PBMCs in AD subjects.

The transmembrane TLR and the cytoplasmic NLR, responsible for sensing the presence of LPS and PGN, were up-regulated in AD subjects, except for TLR5, which recognizes flagellin from flagellated bacteria (data not shown). PGN-associated components stimulate TLR2 receptors on the plasma membrane and MDP, the minimal bioactive structure of PGN, is commonly detected by the intracellular receptor NOD2 (22,42). From the observation of increased plasma PGN, TLR2, and NOD2 expression in AD patients, it can be concluded that PGN likely crossed the gut barrier and stimulated specific inflammatory pathways in PBMCs, which has not been reported to date. Activation of the receptors was confirmed by exposure of PBMCs in culture to physiologically relevant doses of LPS or PGN during a short time period, showing increased TNFα and IL-1β gene transcription and protein concentrations in culture medium in AD subjects. These observations suggest that inflammatory pathways are more intensely activated by LPS and PGN in AD than in control cells.

We next assessed the intracellular signaling pathways regulated by these receptors. TLRs activate MyD88-dependent and independent intracellular signaling pathways and usually involve the NFκB proinflammatory pathway leading to TNFα and IL-6 transcription, and the MAPK pathway culminating in AP-1 activation (19). MyD88 mRNA levels and p-IRAK1 protein expression were increased in PBMCs of actively drinking patients, suggesting that chronic exposure to alcohol and TLR ligands stimulate MyD88-dependent pathways. Surprisingly, several elements indicated inhibition of the NFκB proinflammatory cytokines pathway in AD subjects: 1) mRNA levels of NFκB p105 (the p50 precursor) and p65 subunits did not change; 2) IκBα, which inhibits NFκB nuclear translocation, was strongly up-regulated; 3) the DNA binding activity of the p65 subunit was strongly decreased; 4) TNFα and IL-6 mRNA, which are cytokines tightly regulated by NFκB, were reduced or unchanged, respectively. In line with the inhibition of the NFκB proinflammatory pathway are reports of suppression of NFκB activity in response to LPS in animal and in vitro models of chronic alcohol exposure (43,44).

On the other hand, mRNA levels of the cytokines IL-1β and IL-18 and of the chemokine IL-8 were up-regulated in AD subjects, which could not be ascribed to NFκB activation. We observed activation of the MAPK (p38, JNK) and of the AP-1 (c-Fos/c-Jun) pathways, known to be associated with IL-1β, IL-8, and IL-18 transcription (45-47). IL-1β

and IL-18, synthesized as nonfunctional pro-IL-1β and pro-IL-18, need to be cleaved into bioactive cytokines by caspase-1. Caspase-1 belongs to the multiprotein complex inflammasome, containing the adaptor proteins ASC and NLRP3, which may serve as an inflammasome activation marker. Increased NLRP3, IL-1β, and IL-18 mRNA expression, plasma IL-1β protein up-regulation, and secretion of the active form of the IL-1β protein in the culture medium of PBMCs from AD subjects provide evidence for activation of the inflammasome. However, in our study, the ATP/P2X7 receptor pathway did not seem to be involved in inflammasome activation. Finally, we did not find evidence in favor of the activation of the interferon pathway, which can be triggered by TLR4.

The strong correlations observed between these inflammatory factors and the amount of alcohol consumed as well as the total or partial recovery of several factors observed after 18 days of abstinence, suggest that alcohol is likely the main factor in inducing the inflammatory response. The incomplete recovery of the PGN-receptors TLR2 and NLRP3, as well as the absence of modification of NOD2 expression suggest that longer term abstinence might be required to obtain PGN clearance and concomitant down-regulation of PGN-receptors.

Interestingly, mRNA expression of the molecules belonging to the activated inflammatory pathways was positively correlated with alcohol craving and more particularly with the obsessive dimension of craving at T1. IL-8 was found to be the best predictor of the outcome. The correlations disappeared at T2, but positive correlations between difference scores (Δ = T1-T2) of inflammation and craving were observed, suggesting that large recovery of inflammation during withdrawal is associated with a large improvement in alcohol craving (48). This specific relation between the obsession of drinking and inflammation supports the general hypothesis of an involvement of inflammation in anxious symptoms of alcohol dependence (49), as previously observed in other anxious disorders (50).

Our data are consistent with involvement of these gut-activated inflammatory pathways, in particular the PGN-activated pathway, in the pathophysiology of alcohol dependence, at least in active drinkers. It might be less relevant after they have stopped drinking. However, our observation of increased plasma levels of IL-6 and TNFα but inhibition of mRNA levels of these cytokines in PBMCs suggests that PBMCs are not the only source of circulating cytokines. Inflammation likely also arises from other sources as, for instance, in the liver and/or in the gut wall itself. Additional analyses in liver and/or small

bowel biopsies would be required to determine the respective contribution of each compartment to the inflammatory process.

Furthermore, alcohol might also stimulate inflammation directly at the brain level (2). Previous studies performed on animal models supporting a role for inflammation in alcohol dependence have all supported a specific role for LPS, TLR4, and NFKβ pathways (51,52). Furthermore, Alfonso-Loeches et al. (52) showed that heavy alcohol consumption failed to induce AD behaviors and to activate inflammation in the cortex of TLR4-/- knockout mice. Our data, however, suggest a stronger role for PGN than the LPS-activated pathway in human populations. Together, the results of this study support that gut-derived inflammatory pathways are interesting targets to study in order to improve our understanding of the pathophysiology of alcohol dependence. They also represent potent sites of interventions for the treatment of the disease, perhaps by the use of probiotics or prebiotics that might change the composition of the gut microbiota.

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2.7 Supplemental information

Diagnosis of cirrhosis

Liver biopsy is still regarded as the gold standard for grading and staging liver disease. However, this invasive procedure carries the risk of potential life-threatening complications (e.g., bleeding); therefore, for obvious ethical reasons, liver biopsy cannot be justified in all patients for study purposes. All of the subjects were instead systematically evaluated by blood tests (biochemistry, hematology, coagulation), by gastrointestinal endoscopy (to check for signs of portal hypertension such as esophageal and gastric varices), by liver Doppler-ultrasound (to evaluate the shape of the liver, splenomegaly with collateral intra-abdominal venous circulation, or slowed down or inverted portal venous blood flow), and by measurement of transient liver elastography (Fibroscan®). This new noninvasive technique has been designed to quantify liver stiffness, which has been correlated to liver fibrosis grades based on the METAVIR classification system of fibrosis. A validated cut-off has been published and studies clearly show that this technique is reliable in ruling out significant fibrosis and in confirming cirrhosis (1). Accordingly, we selected only F0 and F1 patients (i.e., absence of significant fibrosis) for the study (2,3). Liver biopsy was performed only in patients in whom advanced fibrosis/cirrhosis was suspected after noninvasive work-up; these patients were, by definition, excluded from the study.

References

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Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were isolated from blood by centrifugation on a Ficoll-Paque Plus gradient (GE Healthcare, Uppsala, Sweden). Twenty mL of blood was diluted with Hank's balanced salt solution (HBSS) (Invitrogen, California, USA). Twenty milliliters of the diluted blood was layered on 15 mL of Ficoll-Paque Plus and centrifuged at 400g for 40 minutes. The plasma layer was discarded and the PBMCs layer at the interface was collected, washed with HBSS, and centrifuged at 100g for 10 minutes. The supernatant was discarded and the cell pellet was washed with HBSS and centrifuged at 100g for 10 minutes. PBMCs were then suspended in 1 mL of TRIzol Reagent (Invitrogen) and kept at -80°C until analysis.

Short-term Cell Culture and In Vitro Stimulation

PBMCs from four AD patients and four healthy controls were used to test TLR activation. Isolated PBMCs were cultured in six-well plates in RPMI-1640 culture medium (700,000 cells/mL) containing L-glutamine (Sigma-Aldrich, Schnelldorf, Germany), 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 µg/mL) (all Life Technologies) in a 5% CO2-humidified incubator at 37°C for 3 h. Thereafter, cells were stimulated with 1, 5, and 10 ng/mL of either LPS from Escherichia coli (Sigma-Aldrich) or PGN from Micrococcus luteus (Sigma-Aldrich) for 6 h and resuspended in TRIzol Reagent for determination of TNFα (LPS stimulation) and IL-1β (PGN stimulation) mRNA levels. Culture medium was recovered for measurements of TNFα and IL-1β protein levels (QuantiGlo R&D Systems, Minneapolis, MN, USA).

Plasma ATP measurement

Blood was drawn from antecubital vein and collected into EDTA tube. Plasma was diluted 1:25 using Hepes-MgCl2-KOH buffer (pH 7.75) and ATP was measured with the ATP Bioluminescence Assay Kit CLS II (Roche, Mannheim, Germany) following manufacturer's instructions.

Assessment of alcohol craving

The Obsessive-Compulsive Drinking Scale (OCDS) is a self-report questionnaire that measures the cognitive aspects of alcohol craving during the preceding 7 days. This questionnaire comprises a total of 14 items, which can be divided into two subscales, a 6-item "obsessive" subscale (e.g., How much of your time where you are not drinking is occupied by

ideas, thoughts, impulses, or images related to drinking?) and an 8-item "compulsive" subscale (e.g., How much of an effort do you make to resist consumption of alcoholic beverages?). Participants responded to each OCDS item on a Likert scale ranging from 0 to 4. Four compulsive items are related to alcohol consumption (e.g., How many drinks do you drink each day?). Because alcohol was prohibited during withdrawal, these items were eliminated and a modified 4-item "compulsive" subscore and a modified 10-item "total" score were computed. Correlations between alcohol craving and inflammation were performed with the modified scores to avoid bias linked to alcohol consumption. A validated French version was administered to all participants (4).

Reference

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Statistical analysis

Data for transcription factor activation, plasma PGN concentrations, and densitometry were analyzed with non-parametric tests (Mann-Whitney test to compare AD to CT subjects). When the levels of plasma cytokines were below the minimal detectable concentration, we drew a 2 X 2 contingency table in which numeric values were transformed into categorical variables (below and above the limit of assay sensitivity), and Fisher's exact test on frequencies was applied to compare AD and CT.

As several possible parameters were found to predict the craving, a more detailed statistical analysis to test the association between craving (outcome) and inflammation (predictor) using a multiple regression model was performed. In order to limit the number of predictors to improve the quality of the model, it was decided to enter in our model only the inflammatory parameters that were significantly associated with alcohol craving (IL-1β, IL-8, c-Fos, c-Jun, NLRP3, NOD2 and IκBα) and a Stepwise method was used which is the appropriate method for exploratory model building.

Supplemental table 1 : Primer sequences used for real-time quantitative PCR

Target Gene	Access Number	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
CD14	NM_000591	CGCTCCGAGATGCATGTG	AGCCCAGCGAACGACAGA
c-Fos	NM_005252	CGAGCCCTTTGATGACTTCCT	GGAGCGGGCTGTCTCAGA
c-Jun	NM_00584	GTGAAAACCTTGAAAGCTCAGAACT	TTAACGTGGTTCATGACTTTCTGTTTA
IFNβ	NM_002176	CCAACAAGTGTCTCCTCCAAATT	GTAGGAATCCAAGCAAGTTGTAGCT
IL-18	NM_001562	CCAAGGAAATCGGCCTCTATT	CTTCACAGAGATAGTTACAGCCATACCT
IL-1β	NM_000576	GCACGATGCACCTGTACGAT	AGACATCACCAAGCTTTTTTGCT
IL-6	NM_000600	TGTAGCCGCCCCACA CA	CCGTCGAGGATGTACCGAAT
IL-8	NM_002228	CTAGGACAAGAGCCAGGAAGAAAC	CGGCCAGCTTGGAAGTCA
IRF3	NM_001571	AGGCCACTGGTGCATATGTTC	GGGCAGAGCGGAAATTCC
IRF7	NM_001572	TACCATC TACCTGGGCT TCG	AGGGTTCCAGCTTCAC
ISG6-16	NM_002038	CAGCAGCGTCGTCATAGGTAAT	CCTCATCCTCCTCACTATCGAGAT
IκBα	NM_020529	GCTGAAGAAGGAGCGGCTACT	TCGTACTCCTCGTCTTTCATGGA
MyD88	NM_002468	CATATGCCTGAGCGTTTCGA	CATCGCGGTCAGACACACA
NLRP3	NM_001079821	TGCCCCGACCCAAACC	GAAGCCGTCCATGAGGAAGA
NOD2	NM_0221621	GCCACGGTGAAAGCGAAT	GGAAGCGAGACTGAGCAGACA
OAS1	NM_001032409	CCACCTGCTTCACAGAACTACAGA	AGGCGGATGAGGCTCTTGA
p105	NM_003998	GGCTACACCGAAGCA ATTGAA	CAGCGAGTGGGCCTGAGA
p65	NM_021975	CTGTGCGT GTCTCCATGCA	TCGTCTGTATCTGGCAGGTACTG
P2X7	NM_002562	TCTTCGTGATGACAAACTTTCTCAA	GTCCTGCGGGTGGGATACT
PANX1	NM_015368	GCTGCATAAGTTTTTCCCCTACA	TGCGAAACGCCAGAACAG
TLR2	NM_003264	TCCTGTGCCACC GTTTCC	GA GCTTTCCTGG GCTTCCTT
TLR4	NM_138554	GATTGCTCAGACCT GGCAGTT	TGTCCTCCC ACTCCAGGTAAGT
TNFα	NM_000594	GGAGAAGGGTGACCGA CTCA	TGCCCAGACTCGG CAAAG

Supplemental table 2 : Antibodies and working conditions for Western blotting

Antibody	Working Conditions	Source
IRAK1 Rabbit mAb	1:500, overnight, 4°C	Cell Signaling Technology
Phospho-IRAK1 Rabbit Polyclonal Ab	1:1000, overnight, 4°C	Santa Cruz
IκBα Rabbit mAb	1: 500 overnight, 4°C	Cell Signaling Technology
p38 Rabbit Polyclonal Ab	1:500, overnight, 4°C	Cell Signaling Technology
Phospho-p38 MAPK Rabbit mAb	1:500, overnight, 4°C	Cell Signaling Technology
JNK Rabbit Polyclonal Ab	1:1000, 2 h, RT	Santa Cruz
Phospho-SAPK/JNK Rabbit mAb	1:500, overnight, 4°C	Cell Signaling Technology
IRF3 Rabbit mAb	1:1000, overnight, 4°C	Cell Signaling Technology
phospho-IRF3 Rabbit mAb	1:1000, overnight, 4°C	Cell Signaling Technology
Peroxidase Conjugated Affinipure Goat Anti-rabbit IgG	1:20000, 1 h, RT	Jackson Immunology Research

RT, room temperature.

2.8 Complementary discussion and conclusion

The increased mRNA expression of TLR4 and TLR2 receptors on PBMCs leads us to suggest that LPS and PGN were both present in blood. Plasma LPS levels in alcohol-dependent subjects have already been assessed in our previous study (Chapter 1) and were found to be higher than in healthy subjects. However, the presence of PGN in blood of alcohol-dependent subjects has been poorly described in the literature. We measured, in PBMCs, the mRNA expression of NOD2 which is an intracellular marker of the presence of PGN and found that it was largely increased in alcohol-dependent subjects, which suggest that PGN were actually circulating in blood of patients. Finally, we assessed the plasma PGN by using a commercially available ELISA kit. The results of the assay indicated that plasma PGN levels were significantly higher in the alcohol-dependent group compared to controls. Information provided by the manufacturer revealed that the specificity of the assay is limited by the fact that cross-reactivity with analogue compounds is possible. Therefore, we are not sure to measure reliably the PGN concentrations in blood as we are limited by the current technical skills. This kit was the only way to assess the PGN levels, but indirect markers (TLR2 and NOD2) confirm our hypothesis that PGN may contribute to the systemic inflammation in alcohol-dependent subjects.

Although LPS and PGN are present in the systemic circulation, it was essential to check whether they actually activate their receptors expressed on PBMCs. Therefore, PBMCs of alcohol-dependent and control subjects were cultured in presence of very low concentration of LPS and PGN and pro-inflammatory cytokines were then measured in the medium or in the cells to evaluate receptors activation. We were attentive to minimize the duration of culture and the cells were stimulated with expected “physiological” doses of LPS and PGN ranking from 0,1 to 10 ng/ml which were at least 1000 times less concentrated than doses commonly used (10µg/ml) in other studies to assess the inflammatory response. As alcohol-dependence is a condition that “naturally” increased the plasma levels of LPS and PGN, the cells that we extracted from the patients have already been in contact and “primed” by the bacterial toxins. This may explain why it was not necessary to over-accentuate the inflammatory response with extra artificial pathogen stimulation to observe a response. In keeping with our observation, shortly after the publication of this study, this concept of physiological stimulation was applied by American researchers where they tested the effect of acute binge drinking on bacterial translocation and immune response in healthy humans and showed that physiological

dose of LPS (100 pg/ml) is sufficient to induce an increase in inflammatory cytokines [250]. In the second part of the study, the expression of molecules involved in the inflammatory pathways (receptors, adaptor molecules, kinases, transcription factors, cytokines) were always measured under naturalistic conditions, without culturing cells or applying bacterial toxin stimulation. Our results are therefore expected to reflect the real and the pathophysiological impact of gut-derived bacterial products on the inflammatory response.

In these conditions, we found strong evidences of NFκB-pathway inhibition, which is in contrast with a lot of previous experimental studies that consider NFκB as the key transcription factor responsible for inflammation, in particular in animal model of chronic alcohol consumption. This discrepancy reminds us that *in vitro* models could be far away from physiological conditions and that results obtained in animal models are not inevitably transposable to humans, particularly in psychiatric diseases such as alcohol-dependence. On the other hand, the MAPK/AP-1 pathway was largely activated in alcohol-dependent subjects and was suggested to up-regulated the synthesis of IL-1β and IL-8.

As for the study described in Chapter 1, we assessed the effect of a short-term alcohol withdrawal on the inflammatory pathways. We found that abstinence was associated with a total recovery of LPS-dependent receptors CD14/TLR4. This is consistent with the total recovery of plasma LPS levels observed in the first study. By contrast, the PGN-dependent receptors TLR2, NOD2 and NLRP3 remained largely expressed after 3 weeks of abstinence. This suggests that PGN are still present in blood at the end of the detoxification program and could contribute to the persistence of plasma inflammatory cytokines at T2. Unhappily, the direct measure of PGN in the plasma of abstinent subjects was not performed in the study because we did not have plasma samples collected in an appropriate way.

Interestingly, the decrease in inflammatory markers, in particular IL-8, during alcohol withdrawal correlated with the improvement of craving scores, suggesting that inflammation might play an important role in the drinking behavior. IL-8 is also called “neutrophil chemotactic factor” meaning that it is able to induce chemotaxis of neutrophils and other inflammatory cells which therefore move toward the site of infection. In this study, we analyzed the inflammatory pathways in PBMCs, which consist in monocytes and lymphocytes. These cells were obtained by using a Ficoll gradient that excluded neutrophils from the whole blood. However, this important class of immune cells which express TLRs

could also play a role in the induction of inflammation following LPS and PGN exposure. Monocytes levels are higher in alcohol-dependent patients compared to healthy controls while the levels of neutrophils did not differ significantly from both groups (fig 1-1). However, after splitting the AD subjects into "high" and "low" intestinal permeability groups (see Chapter 3 for detailed explanations), we found that the monocytes and neutrophils levels were actually higher in subjects with "high" intestinal permeability compared with controls (fig 1-1). Also, among all white blood cells, monocytes and neutrophils positively correlated with gut permeability ($r = 0.37$, $p < 0.05$ for monocytes; $r = 0.42$, $p < 0.05$ for neutrophils, $n = 28$).

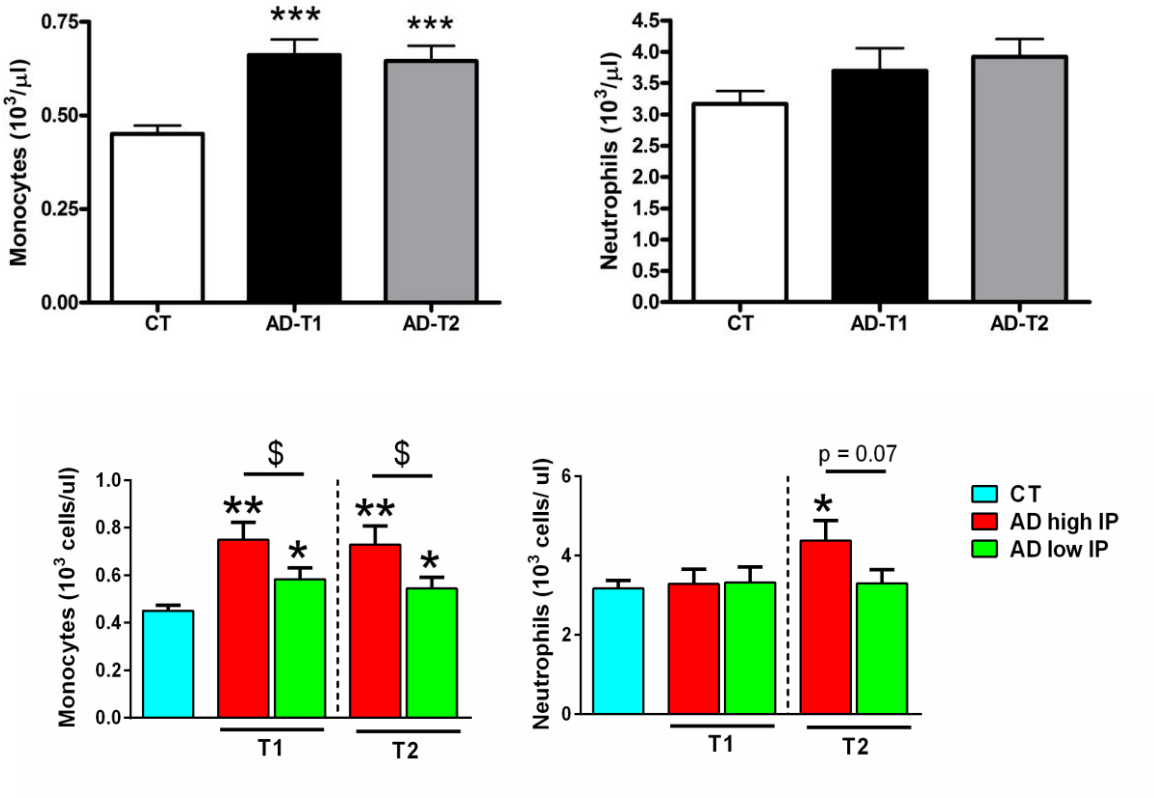

Figure 1-1. Blood monocytes and neutrophils levels (absolute values) measured in healthy controls (CT) and alcohol-dependent subjects (AD) with high (red) and low (green) intestinal permeability (IP) at the beginning (T1) and end (T2) of alcohol withdrawal. * $p < 0.05$ vs CT, ** $p < 0.01$ vs CT, *** $p < 0.001$ vs CT. $ $p < 0.05$ AD high IP vs AD low IP.

Regarding the significant correlations between inflammation and craving, we decided to investigate whether inflammation could have an impact on cognitive and executive functions that are known to be involved in drinking motivation. Experiments are in progress.

The conclusions and perspectives of Chapter 2 are the following:

- In addition to LPS, the peptidoglycans cross the gut barrier and stimulate specific inflammatory pathways in PBMCs.
- Under “natural” physiopathological conditions, NF-κB is strongly inhibited whereas MAPK/AP-1 pathway and inflammasome are activated.
- PBMCs may contribute to the enhanced plasma level of IL-1β and IL-8, but are not the source of systemic TNFα and IL-6 observed in AD subjects. Additional analyses of liver tissue and intestinal biopsies would be required to determine the potential source of systemic TNFα and IL-6.
- The improvement of craving score during detoxification correlates with the decrease in inflammatory markers, in particular IL-8. This suggests that inflammation may influence drinking behavior.
- As IL-8 is a “neutrophils chemotactic factor”, we suggest testing in future studies the inflammatory pathways in neutrophils and their potential contribution in behavioral changes.

3 CHAPTER 3

INTESTINAL PERMEABILITY, GUT BACTERIAL DYSBIOSIS, AND BEHAVIORAL MARKERS OF ALCOHOL DEPENDENCE SEVERITY

Recent findings support that the gut microbiota plays a key role in the onset of gut barrier alterations and inflammation. Dysbalance of the gut microbiota composition has been reported in somatic diseases such as obesity and metabolic disorders, intestinal bowel diseases, allergy. More recently, dysbiosis has also been shown in animal models of autism and alcohol dependence as well as in psychiatric subjects. These observations support the hypothesis that the gut bacteria may interact with the central nervous system and influence mood and behavior.

The originality of this study resides in the fact that we have analyzed in parallel the gut microbiota – from a taxonomic and metabolomics point of view, the intestinal permeability, the systemic inflammation and the psychological symptoms of alcohol-dependent subjects. To our knowledge, this is the first report to combine all these parameters in one clinical study. The main strength of this study is that gut microbiota has been analyzed in details. On the one hand, the composition of gut bacteria has been investigated by using two complementary culture-independent approaches: the next-generation sequencing of the 16S rRNA gene and the quantitative PCR. On the other hand, the functionality of the bacteria inhabiting the gut has been assessed by using metabolomics analysis. Identifying the bacterial changes that occurs upon chronic alcohol consumption is an important step. However, it is likely that the gut-brain interactions are mediated by the metabolites produced by the bacteria rather than bacteria themselves. Therefore, the metabolomic analysis was useful to identify bacterial products that are suspected to have beneficial or detrimental effects on the host health.

The results related to this chapter have been published in *Proceedings of the National Academy of Sciences* (PNAS) *21;111(42):E4485-93.*

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3.1 Abstract

Alcohol dependence has traditionally been considered a brain disorder. Alteration in the composition of the gut microbiota has recently been shown to be present in psychiatric disorders, which suggests the possibility of gut-to-brain interactions in the development of alcohol dependence. The aim of the present study was to explore whether changes in gut permeability are linked to gut microbiota composition and activity in alcohol-dependent subjects. We also investigated whether gut dysfunction is associated with the psychological symptoms of alcohol dependence. Finally, we tested the reversibility of the biological and behavioral parameters after a short-term detoxification program. We found that some, but not all, alcohol-dependent subjects developed gut leakiness, which was associated with higher scores of depression, anxiety, and alcohol craving after 3 weeks of abstinence, which may be important psychological factors of relapse. Moreover, subjects with increased gut permeability also had altered composition and activity of the gut microbiota. These results suggest the existence of a gut-brain axis in alcohol dependence, which implicates the gut microbiota as an actor in the gut barrier and in behavioral disorders. Thus, the gut microbiota appears to be a new target in the management of alcohol dependence.

3.2 Introduction

Alcohol consumption is the world's third largest risk factor for disease and disability and accounts for 5.9% of all deaths worldwide (1). Although alcohol exerts large deleterious effects on health, studies to date on the pathophysiology of alcohol dependence have mainly focused on the influence of alcohol consumption on neuronal functions in the brain (2). A limited number of studies have, however, suggested that gut functions might also be altered by chronic alcohol consumption (3,4). Accordingly, we and others have shown that actively drinking alcohol-dependent (AD) subjects exhibited increased intestinal permeability (IP) and increased plasma levels of gut-derived bacterial products such as lipopolysaccharides and peptidoglycans (5–8). These bacterial products activate specific inflammatory pathways that partially recover after a 3-week period of alcohol abstinence (5,6). These recent observations indirectly suggest the possibility that the composition of gut microbiota could be altered in AD subjects and related to behavioral symptoms.

The human gut microbiota consists of a complex community exceeding 100 trillion microorganisms (9) whose collective genome—the microbiome—encodes 100 times more genes than the human genome (10). It is now widely accepted that the gut microbiota should be considered an "exteriorized" organ placed within the body, which provides important physiological functions and is indispensable for human life (10–12). However, the microbial composition or activity of the gut can be modified by diet, antibiotic use, host genetics, and other environmental factors (13). Data suggest that an imbalance of the intestinal microbiota, known as dysbiosis, may contribute to a variety of somatic diseases such as obesity (14), type 2 diabetes (15), inflammatory bowel diseases (16,17), and allergy (18).

Recent studies suggest that the gut bacteria also influence brain functions and behavior and may therefore play a role in the development of psychiatric disorders (19). Indeed, in experimental studies, researchers observed that germ-free mice displayed reduced anxiety-like behavior compared with mice with normal gut microbiota, demonstrating evidence of gut-to-brain interactions (20,21). Further studies brought forward evidence that the pathways underlying the gut-brain axis are multiple and highly complex, involving brain biochemistry, the vagus nerve, pro-inflammatory cytokines and tryptophan metabolism, (22). Furthermore, inflammation and tryptophan/kynurenine pathways have been related to the development of

depression-like behavior (23–26). In addition, gut bacteria produce neurotransmitters (serotonin, GABA, dopamine, acetylcholine), and bacterial fermentation of dietary fiber induces the release of short-chain fatty acids, which are metabolites with potential neuroactive properties (22). Recent evidence also suggests that *Bacteroides fragilis* may prevent autism spectrum disorder in a mouse model (27) and administration of probiotic *Bifidobacterium infantis* may have anti-depressant properties in rats through changes in tryptophan/kynurenine pathway (26). Although several animal studies support a relation between the gut microbiota and behavior, major questions remain regarding this relation in human health.

Depression and anxiety frequently develop in actively drinking AD subjects and play an important role in the negative reinforcement of drinking tendency (28). These factors are strongly related to the urge to drink, hereafter referred to as alcohol craving (29,30), an important predictor of relapse after detoxification (31). The possibility that these psychological symptoms of addiction are related to a dysbiosis has so far never been investigated. The aim of the present study was to determine whether gut permeability could be associated to the severity of psychological symptoms (depression, anxiety and craving) developed by human AD subjects. Then, we assessed the composition and activity of the gut microbiota and tested whether they are related to gut permeability. Finally, we analyzed whether alterations in gut permeability, microbiota composition and metabolome are reversible after 3 weeks of alcohol withdrawal, which is known to induce partial recovery of psychiatric symptoms (32).

3.3 Results

IP is increased in a subset of AD subjects

Intestinal permeability was measured by using the ^{51}Cr-EDTA method. Results revealed that, at the 2nd day of alcohol withdrawal (T1), 26 of 60 (43%) patients had elevated gut permeability, whereas the remaining 34 (57%) patients had normal gut permeability compared with control subjects (Figure 1A). Subjects were therefore split into 2 groups: AD patients with “high” IP and AD patients with “low” IP. This separation of subjects was calculated according to a deviance criterion at a threshold of 1.65 standard deviations of the mean of the control group. In a normal distribution, this corresponds to the 5th percentile, which is a common threshold to highlight deviance from the mean. The gut permeability level was not related to the amount of alcohol consumed, which was similar in both subgroups of patients (P = 0.72).The demographic characteristics of the subjects included in this preliminary study are shown in Table 1. After 19 days of alcohol abstinence (T2), the gut permeability of AD subjects with high IP decreased significantly, the mean being equivalent to that observed in the control group and in the group of AD subjects with low IP at T1 (Figure 1B). The detailed results of small bowel and colon permeabilities are presented in Table S1.We also examined the type of alcoholic beverages consumed by each subject and found that, on average, the consumption of beer was similar in both groups of AD patients (with high and low IP). However, the consumption of wine tended to be lower in AD subjects with high IP and the consumption of spirits tended to be higher in AD subjects with high IP (Figure S1).

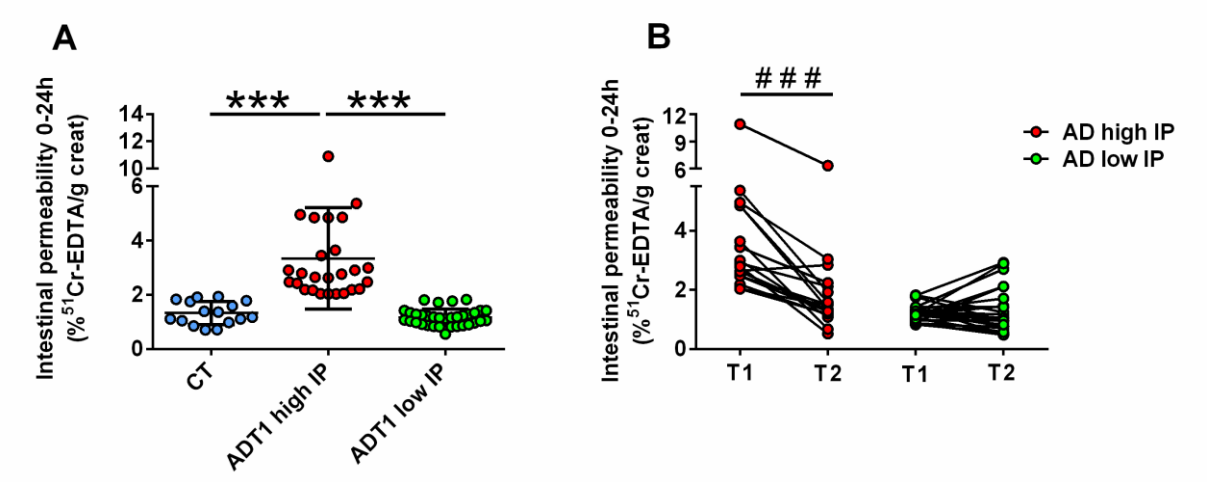

Figure 1 : Intestinal permeability was measured by using the ^{51}Cr-EDTA method. (A) Results revealed that, at T1, 26 of 60 patients had elevated gut permeability, whereas the remaining 34 patients had normal gut permeability compared with control subjects. Subjects were therefore split into 2 groups: AD patients with "high" IP and AD patients with "low" IP. (B) A 3-week alcohol withdrawal induced a total recovery of gut permeability in AD subjects with high IP. Subjects that relapsed during the detoxification program were excluded from analysis and results were obtained in 43 subjects. CT: control subjects; AD: alcohol dependent subjects; IP: intestinal permeability. T1 and T2 refer to the beginning and end of alcohol withdrawal, respectively. *** and ### $P < 0.001$

Table 1: Demographic and clinical characteristics of the control and alcohol-dependent groups in the preliminary and main studies. Data are means ± SD. AD high IP and AD low IP refer to alcohol-dependent subjects with high and low intestinal permeability, respectively. CT refers to the control group. M: male; F: female; nd: not defined.*** $P < 0.001$ (AD vs. CT).

Study data	CT	AD high IP	AD low IP
Preliminary study			
No. of subjects	15	26	34
Sex	8M/7F	19M/7F	28M/6F
Age, y	48 ± 11	48 ± 11	48 ± 10
BMI, kg/m^2	26.1 ± 3.1	23.3 ± 3.9	24.8 ± 4.7
Alcohol intake, g/d	11 ± 7	188 ± 84***	177 ± 104***
Main study			
No. of subjects	15	6	7
Sex	8M/7F	3M/3F	5M/2F
Age, y	48 ± 11	52 ± 9	52 ± 10
BMI, kg/m^2	26.1 ± 3.1	24.6 ± 4.5	26.4 ± 5.3
Alcohol intake, g/d	11 ± 7	126 ± 55 ***	145 ± 86 ***
Albumin, g/dL	nd	4.40 ± 0.36	4.46 ± 0.30
Prealbumin, mg/dL	nd	30.7 ± 6.8	34.4 ± 5.4

Gut barrier alteration is associated with the persistence of psychological symptoms at the end of alcohol withdrawal

We assessed the psychological status of AD subjects, as alcohol dependence is firstly a psychiatric disorder. At the beginning of detoxification, all psychological scores (depression, anxiety, and alcohol craving) were higher in AD subjects than in controls, as described in our previous study (4). Alcohol withdrawal is known to be associated with an improvement in psychological symptoms. Indeed, we found that at the end of the detoxification, the depression and anxiety scores of AD subjects with low IP recovered completely and returned to the same level as that of controls. However, the AD subjects with high IP were still characterized by higher levels of depression, anxiety, and craving (Figure 2A). Correlation analysis revealed that IP measured at the beginning of withdrawal was positively associated with all of the psychological symptoms measured at the end of the detoxification program (Figure 2B). These results suggest that the gut barrier function could be involved in the persistence of psychological symptoms after detoxification.

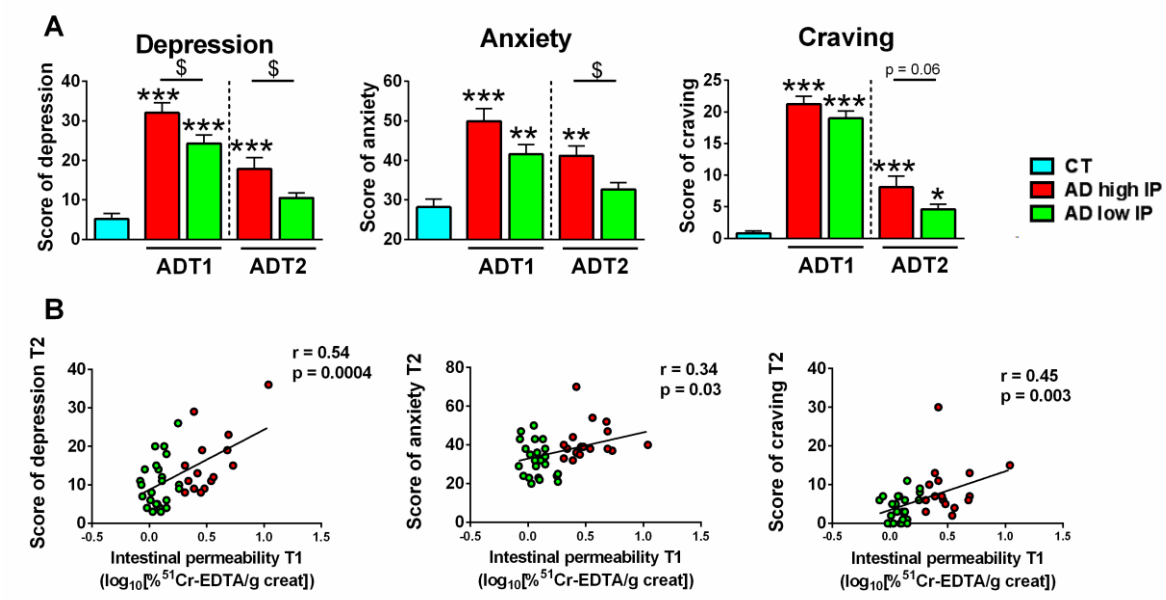

Figure 2: Increased intestinal permeability of AD subjects was associated with the persistence of psychological symptoms at the end of alcohol withdrawal. (A) Scores of psychological factors in CT and AD subjects at the beginning (T1) and end (T2) of withdrawal showing that AD subjects with high IP had higher score of depression, anxiety, and alcohol craving at T2. * $P < 0.05$ vs. CT; ** $P < 0.01$ vs. CT; *** $P < 0.001$ vs. CT; \$ $P < 0.05$. (B) Associations of IP measured at T1 with psychological factors assessed at T2. Values are Pearson's moment correlation coefficients. AD subjects with high IP and low IP are depicted in red and green, respectively. IP: intestinal permeability; CT: control; AD: alcohol dependent.

The gut microbiota profile is altered in AD subjects with high IP

In a subset of 13 AD subjects, we analyzed the gut microbiota composition and functionality and tested whether they could be related to the gut permeability. The demographic and clinical characteristics of these 13 subjects, which were also split into high and low IP groups, are shown in Table 1. Both groups of AD subjects had elevated concentrations of all inflammatory markers (TNFα, IL-1β, IL-6, IL-8 and IL-10) and of high-sensitivity C-reactive protein (Figure S2). However, the level of IL-8 was significantly higher in AD subjects with high IP than it was in AD subjects with low IP, and this cytokine was positively correlated with IP ($r = 0.79$, $p = 0.01$).

The overall gut microbiota composition was analyzed by pyrosequencing of the 16S rRNA gene, and profiles of microbial abundance were obtained from each subject. Nonmetric multidimensional scaling revealed that the bacterial profiles of AD subjects with a high IP differed from those of the controls and the AD subjects with low IP (Figure 3A). We then investigated which bacterial groups were responsible for the changes observed in the profile of AD subjects with high IP. We did not find significant differences between the 3 groups of subjects at the phylum level of the bacteria. However, at the family level, bacteria from Ruminococcaceae and Incertae Sedis XIII were less abundant, while those from Lachnospiraceae and Incertae Sedis XIV were more abundant in AD subjects with high IP compared with AD subjects with low IP and controls (Figure 3B). At the genus level of the bacterial groups, AD subjects with high IP had a drastic decrease in the abundance of *Ruminococcus*, *Faecalibacterium*, *Subdoligranulum*, *Oscillibacter*, and *Anaerofilum*. All of these genera belong to the Ruminococcaceae family. The abundance of *Dorea*, which belongs to the family Lachnospiraceae, was increased in AD subjects with high IP. Additionally, the genera *Blautia* and *Megasphaera* were increased, whereas Clostridia was decreased in AD subjects with high IP (Figure 3B). These analyses also revealed that the relative abundance of the taxa mentioned above was similar in AD subjects with low IP and the controls.

The abundance of common bacterial species was also assessed by using quantitative PCR (qPCR) (Figure 3C). We found that the total amount of bacteria was significantly lower in AD subjects with high IP compared with the other 2 groups. We then quantified the level of *Faecalibacterium prausnitzii*, a bacterial species known for its anti-inflammatory properties (33), and found that it was drastically decreased (up to 4 log units) in AD subjects with high IP. This result is consistent with those obtained with the pyrosequencing approach. In

addition, *F. prausnitzii* was negatively correlated with plasma IL-8 levels (r = -0.65, P = 0.003) Finally, we assessed the levels of *Bifidobacterium* spp. and *Lactobacillus* spp., and found that the level of *Bifidobacterium* spp. was significantly lower in AD subjects with high IP compared with controls and AD subjects with low IP. The decrease was not significant for the level of *Lactobacillus* spp.

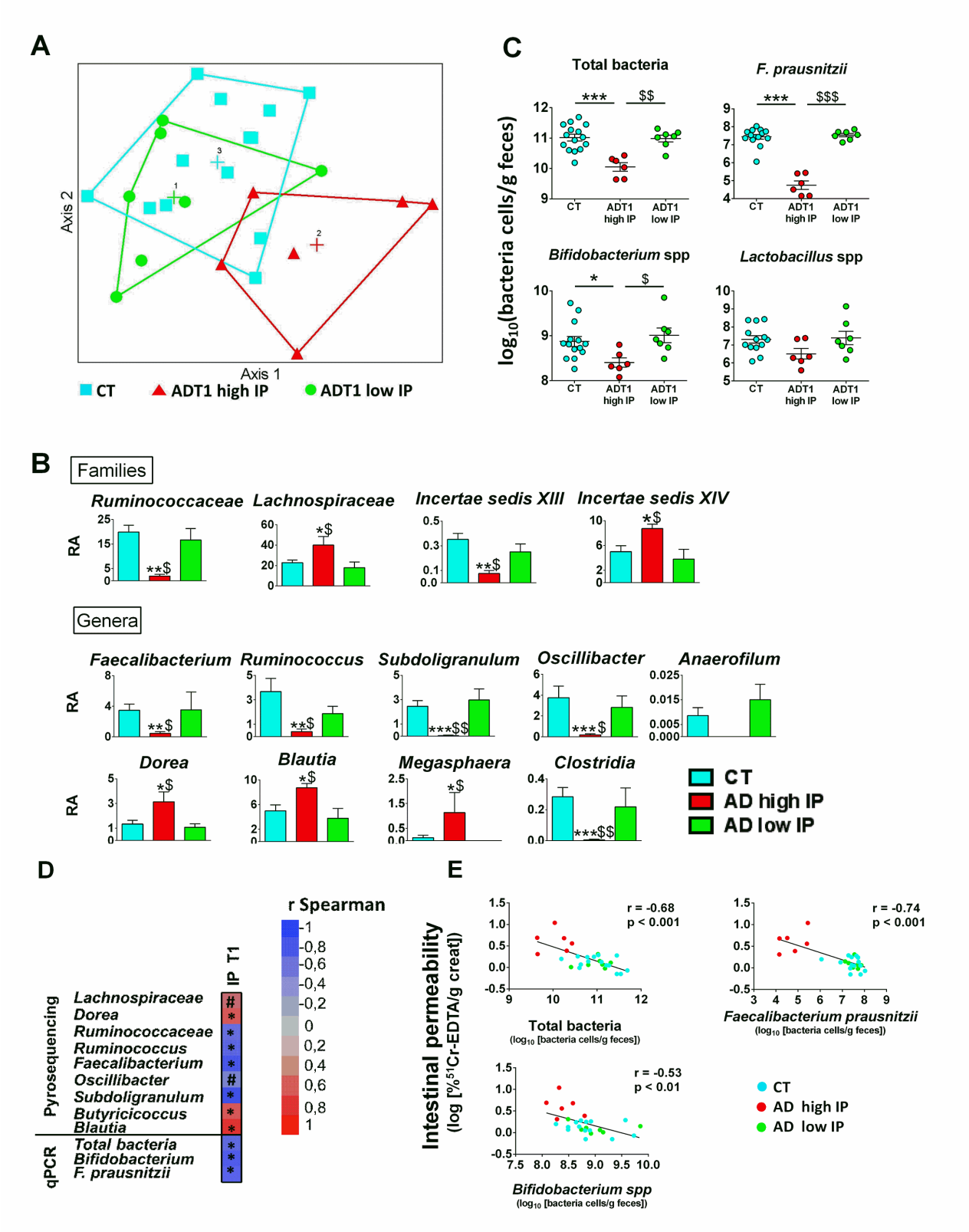
A
Axis 2
Axis 1
CT
ADT1 high IP
ADT1 low IP
C
Total bacteria
F. prausnitzii
Bifidobacterium spp
Lactobacillus spp
log10(bacteria cells/g feces)
B
Families
Ruminococcaceae
Lachnospiraceae
Incertae sedis XIII
Incertae sedis XIV
RA
Genera
Faecalibacterium
Ruminococcus
Subdoligranulum
Oscillibacter
Anaerofilum
Dorea
Blautia
Megasphaera
Clostridia
CT
AD high IP
AD low IP
D
r Spearman
IP T1
Pyrosequencing
qPCR
Lachnospiraceae
Dorea
Ruminococcaceae
Ruminococcus
Faecalibacterium
Oscillibacter
Subdoligranulum
Butyricicoccus
Blautia
Total bacteria
Bifidobacterium
F. prausnitzii
E
Intestinal permeability
(log [%51Cr-EDTA/g creat])
r = -0.68
p < 0.001
Total bacteria
(log10 [bacteria cells/g feces])
r = -0.74
p < 0.001
Faecalibacterium prausnitzii
(log10 [bacteria cells/g feces])
r = -0.53
p < 0.01
Bifidobacterium spp
(log10 [bacteria cells/g feces])
CT
AD high IP
AD low IP

Figure 3: Gut microbiota profiles of AD subjects with high and low intestinal permeability at the beginning of alcohol withdrawal. (**A**) Gut bacterial profiles were calculated for each subject using the abundance of the bacterial families from 454 pyrosequencing data. Bacterial taxa for which the sum of sequences in all the samples was less than 0.01% of the total number of sequences were removed from the analysis. Hellinger transformation was applied to the resulting matrix. Subjects were plotted in the map by using nonmetric multidimensional scaling. (**B**) Relative abundance of bacterial families and genera at the beginning of withdrawal. No significant differences were observed between AD subjects with low IP and controls. Differences observed between AD subjects with high IP and the other 2 groups are depicted. Results of relative abundance obtained from pyrosequencing are expressed in % sequences/taxon. * $P < 0.05$ vs. CT, ** $P < 0.01$ vs. CT, *** $P < 0.001$ vs. CT, $ $P < 0.05$ vs. ADT1 low IP, $$ $P < 0.01$ vs. ADT1 low IP. (**C**) Total bacteria, *F. prausnitzii*, *Bifidobacterium* spp., and *Lactobacillus* spp. were quantified by qPCR in CT and AD subjects at the onset (T1) of alcohol withdrawal. * and $ $P < 0.05$, $$ $P < 0.01$, *** and $$$ $P < 0.001$. (**D**) Chart depicting the correlations between IP and gut bacteria detected by pyrosequencing and qPCR methods at the beginning of detoxification. * indicates significant correlations ($P < 0.05$) and # $0.1 < P < 0.05$. (**E**) Correlations between IP at T1 and gut bacteria measured by qPCR. r indicates Pearson's coefficient. AD subjects with high IP and low IP are depicted in red and green, respectively. IP: intestinal permeability; RA: relative abundance; AD: alcohol dependent; CT: control. T1 refers to the beginning of alcohol withdrawal.

An altered microbiota composition is associated with gut barrier dysfunction

As only the AD subjects who presented increased IP had an altered gut microbiota composition compared with control subjects, we hypothesized that some bacteria could be involved in the regulation of the gut barrier function. We therefore tested the correlations between gut bacteria and IP at T1 (Figure 3D,E). Our analysis revealed a negative correlation between IP and the total amount of bacteria, indicating that subjects with a low number of bacteria in the gut had a higher IP. Negative correlations were also found for the bacteria belonging to the Ruminococcaceae family, especially for *F. prausnitzii*. *Bifidobacterium* was also negatively correlated with IP. The genera *Dorea* and *Blautia* that were increased in AD subjects with high IP were positively correlated with IP. These results support the hypothesis that the microbiota composition may influence gut barrier function.

Effect of short-term alcohol withdrawal on gut microbiota composition

The microbial composition of AD subjects was also assessed at the end of a 3-week detoxification program (T2). We found that alcohol abstinence induced a significant increase in Ruminococcaceae in subjects with high IP. The genera *Ruminococcus* and *Subdoligranulum* also increased at T2 although not significantly. However, the family Erysipelotrichaceae and the genus *Holdemania* decreased significantly from T1 to T2 in all AD subjects (Figure 4A). Alcohol withdrawal had no impact on the abundance of the other families or genera that were found to be modified in AD subjects with high IP at T1. However, qPCR analysis revealed that the total amount of bacteria, as well as the levels of *Bifidobacterium* spp. and *Lactobacillus* spp., increased significantly during withdrawal in AD subjects with high IP and returned to the levels of controls (Figure 4B). In contrast, the levels of *F. prausnitzii* remained unchanged at the end of the detoxification program (Figure 4B).

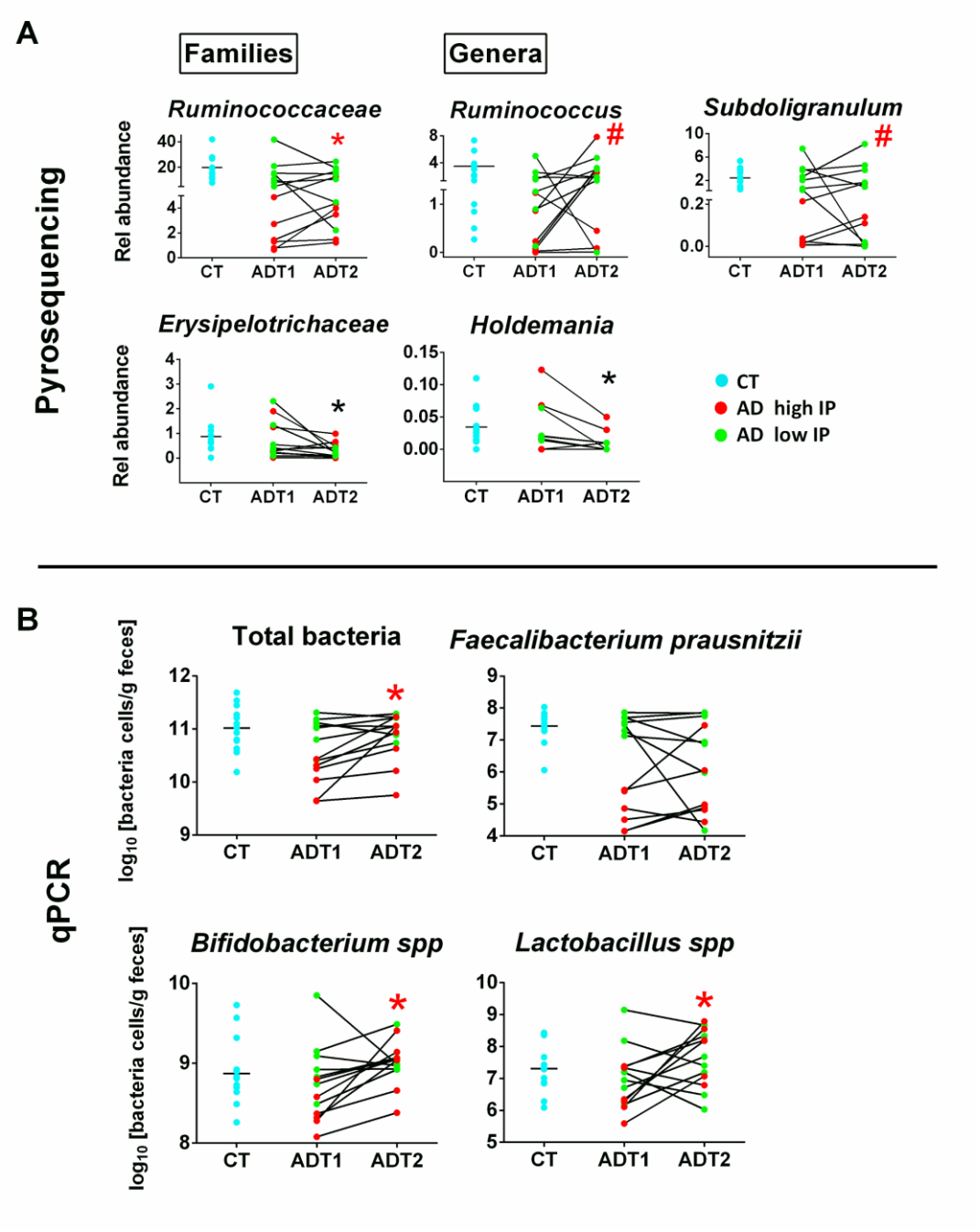

Figure 4: Effect of alcohol withdrawal on gut microbiota composition. (**A**) A significant increase in Ruminococcaceae was observed from T1 to T2 in AD subjects with high IP (*P < 0.05). The genera *Ruminococcus* and *Subdoligranulum* also increased during withdrawal in AD subjects with high IP but not significantly (#P = 0.11). The family Erysipelotrichaceae and the genus *Holdemania* decreased significantly during withdrawal (*P < 0.05) in all subjects. Results of relative abundance obtained from pyrosequencing are expressed in % sequences/taxon. (**B**) Abundance of total bacteria, *F. prausnitzii*, *Bifidobacterium* spp., and *Lactobacillus* spp. after 3 weeks of alcohol abstinence as measured by qPCR. * P < 0.05 in AD high IP from T1 to T2. AD high IP and low IP are depicted in red and green, respectively. AD: alcohol dependent; CT: control; IP: intestinal permeability. T1 and T2 refer to the beginning and end of alcohol withdrawal, respectively.

The metabolic profile is altered in AD subjects with gut barrier dysfunction

Metabolomic analyses of fecal samples were also performed in the subset of 13 AD subjects in order to investigate whether bacterial metabolites could be associated with the altered gut barrier function. A total of 155 volatile organic compounds (VOCs) were identified. Subject-specific compounds and metabolites present in less than 20% of subjects in both groups (AD and control) were discarded from statistical analysis. Ninety-nine VOCs remained and were considered in the analysis of metabolic profiles. No outlier was detected with principal component analysis. Thirty-eight compounds were common to 80% of the samples (Table S2). Some metabolites, including 2-methyl-1-butanol and methanethiol, were commonly found in controls but were absent in AD subjects (Table S3). On the other hand, metabolites belonging to alcohols, alkanes, and benzenes were found only in AD and not in control subjects (Table S4).

We then performed additional analyses that considered the gut barrier function of the individuals. Partial least squares-discriminant analysis (PLS-DA) revealed that the metabolic profiles of AD subjects with high IP differed from those with low IP (Figure 5A). The corresponding loading plot (Figure S3), showing the metabolites, was used to identify discriminating metabolites, whose relative indices are shown in Figure S4A-G. Among them, phenolic and indolic compounds, which arise from the metabolism of aromatic amino acids, were found to be associated with the gut barrier status. Phenol was present in high amount in patients with high IP, whereas it was almost absent in subjects with low IP (Figure 5B). However, the level of 4-methyl phenol was higher in AD subjects with low IP compared with AD subjects with high IP (Figure 5B). Indole and 3-methyl indole were present in high amounts in AD subjects with low IP, but were lower or almost totally absent in AD subjects with high IP (Figure 5C).

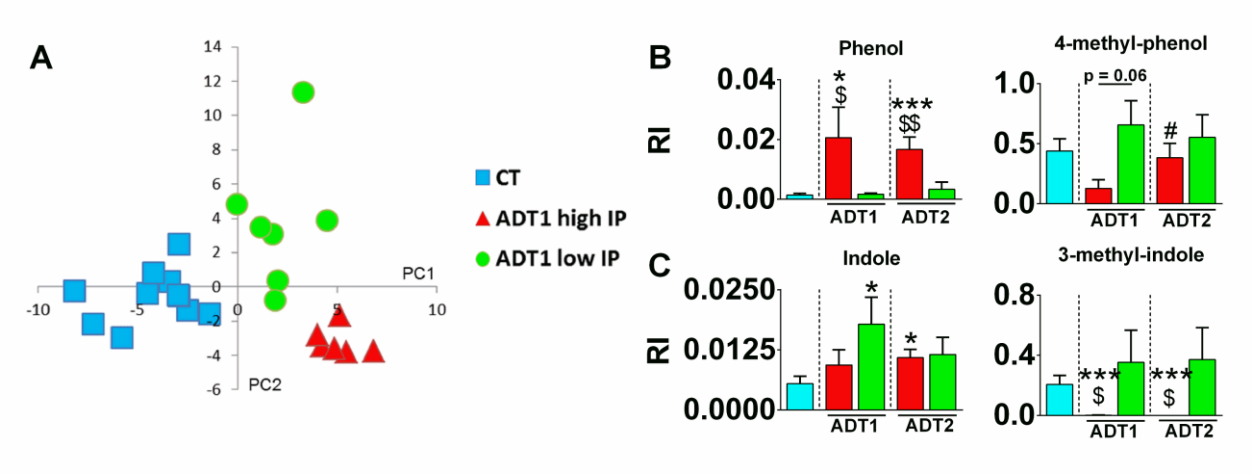

Figure 5: Metabolomic profiling of AD subjects with high and low IP. (**A**) Score plots showing clustering of the metabolite profiles analyzed with partial least squares-discriminant analysis. (**B-C**) Volatile organic compounds belonging to the chemical classes (**B**) phenols, (**C**) indoles. * $P < 0.05$ compared with CT, *** $P < 0.001$ compared with CT, $ $P < 0.05$ AD high IP vs. AD low IP at the same study time, $$ $P < 0.01$ AD high IP vs. AD low IP at the same study time, # $P < 0.05$ compared with ADT1 high IP. AD subjects with high IP and low IP are depicted in red and green, respectively. CT subjects are depicted in blue. IP: intestinal permeability; RI: relative indices; CT: control subjects; AD: alcohol-dependent subjects. T1 and T2 refer to the beginning and end of alcohol withdrawal, respectively.

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3.4 Discussion

Gut permeability is associated with the severity of behavioral markers of alcohol dependence

A limited number of human studies (7,34–36) have analyzed gut permeability in AD subjects, who are often also diagnosed with alcoholic liver disease . Most of these studies reported an increase in small bowel permeability, while a few pointed to increased colon permeability. We have observed two different clusters of AD subjects with distinct permeability features. The high IP group of subjects had a large increase in small bowel and colon permeabilities that recovered after a detoxification program. In the low IP group, the gut barrier function remained normal throughout the process.

AD subjects also presented with psychological symptoms, including depression, anxiety, and alcohol craving, which contribute to the negative reinforcement process, a major mechanism involved in the persistence of alcohol dependence (38) that is related to a higher probability of relapse after detoxification. In our previous studies (5,6), we observed that alcohol withdrawal induced only a partial recovery of these behavioral markers. The present study shows that the recovery of these markers is not evenly distributed among AD subjects. AD subjects with low IP recovered completely at T2 for depression and anxiety. This population seems to present with a less severe form of dependence where affective symptoms recover after detoxification. Conversely, in AD subjects with high IP, the scores of depression, anxiety and craving remained largely increased, even when they had stopped drinking for more than two weeks. These observations suggest that gut permeability is related to psychological status at the end of alcohol withdrawal.

If gut permeability does play a role in behavioral changes, one must pay attention to the potential mechanisms by which alterations of the gut barrier function occur. The possibility of a toxic effect of ethanol on the small bowel epithelium has been described in healthy subjects (34,39) and in in vitro studies (40–43). However, patients from both groups consumed the same amount of alcohol, which lessens the implication that ethanol itself is involved in permeability disturbance. The difference in IP in this context might be ascribed to changes in microbial composition and activity. This hypothesis is supported by a rodent study in which antibiotic treatment abolished the ethanol-induced increase in colonic paracellular permeability (44).

Increased gut permeability is associated with dysbiosis in AD subjects

Consistent with the hypothesis that dysbiosis is linked to gut barrier alteration, only the subgroup of AD subjects with high IP had altered gut microbiota composition. It consisted of a large decrease in the overall bacterial load, a drastic decrease in abundance in the Ruminococcaceae family (*Ruminococcus*, *Faecalibacterium*, *Subdoligranulum*, *Oscillibacter*, *Anaerofilum*), and an increase in abundance in the Lachnospiraceae family (*Dorea*) and the genus *Blautia*.

Preclinical studies have shown that chronic ethanol administration induces in rats a dysbiosis (45), and in mice a decrease in the level of Ruminococcaceae (46), or a decrease in the level of Firmicutes and an increase in Bacteroidetes (47). In humans, few studies have evaluated the gut microbiome of AD subjects and never in relation to gut permeability. Kirpich et al. (48) observed a decrease in *Bifidobacterium* and *Lactobacillus* in the stool cultures of AD subjects compared with those of healthy controls. In 2012, Mutlu et al. showed alterations of the mucosal-associated colonic microbiome in only a subset (31%) of AD subjects (49), indicating that not all alcoholics were dysbiotic, which is in line with our observation that only part of the AD patients had an altered gut microbiota profile. Furthermore, in the same study, the dysbiotic group included actively drinking and sober alcoholics (>1 month), suggesting long-lasting dysbiosis, which is consistent with the incomplete recovery of the gut microbiota that we observed after 3 weeks of abstinence. Interestingly, we observed that *Lactobacillus* spp. and *Bifidobacterium* spp., as well as bacteria from the family Ruminococcaceae, increased during alcohol abstinence, suggesting that these bacteria, known to have a beneficial impact on gut barrier function (50), could contribute to the recovery of IP at T2. This suggestion is supported by the strong negative correlations that we observed between IP and *Bifidobacterium* as well as Ruminococcaceae bacteria, particularly *F. prausnitzii*. This species is also depleted in Crohn's disease (33) and ulcerative colitis (51) and has been shown to have anti-inflammatory properties both in vitro and in vivo (33) and therefore seems to be crucial for gut homeostasis. Indeed, supernatants from *F. prausnitzii* cultures inhibited IL-8 secretion and NF-κB activation in Caco-2 cells stimulated with IL-1β (33). In our study, AD patients who presented with low levels of *F. prausnitzii* had higher plasma IL-8 levels, and these variables were significantly and negatively correlated. Taken together, our results show that alterations in microbial

composition are associated with increased IP and increased plasma levels of pro-inflammatory cytokines.

We also hypothesize that metabolites produced by gut bacteria might be at the origin of gut barrier dysfunction and inflammation. The metabolic profiles calculated by PLS-DA were clearly distinct between control, low IP, and high IP AD subjects. Overall, this observation supports the existence of a relation between metabolites and IP. Basically, 2 types of microbial fermentation occur in the colon: saccharolytic fermentation and proteolytic fermentation (52). The former is generally considered to be beneficial to the host, and the latter is presumed to be detrimental and might be involved in the etiology of colon cancer and ulcerative colitis (53). The main products of carbohydrate fermentation, i.e., SCFA, which present with beneficial functions (54), were not different between AD and control subjects and unrelated to IP. Metabolite differences among groups hence resulted mainly from the protein fermentation that leads to the formation of branched-chain fatty acids, indolic compounds, and potentially toxic metabolites such as phenolic and sulfur-containing compounds. The production of phenolic compounds in the gut depends on microbial composition (55) or microbial metabolic activities (56). Phenol that derives from tyrosine breakdown was largely increased in high IP AD subjects compared with the other 2 groups. The toxic effect of phenol on intestinal epithelial cells has been demonstrated in 2 independent in vitro studies (57,58) suggesting that phenol is a potential driver of gut barrier alterations. Another phenolic compound, 4-methyl phenol (also called p-cresol), was decreased in AD subjects with high IP compared with the other 2 groups and increased upon alcohol withdrawal; the latter effect could be associated with the increase in *Lactobacillus* spp., *Bifidobacterium* spp., and Ruminococcaceae from T1 to T2 observed in AD subjects with high IP, as these genera and this family have been shown to be related to the production of p-cresol (59–62). Tryptophan bacterial metabolism results in a large variety of indolic compounds (62–65). In in vitro studies, indolic compounds were shown to improve intestinal cell barrier function and to decrease pro-inflammatory IL-8 expression (66,67). Interestingly, the level of 3-methylindole was completely blunted in high IP AD subjects who also presented with a higher IL-8 plasma level. Moreover, a study showed that the beneficial impact of probiotics on gut barrier function was induced by a protein factor (50). Taken together, these observations are consistent with a protective role of gut microbes that produce indolic compounds and p-cresol on the gut barrier and inflammation and with a detrimental role of bacteria producing phenol.

Study limitation

The existence of distinct populations of AD subjects with differences in intestinal permeability that are related to differences in psychological symptoms could be established on a large group. There is however one limitation of this study that is the size of the sample for which we could obtain large details on the composition and function of the gut microbiota and on inflammation. The small size of the sample is due to the fact that deep analysis of gut microbiota are expensive and time consuming and that we used a test retest design that doubles the number of samples.

In conclusion, this is the first study to investigate gut permeability, gut microbiota composition and activity and their relationship with the behavioral markers of addiction severity in AD subjects (Figure 6). The observation that some, but not all, AD subjects develop gut leakiness indicates that chronic alcohol dependence is necessary but not sufficient to cause gut dysfunction. Thus, other cofactors besides direct toxicity of alcohol may be involved. Here we showed that AD subjects presenting with increased IP also had altered gut microbial composition and activity. These results strongly suggest that the bacteria present in the gut and/or the metabolites produced by the bacteria may be involved in the regulation of the gut barrier function and could therefore contribute to the indirect toxicity of alcohol consumption. Moreover, we showed that AD subjects with gut dysfunction had higher scores for depression, anxiety, and alcohol craving at the end of the detoxification program, which is expected to influence the probability of relapse through a negative reinforcement mechanism. These results suggest the existence of a gut-brain axis in alcohol dependence, in which the gut microbiota could alter the gut barrier and influence the severity of alcohol-dependence behaviors. However, the mechanisms underlying the communication between the gut and the brain have not been analyzed in this study but deserve further investigation, in particular the involvement of the vagus nerve as well as the tryptohan/kynurenine pathway in alcohol-dependent subjects.

Overall, the gut microbiota appears to be a new target in the management of patients who are being treated for alcohol dependence. In view of the bacteria that were modulated upon alcohol consumption, which characterize increased gut permeability, we propose that more attention be paid to the nutritional follow-up of abstinent patients. Probiotics and prebiotics are known to improve the composition of the gut microbiota in favor of bacteria

that decrease gut barrier alterations (50,68) and inflammation (13,69), and they may improve mood and behavior in several pathological contexts (27,70–73). Therefore, the use of these nutritional tools to improve gut function and mental health in patients diagnosed with alcohol use disorders deserves interest.

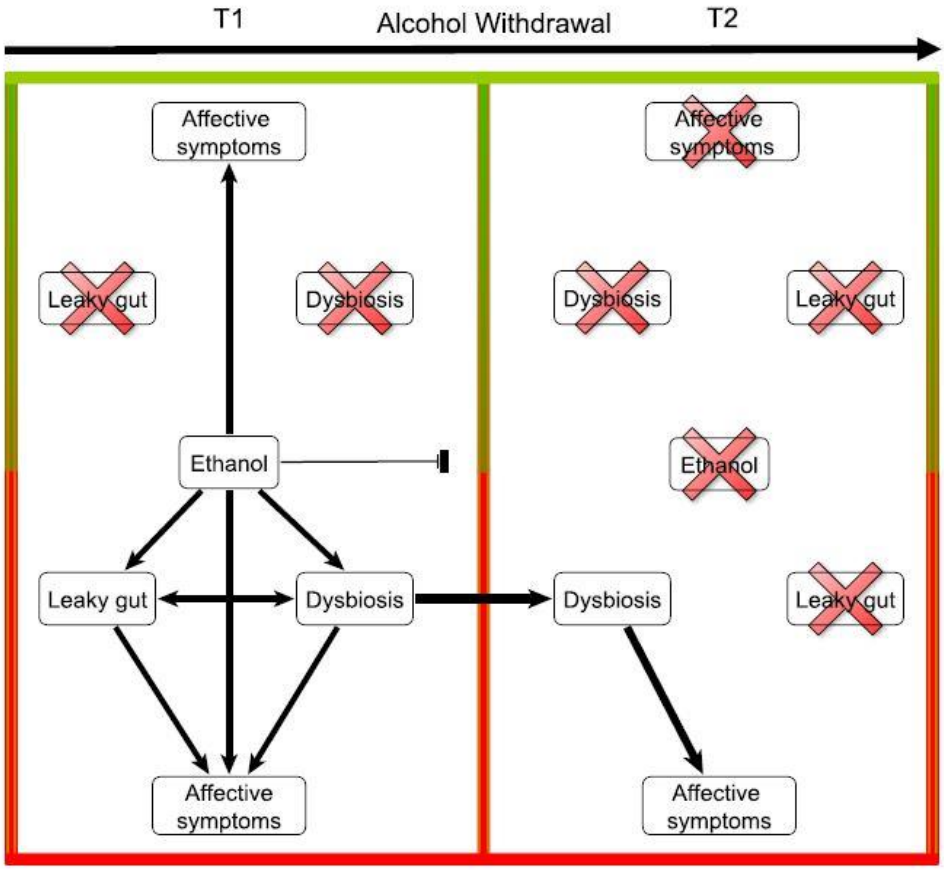

Figure 6: Model representing the relationship between alcohol consumption, gut dysfunction and affective symptoms in the two subsets of alcohol-dependent subjects at both times of alcohol withdrawal. At the beginning of withdrawal (T1), a subset of AD subjects developed affective symptoms that were likely induced by ethanol and that were not associated with gut disorders. In the other subset of AD subjects, alcohol consumption was associated with gut leakiness, gut microbiota alterations and affective symptoms. After 3 weeks of abstinence (T2), affective symptoms recovered completely in the subset of AD subjects that did not present with gut dysfunction at T1. In the other subset of AD subjects, the gut barrier was restored upon abstinence, but gut dysbiosis was still present at T2 and might be responsible for the persistence of affective symptoms. From this, we hypothesize that gut microbiota alterations could be associated with a more severe form of alcohol-dependence and a higher probability of relapse through negative reinforcement mechanisms linked to higher levels of depression, anxiety and alcohol craving.

3.5 Material and methods

Subjects

A group of AD subjects presenting with a diagnosis of alcohol dependence according to the Diagnostic and Statistical Manual of Mental Disorders (4th ed.) (74) who were clinically evaluated by a psychiatrist (PdT), were incorporated in to the study when admitted to the gastroenterology ward for a 3-week detoxification and rehabilitation program. Subjects had kept drinking until the day of admission to the detoxification ward, or the day before, and were tested twice, on the day following admission (T1) and on day 19 (T2) corresponding to the last day of detoxification. Patients who had metabolic disorders such as obesity (BMI > 30 kg/m2) and diabetes, inflammatory bowel disease, other chronic inflammatory diseases (such as rheumatoid arthritis), or cancer, as well as those who took antibiotics, probiotics, glucocorticoids, or nonsteroidal anti-inflammatory drugs during the 2 months preceding enrollment were excluded from the study. Transient liver elastography (Fibroscan) was performed in all patients on the day of admission in order to quantify liver stiffness, which correlates with fibrosis grades according to the METAVIR classification system of fibrosis (75). From this evaluation, only patients without significant fibrosis (F0 and F1 scores) were selected. Patients with overt cirrhosis, on the basis of laboratory and imaging tests, were excluded (see Supplemental Material). A complete medication and medical history was taken at admission, as well as basic demographic and clinical data related to nutritional status. For a preliminary study, we could select a group of 60 AD subjects that were tested at T1 for intestinal permeability and psychological symptoms. Among them, 44 subjects who had remained abstinent until the end of the detox period were also tested at T2. This sample of subjects was split into high and low IP groups. From this preliminary group, a subset of 13 subjects (8 men and 5 women) was tested for additional gut microbiota composition and functionality measurements both at T1 and T2. AD subjects were compared for intestinal permeability, psychological dimensions, gut microbiota composition and functionality with 15 age-, gender-, and BMI-matched controls who socially consumed less than 20 g of alcohol per day.

Assessment of alcohol consumption

The amount of alcohol consumed the week prior to hospitalization was evaluated with the time-line follow-back approach (76), as detailed in de Timary et al. (77).

Measurement of IP

IP was assessed by using the radioactive probe 51Cr-EDTA as described previously (5). Briefly, after an overnight fast and emptying of the bladder, patients drank a Nutridrink (200 ml, 150 kcal/100 ml) (Nutricia, Brussels, Belgium) containing 50 µCi (1.85 MBq) 51Cr-EDTA. Urine was collected for 24 hours. Radioactivity was measured in urine collections with a gamma counter (Cobra5003 Canberra Packard, Downers Grove, IL). Urinary excretion was expressed as the percentage of the ingested dose (ID) normalized to creatinine concentration (% ID/g creatinine).

Gut microbiota analysis

Gut microbiota was analyzed by using 2 culture-independent methods; pyrosequencing and qPCR of 16S rDNA. These methods are complementary, since pyrosequencing allows the creation of a qualitative bacterial profile that considers most of the bacteria present in the gut, whereas qPCR is used to quantify specific bacterial targets of particular interest. Moreover, qPCR is more sensitive and can quantify some bacteria that are not detected with pyrosequencing, such as *Bifidobacterium* spp.

Fecal samples were collected in a sterile container and immediately stored at -80oC until further processing. Bacterial DNA extraction was performed by using the repeated bead beating procedure with a modified protocol for the QIAamp Stool DNA Mini Kit (Qiagen, Hilden, Germany).

A detailed description of both methods is provided in the supplemental material and primer sequences are mentioned in Supplemental Table 5.

Analysis of VOCs in fecal samples

Stool samples were transferred to a headspace vial and VOCs were analyzed on a gas chromatography-mass spectrometry quadrupole (Trace GC Thermoquest, Rodano, Italy and DSQ II, Thermo Electron, San José, CA), which was coupled online to a purge-and-trap system. Before analysis, 125 mg fecal aliquots were suspended in 5 ml water. Diethyl acetic

acid (1.5 mg/L) was added as an internal standard. A magnetic stirrer, sulfuric acid, and a pinch of sodium sulphate were added to the sample to acidify and salt out the solution. The chromatograms thus obtained were processed by using AMDIS (Automatic Mass Spectral Deconvolution and Identification Software version 2.71) provided by the US National Institute of Standards and Technology (NIST, Gaithersburg, MD). Identification of the metabolites in the samples was achieved by manual visual inspection of the mass spectra of unknown peaks with the NIST library. All compounds were relatively quantified compared with diethyl acetic acid. A detailed description of the method is provided in the supplemental material.

Assessment of mood and psychological symptoms

At the beginning and end of detoxification, all patients were tested for depression, anxiety, and alcohol craving with the French versions of self-reported questionnaires: the Beck Depression Inventory, the State-Trait Anxiety Inventory (form YA), and the Obsessive-Compulsive Drinking Scale. These tests have been described in detail previously (5).

Statistical analysis

Statistical analyses were performed by using SPSS version 20.0 (IBM Corp, Armonk, NY). Assumptions of normality and equality of variances were checked with the Kolmogorov-Smirnov and Levene tests, respectively. If the assumptions were not met, Kruskal-Wallis tests were used to compare controls, AD subjects with high IP, and AD subjects with low IP. If the assumptions were met, parametric ANOVAs were performed. Significant ANOVA results were followed by Bonferroni's post hoc test for pairwise comparisons. The effect of alcohol withdrawal was assessed by using Wilcoxon or paired t-tests to compare data at T1 and T2. Bivariate correlations were performed with Spearman's rho or Pearson's product-moment correlation coefficient, depending on data assumptions.

The absolute number of sequences for identified and unclassified taxa obtained by pyrosequencing in each sample was transformed by using the Hellinger method after removing taxa representing less than 0.01% of total abundance. The resulting matrix was used to construct nonmetric multidimensional scaling by using Bray-Curtis dissimilarity with PCOrd V6.08 (MjM Software, Gleneden Beach, OR, USA). The Bray-Curtis coefficient is a statistic used to quantify the compositional dissimilarity between 2 different bacterial profiles.

Relative abundances of taxa were analyzed by using Kruskal-Wallis tests and qPCR data were analyzed with ANOVA after log-transformation.

Unscrambler X Version 10.2 (CAMO A/S, Trondheim, Norway) was used to perform cluster analyses of metabolite profiles. Subject-specific compounds (those detected in only 1 person) and metabolites present in less than 20% of subjects in both groups (AD and control) were discarded from statistical analysis. 10 control samples were available for this analysis. Principal component analysis was applied to detect outliers. Clustering of similar metabolite patterns of the samples according to control, AD with high IP, and AD with low IP groups at T1 was then performed by using PLS-DA and was presented as a score plot. The corresponding loading plot, showing the metabolites, was used to identify discriminating metabolites. Kruskal-Wallis tests were applied to compare the relative indices of metabolites between controls, AD subjects with high IP, and AD subjects with low IP.

Study approval

The study protocol was approved by the local Ethical Committee and all subjects signed an informed consent form prior to the investigation (B40320096274, Commission d'éthique biomédicale hospitalo-facultaire de l'UCL).

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3.7 Supporting information

3.7.1 Supporting material

Diagnosis of cirrhosis

Liver biopsy is still regarded as the gold standard for grading and staging liver disease. However, this invasive procedure carries the risk of potential life-threatening complications (e.g., bleeding); therefore, for obvious ethical reasons, liver biopsy cannot be justified in all patients for study purposes. All of the subjects were instead systematically evaluated by blood tests (biochemistry, hematology, coagulation), by gastrointestinal endoscopy (to check for signs of portal hypertension such as esophageal and gastric varices), by liver Doppler-ultrasound (to evaluate the shape of the liver, splenomegaly with collateral intra-abdominal venous circulation, or slow or inverted portal venous blood flow), and by measurement of transient liver elastography (Fibroscan). This new noninvasive technique has been designed to quantify liver stiffness, which has been correlated to liver fibrosis grades based on the METAVIR classification system of fibrosis. A validated cut-off has been published and studies clearly show that this technique is reliable in ruling out significant fibrosis and in confirming cirrhosis (1). Accordingly, we selected only F0 and F1 patients (i.e., absence of significant fibrosis) for the study (2, 3). Liver biopsy was performed only in patients in whom advanced fibrosis/cirrhosis was suspected after noninvasive work-up; these patients were, by definition, excluded from the study.

Gut microbiota analysis by pyrosequencing and qPCR

Fecal microbiota composition was studied by pyrosequencing of the V1-V2 region of the 16S rRNA gene. 16S rDNA was amplified by using 27F and 338R primers fused with 454 titanium sequencing adapters. 338R primers contained unique error-correcting 12-base barcodes that allowed us to tag PCR products from different samples (4). Each sample was amplified in triplicate in a reaction volume of 25 μL containing 1.5 U of FastStart Taq DNA Polymerase (Roche), 0.2 μM of each primer, and 20 ng of genomic DNA. PCR was carried out under the following conditions: initial denaturation for 3 min at 95°C, followed by 25 cycles of denaturation for 20 s at 95°C, annealing for 20 s at 52°C and elongation for 60 s at 72°C, and a final elongation step for 8 min at 72°C. The amplification of the 16S rDNA was

not successful for 2 control samples, which were therefore excluded from the pyrosequencing analysis. Triplicates were combined, purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany), and then quantified by using the Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA). Purified PCR products were diluted to a concentration of 20 ng/µl and pooled in equal amounts. The pooled amplicons were purified again with the Ampure magnetic purification beads (Agencourt, Danvers, MA) to remove short amplification products. Sequencing was performed by using 454 GS FLX titanium chemistry at GATC Biotech (Konstanz, Germany).

Raw data were quality filtered to remove sequences that were shorter than 200 nucleotides or longer than 1000 nucleotides, or that contained primer mismatches, ambiguous bases, uncorrectable barcodes, or homopolymer runs in excess of 6 bases. Quality-filtered reads were trimmed of 454 adapter and barcode sequences and analyzed with the software package Quantitative Insights Into Microbial Ecology (QIIME) (5) (version 1.6.0). A total of 517808 sequences were obtained for the 39 samples (13 controls and 13 AD subjects sampled before and after alcohol withdrawal). Sequences were demultiplexed and an average of 13277 sequences were attributed to each sample (range: 6636-16482 sequences). Sequences were assigned to operational taxonomic units (OTUs) by using UCLUST with a 97% threshold of pairwise identity. The most abundant sequence was picked as representative for each OTU and was given a taxonomic assignment by using the Ribosomal Database Project (RDP) Classifier (6). Representative OTUs were aligned with Pynast (7). Sequences were checked for the presence of chimeras by using ChimeraSlayer, and chimeric sequences were excluded from all downstream analyses. Similarly, sequences that could not be aligned with Pynast were also excluded.

qPCR of 16S rDNA was used to quantify the abundance of selected members of the gut microbiota. The primers used to detect total bacteria, Bifidobacterium spp., Lactobacillus spp., and Faecalibacterium prausnitzii are mentioned in Supplemental Table 5. PCR amplification was carried out as follows: 10 min at 95°C, followed by 45 cycles of 3 s at 95°C, 26 s at 58°C or 60°C, and 10 s at 72°C. Detection was achieved with the STEP one PLUS instrument and software (Applied Biosystems, Foster City, CA) using the MESA FAST qPCR MasterMix Plus for SYBR Assay (Eurogentec, Verviers, Belgium). BSA was added to samples. Each assay was performed in duplicate in the same run. For construction of

standard curves, 5-fold dilution series from target species genomic DNA preparations (DSMZ, Braunschweig, Germany) were applied to the PCR.

Analysis of volatile organic compounds (VOCs) in fecal samples by GC-MS

VOCs from stool samples were analyzed on a gas chromatography-mass spectrometry (GC-MS) quadrupole (Trace GC Thermoquest, Rodano, Italy and DSQ II, Thermo Electron, San José, CA), which was coupled online to a purge-and-trap system. Before analysis, 125 mg fecal aliquots were suspended in 5 ml water. Diethyl acetic acid (1.5 mg/L) was added as an internal standard. A magnetic stirrer, sulfuric acid, and a pinch of sodium sulphate were added to the sample to acidify and salt out the solution.

Briefly, VOCs were purged out of the sample with a helium flow (high purity (>99.99%)) at a rate of 40 ml/min for 20 min at 70 °C. Consequently, helium was carried over a "dry flow" column (Trap Tenax, Velocity, Interscience, Louvain-la-Neuve, Belgium) to control moisture transfer, and VOCs were concentrated on a second polar trap column (Trap Vocarb, Velocity, Interscience, Louvain-la-Neuve, Belgium). By raising the temperature to 250 °C, the VOCs were desorbed from the column to the injector of the GC, where they were separated on an analytical column (AT Aquawax DA, 30 m × 0.25 mm I.D., 0.25 µm film thickness, Grace, Deerfield, IL). The oven starting temperature was 35 °C for 1 min and increased by 5 °C/min to 100 °C and by 10 °C/min to 240 °C. The final temperature was held constant for 5 min. Masses between m/z 33 and m/z 200 were detected in full scan mode at 1.5 scans/s. XCalibur software (Version 1.4 SR1, Thermo Electron) was used for automatization of the GC-MS and for data acquisition.

The chromatograms that were obtained were processed by using AMDIS (Automatic Mass Spectral Deconvolution and Identification Software version 2.71) provided by the US National Institute of Standards and Technology (NIST, Gaithersburg, MD). This software provides quality matching by using advanced spectral algorithms, adjacent peak deconvolution, and background subtraction, which enables unambiguous identification together with quantitative indication of the metabolite levels. Identification of the metabolites in the samples was achieved by manual visual inspection of the mass spectra of unknown peaks with the NIST library. All compounds were relatively quantified compared with diethyl acetic acid.

Supporting references

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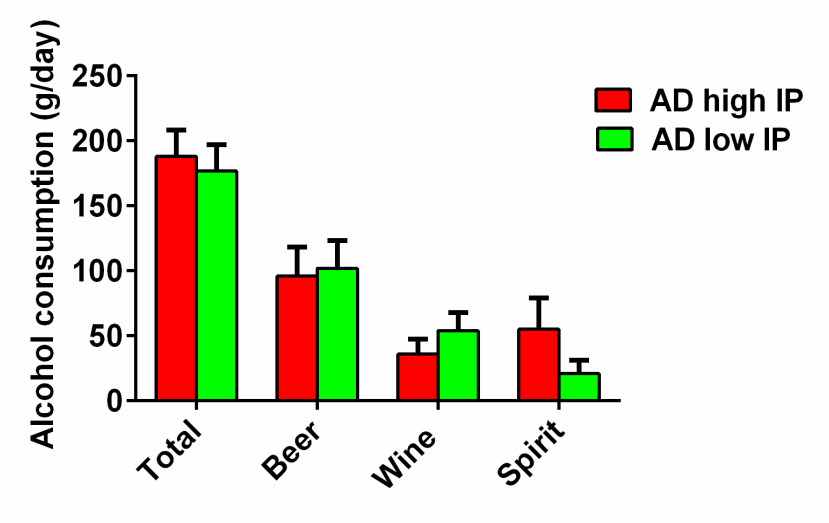
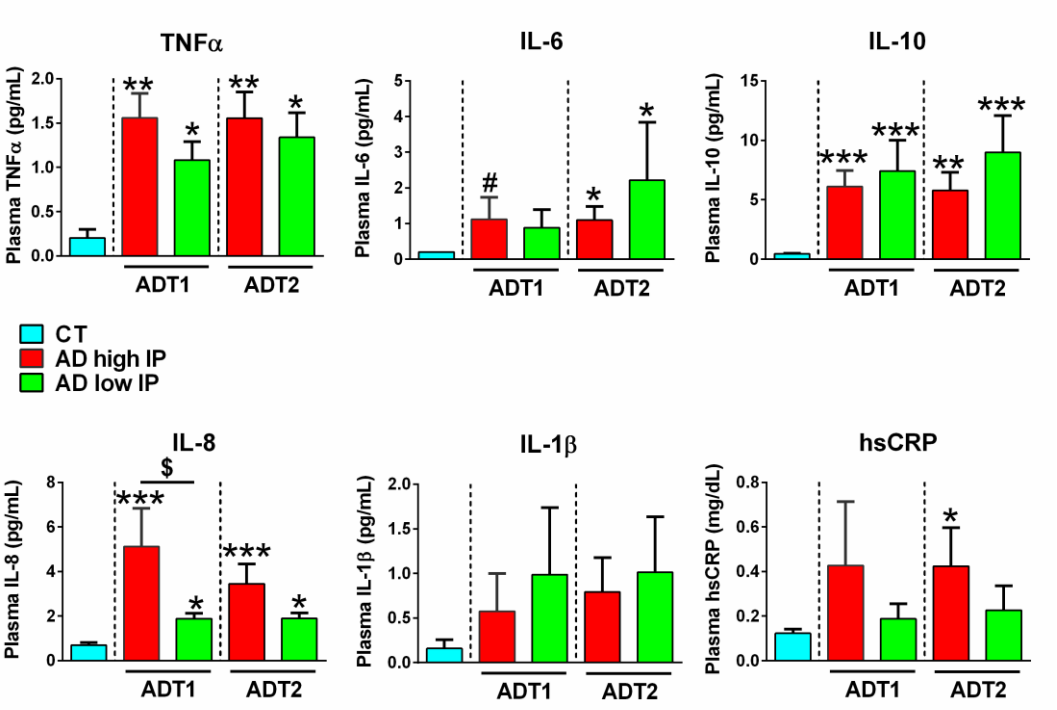
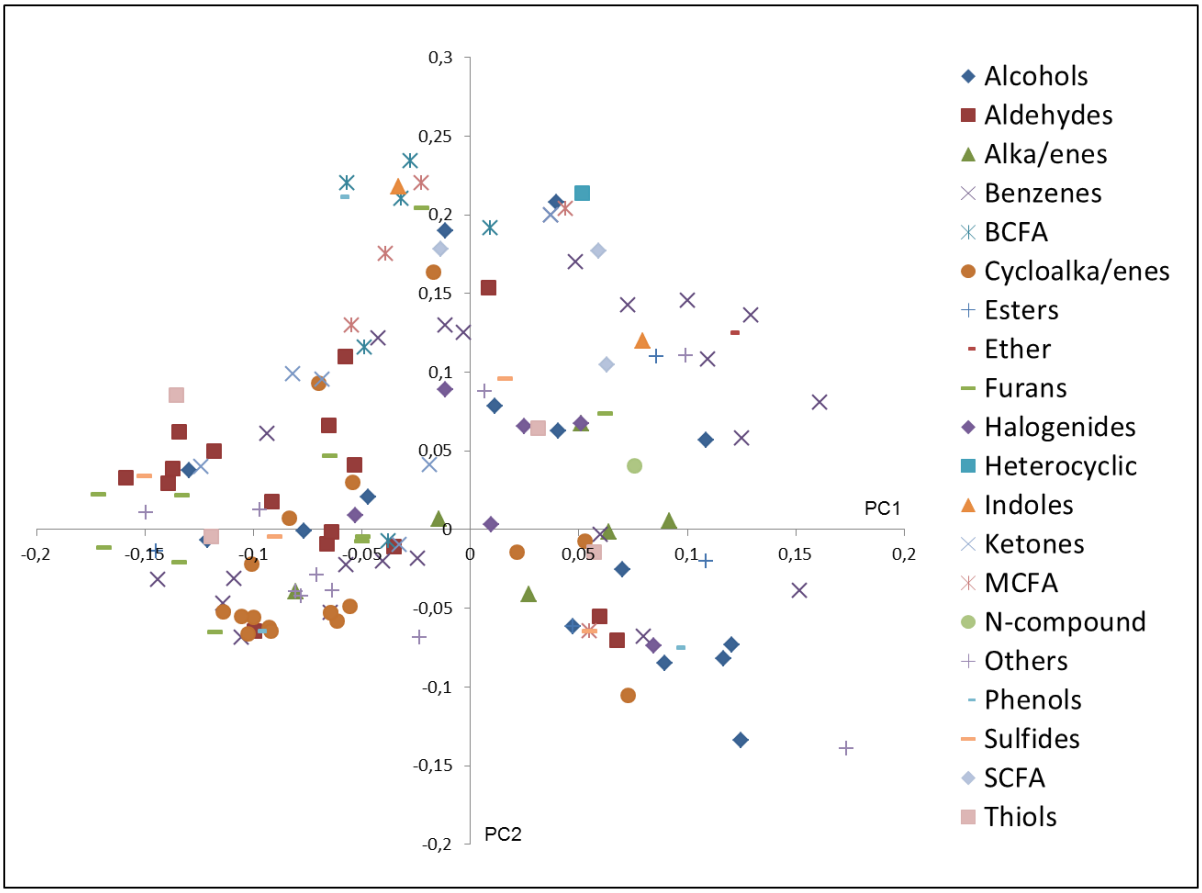
3.7.2 Supporting figures



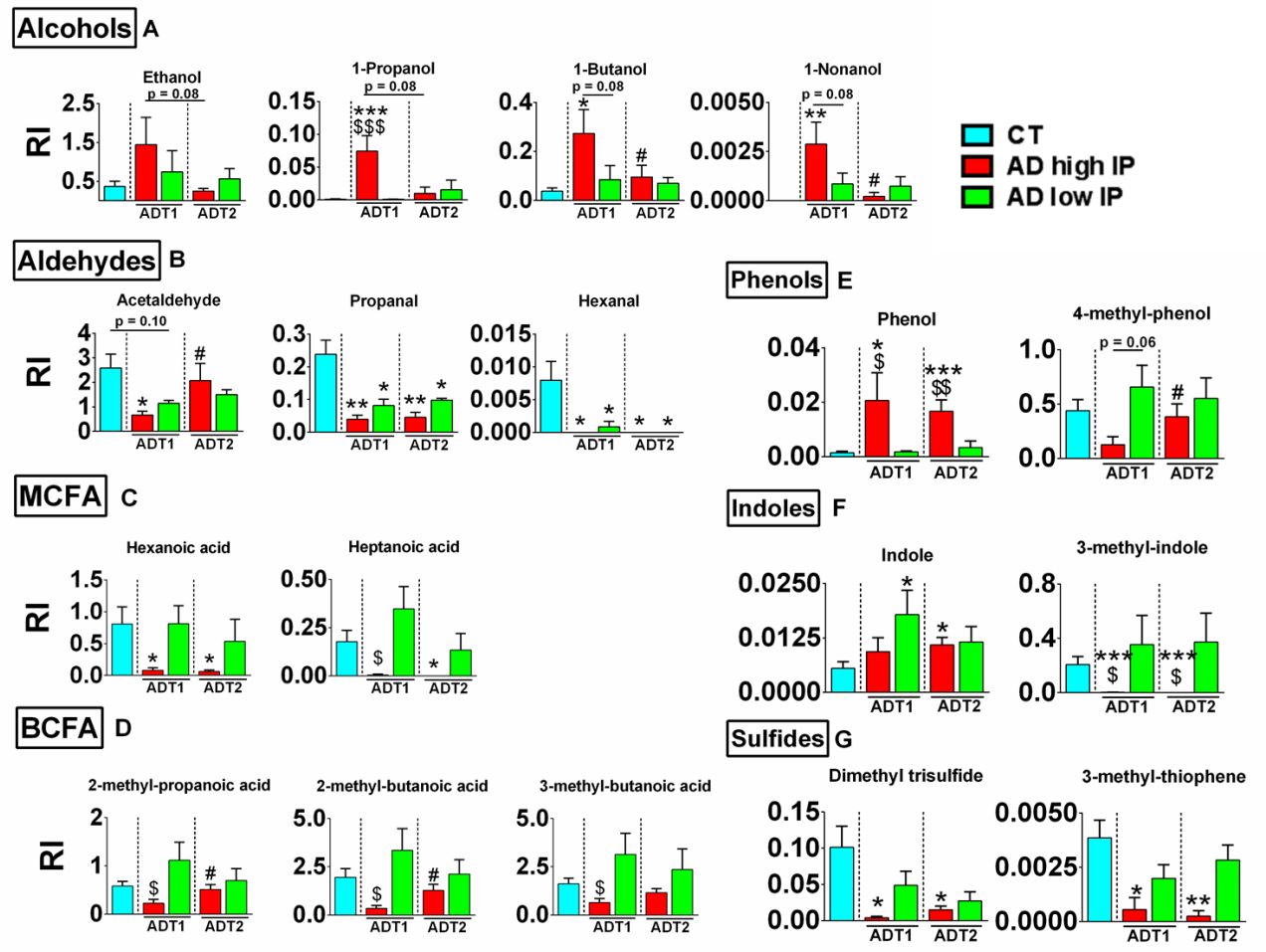
Supplemental figure 1 : Types of alcoholic beverages consumed by AD subjects with high and low IP. Alcohol consumption was calculated by using the time-line follow-back approach and is based on the week prior to the hospitalization. AD: alcohol dependent; IP: intestinal permeability.



Supplemental figure 2 : Increased plasma inflammatory markers in AD subjects at the beginning and end of detoxification (T1 and T2). # P < 0.10 vs. CT, * P < 0.05 vs. CT, ** P < 0.01 vs. CT, *** P < 0.001 vs. CT. CT: control; AD: alcohol dependent; IP: intestinal permeability.



Supplemental figure 3 : Loading plot resulting from PLS-DA analysis of metabolite profiles. This plot, showing the metabolites listed according their chemical class, was used to identify discriminating metabolites.



Supplemental figure 4: Volatile organic compounds belonging to the chemical classes (A) alcohols, (B) aldehydes, (C) medium-chain fatty acids (MCFAs), (D) branched-chain fatty acids (BCFAs), (E) phenols, (F) indoles, and (G) sulfides. * P < 0.05 compared with CT, ** P < 0.01 compared with CT, *** P < 0.001 compared with CT, $ P < 0.05 AD high IP vs. AD low IP at the same study time, $$ P < 0.01 AD high IP vs. AD low IP at the same study time, $$$ P < 0.001 AD high IP vs. AD low IP at the same study time, # P < 0.05 compared with ADT1 high IP. AD subjects with high IP and low IP are depicted in red and green, respectively. CT subjects are depicted in blue. IP: intestinal permeability; RI: relative indices; CT: control subjects; AD: alcohol-dependent subjects. T1 and T2 refer to the beginning and end of alcohol withdrawal, respectively.

3.7.3 Supporting tables

Supplemental table 1. Average values of intestinal permeability (IP) in CT and AD subjects at the beginning (T1) and end (T2) of alcohol withdrawal.

Time	IP	CT	AD high IP	AD low IP
T1	0–4 h	2.36 ± 0.87	7.81 ± 5.46 ***/$$$	2.58 ± 0.79
	4–24 h	1.08 ± 0.37	2.79 ± 1.73 ***/$$$	0.90 ± 0.26
	0–24 h	1.34 ± 0.43	3.71 ± 2.15 ***/$$$	1.22 ± 0.28
T2	0–4 h	nd	3.26 ± 2.54 $	1.93 ± 1.11
	4–24 h	nd	1.46 ± 1.01	1.00 ± 0.63
	0–24 h	nd	1.86 ± 1.34	1.23 ± 0.75

Results are expressed as the percentage of the ingested dose of ^{51}Cr-EDTA found in urine normalized for creatinine. Urine was collected for 24 h during 2 periods that were expected to reflect small bowel (0-4 h) and colon (4-24 h) permeability. From the total intestinal permeability value at T1, AD patients were split into high IP and low IP groups. Data are means ± SD. *** $P < 0.001$ vs. CT; $$ $P < 0.01$ vs. AD-Low IP; $$$ $P < 0.001$ vs. AD-Low IP. CT: control; AD: alcohol dependent; nd: not defined.

Supplemental table 2. Volatile organic compounds found in more than 80% of the CT and AD study subjects.

Chemical class	Metabolite	% presence in CT	% presence in ADT1
Alcohol	1-Butanol	100	100
Aldehyde	Acetaldehyde	100	100
Aldehyde	Propanal	100	100
Aldehyde	Propanal, 2-methyl-	91	85
Aldehyde	Aldehyde RT 19,73	82	100
Aldehyde	Aldehyde RT 22,42	91	85
Aldehyde	Aldehyde RT 23,23	100	100
Benzene	Benzene	82	85
Benzene	Toluene	100	100
Benzene	x-Xylene RT 7,07	91	100
Benzene	Benzene, RT 9,86	100	100
Benzene	Benzene, RT 10,27	82	100
Cycloalkene	D-Limonene	100	100
Cycloalkene	3-Cyclohexene-1-methanol, .alpha.,.alpha.4-trimethyl-	91	85
SCFA	Acetic acid	100	100
SCFA	Propanoic acid	100	100
SCFA	Butanoic acid	100	100
BCFA	Propanoic acid, 2-methyl-	100	100
BCFA	Butanoic acid, 3-methyl-	100	100
BCFA	Butanoic acid, 2-methyl-	100	100
BCFA	Pentanoic acid, 4-methyl-	100	85
MCFA	Pentanoic acid	100	100
MCFA	Hexanoic acid	100	100
Ether	Ethyl ether	100	100
Furan	Furan	100	85
Furan	Furan, tetrahydro-	100	92
Halogenide	Trichloromethane	100	92
Halogenide	Bromochloronitromethane	100	100
Halogenide	Methane, tribromo-	91	92
Indole	Indole	100	100
Phenol	Phenol	82	92
Phenol	Phenol, 4-methyl-	100	100
Sulfide	Carbon disulfide	100	100
Sulfide	Disulfide, dimethyl	100	100
Sulfide	Dimethyl trisulfide	100	92
Thiol	3,4-Dimethylthiophene	100	100
Other	Benzaldehyde	100	100
Other	Benzoic acid, 4-ethoxy-, ethyl ester	100	100

Some aldehydes and benzenes were not identified. CT: control; AD: alcohol dependent; RT: retention time; SCFA: short-chain fatty acid; BCFA: branched-chain fatty acid; MCFA: medium-chain fatty acid. T1 refers to the beginning of alcohol withdrawal.

Supplemental table 3. Volatile organic compounds found in CT subjects but in less than 10% of alcohol-dependent AD subjects.

Chemical class	Metabolite	% presence in CT	% presence in ADT1
Alcohol	1-Butanol, 2-methyl-	64	0
Aldehyde	Butanal, 3-methyl-	36	< 10
Aldehyde	Hexanal	55	< 10
Benzene	Benzene, RT 11,26	27	0
Cycloalkane	Cyclopentane, methyl-	27	0
Furan	Furan, 2-ethyl-5-methyl-	27	< 10
Ketone	2-pentanone	27	< 10
Thiol	Methanethiol	55	0
Other	Anisole, p-allyl-	36	< 10

The metabolite benzene RT 11,26 was not identified. CT: control; AD: alcohol dependent; RT: retention time. T1 refers to the beginning of alcohol withdrawal.

Supplemental table 4. Volatile organic compounds found in AD subjects but in less than 10% of CT subjects.

Chemical class	Metabolite	% presence in CT	% presence in ADT1
Alcohol	1-Propanol	< 10	46
Alcohol	1-Nonanol	0	46
Benzene	Benzene, RT 9,23	< 10	31
Benzene	Styrene	0	92
Benzene	Benzene, RT 10,66	< 10	31
Benzene	Benzene, RT 11,82	0	46
Benzene	Benzaldehyde, 3,5-dimethyl-	0	100
Benzene	4-Hydroxy-2-methylacetophenone	< 10	31
Alkane	Hexane	0	46
Ester	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	0	31
Furan	Furan, 2-ethyl-5-methyl-	< 10	23

The metabolites benzene RT 9,23, benzene RT 10,66, and benzene RT 11,82 were not identified. CT: control; AD: alcohol dependent; RT: retention time. T1 refers to the beginning of alcohol withdrawal.

Supplemental table 5. Primer sequences used for qPCR analysis

Target	Forward and reverse primers	T° annealing (°C)
Total bacteria	F: ACT-CCT-ACG-GGA-GGC-AGC-AG R: ATT-ACC-GCG-GCT-GCT-GG	60
Bifidobacterium spp.	F: GAT-TCT-GGC-TCA-GGA-TGA-ACG-C R: CTG-ATA-GGA-CGC-GAC-CCC-AT	60
Lactobacillus spp.	F: AGC-AGT-AGG-GAA-TCT-TCC-A R: CAC-CGC-TAC-ACA-TGG-AG	58
Faecalibacterium prausnitzii	F: GAG-CCT-CAG-CGT-CAG-TTG-GT R: CCA-TGA-ATT-GCC-TTC-AAA-ACT	60

3.8 Complementary discussion and conclusion

In this complementary discussion, we will have the opportunity to discuss into details the data presented in the article, where several important aspects were only shortly discussed due to word count limitation.

Discussion of the data of the intestinal permeability

In Chapter 1, we found that intestinal permeability of alcohol-dependent subjects was, on average, increased at the beginning of alcohol withdrawal and recovered totally at the end. We also found that, on average, the psychological symptoms of alcohol dependence recovered partially during the detoxification program. In this Chapter 3, we found that only a subset of alcohol-dependent subjects developed gut leakiness. Those patients presented with high score of depression, anxiety and craving at the end of withdrawal whereas the patients which had normal intestinal permeability recovered completely for depression and anxiety.

Discussion on metabolomics data

In vitro studies have shown that ethanol can induce a decrease in epithelial resistance of Caco-2 cells [79]. However, the authors used a high dose of ethanol to see a small effect on epithelial cells permeability. On the other hand, acetaldehyde has been reported to have more deleterious effect on paracellular permeability than ethanol in vitro [86]. These results were confirmed in colonic tissues extracted from rats after alcohol intake [75]. In our study, both patients with high and low intestinal permeability drank similar amounts of alcohol. All these observations suggest that a direct effect of ethanol on epithelial cells is not the main mechanisms involved in vivo upon chronic alcohol consumption. Ferrier *et al.* suggested that the increase in colonic permeability of rats after alcohol intake was induced by ethanol oxidation into acetaldehyde by the colonic microflora [75]. In their study, antibiotic treatment abolished the ethanol-induced increase of intestinal permeability and reduced colonic acetaldehyde concentration. In our study, luminal acetaldehyde levels of both low and high intestinal permeability alcohol-dependent patients were lower than that of controls. However, as human cells and gut bacteria possess ADH (to oxidize ethanol into acetaldehyde as well as to reduce acetaldehyde into ethanol) and ALD (to oxidize acetaldehyde into acetate or to reduce acetate into acetaldehyde) make the results of acetaldehyde levels found in feces

difficult to interpret. Furthermore, at the time of feces collection, the AD subjects had not ingested ethanol for more than 24 hours that make the importance of acetaldehyde for gut permeability difficult to conclude. Whatever the case, the fact that only dysbiotic subjects developed gut leakiness suggests that ethanol induces gut barrier alteration by an indirect mechanism involving the gut microbiota. Phenolic and indole compounds which are metabolites produced by the gut microbiota are good candidates to play a role in the regulation of the gut barrier function. The problem is that many different bacterial genera and species could be involved in the production of these metabolites. A key issue would be to identify which bacteria produce which metabolites.

Short-chain fatty acids (SCFAs) like acetate, propionate and butyrate are known to play important role in the regulation of immune system, gut barrier and gut physiology. We did not find differences in the levels of SCFAs between controls and alcohol-dependent groups. However, the SCFAs measured in fecal samples do not necessary represent the real production in the upper part of the intestine. Indeed, SCFAs, mainly butyrate, are important source of energy for colonocytes and are therefore absorbed and metabolized and the concentrations obtained in fecal samples are the remaining non-absorbed SCFAs found in the most distal part of the gut. It is plausible that the SCFAs produced in the proximal intestine could have an effect on gut barrier and immunity but their concentrations are much more difficult to obtain in humans. Therefore, the data related to the fecal levels of metabolites must be carefully interpreted.

Discussion on psychological data

We found that intestinal permeability was correlated with the psychological markers of addiction severity such as depression, anxiety and alcohol craving. This suggests that the gut may influence the brain functions and emotions. We also measured a personality factor called emotional intelligence in alcohol-dependent and controls subjects. Emotional intelligence reflects the ability to adaptively perceive, understand, regulate and utilize one’s emotions and those of others [251]. Alcohol-dependent and control subjects filled in a self-reported questionnaire consisting in 75 items and that encompasses four factors: well-being, self-control, emotionality and sociability. The scores of all factors were lower in alcohol-dependents subjects compared with controls (figure 3-1).

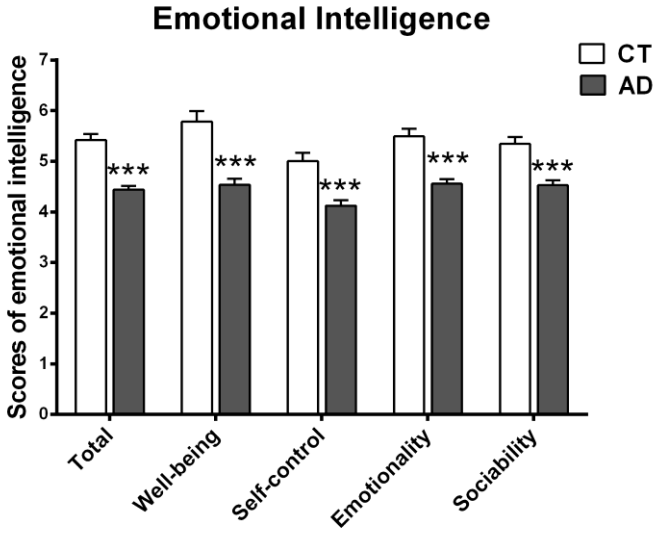


Figure 3-1 : Score of emotional intelligence in alcohol-dependent (AD) and control (CT) subjects.

We found that intestinal permeability was negatively correlated with the sociability factor of emotional intelligence, suggesting a possible relation between intestinal permeability and the expression of a personality trait, that may be relevant for interpersonal relations (figure 3-2). The personality trait sociability refers to social competence, emotion management and assertiveness. High scorers for this trait usually perceive themselves as accomplished networkers with excellent social skills and capable of influencing other people's feelings whereas low scorers considered themselves as followers rather than leaders. Furthermore, in a limited number of subjects for which we have analyzed the gut microbiota, we observed a positive correlation between the sociability factor and the genus *Bifidobacterium* spp (figure 3-3). This bacterium was also negatively correlated with the score of alcohol craving. The observation of these relations does not allow to prove a causal link between gut permeability or gut bacteria and personality. However, if this came to be verified, it would mean that strategies aiming at improving the gut microbiota and restoring gut barrier function might help patients to improve their psychological well-being and might potentially influence personality trait.

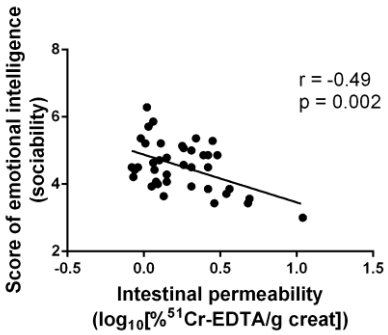


Figure 3-2: Association of intestinal permeability and the sociability factor of emotional intelligence

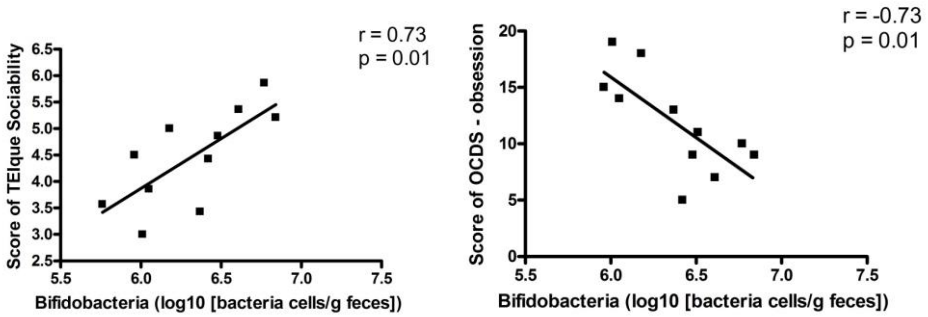


Figure 3-3: Association of *Bifidobacterium* spp with the sociability factor of emotional intelligence and with the score of alcohol craving (OCDS).

The conclusions of Chapter 3 are the following:

- Only a subset of alcohol-dependent subjects presents gut leakiness. A direct toxic effect of ethanol on intestinal epithelial cells is likely not to be the main mechanism upon chronic alcohol consumption, since subjects with high and low intestinal permeability exhibit similar amount of alcohol intake.

- Only subjects with leaky gut have gut dysbiosis. This result suggests that alteration of the gut microbiota is an indirect mechanism by which ethanol induces increase in intestinal permeability.

- Alteration of the gut microbiota composition consists mainly in reduced abundance of Ruminococcaceae (*Ruminococcus*, *Faeacalibacterium*, *Suboligranulum*, *Oscillibacter and Anaerofilum*) and *Bifidobacterium* and increased abundance of Lachnospiraceae (*Dorea*) and *Blautia*.

- Alterations of the gut microbiota functionality are revealed by using fecal metabolomics and consist mainly in significant changes in the level of phenolic and indole-derived compounds. Phenol is considered to be detrimental for intestinal epithelial cells while indole-derived compounds and para-cresol are more susceptible to exert beneficial effects on the gut barrier.

- Higher intestinal permeability is associated with the persistence of psychological symptoms at the end of alcohol withdrawal. Patients with leaky gut suffer from a more severe addictive behavior and may be more susceptible to relapse after a detoxification program.

GENERAL CONCLUSIONS AND PERSPECTIVES

1 GENERAL CONCLUSIONS

The harmful use of alcohol ranks as the leading risk factor for death and disability when considering people aged 15-59 years [2]. The prevalence of alcohol use disorders reaches 7.5 and 6% of the population in Europe and America, respectively, and all social classes are concerned [2]. Alcohol addiction has traditionally been considered a brain disease. Neurobiological studies have identified ethanol-induced changes in the concentration of neurotransmitters involved in the brain reward circuit. However, pharmacological treatments that target the brain neurotransmitters have been disappointing and the relapse rate in this disease is still very high. That is why alcohol dependence is defined as a chronic relapsing disease. In addition to the deleterious effect of ethanol on the brain, many other peripheral organs are damaged. In this PhD work, we have focused on the effect of heavy and chronic alcohol consumption on the gut and more particularly on the gut microbiota which is now seen as an "exteriorized" organ placed within the body, which plays a key role in the regulation of host physiology and behavior. Abnormal composition of the gut microbiota has been reported in several somatic diseases such as obesity and metabolic disorders, Crohn's disease, ulcerative colitis and more recently in animal models of autism and alcohol dependence [90, 160]. Using germ-free animals and interventions that modify the gut microbiota composition (pro- and prebiotics, antibiotics) as well as behavioral tests that mimic human depression and anxiety, experimental researches of the last few years have suggested several mechanisms underlying the communication between the gut bacteria and the central nervous system. These data evoked a role for the inflammatory cytokines, the HPA axis, the vagus nerve, the metabolites produced by the bacteria and the alterations of tryptophan metabolism by inflammation as potential mediators [164]. However, it remains an intriguing idea that the composition of the gut microbiota might be associated with psychiatric conditions and data showing associations between the gut and the brain are scarce in humans [191–194]. The aim of this thesis was to test whether alterations in the gut microbiota composition and functions occur in alcohol-dependent subjects and whether these changes could be related to psychological symptoms of addiction.

In the first study, we developed the ^{51}Cr-EDTA method to assess both small bowel and colon permeability in alcohol-dependent subjects that had not developed liver damage. We

found that these patients presented with leaky gut, elevated plasma LPS levels and low-grade systemic inflammation. A short term alcohol withdrawal permitted the total recovery of leaky gut and endotoxemia but inflammation was still present. This suggests that LPS is not the only agent inducing an inflammatory response. Furthermore, this study points out for the first time an important relationship between inflammatory markers and alcohol craving which play a central role in the drinking behavior.

As inflammation may be a communication pathway between the gut and the brain, we looked for the origin of systemic inflammation in alcohol-dependent subjects. In the second chapter, we found that, in addition to LPS, another bacterial product called peptidoglycan that derived from Gram positive bacteria also crossed the gut barrier and reached the systemic circulation. These gut-derived bacterial toxins activated the peripheral blood mononuclear cells (PBMCs) which may contribute to the elevated plasma pro-inflammatory cytokines IL-1β and IL-8. Activated inflammatory pathways in PBMCs were related to the amount of alcohol consumed. While a short-term alcohol withdrawal was associated with LPS recovery, peptidoglycan seemed to be still present in the circulation, in parallel with the persistence of inflammatory markers at the end of the detoxification program. The improvement of alcohol craving score during the withdrawal was associated with the decrease in inflammatory markers, and IL-8 was found to be the best predictor of the psychological outcome.

In the last chapter, our attention was paid on the role of the gut on psychological and emotional disturbances. We observed that some, but not all, alcohol-dependent subjects developed gut leakiness. Direct toxicity of ethanol does not seem to be the causal factor of the increase in intestinal permeability as both subjects with and without leaky gut drank similar amount of alcohol. However, deep analysis of the gut microbiota composition and functionality revealed that subjects with leaky gut had also altered gut microbiota. Some specific bacteria like Ruminococcaceae and Lachnospiraceae or bacterial metabolites like phenolic and indole compounds may be involved in the regulation of the gut barrier function. Therefore, alteration of the gut microbiota is suggested to be an indirect mechanism by which ethanol would increase intestinal permeability. More importantly, subjects with leaky gut and altered gut microbiota presented with a more severe form of dependence including higher levels of depression, anxiety and alcohol craving compared to subjects without dysbiosis. The results of this third study showed, for the first time in human alcohol-dependent subjects, an association between the gut and the psychological symptoms of addiction.

All these data are in favor of the existence of gut-brain axis in alcohol-dependent subjects, where inflammation could play a role in the communication between the organ originally devoted “simply” to absorption or fermentation of nutrients, and the central nervous system. The high fraction of patients who resume alcohol drinking few months, few weeks or even directly after a detoxification program (which is supposed to restore a neurochemical equilibrium and suppress the dependent state) is a reality difficult to accept for the medical community, for the patients and their family. The persistence of inflammation and dysbiosis observed at the end of detoxification supports the idea that new strategies, targeting the gut and not the brain, might be beneficial in the treatment of alcohol dependence. More particularly, the use of nutritional factors called prebiotics appears to be the most promising strategy to improve, naturally and in a safe way, gut and psychological health in alcohol-dependent subjects.

2 DISCUSSION AND PERSPECTIVES

In this section, we will provide additional elements of discussion concerning the overall principles of the rationale of the PhD work, but also concerning results that we had obtained during the course of the different studies presented in the results section. We will also present and briefly discuss additional preliminary data that offer new perspectives for future studies.

Specificity of our study design

During this PhD work, all tests were performed in human subjects. Alcohol-dependent subjects were recruited the day of their admission at the hospital for a detoxification program. One advantage of the studies presented here resides in the fact that we have not only analyzed the effect of heavy and chronic alcohol consumption but also the effect of short-term alcohol abstinence on biological and psychological parameters related to alcohol dependence. The effect of alcohol withdrawal has, indeed surprisingly, been very poorly described in the literature while it is a critical period that occurs repeatedly during this chronic relapsing disease. Contrary to experimental studies on animal models, we only have few possibilities of interventions in humans to test specific hypothesis. Alcohol withdrawal is a very specific condition that allows the distinction between the effects of intoxication and those of chronic alcohol exposure that can persist even after several weeks of abstinence. The patients incorporated into our studies were tested twice, at the beginning and at the end of the detoxification program using the same tests, methods and conditions. Within the test re-test period, all patients have maintained abstinence during a period of 18 days. Moreover, they received the same hospitalization program, the same psychological support and they went back home after the same period of time. It is therefore a highly controlled design, and these conditions have generally not been met in the previous human studies.

Investigation of the gut barrier function

Alteration of the gut barrier has been analyzed using in vivo permeability test and results showed that some, but not all alcohol-dependent subjects developed leaky gut. However, it would be interesting to analyze other parameters that are associated with the gut barrier function such as TJs expression, antimicrobial peptides and mucins. During the detoxification program, alcohol-dependent inpatients underwent small intestinal biopsies usually 2 days after the last drink. We therefore have material for deeper analysis of the gut barrier function. The gene expression of TJs that regulate the paracellular permeability has been assessed by quantitative PCR. Results showed that the expression of claudin-1, occludin and ZO-1 in intestinal epithelial cells of alcohol-dependent subjects did not differ from that of controls (figure 2-1). However, if alcohol exerts a direct toxic effect on epithelial cells, one could expect that the protein expression rather the gene expression would be altered. Western-blot and immunohistochemistry targeting the TJs are in progress.

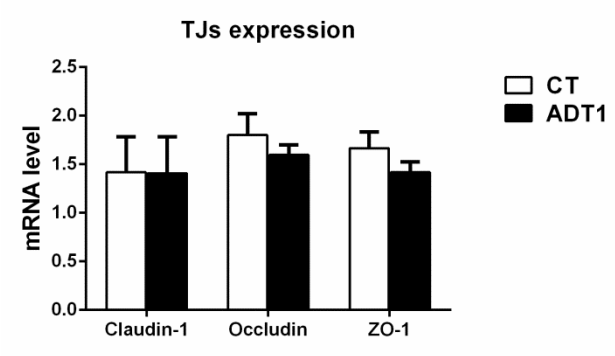


Figure 2-1: TJs mRNA expression in small bowel biopsies obtained from alcohol-dependent subjects and controls. Experiments performed by Peter Stärkel and Christine de Saeger, data not published.

Regarding antimicrobial compounds, Yan *et al.* [90] observed that the gene and protein expressions of Reg3b and Reg3g were reduced in the small intestine of mice fed alcohol compared to control mice. The authors suggested that the decrease in antimicrobial proteins might contribute to the enteric dysbiosis following alcohol administration. They also showed that prebiotic fructo-oligosaccharides restored Reg3g levels. Moreover, the same authors reported a decrease in Reg3g gene and protein expression in duodenal biopsies obtained from actively drinking alcoholic patients.

Concerning the intestinal mucus layer, which forms a physical barrier between the underlying epithelium and the gut lumen, Hartmann *et al*. [252] recently investigated its functional role, and in particular the role of Muc2, using a mouse model of alcoholic liver injury. Muc2 is the most abundant secreted mucin in the gastrointestinal tract and its absence results in a significantly thinner mucus layer. The results of this study showed that, following alcohol administration, knockout $Muc2^{-/-}$ mice exhibited lower plasma LPS levels despite an increased intestinal permeability compared with wild-type mice. This could be explained by the fact that $Muc2^{-/-}$ mice are protected from intestinal bacterial overgrowth and alcohol-induced dysbiosis, potentially through up-regulation of antimicrobial factors such as Reg3b and Reg3g. They tested the hypothesis in alcoholic patients and observed an increased thickness of the mucus layer on duodenal biopsies compared with healthy humans. The authors speculated that following chronic alcohol consumption, the increase in mucus thickness (which could be seen as a good reaction of the intestine) prevent the bacterial ligands to access enterocytes and therefore prevent the production of antimicrobial compounds. Less antimicrobial molecules reach the lumen and the proliferation of intestinal bacteria is less controlled. We could therefore expect that Muc2 is up-regulated in intestinal biopsies of alcoholic patients but this result has never been reported.

We propose to measure the expression of antimicrobial compounds and Muc2 in intestinal biopsies of alcoholic patients in parallel with the in vivo permeability test that showed that only a subset of patients developed leaky gut and dysbiosis. We also propose to test the effect of short-term alcohol abstinence (which is associated with decreased LPS levels and intestinal permeability) on mucins and antimicrobial peptides. One limitation of these proposed studies is that although we can easily have small bowel biopsies as this medical examination is integrated in the patient's hospitalization program, we can hardly have access to colonic mucosa which would be more influenced by the gut microbes.

The gut microbiota and its relation with intestinal permeability

Our study is the first to assess, in human alcohol-dependent subjects, the intestinal permeability, the gut microbiota composition and the fecal bacterial metabolites. Our results showed that only a subset of alcohol-dependent subjects developed gut leakiness that was associated with alteration of the gut microbiota. Dysbiosis, consisting mainly in decrease in Ruminococcaceae and increase in Lachnospiraceae, was suggested to influence the gut barrier

function possibly through microbially-derived metabolites such as phenolic and indole compounds which have been proved to modify the permeability of intestinal epithelial cells in vitro. The levels of other metabolites described in the supplemental section of Chapter 3 of the results section, and in particular medium chain fatty acids and branched chain fatty acids were also very different between subjects with high and low intestinal permeability. However, until now, no experimental data exist in the literature to confirm their involvement in the gut barrier regulation.

We suggest that alcohol induces gut dysbiosis that in turn induces an increase in intestinal permeability. However, the correlations between gut bacteria and permeability do not imply a cause-effect relationship and it could be possible that alcohol alters the intestinal epithelial cells (that form the gut barrier) which results in dysbiosis through, for instance, a reduced production of antimicrobial compounds, as shown in animal study [90]. To test whether gut dysbiosis is responsible for increased intestinal permeability, we could design an experiment in which the gut microbiota of an alcoholic with dysbiosis is introduced in a germ-free mouse, in parallel with another germ-free mouse which receives the gut microbiota of an alcoholic without dysbiosis, and then measure the intestinal permeability of these mice.

The potential pathways underlying the gut microbiota-brain axis

1) The bacterial metabolites

The bacterial metabolites have been considered a potential mechanism underlying gut-brain axis [164]. We assess, in our study, the fecal metabolome of alcohol-dependent subjects. As fecal metabolites may likely influence the gut barrier function, we found consistent to test the association between specific fecal bacterial metabolites and the host intestinal permeability. However, it is unlikely that fecal metabolites directly influence brain functions but it is possible that the metabolites produced in the gut lumen stimulate intestinal cells which in turn secrete other neuroactive compounds that could be transmitted to the brain. Also, fecal metabolites are absorbed through the gut mucosa and then access the blood compartment and are further metabolized by host enzymes (liver, kidney). As shown in a recent study using mouse model of autism [253], it is possible that blood metabolites, that initially originate from the gut microbes, influence brain functions and behavior.

It would therefore be interesting to assess in parallel fecal and blood metabolomes and to test the association between blood metabolites and brain functions.

2) The tryptophan metabolism

In the gut lumen, tryptophan (TRP) is metabolized by the bacteria that form the end-products indole and 3-methyl indole (skatole) which seem important regarding to gut barrier function. Gut bacteria are also able to convert TRP into kynurenine (KYN) but this compound was not detected in our GC-MS fecal analysis. However, TRP can cross the gut mucosa and reach the systemic circulation where it can be converted into either serotonin (5-HT) or KYN depending on the inflammatory conditions. As described in the introduction, TRP is the precursor of 5-HT an important neurotransmitter involved the regulation of emotional states. Pro-inflammatory cytokines can activate the enzyme indoleamine-2,3-dioxygenase (IDO) expressed in immune cells to degrade TRP into KYN and other metabolites which are neuroprotective (kynurenic acid, KYNA) or rather neurotoxic (quinolinic acid, QUIN) (figure 2-2).

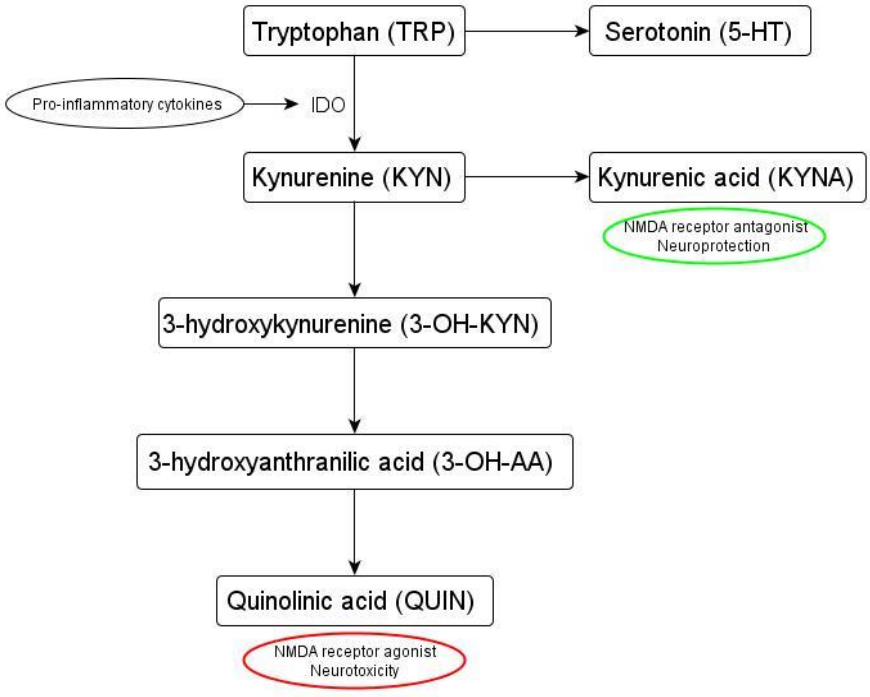


Figure 2-2 : Tryptophan metabolism pathway. Tryptophan is the precursor of serotonin but upon inflammation, tryptophan is converted into kynurenine.

In collaboration with Dr. Aye-Mu Mynt (Ludwig-Maximilian University, Germany), we obtained data on the blood concentration of the tryptophan metabolites in 55 alcohol-dependent subjects at the beginning (T1) and end (T2) of a 3-week alcohol withdrawal. The results are shown below (figure 2-3).

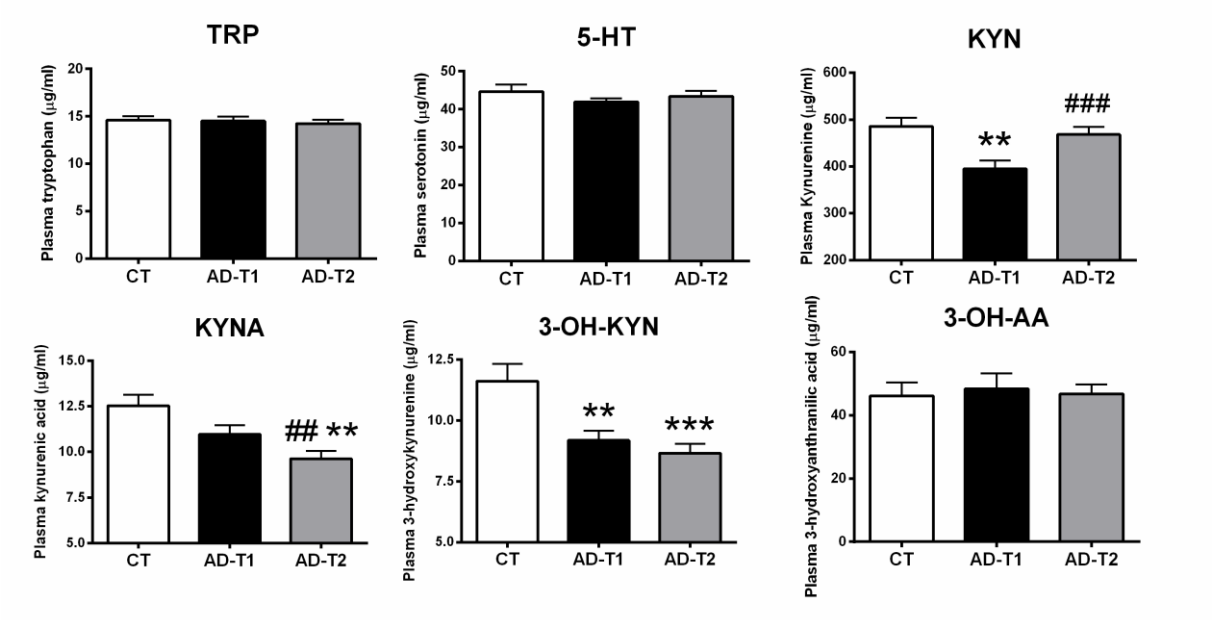


Figure 2-3: Plasma levels of tryptophan metabolites in alcohol-dependent (AD) and control (CT) subjects at the beginning (T1) and end (T2) of a 3-week detoxification program. ** $p < 0.01$ vs CT, *** $p < 0.001$ vs CT, ## $p < 0.01$ vs ADT1 and ### $p < 0.001$ vs ADT1.

We found that the levels of TRP and 5-HT in alcohol-dependent subjects were similar to those of controls. However, the levels of KYN and of 3-hydroxykynurenine (3-OH-KYN) were significantly decreased in the patients at the beginning of withdrawal. Therefore, the ratio KYN/TRP was significantly decreased in alcohol-dependent subjects. In experimental studies in which inflammatory response was induced by a pathogen stimulus, the ratio KYN/TRP is generally increased, due to conversion of TRP into KYN by IDO.

The results of the plasma concentrations of TRP and KYN metabolites are difficult to interpret because they did not necessary reflect the brain concentrations. Also, the surprising decreased plasma level of KYN may be due to:

1) Its further conversion into 3-OH-KYN and then into 3-hydroxyanthranilic acid (3-OH-AA). The latter metabolite is the precursor of QUIN which has been shown to be neurotoxic but this end-product metabolite was not yet measured in our study.

2) The fact that KYN enters the brain via large amino acid transporter, which also allows the transport au TRP across the blood-brain barrier. We therefore could measure the plasma levels of other large neutral amino acids such as tyrosine, valine, leucine, isoleucine, phenylalanine which also compete for the transporter and could affect the availability of brain TRP.

As mentioned above, it is possible that some blood metabolites might influence brain function and behavior. We therefore tested the correlation between the tryptophan metabolites and the psychological symptoms developed by alcohol-dependent but we did not find convincing results suggesting that this pathway does not seem to have significant effect on behavioral dimensions tested so far.

3) The vagus nerve

The importance of the vagus nerve in gut-brain interactions is controversial. Some studies have shown that the effects of gut microbiota or potential probiotics on brain functions are dependent of vagal activation [182, 183]. However, the mechanisms through which vagal afferents become activate by gut bacteria are still unclear. Vagus-independent mechanisms have also been demonstrated. For instance, infection with parasite *Trichuris muris* induced gastrointestinal inflammation and anxiety-like behavior in mice. Vagotomized mice infected with the parasite display similar anxiety-like behavior [184]. In another study, mice received orally a mixture of antimicrobials that altered the composition of the microbiota and the exploratory behavior. The effect of antimicrobials on behavior was still present in previously vagotomized mice [169].

In humans, it is not an easy task to determine the role of the vagus nerve in the communication between the gut and the brain but it is possible to obtain, quite easily, data on the vagus nerve activity which is reflected by the heart rate variability (HRV). In a continuous electrocardiographic record, each QRS complex is detected and the so-called normal-to-normal intervals, i.e. all intervals between adjacent QRS complexes resulting from sinus node depolarization, are determined [254]. We therefore performed on alcohol-dependent subjects electrocardiograms from which we extracted two indices of the HRV called time-domain measures: Standard Deviation of Normal-to-Normal intervals (SDNN) and Root Mean Square of Successive Differences between normal-to-normal intervals (RMSSD).

Our preliminary results showed a decrease in HRV in alcohol-dependent subjects (SDNN: 26 ±14 ms; RMSSD: 29 ± 17 ms) compared to values obtained in healthy population (SDNN: 50 ±16 ms; RMSSD: 42 ± 15 ms) [255]. The results are consistent with a recent meta-analysis that revealed that alcohol dependence is associated with a reduced HRV [256] . Interestingly, we found significant negative correlations between HRV and pro-inflammatory cytokines, particularly TNFα (table 2-1). This result is in line with the anti-inflammatory capacity of vagal activation [257].

Table 2-1 : Correlation between heart rate variability (HRV), alcohol consumption and cytokine TNFα.

HRV vs.	Spearman r	p-value	n
Alcohol consumption (g/day)	- 0.584	0.011	18
Plasma TNFα (pg/ml)	- 0.595	0.006	20

To summarize the data, we observed that high alcohol consumption is related to reduced vagal activity. The more reduced the vagal activity, the higher the inflammation. The higher the inflammation, the higher the depression and craving. From this data, we can hypothesize that the vagus nerve could mediate the effect of peripheral inflammatory cytokines on the psychological symptoms of alcohol dependence.

The effect of a short-term alcohol withdrawal on vagus nerve activity is currently under investigation, in collaboration with Dr Yori Gidron, from Vrije Universiteit van Brussels.

4) The inflammatory cytokines

Alcohol-dependent subjects are characterized by a chronic low-grade inflammation which was shown to be associated with psychological dimensions like depression and alcohol craving. It is challenging to investigate the behavioral consequences of chronic inflammatory process. While many studies have been performed in animals, data in humans are limited. Behavioral tests (force swim test, tail suspension test, elevated-plus maze, open field test) have been designed to assess mood changes in animals. Overall, they are limited to the measurement of depression-like or anxiety-like behavior. In patients, mood assessment is

done by questionnaires and it is possible to distinguish a lot of symptoms associated with mood such as feeling of guilt, anhedonia, fatigue, irritability, suicidal thoughts, feeling of helplessness. It is obvious that many aspects of human behavior and psychology cannot be modeled in laboratory animals. To counteract this problem, human models of systemic inflammation have been proposed. One model, developed by Capuron *et al*., is based on the therapeutic administration of cytokines (IFNα or IL-2) in cancer patients and then analysis of anxious and depressive symptoms [209]. IFN therapy is also commonly proposed in the treatment of hepatitis C and is associated with the development of major depressive episodes [258, 259]. However, injecting a single cytokine does not very much mimic a natural immune response induced by a pathogen. Another model consists in injecting intravenously bacterial endotoxins (LPS) to raise circulating cytokines levels in healthy volunteers and then analyzing mood and neuropsychological competences [214, 215, 260–263]. The main limitations of these models reside in the fact that LPS injection induces an acute and transient inflammatory response and therefore do not reproduce chronic inflammatory conditions which are more frequently encountered in the clinic. Moreover, one drawback of such type of study is the potential bias linked to the patient's subjectivity or social desirability when filling in psychological questionnaires and more importantly, these types of studies raise ethical concerns and are therefore not easily approved. On the other hand, another important point has been raised recently by Seok *et al.* regarding the extensive use of murine models in biomedical research [264]. They found very low correlation of expression changes between human genes and their mouse orthologs in several inflammatory conditions demonstrating that the genomic responses to inflammatory stimuli in mouse models cannot be extrapolated to human conditions. Therefore, it is essential to design experimental studies in animal models to understand the complex interactions between peripheral immune response and brain functions and behavior but it is also important to confirm data obtained in animals models in humans whenever possible and give higher priority to translational medical research.

In this PhD work, we had the opportunity to measure inflammatory response elicited by chronic alcohol drinking under naturalistic conditions. We provided data on the relationship between peripheral inflammatory markers and the severity of depression and alcohol craving in alcohol-dependent subjects. As pro-inflammatory cytokines are known to influence many brain functions, we are currently performing studies in order to test the association between inflammatory markers and cognitive and executive functions such as working memory, selective attention, inhibition, flexibility and decision making in alcohol-dependent subjects.

These functions are, as described in the introduction, other important factors playing a role in the loss of control over drinking and other behaviors observed in alcohol-dependent subjects. Most of these functions cannot be tested on laboratory animals and up to now human data are lacking.

Also, as mood disorders are recognized as having a strong association with a pro-inflammatory state, one could therefore imagine that reducing inflammation may have a positive impact on mood. The first strategy aiming at reducing inflammatory state is the use of anti-inflammatory agents such as non-steroidal anti-inflammatory drugs (NSAID), minocycline (a tetracycline antibiotic) and anti-TNFα agents (etanercept, infliximab). The use of these anti-inflammatory drugs to treat mood disorders has shown controversial results [265] and they are definitely not recommended in alcohol-dependent subjects for several reasons: NSAID has been demonstrated to increase the gut permeability [64], antibiotics are known to strongly alter bacterial diversity [266] and more importantly, anti-inflammatory compounds have the potential to compromise resistance to infection. However, another, more interesting, indirect strategy to reduce inflammation would be the use of pro- or prebiotics which can target the origin of inflammation. Indeed, these dietary compounds have been shown to improve gut microbiota composition [267] and reduce gut leakiness as well as endotoxemia and inflammation [126, 177, 268] also in the context of chronic alcohol consumption [92, 93, 269]. Moreover, recent findings support the idea that they can have beneficial effect for mental health in animal models [182, 253, 270] and humans studies [191, 193, 194]. However, exposing an extremely leaky intestine to additional live bacteria, albeit apparently “friendly” probiotics may be unsafe. Therefore, the use of prebiotics appears to be the most promising strategy to improve, naturally and in a safety way, gut and mental health in alcohol-dependent subjects.

Potential factors that could be responsible for the development of gut leakiness and dysbiosis in a subset of alcohol-dependent subjects.

Despite the demonstration of a direct toxicity of ethanol on intestinal epithelial cells in vitro, we failed to found a relation between ethanol intake and intestinal permeability in our study on human alcohol-dependent subjects. Hence, an indirect mechanism involving changes in the gut microbiota may underlie ethanol-induced increase in intestinal permeability. However, we still do not know why some subjects resist to microbial changes upon heavy alcohol consumption whereas other patients presented with drastic dysbiosis. A major challenge was therefore to determine the factors that are responsible for the development of gut leakiness and dysbiosis in only a subset of alcoholics, or conversely factors that may protect alcohol-dependent subjects from gut dysfunction.

It is well established that changes in the microbial composition could be induced by several environmental factors including antibiotics use, life-style, stress, diet, as well as age and genetics. We were able to control two of these factors: subjects with high and low intestinal permeability did not differ for age and subjects who took antibiotics were excluded from the study. Concerning the dietary intakes we failed to control them in the study presented in chapter 3. This is important information missing in our study since we know that nutritional intakes are strongly altered in alcohol dependence [271]. However, this has been evaluated in a previous, independent study conducted on a population of alcohol-dependent subjects recruited in the same conditions [271]: on average, alcohol accounted for almost 40% of caloric intake and proteins, lipids and carbohydrates intakes were significantly reduced compared to those of healthy controls. This observation of frequent dietary deficits in this population could be responsible for the development of dysbiosis observed in a subset of patients. However, the two groups of patients, although presenting with large differences in microbiota composition, consumed similar amounts of ethanol. If diet was a major cause for differences in gut microbiota, this would happen independently of the amount of ethanol consumed. This hypothesis has never been tested so far. Mutlu *et al*. [94] reported no difference in fat and fiber intakes between non-dysbiotic and dysbiotic alcohol-dependent subjects, even if they admit that nutritional assessments were performed on very limited number of subjects. In our study, plasma pre-albumin level which could be seen as a marker of nutritional status [272], did not differ between low and high intestinal permeability groups.

These observations do not support a major influence of diet to distinguish dysbiotic from non-dysbiotic alcohol-dependent subjects.

Other factors may explain dysbiosis in alcohol-dependent subjects: genetics, life-style and stress. It is plausible that genetic variation exist between subjects with dysbiosis and subjects with a normal gut microbiota. Moreover, evidence of genetic influences on addiction has been provided by adoption or twin studies which support the conclusion that the proportion of risk for alcohol dependence explained by genes is between 40 and 60% [273]. American researchers are currently testing the hypothesis that disrupted circadian rhythms may contribute to the differential susceptibility for alcohol-induced gut leakiness in a subset of alcoholics [81, 274, 275]. Circadian rhythms control a variety of biological processes including sleep/wake cycles, body temperature, hormone secretion, glucose homeostasis, immune function as well as intestinal function since circadian genes control the expression of TJs genes involved in the regulation of intestinal permeability [276]. Disruption of circadian rhythms has deleterious consequences for health and has been associated with metabolic syndrome, cardiovascular disease, cancers and intestinal disorders. Life-style and environmental factors such as shift work, late-night activity, jet lag but also timing of food intake and alcohol consumption are obvious disrupters of circadian rhythmicity [277]. An in vitro study [81] has shown that alcohol disrupts intestinal epithelial cells permeability in parallel with up-regulation of protein levels of circadian clock genes CLOCK and PER2. Moreover, knock down of these genes expression by siRNA prevent alcohol-induced hyperpermeability in Caco-2 cells [81]. Further studies are therefore needed to determine whether the expression of intestinal circadian genes is altered in alcoholic patients and whether change in circadian gene expression can differentiate alcoholics with and without leaky gut.

Finally, the question of stress also deserves interest since changes of microbial composition under stress conditions have been reported in animal studies [172, 278] and in humans [279, 280]. In some individuals, alcohol drinking is an attempt to cope with stress [281]. Life-stress has been shown to alter the activity of the HPA axis. Indeed, stress triggers the release of corticotropin-releasing factor (CRF) in the hypothalamus, causing adrenocorticotrophic hormone (ACTH) secretion from the pituitary gland and subsequently glucocorticoids (corticosterone in rodents, cortisol in humans) from the adrenal glands. In

animal models, maternal separation (MS) during the neonatal period is used to mimic early-life stress. MS in rodents is associated with enhanced activity of the HPA axis resulting in increased CRH content in hypothalamic neurons [282] and elevated levels of serum corticosterone [283]. CRF has been also implicated in stress-induced intestinal abnormalities [284] such as increased colonic permeability [285] and colonic mucin release [286]. Probiotics (*L. rhamnosus* and *L.helveticus*) treatment of MS rat pups was reported to normalize corticosterone levels and ameliorate colonic dysfunction [283]. In adult rats, chronic peripheral administration of CRF, mimicking chronic stress, also caused increase in serum corticosterone levels and colonic barrier dysfunction [287]. In clinic, elevated plasma cortisol concentrations and enhanced CRH levels in cerebrospinal fluid have been repeatedly observed in patients suffering from depression and anxiety [282, 288]. Therefore, we assessed the salivary cortisol level in alcohol-dependent and controls subjects. Our preliminary data revealed that patients with high intestinal permeability had higher cortisol level than subjects with low intestinal permeability at both times of withdrawal (figure 2-4). Moreover, cortisol level was positively correlated with intestinal permeability ($r = 0.43$, $p = 0.03$).

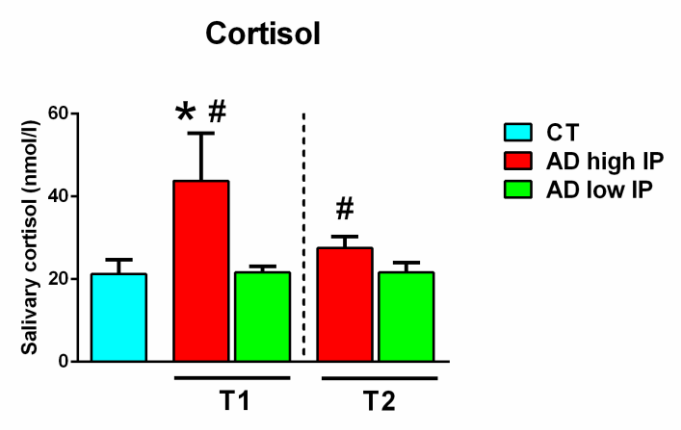


Figure 2-4 : Salivary cortisol levels of alcohol-dependent (AD) and control (CT) subjects at the beginning (T1) and end (T2) of alcohol withdrawal. * $P < 0.05$ vs CT, # $P < 0.05$ vs AD low IP at the same study time point.

From these results, we can hypothesize that gut microbiota and gut barrier alterations associated with higher activity of the HPA axis could reflect a higher stress state or a decreased potential to cope with stress in a subset of alcohol-dependent subjects.

M**ain conclusions**:

- Alcohol-dependent subjects are characterized by a chronic low-grade systemic inflammation. Plasma pro-inflammatory markers positively correlated with depression and craving.

- Leaky gut permits the translocation of gut-derived bacterial toxins like LPS and peptidoglycans which reached the systemic circulation and stimulated peripheral blood mononuclear cells to induce the expression of IL-1β and IL-8. The activated inflammatory pathways in these immune cells are correlated with alcohol consumption and alcohol craving.

- Only a subset of alcohol-dependents subjects developed gut leakiness which is related to alteration of the gut microbiota. Dysbiosis is suggested to be an indirect mechanism by which ethanol induced increased intestinal permeability. Leaky gut and dysbiosis are associated with the persistence of psychological symptoms at the end of alcohol withdrawal. Gut dysfunction is therefore related to a more severe form of dependence and could influence the probability of relapse after detoxification.

- A short term alcohol-withdrawal is associated with the total recovery of intestinal permeability and plasma LPS levels whereas peptidoglycans, inflammatory markers and gut dysbiosis persist. The psychological symptoms of alcohol-dependence improved largely during alcohol withdrawal but are still present, at the end of the detoxification program, only in alcohol-dependent subjects with altered gut microbiota.

Main perspectives:

- To analyze the blood metabolome and test association between blood metabolites and psychological symptoms.

- To analyze other components of the gut barrier function such TJs protein, antimicrobial peptides and mucins expression in small bowel biopsies at the beginning and end of alcohol withdrawal. To check their relation with in vivo permeability test showing that only a subset of alcohol-dependent subject develop gut barrier alterations.

- To test the association between inflammatory markers and cognitive and executive functions.

- To perform a randomized, double-blind, placebo-controlled clinical trial to test the effect of prebiotic supplementation on gut microbiota, intestinal permeability, systemic inflammation, and psychological symptoms during a detoxification program, and evaluate the relapse rate within 6 months. Detailed nutritional assessments should be reported.

- To test the effect of alcohol withdrawal on vagus nerve activity.

- To measure plasma quinolinic acid level, a neurotoxic metabolite and end-product of the TRP/KYN pathway.

- To determine whether the expression of intestinal circadian genes is altered in alcoholic patients and whether change in circadian gene expression can differentiate alcoholics with and without leaky gut.

"*The thing that was the most exciting about it all is that I found that without drugs, I was still writing very well, and that was probably the most rejuvenating aspect of it all, is that you don't need to get stoned out of your gourd to write well. It was an incredibly important period for me. I can't say it was like night and day that suddenly I was this different person. It was not. It took quite a long time. But I think I did see light at the end of the tunnel, and it was not a train."*

David Bowie.

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APPENDIX

Appendix

1 ABREVIATION LIST

AD	alcohol-dependent
ADH	alcohol dehydrogenase
AJs	adherens junctions
ALD	alcoholic liver disease
ALDH	aldehyde dehydrogenase
ANOVA	analysis of variance
AP-1	activator protein 1
BAWL	batterie d'attention de William Lennox
BCAAs	branched-chain amino acids
BCFAs	branched-chain fatty acids
BDI	beck depression inventory
BDNF	brain-derived neurotrophic factor
BMI	body mass index
CD14	cluster of differentiation 14
CNS	central nervous system
CRP	C-reactive protein
CT	controls
DSM	diagnostic and statistical manual of mental disorders
FOS	fructo-oligosaccharide

GC-MS	gas chromatography - mass spectrometry
GLP-2	glucagon-like-peptide 2
HPA	hypothalamus-pituitary-adrenal (axis)
hsCRP	high-sensitivity C-reactive protein
IDO	indoleamine-2,3-dioxygenase
IFNβ	interferon-beta
IFNγ	interferon-gamma
IL	interleukine
iNOS	inducible nitric oxide synthase
IRAK1	IL-1 receptor-associated kinase
IRF3	interferon regulatory factor 3
JNK	c-Jun amino-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MDP	muramyl dipeptide
MLCK	myosin-light chain kinase
MUC2	mucin 2
MyD88	myeloid differentiation factor 88
NFκB	nuclear factor kappa-B
NLRs	nucleotide oligomerization domain receptors
NLRP3	NLR family, pyrin domain containing 3
NMR	nuclear magnetic resonance spectroscopy

NOD2	nucleotide-binding oligomerization domain containing 2
OCDS	obsessive-compulsive drinking scale
PAMPs	pathogen-associated molecular patterns
PBMCs	peripheral blood mononuclear cells
PCA	principal component analysis
PEG	polyethylene glycol
PGN	peptidoglycan
PLS-DA	principal least square-discriminant analysis
PPARγ	peroxisome proliferator-activated receptor-γ
PRRs	pattern recognition receptors
qPCR	quantitative polymerase chain reaction
Reg3g	regenerating islet-derived 3-gamma (mRNA)
RegIIIγ	regenerating islet-derived 3-gamma (protein)
ROS	reactive oxygen species
RT	retention time
SCFAs	short-chain fatty acids
SD	standard deviation
SEM	standard error of the mean
STAI	state-trait anxiety inventory
TJs	tight junctions
TLFB	time-line follow-back

TLR2	toll like receptor 2
TLR4	toll like receptor 4
TLRs	toll-like receptors
TNFα	tumor necrosis factor-alpha
TRIF	TIR domain-containing adapter inducing IFN-beta
VOCs	volatile organic compounds
ZO-1	zonula occludens 1

Appendix