Susceptibility to chronic liver disease
- Role of environmental and genetic factors

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*Nihil difficile volenti*
Susceptibility to chronic liver disease
- Role of environmental and genetic factors

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ABSTRACT

The onset and the progression of chronic liver disease involve environmental and genetic factors. Hepatic stellate cells (HSCs) are important players in these processes and are the main storage site for retinol. We studied the role obesity, alcohol and patatin-like phospholipase domain-containing 3 (PNPLA3) I148M variant on the susceptibility to chronic liver disease. Moreover, we tried to understand the molecular mechanism underlying the association between PNPLA3 and chronic liver disease.

In paper I we analysed the long-term effect of weight loss due to bariatric surgery on liver damage in a large prospective controlled cohort, the Swedish Obese Subjects study. We analysed changes in serum transaminases between follow-up and baseline values in the bariatric surgery and control groups. Serum transaminases at 2- and 10-year follow-up were lower in the bariatric surgery than in the control group. The transaminase reduction was proportional to the degree of weight loss. In addition, the prevalence of severe liver disease was lower in the surgery than in the control group during the follow-up.

In paper II we examined the effect of age at onset of at-risk alcohol intake and PNPLA3 I148M variant on the incidence of alcoholic cirrhosis. Both variables were independent risk factors for the onset of alcoholic cirrhosis. However, the risk conferred by the 148M variant was higher in subjects who started at-risk drinking earlier than in those who started later.

In paper III, we tested the hypothesis that PNPLA3 is involved in the retinol release from HSCs. We found that PNPLA3 is regulated by the availability of retinol in HSCs and that it has an esterase activity on retinyl palmitate, which is impaired in the 148M mutant protein.
In conclusion, our data show that modifying environmental factors may affect the natural history of chronic liver disease and that the interplay between environmental and genetic factors defines the individual risk to the disease. Specifically, obesity-related chronic liver damage is reduced by sustained weight loss after bariatric surgery and this may prevent the onset of severe liver disease. Age of exposure to alcohol affects the degree of the risk conferred by PNPLA3 I148M variant. In addition, we suggest that the retinol release from HSCs mediated by PNPLA3 may be one important step in the onset of chronic liver disease.

**Keywords:** chronic liver disease, susceptibility, human genetics, NAFLD, ALD, PNPLA3

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Kronisk leversjukdom är benämningen på en grupp sjukdomar som drabbar levern och utvecklas när levern har skadats. Den orsakas av både miljö- och genetiska faktorer. I denna studie undersökte vi till vilken utsträckning fetma, hög alkohol konsumtion och en genetisk variant av patatin like phospholipase domain containing 3 (PNPLA3) bidrar till att patienter utvecklar kronisk leversjukdom. Dessutom, försökte vi förstå den bakom liggande mekanismen som länkar PNPLA3 och kronisk leversjukdom.


Vi upptäckte även att ålder samt PNPLA3 I148M varianten ökar risken för uppkomsten av alkoholisk levercirros. Dock observerade vi att patienter som började dricka tidigt och har PNPLA3 148M genen drabbades in mindre utsträckt av levercirros än patienter med genen som började dricka sent i livet.

Till slut undersökte vi mekanismen som ligger bakom sambandet mellan PNPLA3 och kronisk leversjukdom. Stellateceller är en typ av celler som finns i levern, lagrar retinol och har en viktig roll i utveckling av kronisk leversjukdom. Vi föreslår att sekretion av retinol från stellateceller förmedlas av PNPLA3, samt att 148M varianten försämrar denna funktion. Detta resultat pekar på en viktig ny mekanism i uppkomsten av kronisk leversjukdom.
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<td>Alcoholic liver disease</td>
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<tr>
<td>ALT</td>
<td>Alanine transferase</td>
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<td>AST</td>
<td>Aspartate transferase</td>
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<td>BMI</td>
<td>Body mass index</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>FFAs</td>
<td>Free fatty acids</td>
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<td>GWAS</td>
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<td>HBV</td>
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<td>HCC</td>
<td>Hepatocellular carcinoma</td>
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<td>HCV</td>
<td>Hepatitis C virus</td>
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<tr>
<td>MAF</td>
<td>Minor allele frequency</td>
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<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
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<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
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<tr>
<td>ORO</td>
<td>Oil Red O</td>
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<tr>
<td>PNPLA3</td>
<td>Patatin-like phospholipase domain-containing 3</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>SOS</td>
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<td>VLDLs</td>
<td>Very low density lipoproteins</td>
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1 INTRODUCTION

Chronic liver disease is a growing cause of morbidity and mortality. In the United States, the prevalence of chronic liver disease has progressively increased in the last 20 years, affecting now about 15% of individuals. The definition of chronic liver disease includes a wide spectrum of liver injury from liver damage to inflammation, necrosis, fibrosis, and carcinogenesis. Liver cirrhosis and hepatocellular carcinoma (HCC) are the main responsible for liver-related mortality and impairment in the quality of life. Liver cirrhosis is the fourteen worldwide, and the fourth in central Europe, cause of death in adults. More than half of the indications for liver transplantation in Europe are represented by cirrhosis.

The onset and the progression of chronic liver disease involve many different factors, such as environmental and genetic factors. The main causes of chronic liver disease are alcohol consumption, hepatitis B (HBV) and C (HCV) virus, and obesity. These factors may lead to the full spectrum of chronic liver damage, from the simple hepatocellular damage (indicated by an increase of serum transaminases) to the end-stage diseases, such as cirrhosis and HCC.

1.1 Environmental factors

In this paragraph the main environmental factors that influence the onset and progression of chronic liver diseases are examined.

1.1.1 Obesity

According to the World Health Organization (WHO) obesity is defined as an abnormal or excessive fat accumulation that may impair health and corresponds to a body mass index (BMI) greater than or equal to 30. The prevalence of obesity has increased in the last three decades and to date it affects 35% of the adult population in the United States. Specifically, obesity prevalence has increased from 14% in the 1980s to 36% in 1990s while, subsequently, it did not show further significant increase, as shown by data from the National Health and Nutrition Examination Survey.

Obesity has become a burden for health care in Western Countries because it is associated with increased morbidity and mortality. A population study carried out on more than 83,000 subjects showed that obese subjects had a higher risk of mortality (due to all the causes) than those with normal-weight. In addition, obese subjects who remain obese have a 24% increased risk of mortality than obese subjects who lose weight after bariatric surgery. The most common diseases associated with obesity are dyslipidaemia, insulin resistance, cardiovascular diseases, and cancer. However,
several studies have also associated obesity to the risk of chronic liver diseases. The most common hepatic alteration found in obese individuals is the accumulation of lipids in the liver, which is defined as fatty liver or hepatic steatosis. In obese subjects undergoing bariatric surgery, the prevalence of steatosis is greater than 80-90% and many subjects have also a more advanced liver damage (prevalence of steatohepatitis 24-98% and cirrhosis 1-7%).

Steatosis is one feature of the so-called non-alcoholic fatty liver disease (NAFLD), which obesity is one of the main risk factors for. NAFLD is a broad disease that includes the entire spectrum of liver damage from the simple steatosis to inflammation (non-alcoholic steatohepatitis or NASH), fibrosis (the latter stage of which is cirrhosis) and carcinogenesis. It assumes the presence of steatosis in absence of other causes of liver damage. The molecular mechanisms underlying the pathophysiology of NAFLD are not fully understood yet. In general, the accumulation of lipids in the liver (i.e., steatosis) may be due to two main causes: increased production or decreased catabolism of lipids. So the main mechanisms involved are: increased dietary intake of free fatty acids (FFAs); increased lipolysis from adipose tissue; increased de novo lipogenesis; decreased β-oxidation; decreased hepatic secretion of very low-density lipoproteins (VLDLs). However, not all the subjects affected by steatosis will progress to a more severe stage. The so-called “two-hit model” hypothesises that after a first hit (i.e., steatosis) another hit is needed to develop NASH. More recently, a multiple parallel hits model has been proposed. In that model, the first hit is insulin resistance, which leads to steatosis. The steatotic liver is more vulnerable to a series of hits (e.g., oxidative stress, adipokines) leading to damage, inflammation and ultimately to fibrosis.

Obesity is also a risk factor for progression of liver diseases. In 2007, a meta-analysis on excess body weight and liver cancer examining 11 cohort studies showed that the risk of this cancer was higher in overweight and obese subjects (17% and 89% increase of the risk, respectively) than in those with normal weight. Another study analysed approximately 11,500 subjects from the United States to determine the effect of obesity on death or hospitalization due to cirrhosis. After 13-year follow up, the risk of cirrhosis-related death or hospitalization was greater in obese than in normal-weight subjects. In particular obese subjects had a 69% increase of the risk of cirrhosis-related death or hospitalization. In addition, a prospective study carried out in more than 18,000
men\textsuperscript{36}, which were follow up for a maximum of 38 years, found that the mortality due to liver disease increased proportionally to body weight. After adjusting for other risk factors, overweight and obese men had a 1.37 and 2.28-fold, respectively, higher risk of liver disease mortality than normal-weight men.

Furthermore, obesity is not only a risk factor for liver disease \textit{per se}, but it accelerates the progression of liver disease when other aetiological factors are present. As an example, a retrospective study\textsuperscript{37} analysed 324 subjects with chronic HCV infection to identify the risk factors for hepatic steatosis, including excess body weight. That study found that the risk of steatosis was higher (4-fold) in obese subjects than in those non-obese. Particularly, in obese subjects, the risk of severe steatosis was even higher reaching a 5-fold increase.

Bariatric surgery generally refers to a surgical procedure performed to obtain weight loss in obese individuals. It includes different procedures\textsuperscript{38} that are classified as: malabsorptive, if they determine a condition of malabsorption; restrictive, if they lead to reduce the stomach size; mixed, if they combine malabsorptive and restrictive effects. To date, bariatric surgery is the most effective treatment to achieve sustained weight loss in severely obese subjects and prevent morbidities and mortality associated with obesity, such as myocardial infarction, cancer and stroke\textsuperscript{14,39,40}. Previous studies show a reduction of steatosis after bariatric surgery\textsuperscript{41,42} and a beneficial effect on transaminase levels\textsuperscript{43}. However those studies have some flaws: small sample size, lacking of a control group, short follow-up. The long-term effect on chronic liver disease prevalence and incidence has not been studied in large case-control studies with long follow-up.

\subsection*{1.1.2 Alcohol}
At-risk alcohol consumption is an important cause of morbidity and mortality, accounting for the 4.6\% of the global burden of disease and injury and 3.8\% of death worldwide\textsuperscript{44}. It is probably the oldest type of chronic liver disease because fermented drinks were present already in the 10,000 BC\textsuperscript{45}. To date, it is one of the most common causes of liver disease worldwide\textsuperscript{44}. In Europe, it represents about 1/3 of the causes of cirrhosis in subjects who underwent liver transplantation\textsuperscript{1}.

The entire spectrum of liver disorders due to alcohol use is defined as alcoholic liver disease (ALD). ALD includes steatosis, inflammation (i.e., hepatitis), fibrosis (the latter stage of which is liver cirrhosis) and carcinogenesis. Steatosis is present in more than 90\% of at-risk drinkers but not all progress to more advanced stage of the disease. If the alcohol consumption is continued, 20–40\% of subjects with steatosis will develop steatohepatitis; of these \~16\% will develop cirrhosis\textsuperscript{46}. The risk of cirrhosis greatly increases with steatohepatitis and 16\% of patients with steatohepatitis develop cirrhosis within five years as compared to 7\% of subjects with simple steatosis\textsuperscript{47}. 

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An important factor that determines the severity of liver damage is the amount of alcohol intake. There is a proportional dose-effect relation between alcohol intake and liver damage. The prevalence of both chronic liver damage and the more advanced cirrhosis increases with increasing amount of alcohol intake. An Italian population study, which was carried out on approximately 6,500 subjects, showed that the prevalence of cirrhosis was 0.15% when the alcohol intake was below 30 g/day but it increased up to 6% when the intake was greater than 120 g/day. Similarly the prevalence of chronic liver disease (at an earlier stage) increased from 0.5% to 8% with alcohol intake below 30 and greater than 120 g/day, respectively. Thus, subjects with alcohol intake greater than 120 g/day had a risk of developing chronic liver disease or cirrhosis that was 36 or 62-fold, respectively, higher than those abstainers. Even though the amount of alcohol intake plays a pivotal role in the onset of ALD, there are other factors that may affect the evolution of alcoholic liver diseases, such as drinking patterns, the duration of at-risk alcohol consumption, and genetics. Drinking also outside mealtimes and the use of different beverages increases the risk of ALD by 3-5 and 23 times, respectively.

Moreover, genetic factors are involved in the pathogenesis of ALD. This aspect will be examined in the paragraph 1.3. Furthermore, alcohol use accelerates the progression of liver diseases due to other aetiological factors5,49.

Over the years, several mechanisms have been proposed to explain the pathogenesis of ALD30,51. Proposed mechanisms for the onset of steatosis include increased lipogenesis by the activation of the sterol regulatory element binding protein 1c (SREBP-1c) and reduced β-oxidation by down-regulation of peroxisome proliferator-activated receptors (PPAR) α. Regarding the progression of liver damage, the classical mechanisms involve alcohol metabolites and redox imbalance. Indeed, the liver metabolises most of the ingested alcohol and the produced metabolites, especially acetaldehyde and acetate, may induce liver damage. Chronic alcohol abuse increases the production of reactive oxygen species (ROS) and reduces that of antioxidants52, such as mitochondrial glutathione. ROS leads to the oxidation of lipids, proteins, and DNA and the activation of immune system and inflammation. In addition acetaldehyde induces the pro-fibrogenic pathway in hepatic stellate cells (HSCs) and acetate increases histone acetylation, thus affecting the expression of several genes amongst which pro-inflammatory cytokines. Chronic abuse of alcohol also leads to the depletion of S-adenosylmethionine, an important molecule in methylation, which is involved in several intracellular functions, such as signalling. Recently, other complementary mechanisms have been proposed90. Alcohol induces the overgrowth of gut bacteria, dysbiosis, and an increase of the permeability of the intestinal wall93. So endotoxin, produced by bacteria in the gut, may reach the liver. In the liver, endotoxin induces an inflammatory response with the production of ROS, oxidative stress and so liver damage.
1.1.3 Others

Other environmental factors may affect the risk for chronic liver disease, such as hepatitis viruses, gut microbiota.

Hepatitis viruses

Chronic hepatitis C virus (HCV) affects approximately 3% of the population worldwide and it is one of the major causes of chronic liver disease in the Western countries\(^5\). Most of the infected HCV patients (~80%) will progress to a chronic liver disease. Among those subjects, 20% will develop liver cirrhosis, approximately 20 years after the infection and 25% of the subjects with HCV-related cirrhosis will develop sequelae of end stage liver disease, such as hepatocellular carcinoma. The effective treatment of the HCV may reduce the progression to cirrhosis but it does not seem to reduce the onset of HCC\(^5\). To date, virus-related cirrhosis is the main cause of liver transplantation in Europe\(^3\). The mechanisms of HCV-induced liver damage have been studied widely. The virus is a RNA virus, which can replicate in the host hepatocytes slowly. Several intracellular pathways are involved in the pathogenesis of HCV-induced liver damage, such as oxidative stress, endoplasmic reticulum stress, and cellular apoptosis. Oxidative stress seems to play an important role in viral genome heterogeneity, which is likely a mechanism used by the virus to escape from the immune system\(^5\). HCV induces a chronic inflammation in the liver that is associated with continuous generation of ROS and thus increased oxidative stress\(^5\). In addition, HCV directly interacts with the endoplasmic reticulum (ER) and affects protein trafficking leading to ER stress\(^5\). Cellular apoptosis might be mediated by the virus itself or by the complex interaction between the host and the virus. Subjects with HCV infection have increased expression of Fas antigen, a marker of apoptosis, in the liver\(^5\). In addition T-lymphocytes, activated by the virus, produce different cytokines that can induce pro-apoptotic pathways\(^5\).

Hepatitis B virus (HBV) affects approximately 2 billion people worldwide and 350 million are chronic carriers\(^6,7\). In 2010 HBV infection was the 10\(^{th}\) cause of death and the cause of approximately half of the total liver cancer mortality\(^2\). HBV is a DNA virus from the hepadnaviridae family and is able to integrate in the DNA of the host\(^6\). The HBV infection is characterised by an initial immune tolerant phase, during which the virus replicates in the hepatocytes\(^6\). Next, an immune reactive phase occurs, characterised by a reaction of the immune system against the infected hepatocytes\(^6\). If the immune response is ineffective, subjects with a chronic HBV infection may develop a more severe disease, such as cirrhosis and HCC. The 5-year incidence of cirrhosis in chronic HBV infected subjects is 8-20\(^{\%}\)\(^6\). Different factors have been involved in the onset of HBV-related HCC and include host and viral factors, such as viral DNA integration, expression of oncogenic proteins, chronic immune mediated inflammation.
**Gut microbiota**

Gut microbiota indicates all the microbes that are present in the gastrointestinal tract. In the last years growing literature associated gut microbiota to health and illness in humans. Gut microbiota has also been involved in the pathogenesis of liver disease and its sequelae. Data in humans show that obese subjects have more bacterial overgrowth and NAFLD subjects have increased intestinal permeability than controls. In addition, chronic alcohol consumption is associated with dysbiosis, bacterial overgrowth and higher intestinal permeability. Subjects with cirrhosis have altered permeability of the intestinal wall and higher bacterial translocation. Indeed, those subjects show increased bacterial DNA and antibodies against microbes in the blood. Dysbiosis, bacterial overgrowth, and increased intestinal permeability facilitate the transfer of endotoxin into the portal blood and so in the liver. In the liver, endotoxin induces pro-inflammatory genes and activation of HSCs, leading to hepatic damage and fibrosis. However, further studies are needed to understand deeply the role of gut microbiota in the onset and progression of chronic liver disease.

### 1.2 Genetic factors

Ethnic differences have been reported in the susceptibility to develop chronic liver disease. For example, the prevalence of NAFLD is higher in Mexican-Americans (~24%) than in non-Hispanic whites (~18%) and non-Hispanic blacks (14%) . The concordance in the prevalence of alcoholic cirrhosis among monozygotic twins is greater than in dizygotic twins. Furthermore, only a subset of subjects with a mild chronic liver disease progresses to a more severe disease. In the last decade, the latest technologies for genetic analyses led to the discovery of new genetic loci involved in the susceptibility to chronic liver disease.

#### 1.2.1 Mendelian disorders and steatosis

Among other causes, liver fat accumulation may be due to some diseases that show a Mendelian inheritance and affect the efflux of triglycerides from the liver, lipid metabolism, fatty acid oxidation, or insulin signalling. These disorders are usually associated with systemic symptoms. Some of these disorders are reported below.

Two diseases that alter the efflux of triglycerides from the liver are: abetalipoproteinemia and familial hypobetalipoproteinemia. Abetalipoproteinemia is an autosomal recessive disorder of lipoprotein metabolism. It is caused by mutations in the microsomal triglyceride transfer protein (MTTP), which is a protein responsible for the formation of VLDLs. Specifically, MTTP catalyses the incorporation of triglycerides with apolipoprotein B (ApoB) molecules to form VLDLs. The disease is characterized by the almost complete absence of ApoB-containing particles in the blood, reducing the triglyceride efflux mediated by the VLDLs and
leading to steatosis. Familial hypobetalipoproteinemia is an autosomal dominant disorder caused by mutations in the ApoB protein leading to truncated protein or impaired folding and secretion. The impaired ApoB function leads to a reduction in the VLDL packing and the accumulation of lipids in the liver.

Familial lipodystrophies are a heterogeneous group of metabolic alterations that are characterized by abnormal distribution of subcutaneous adipose tissue that begins early in life. Specifically, they are characterized by losing fat from the upper and lower limbs. In some cases, they may be associated with steatosis. Several genetic alterations have been identified as causative factors leading to the definition of different types of lipodystrophy (e.g., familial partial lipodistrophy type 2 and mutations in the nuclear laminin A/C gene).

Another example of Mendelian disorder that leads to steatosis is the neutral lipid storage disorder, which is caused by homozygous or compound heterozygous mutations in the patatin-like phospholipase domain-containing 2 (PNPLA2) gene and has an autosomal recessive inheritance. This gene encodes for the protein adipose triglyceride lipase (ATGL), which is responsible for the hydrolysis of triglycerides from the adipose tissue. The accumulation of lipids in the liver is a consequence of a reduced hydrolysis of triglycerides due to the production of a truncated ATGL protein, which has no enzymatic activity or cannot bind to the lipid droplets.

### 1.2.2 Common polymorphisms and steatosis

In most cases, steatosis is not due to a Mendelian disorder of the lipid metabolism but it is influenced by common genetic variants. In the last decade, the development of new technologies for genetic studies led to the discovery of common genetic variants involved in the onset of steatosis. Several genes have been investigated and some are reported below in this paragraph.

**Patatin-like phospholipase domain-containing 3 (PNPLA3)**

In 2008, a genome-wide association study (GWAS) identified one genetic variant (rs738409) that was associated with liver steatosis. This variant results in an isoleucine (I) to methionine (M) substitution at position 148 in the patatin-like phospholipase domain-containing 3 (PNPLA3) protein. To date, it is the most widely recognized mutation for liver disease in humans. This gene and its I148M variant will be discussed in more detail in paragraph 1.3.

**Transmembrane 6 superfamily member 2 gene (TM6SF2)**

A recent exome-wide association study found that the rs58542926 variant of the transmembrane 6 superfamily member 2 gene (TM6SF2) is associated with hepatic lipid content. This variant encodes for a lysine (E) to glutamic acid (K) substitution at the
amino acidic position 167 (E167K). Specifically, that study found that carriers of the 167K variant have higher lipid content in the liver. Next, two studies\(^2\)\(^3\) carried out on Europeans deeply analysed the effect of this genetic variant on NAFLD prevalence and histological features. Both studies showed that the TM6SF2 rs58542926 variant is associated with NAFLD and advanced fibrosis/cirrhosis and that this association is independent from other known risk factors. Another study\(^4\), carried out on an Italian population of 148 subjects with chronic HCV infection, found that carriers of the TM6SF2 167K variant have higher prevalence of severe steatosis. TM6SF2 is highly expressed in the small intestine, liver, and kidney. This gene encodes for a protein of 351 amino acids, which is localized in the ER and the ER-Golgi intermediate compartment of hepatocytes. An in vitro study\(^5\) showed that silencing of TM6SF2 reduces the secretion of VLDLs, increases the intracellular content of triglycerides and lipid droplets. Conversely, the overexpression of TM6SF2 determines a reduction in the intracellular lipid accumulation\(^6\). Thus these data suggest that TM6SF2 affects the onset of steatosis by modulating triglyceride secretion from the liver.

**Apolipoprotein C3 (APOC3)**

Two SNPs in APOC3 have been associated with NAFLD and insulin resistance\(^6\). A study carried out on 95 healthy Asian-Indian men examined the effect of APOC3 rs2854116 and rs2854117 on NAFLD e metabolic parameters. NAFLD was present in 38% of carriers of APOC3 variant allele, while it was absent in all the wild-type homozygotes. In addition, those two variants were associated with increased plasma APOC3 levels, increased plasma triglyceride and reduced triglyceride clearance. The increased levels of APOC3, which inhibits lipoprotein lipase, may lead to an increase in chylomicron remnants that are taken up by the liver, resulting in hepatic lipid accumulation.

**Glucokinase Regulator (GCKR)**

The glucokinase regulator is a protein that regulates the function of the enzyme glucokinase, which is responsible for the glucose storage by regulating the phosphorylation of glucose to glucose-6-phosphate. The GCKR rs1260326 was associated with higher intrahepatic content of triglycerides in different ethnic groups\(^7\). When the joint effect of GCKR rs1260326 and PNPLA3 I148M was tested, the two variants could explain 32% of the liver fat in Europeans, 39% in African Americans, and 15% in Hispanics\(^7\). In addition, the enzymatic activity mediated by fructose-6-phosphate of the purified human GCKR mutant protein was lower than the wild-type protein, leading to an increase of the gluokinase activity. The enhanced activity of gluokinase activity may increase the glycolysis and malonyl-CoA levels, leading to an increase of de novo lipogenesis, inhibition of β-oxidation and, ultimately, to liver fat accumulation\(^8\).
Peroxisome proliferator-activated receptors (PPARs)

PPARs are nuclear receptors that are involved in the regulation of several cellular processes such as cell differentiation, carcinogenesis, glucose, and lipid metabolism. A study, carried out on 79 NAFLD subjects and 63 healthy subjects, found that the PPARα Val227Ala variant was associated with NAFLD, and, specifically, the prevalence of 227Ala allele was lower in NAFLD subjects than in controls. Another study, carried out in 2010 on 622 subjects, examined the effect of the Pro12Ala variant in PPARγ2 on histological features in NAFLD and ALD. The presence of severe steatosis was not associated with the variant, but ALD subjects carrying the 12Ala allele had higher necroinflammation than those carrying the Pro12 homozygotes. Conversely, a study on a Chinese population showed that the PPARγ C161T variant was an independent risk factor for NAFLD, with homozygotes for the T allele and the heterozygotes having a about 5-fold and 3-fold higher risk than homozygotes for the C allele. In addition, the PPARγ coactivator 1α gene (PPARGC1A) rs2290602-T allele has been associated with a 2.73-fold increased risk of steatosis in a Japanese population. On the other hand, a Chinese study showed that the prevalence of the PPARGC1A Gly482Ser was not different between NAFLD subjects and controls.

1.2.3 Other genetic variants involved in ALD

In addition to genetic variants that affect the onset of steatosis, other variants may affect the susceptibility to ALD by modulating other factors, such as alcohol dependence and ethanol metabolism. Some studies suggested that polymorphisms in the gamma-aminobutyric acid receptor alpha-2 (GABRA2) gene might be associated with alcohol dependence. A meta-analysis, which examined 50 genetic case-control studies, found that alcohol dehydrogenase (ADH) 2*1, ADH3*2, and aldehyde dehydrogenase (ALDH)2*1 coding allele were risk factors for alcoholism with a stronger effect in Asians and milder in Europeans.

1.2.4 Genetic variants involved in other chronic liver diseases

The individual genetic background strongly affects the onset and progression of other chronic liver diseases, such as chronic HCV disease and autoimmune diseases. Below are reported some examples.

In the last few years, interleukin (IL) 28B locus has been widely studied for its association with the response to interferon treatment of chronic HCV infection. In 2009 a GWAS carried out on around 1,600 subjects to identify genetic factors that may explain the difference in the rate of response between subjects with Europeans and African American ancestry. That study identified a SNP, rs12979860, located...
upstream to IL28B gene to be associated with the response to the treatment. In particular, the CC genotype was associated with a higher response to the treatment in both Europeans and African Americans. However, the prevalence of the CC genotype was higher in Europeans than in African subjects and this different prevalence may account for 50% of the difference in the response to treatment between the two populations. Next, the association between IL28 rs12979860 and response to antiviral treatment has been confirmed in several independent studies. To date, genetic tests for the IL28 genotype are widely available.

Regarding autoimmune liver disease, the strongest association with SNPs were found within the human leukocyte antigen (HLA) region. Primary biliary cirrhosis (PBC) is characterised by a slow progressive destruction of the small bile ducts. Case-control studies identified DRB1*08 alleles as risk factors for PBC in Europeans, while DRB1*11 has a protective effect against the disease. After the beginning of the GWAS era, some studies tried to identify PBC susceptibility loci. A GWAS in 2009, carried out on 536 North American, identified 13 variants in the HLA class II region and other variants in IL12A and IL12 receptor β2 to be associated with PBC cirrhosis. These results were confirmed in an independent GWAS study carried out in 2010 in an Italian population; that study also identified other non-HLA loci. Another GWAS in 2011 identified 12 novel susceptibility loci for PBC, such as STAT4 and DENND1B, which have been involved in the pathogenesis of other autoimmune disorders. Thus, literature data suggest that both innate and adaptive immune responses are involved in the pathogenesis of PBC. Primary sclerosing cholangitis (PSC) is characterised by chronic inflammation of the bile ducts that leads to their obliterative fibrosis. Over the years, several HLA variants, such as DR3 and HLA-B8, have been associated with the risk of PSC. In 2010, a GWAS carried out in Northern Europeans identified the HLAB locus to be strongly associated with PSC. Two non-HLA SNPs, rs3197999 in MST1 and rs6720394 near BCL2L11, were also identified by another GWAS in 2011. Ulcerative colitis (UC) is present in 60-80% of patients with PSC. Thus many studies have investigated subjects with both diseases. Recently, a GWAS examined more than 4,000 subjects to identify susceptibility loci for only PSC or UC and loci for both diseases. That study identified two novel susceptibility loci, GPR35 and TCF4. The GPR35 variants were associated with both PSC and UC, while TCF4 variant was associated with only PSC. However, the mechanism by which these two loci actually affect the onset of PSC is unknown.
1.3 Patatin-like phospholipase domain-containing 3 (PNPLA3)

PNPLA3 or adiponutrin is a phospholipase that belongs to the family of patatin-like domain-containing phospholipases\textsuperscript{116,117}. The family has this name because the first family member was identified in potatoes\textsuperscript{118}. Members of this family have a highly similar protein structure including an N-terminal region that contains a conserved patatin-like domain. The \textit{PNPLA3} gene has been identified for the first time as a determinant of adipocyte differentiation in mouse pre-adipocytes by a study that aimed to compare changes in mRNA expression before and after differentiation\textsuperscript{119}.

1.3.1 PNPLA3 in human liver diseases

In 2008 a GWAS found that a common genetic variant in \textit{PNPLA3} was associated with hepatic triglyceride content\textsuperscript{86}. This variant, which is identified as rs738409, encodes for an amino acidic change at position 148, from isoleucine (I) to methionine (M). In the same year, an independent GWAS on serum transaminases levels found the same variant to be associated with higher transaminase levels\textsuperscript{120}. Next, several studies investigated whether this genetic variant was also associated with chronic liver disease. To date, \textit{PNPLA3} I148M is the most replicated variant that has been associated with the entire spectrum of chronic liver disease, from simple steatosis\textsuperscript{121-123}, to inflammation (chronic hepatitis)\textsuperscript{121,122}, fibrosis\textsuperscript{124}, cirrhosis\textsuperscript{121,125}, and hepatocellular carcinoma\textsuperscript{90,123,126}. The associations have been showed in individuals with different aetiological factors, such as NAFLD, alcoholic liver disease, hepatitis C virus and in both adults and children\textsuperscript{127,128}.

In particular, one of the first studies on NAFLD showed that each 148M allele confers a 30\% increased risk of moderate/severe steatosis and a 70\% increased risk of NASH and cirrhosis in Europeans\textsuperscript{121}. Similarly, this variant was found to increase the risk of alcoholic cirrhosis by 45\% in Mestizo subjects with history of alcohol abuse\textsuperscript{125}. The association between \textit{PNPLA3} I148M and alcoholic cirrhosis was also confirmed in Europeans\textsuperscript{123,129}. However, those studies on alcoholic liver disease were all performed on cross-sectional cohorts.

\textit{PNPLA3} 148M variant is also associated with an increased risk of HCC. Our group previously showed that obese subjects who are homozygotes for the 148M allele have a 16-fold higher risk of developing HCC than those carrying the 148I allele\textsuperscript{130}. In 2011, a German study showed that, among subjects with alcoholic cirrhosis, those homozygous for the 148M allele had a 4-fold increased risk of HCC\textsuperscript{131}. Another study showed an even stronger effect of the variant: the risk of HCC was 4 and 8-fold higher in subjects
that were heterozygotes and those homozygotes for the 148M allele, respectively, than in those homozygotes for the 148I allele. A recent meta-analysis performed on 2,503 European subjects showed that each PNPLA3 148M allele is associated with 77% increased risk of HCC. The risk was higher for the ALD-related HCC (O.R. = 2.2) than for the HCV-related HCC (O.R. = 1.77).

Taken all together, those data show that PNPLA3 I148M has a relevant role in the pathogenesis of chronic liver disease. However, the molecular mechanisms that lead to the disease onset are not known.

1.3.2 PNPLA3 function, regulation, and I148M variant

Some studies have investigated the function and the regulation of PNPLA3 and how these are affected by the I148M variation. However, some results are conflicting. In vitro studies reported different data on the enzymatic activity of PNPLA3 and the effect of the 148M variant on the function. Some studies showed that PNPLA3 has a lipase activity on the glycerolipids and the 148M variant results in a loss-of-function of this enzymatic activity. A study from our group showed that PNPLA3 is specifically involved in the hepatic secretion of triglycerides and that the 148M variant leads to an impairment of the secretion and accumulation of triglycerides in hepatocytes. In contrast with those results, one study showed that PNPLA3 has a lysophosphatidic acid acyl-transferase activity and the 148M is a gain-of-function variant that leads to intracellular accumulation of fat by increasing this enzymatic activity.

The function of PNPLA3 has also been investigated by using in vivo murine models. The pnpa3 knock-out mouse did not show differences in the liver fat content or in the triglyceride esterase activity. However, the murine and the human PNPLA3 proteins are different: the murine Pnpla3 is shorter (384 amino acids vs. 481 amino acids for the human one) and the gene expression pattern in tissues differs. On the other hand, after overexpressing the human PNPLA3 in mice on a high sucrose diet, the PNPLA3 148M mice have a higher lipid accumulation in the liver. In addition, after incubation with radiolabeled glycerol, primary hepatocytes from 148M transgene mice showed a lower glycerol release than those from 148I transgene mice, suggesting hepatic triglyceride release is impaired in the 148M transgene mice.

All those findings indicate that PNPLA3 has an esterase activity on triglycerides and is involved in the extracellular release of triglycerides. The 148M variant has an impaired lipase activity and leads to the impaired secretion and to the intracellular accumulation of triglycerides.
1.3.3 PNPLA3 and hepatic stellate cells (HSCs)

Hepatic stellate cells (HSCs), previously called Ito cells, are mesenchymal cells that lie within the Disse space, around the hepatic sinusoids. They are the main storage site for vitamin A or retinol and play an important role in retinol metabolism. Moreover, HSCs are key players in the pathophysiology of liver diseases.

In physiological conditions, HSCs are in quiescent state and contain retinol, as retinyl esters (mainly retinyl palmitate), stored in cytoplasmatic lipid droplets. The enzyme that catalyses the esterification between palmitic acid and retinol is the lecithin-retinol acyl transferase (LRAT). The stored retinol can be released when needed. However, the enzyme that catalyses the hydrolysis of retinyl palmitate to retinol and palmitic acid has not yet been identified, even though several possible candidates have been investigated.

In pathological conditions, such as liver damage, HSCs undergo activation, which is characterised by specific changes: they become myofibroblast-like cells and lose the stored the retinol. However, it is not known if the depletion of retinol is a cause or a consequence of the activation process. Activated HSCs are able to contract and secrete collagen that leads to fibrosis. The continued deposition of collagen fibres may progress to severe stages of fibrosis, leading ultimately to the onset of cirrhosis.

Thus, HSCs have an important role in retinol metabolism and liver disease. The link between lipids, retinol and liver disease is not yet known. PNPLA3 is a protein with an enzymatic activity that acts on lipids and it is associated with the onset of liver disease. It is possible to speculate that PNPLA3 might play a role in HSCs regulation of retinol metabolism and onset of liver disease.
2 AIM

The overall aim of this project is to study the susceptibility to chronic liver disease due to environmental and genetic factors.

2.1 Specific aims

• Paper I: To study the long-term effect of weight loss induced by bariatric surgery on chronic liver damage in obese subjects

• Paper II: To determine the single and combined effect of environmental and genetic factors (i.e., *PNPLA3*) on the susceptibility to alcoholic cirrhosis

• Paper III: To study the molecular mechanisms underlying the association between *PNPLA3* and its I148M variant and the onset of chronic liver disease
3 PATIENTS AND METHODS

The patient cohorts and the methods used in this thesis are described in details in the Material and Methods sections of each paper. However, in this section, a brief summary and a more general discussion of the methods are presented.

3.1 Study cohorts

3.1.1 The Swedish Obese Subjects (SOS) study

The SOS study is a well-phenotyped longitudinal matched case-control study designed to assess the effect of bariatric surgery compared to conventional treatment on obesity-associated morbidities and mortality. The bariatric surgery group (2,010 individuals) was matched with a control group (2,037 individuals) that received obesity conventional treatment. The two study groups had identical inclusion and exclusion criteria. Inclusion criteria were age between 37 to 60 years and BMI of \( \geq 34 \) kg/m\(^2\) for men and \( \geq 38 \) kg/m\(^2\) for women. The exclusion criteria were: earlier bariatric surgery, earlier surgery for gastric or duodenal ulcer, alcohol abuse or alcohol/drug problems, other relevant diseases. The incidence of morbidities, including liver disease, is recorded yearly from the National Swedish Inpatient Register.

Methodological considerations: Previous papers reported that weight loss, induced by lifestyle intervention or bariatric surgery, reduces serum transaminase levels and NAFLD. However those papers had different study design (e.g., lack of control group), small sample size, or shorter follow-up. On the other hand, the SOS study offers a unique opportunity to extensively study the effect of weight loss induced by bariatric surgery on serum transaminases. Indeed, the SOS is a case-control study including 4,000 obese subjects with a long (median 14 years) follow-up and biochemical data available at baseline and during the follow up.

3.1.2 Rome cohort

A total of 753 consecutive individuals, admitted to the Outpatient Clinics at the Department of Clinical Medicine, Policlinico Umberto I, Rome (Italy) for alcohol abuse or dependence between 2005 and 2010, were retrospectively examined. After excluding individuals with incomplete data or poor DNA quality, a total of 384 individuals were analysed. Data on patterns and amount of alcohol consumption were obtained using the Life-time Drinking History (LDH). Daily at-risk alcohol consumption was defined as \( \geq 3 \) and \( \geq 2 \) alcohol units for men and women, respectively. The diagnosis of alcoholic
abuse or dependence was established according to the revised text of the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria. Most subjects had a diagnosis of alcohol dependence. The diagnosis of cirrhosis was clinically assessed.

**Methodological considerations:** Some of the data for this study were collected retrospectively and this might have led to selection bias. However, prospective studies on the effect of alcohol consumption and chronic liver diseases would be too unfeasible and randomization, to avoid bias of selection, may not be done for ethical reasons. In this cohort, even if all the subjects were at-risk drinkers, the prevalence of alcohol abuse (as opposed to alcohol dependence) is low. The diagnosis of alcohol abuse and dependence was made based on the DSM-IV criteria\(^{159}\). These criteria define those two conditions as maladaptive pattern of alcohol use but with different peculiar behavioural and psychological characteristics that allow discriminating exactly between them. These criteria do not include the amount of alcohol intake. Moreover, all the individuals included in the current study have been admitted to the Outpatients clinics at the Department of Clinical Medicine in Rome. This Department is the referral centre for alcohol problems for the Lazio region, where the recruitment centre is located. Thus, the lower prevalence of alcohol abuse, compared to alcohol dependence, reflects this condition. This finding is in line with previously published data from referral centres for alcohol problems\(^{160}\) reporting that the prevalence of alcohol dependence was higher than alcohol abuse.

The assessment of drinking habits is often made by self-reports so it is important that the methods used are reliable. A possible bias that originates from using self-reports is the underestimation of alcohol problems. In this study, the subjects were recruited at a referral centre for alcohol problems, so anamneses on drinking habits were extensive and made by well-trained personnel. This also allowed collecting several parameters, such as age at-onset of at-risk drinking, which have not been examined in details in previous studies. The patterns and the amount of alcohol consumption from the onset of at-risk alcohol consumption to the outpatient examination were obtained by interview using the LDH. There are different methods to assess the drinking habits. The LDH collects information on lifetime drinking habits by recalling chronologically the drinking pattern from the adolescence to adulthood. A negative aspect of this method is that data are retrospectively collected and it might not be precise in the assessment of the most recent drinking period\(^{158,161}\). On the other hand, it is highly reliable in the assessment of long-term drinking habits. So it suits perfectly the aims of this study because the long-term overall assessment is required.
3.1.3 Milan cohort

The effect of PNPLA3 I148M on retinol metabolism was examined in a cohort of 146 individuals, with biopsy-proven NAFLD diagnosis, enrolled between 2008 and 2013 at Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico Milano, Italy. Most of the subjects were men, 45% had non-alcoholic steatohepatitis (NASH). Blood samples were collected after an overnight fasting.

Methodological considerations: A limitation of this cohort is that it includes only 146 subjects. However, this cohort is a well-characterised NAFLD population with both histologic and biochemical phenotype available. Our data need to be confirmed in larger populations.

3.2 Genetic analyses

3.2.1 Genotyping method

The genotyping of the PNPLA3 rs738409 variant has been performed by TaqMan® SNP Genotyping Assays. This method is a 5’-nuclease assay that is used to amplify and detect specific genetic variants in the genomic DNA. This assay contains two target-specific primers and two probes with dye label at the 5’ end (VIC® and FAM™ each identifying a specific allele of the SNP), a minor groove binder (MGB) and a nonfluorescent quencher on the 3’ end. The probes hybridize to the specific sequence of DNA between the two primers. During PCR, when the Taq polymerase reaches the probes, it cleaves the quencher and the VIC® or FAM™ dye emits fluorescence, which is specific for one of the alleles. After each PCR cycle, the intensity of fluorescence increases in proportion to the amount of amplicon synthesized.

In Figure 1 an example of the result of a genotyping for a biallelic variant assay is shown by using TaqMan® SNP genotyping and Bio-Rad CFX-real time Detection system. On the two axes the intensity of the fluorescence for VIC® and FAM™ are reported. Based on the fluorescence of the two fluorophores, we observe the presence of three clusters of samples: the cluster with orange dots represents the homozygotes for allele 1 (FAM™), the one with green triangles the heterozygotes, and the one with blue squares the homozygotes for allele 2 (VIC®). The black diamond represents the negative control.
Methodological considerations: Currently, different methods for SNP genotyping are available. We used the 5'-nuclease assay because we needed to genotype only one SNP in around 400 subjects. The SNP is common (MAF in Europeans = 0.22) so the samples were supposed to be enough to allow a clear discrimination of the three genotypes. Moreover, the Taqman® assay does not require particular equipment. This method is quick and its costs are low to genotype a single SNP in 400 subjects. Alternative methods that can be used include direct sequencing and Restriction Fragment Length Polymorphism but they are more time-consuming and less cost-effective to genotype a single SNP in 400 samples.

Abbreviations: SNP, single nucleotide polymorphism; RFU, relative fluorescence unit.
3.2.2 Hardy-Weinberg equilibrium

The genotype frequencies have been tested for deviations from the Hardy-Weinberg equilibrium (HWE). The HWE is a principle stating that allele and genotype frequencies in a population will remain constant across generations when no evolutionary influences are present. In case of a biallelic locus, the genotype frequency follows the formula: \[ p^2 + 2pq + q^2 = 1 \] (\( p \) = major allele frequency; \( q \) = minor allele frequency; \( p^2 \) = common homozygotes; \( 2pq \) = heterozygotes; \( q^2 \) = minor homozygotes).

**Methodological considerations:** HWE can be used as a control for the quality of the genotyping. In absence of evolutionary events or non-random mating, the observed and expected genotype frequencies should not be different; so if a difference is found, it might indicate that errors in the genotyping occurred.

3.2.3 Gene expression analysis

\( PNPLA3 \) mRNA expression was measured in 48 human tissues using TissueScan Human Normal cDNA Array (Origene) and in different cell types after extraction of total RNA. The total RNA was extracted from primary HSCs, primary human hepatocytes, HEPG2 and CACO-2 cells using the RNeasy Mini Kit (Qiagen). cDNAs were synthesized using a reverse-transcription kit (Applied Biosystems). The \( PNPLA3 \) expression was assessed using the delta-delta-CT method and normalized to \( \beta \)-actin. TaqMan® gene expression assay was performed according to the manufacturer’s instruction on a 7900HT Fast Real-Time PCR System.
3.3 Retinol, PNPLA3 function and regulation in HSCs

3.3.1 Hepatic stellate cells (HSCs)

Two types of HSCs were used: LX-2 cells and primary human HSCs. The LX-2 cells are an immortalized line of human HSCs, which were kindly provided by Professor Scott L. Friedman (Mount Sinai School of Medicine, New York, USA). This cell line was obtained by transfecting human HSCs isolated from liver by ultracentrifugation with the SV40 large T antigen (Simian Vacuolating Virus 40 TAg), a proto-oncogene from the polyomavirus SV40. The cell line was selected for growth in medium containing 2% foetal calf serum. Primary human hepatic stellate cells were obtained by ScienCell, plated after coating with Poly-L-Lysine, and grown according to the manufacturer’s instructions.

Methodological considerations: Two types of HSCs were used in the experiments due to the specific characteristics of the cell types. The LX-2 cells, being an immortalized cell line, are easy to grow and last longer in culture, while the primary HSCS, being not immortalized, have a shorter duration in culture but are more similar to normal cells. So the experiments were performed in both the LX-2 cells and in the primary HSCs.

3.3.2 Effect of PNPLA3 on retinol metabolism

The effect of retinol on the intracellular lipid accumulation, on PNPLA3 protein regulation and on the hydrolysis of retinyl palmitate was assessed in HSCs. First, HSCs were incubated with and without retinol and palmitic acid for 0, 12, 24, 48h. Next, the same experiment was performed after up-regulating PNPLA3 by treatment with insulin and after silencing PNPLA3 by siRNA. The intracellular accumulation of lipids and the PNPLA3 protein amount were measured. The accumulation of intracellular lipids was assessed by Oil Red O (ORO) staining and the total ORO-stained area was determined as described previously. Pictures were obtained using Axio KS 400 Imaging System and AxioVision 4.8 Software (Zeiss) at 100X magnification. The intracellular PNPLA3 protein amount was detected by western blot analysis.

To test whether PNPLA3 was involved in the release of retinol, a pulse and chase experiment was performed. HSCs were incubated with [3H]-retinyl palmitate for 36 h and then retinol release was chased for 0, 2, 4, 8, 24 h. Intracellular retinyl palmitate and extracellular retinol were isolated by thin-layer chromatography and the [3H]-radioactivity was measured by scintillation counting.

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3.3.3 Retinyl esterase activity and effect of the 148M variant

To test whether PNPLA3 has a retinyl esterase activity and to study the effect of the 148M variant on this activity, human PNPLA3 148I and 148M proteins were purified by using a yeast model. Then the purified proteins were incubated with retinyl [14C]-palmitate for 30’ at 37°C. The release of [14C]-palmitic acid was measure by scintillation counting and the enzymatic kinetic was assessed according to the Michaelis-Menten model. Moreover, the effect of PNPLA3 I148M on plasma retinol binding protein 4 (RBP4) was assessed by enzyme-linked immunosorbent assay (ELISA) in 146 individuals with NAFLD.

Methodological considerations: In the in vitro experiments, cells were incubated with both retinol and palmitic acid because retinol is stored in HSCs mainly as retinyl palmitate. To test the retinyl esterase activity, we used proteins that were purified in yeasts. We previously used the same method in testing the esterase activity of PNPLA3 on triglycerides. We specifically used the yeast Pichia pastoris to purify the proteins because it is one of the most efficient hosts for the production of the eukaryotic membrane proteins162,163. The measurement of RBP4 was used as an indirect marker of retinol levels. In humans, retinol is bound to RBP4 in the blood164 and RBP4 levels correlates with the plasma levels of retinol165 so it constitutes a reliable index of plasma retinol.

3.4 Statistical analyses

In paper I different statistical methods were used to address the different questions. The analysis of covariance (ANCOVA) was used to compare changes in anthropometric and laboratory variables between the treatment groups. Spearman rank correlation tests were used to assess the relationship between transaminase and weight changes. Logistic regression models were used to assess the risk having high transaminase levels and the possibility to recover from this condition. The prevalence of the ALT/AST ratio <1 in the control and in the surgery group was compared by Fisher’s exact test.

In paper II, linear regression model was used to analyse the differences in the continuous variable across the PNPLA3 I148M genotypes. The cumulative incidence of alcoholic cirrhosis was calculated by using Kaplan–Meier estimates of cumulative incidence rates while the differences in the incidence among groups were tested by log-rank test. To calculate the risk of cirrhosis onset, the Cox-regression analysis was used.
In paper III, data from *in vitro* experiments were analysed using Student’s t-test. Differences in retinol binding protein 4 levels across PNPLA3 I148M genotypes were analysed using linear regression analysis under a recessive genetic model. The post-hoc analyses to compare the differences in retinol binding protein 4 levels between paired genotypes were carried out by Fisher's least significant difference test.

**Methodological considerations:** There are different statistical methods that can be used to analyse differences between two groups. ANCOVA was used because it allows controlling for the effect of covariates (i.e., age, gender, BMI, and parameter at baseline). The linear regression model was chosen because it allows including covariates in the model and analysing data also under an additive genetic model. In paper II we analysed the incidence of alcoholic cirrhosis by calculating a follow-up time for each subject. This follow-up was calculated as the time between age at onset of at-risk alcohol consumption and age at cirrhosis onset or at the clinical examination. So we were able to define the years of exposure to the pathogenic agent (i.e. at-risk alcohol consumption) and, even if all the data were retrospectively examined, we could analyse them in a prospective way.
4 RESULTS

In this section of the thesis, a brief summary of the main results of each paper is presented. For the detailed description of the results, see the full papers at the end of the thesis.

4.1 Paper I

Serum ALT and AST levels were lower in the surgery than in the control group at 2- and 10-year follow-up (both p-values<0.001). The reduction in serum transaminase levels was proportional to the degree of weight loss (Figure 2).

Figure 2 Serum transaminase changes and their relationship to body weight in the follow up.

The incidence of and the remission from high transaminases were higher in the surgery than the control group at 2- and 10-year follow-up (all the p<0.001; Figure 3).
Figure 3 High transaminase incidence and remission in the surgery and control groups in the follow-up.

High transaminase group was defined if one or both transaminase levels were above the following cut-offs: AST ≥ 33 U/L (men) and 29 U/L (women) ALT levels ≥ 43 U/L (men) and 30 U/L (women). Incidence and remission are expressed as proportion of individuals; the bars indicate the 95% confidence intervals. *P value <0.001, surgery vs. control group. A. Control n= 916 and 621 at 2- and 10-year FU; Surgery n= 857 and 618 at 2- and 10-year FU. B. Control n= 519 and 332 at 2- and 10-year FU; Surgery n= 731 and 491 at 2- and 10-year FU. Abbreviations: FU, follow up; AST, aspartate transferase; ALT, alanine transferase.

Similarly, the prevalence of ALT/AST ratio<1 was lower in the surgery than in the control group at both 2-year (p<0.001) and 10-year (p=0.001) follow-up.

Bariatric surgery is associated with long-term reduction of serum transaminases in obese individuals. Our data suggest that sustained weight loss following bariatric surgery reduces liver damage and might prevent the development of long-term chronic liver disease.

4.2 Paper II

The incidence of alcoholic cirrhosis was higher in subjects with older than in those with younger age at onset of at-risk alcohol consumption (p<0.001) and in PNPLA3 148M allele carriers than in subjects with the PNPLA3 148II genotype. By examining the incidence of cirrhosis in the population stratified by both age at onset of at-risk alcohol consumption and by PNPLA3 I148M genotypes (Figure 4), we found that it was higher
in carriers of the PNPLA3 148M allele, irrespective of the age at onset of at-risk alcohol consumption (p<0.001).

Figure 4 Cumulative incidence of alcoholic cirrhosis in subjects according to age at onset of at-risk alcohol consumption and PNPLA3 I148M genotype.

Subjects were stratified in two groups by using the median value of age at onset of at-risk alcohol consumption, which was 24 years. Next each group was divided according to the PNPLA3 I148M genotype. FU indicates the duration (years) of at-risk alcohol consumption, which was defined as a daily intake of ≥3 and ≥2 alcohol units for men and women, respectively. The number of events was: II n = 4, IM n = 13, MM n = 8 (< 24 years); II n = 15, IM n = 26, MM n = 18 (> 24 years). The number at the bottom indicates the number of individuals at risk at the specific time point. Abbreviations: PNPLA3, patatin-like phospholipase domain-containing 3; II, individuals with two 148I alleles; MM, individuals with two 148M alleles; IM, heterozygotes; FU, follow up.

The lowest incidence of cirrhosis was observed in those subjects with lower age at onset of at-risk alcohol intake and the PNPLA3 148II genotype. On the other hand, the highest incidence of cirrhosis was observed in those subjects with higher age at onset of at-risk alcohol intake carrying the PNPLA3 148M allele.
Our data show that older age at onset of at-risk alcohol consumption and the PNPLA3 148M allele are independent risk factors for the onset of alcoholic cirrhosis in at-risk drinkers. This study indicates that environmental factors modulate the risk of chronic liver disease conferred by the genetic background.

4.3 Paper III

PNPLA3 is highly expressed in human hepatic stellate cells (HSCs) and its expression is regulated by the presence of retinyl palmitate. When HSCs were incubated with retinol and palmitic acid up to 48 hours, the accumulation of intracellular lipids (i.e., lipid droplets) increased and PNPLA3 content decreased over time. On the other hand, the depletion of retinol and palmitic acid led to a reduction in the content of lipid droplets and to an increase of PNPLA3 content over time.

PNPLA3 is involved in the storage and release of retinol in HSCs. After silencing PNPLA3 in HSCs and depleting retinol and palmitic acid, the intracellular depletion of and the extracellular release of retinol were lower than those observed when PNPLA3 was present. The I148M variant affects this function. After treatment with retinol and palmitic acid, HSCs overexpressing the PNPLA3 148I protein had lower intracellular accumulation of lipids (retinyl palmitate) than those overexpressing the PNPLA3 148M mutant protein (Figure 5).
To test if PNPLA3 has an esterase activity on retinyl palmitate and to study the effect of the 148M variant, we incubated purified PNPLA3 148I and 148M with radiolabeled retinyl [14C]-palmitate, and assessed the enzymatic kinetic by Michaelis–Menten model. After analysing the amount of the released [14C]-palmitic acid, we found that PNPLA3 148I has a greater esterase activity and a greater affinity for its substrate (i.e., retinyl palmitate) than 148I mutant protein (Figure 6). These data suggest that PNPLA3 148I has retinyl palmitate lipase activity and that the I148M variant leads to a loss of this enzymatic function.
Figure 6 Enzymatic activity of PNPLA3 148I and 148M proteins.

Purified PNPLA3 148I and 148M were incubated with increasing concentrations of radiolabeled retinyl palmitate. Released palmitic acid was measured by scintillation counting. Enzymatic kinetic was assessed according to the Michaelis-Menten model.

Serum retinol binding protein 4 levels were lower in subject with NAFLD carrying the PNPLA3 148M allele than those carrying the 148I allele.

Our data show that PNPLA3 is involved in the storage and release of retinol from HSCs by acting as a lipase on the retinyl palmitate. The 148M variant leads to a loss of this activity and to an intracellular retention of retinol.
5 DISCUSSION

In this section, a discussion of the main results of the papers is reported.

5.1 Chronic liver disease in obese subjects

Obesity is a known risk factor for chronic liver disease and high serum transaminases are commonly associated with obesity\textsuperscript{17,166,167} and with onset of chronic liver disease. Bariatric surgery is the most effective strategy to achieve and maintain weight loss\textsuperscript{14}. In paper I, the effect of bariatric surgery on chronic liver damage was investigated. Data presented on Paper I show that weight reduction after bariatric surgery has a beneficial effect on serum transaminases, markers of liver damage, and that this effect is also present several years after the surgery. Our data suggest that sustained weight loss due to bariatric surgery reduces liver damage and may possibly prevent the onset of chronic liver diseases in obese subjects.

5.1.1 Effect of bariatric surgery on transaminases

Both transaminases showed a sustained reduction in the surgery group over time. This change was significantly different from that observed in the control group. Obese subjects from the control group, whose weight remained unchanged, showed an increase in serum transaminases over time. Transaminase reduction was smaller at 10 years than at 2 years after bariatric surgery. This effect is likely due to weight regain observed 10 years after bariatric surgery\textsuperscript{14}.

Even though some subjects with liver damage may have normal serum transaminases\textsuperscript{168}, serum transaminases strongly correlate with the severity of liver damage\textsuperscript{169}. A Finnish study on non-diabetic subjects used specific cut-offs for transaminases to identify those subjects with steatosis\textsuperscript{170}. Thus we decided to use those cut-offs as an index of steatosis in our population. Moreover, by excluding those subjects with high alcohol intake, we increased the chances of specifically including only subjects affected by non-alcoholic steatosis or NAFLD. Therefore, we analysed the long-term effect of bariatric surgery on the incidence of and the remission from NAFLD. The incidence of and the remission from NAFLD at both 2- and 10-year follow-up were significantly more favourable in the surgery group than in the control group. This suggests that bariatric surgery might prevent the onset of NAFLD and also might reverse this condition in obese subjects.
5.1.2 Correlation between weight loss and transaminases

The relationship between changes in serum transaminases and changes in body weight was analysed. We observed that weight gain was always associated with an increase of transaminases over time.

ALT had a progressive linear reduction with increasing weight loss at 2- and 10-year follow-up. These data indicate that sustained weight loss has a beneficial long-term effect on chronic liver damage and that the effect is proportional to the degree of weight loss. Regarding AST changes, we observed a progressive linear reduction related to weight loss at 2-year follow-up. On the other hand, at 10-year follow-up, the changes were proportional to the weight changes but we did not observe a reduction of serum AST. However, we found that the greater the weight loss the lower the AST increase.

The tissue and intracellular distribution of ALT and AST is different: ALT is highly expressed in the liver and it is located in cytosol and mitochondria; AST is highly expressed in liver, heart, skeletal muscle, kidney and it is located in the cytosol. Thus, the different changes in the two transaminases may be ascribed to factors that changed over time (e.g., age, environmental factors, lifestyle habits, medications) and differently influenced the two transaminases\textsuperscript{171}. Nevertheless, even though we observed an increase of serum AST at 10 years after bariatric surgery despite weight loss, we found that the greater the weight loss the lower the AST increase, suggesting that weight loss still has a beneficial effect reducing chronic liver damage.

5.1.3 Effect of bariatric surgery on severe liver disease

Studying the effect of bariatric surgery on serum transaminases or chronic liver disease was not a predefined endpoint of the SOS study. For this reason, detailed information on liver disease and direct measurement of liver fat are not available. Thus, we decided to use the ALT/AST ratio as an indirect index of severe liver disease\textsuperscript{172} in our cohort. This index has been found to be an independent predictor of fibrosis in subjects with NASH. The prevalence of ALT/AST ratio <1 (i.e., severe liver disease) was lower in the surgery than in the control group at both 2- and 10-year follow-up. These data suggest that bariatric surgery, by reducing hepatocyte damage over time, may prevent the onset of severe liver disease in obese subjects.
5.1.4 Comparison with data in the literature

Our results are in line with previous studies that examined the effect of weight loss due to lifestyle intervention or bariatric surgery on serum transaminases and NAFLD. Those studies showed that serum transaminases and hepatic histologic features improved after weight loss. However, those reports had different study designs (often without a control group) and shorter follow-up. On the contrary, the novelty and the strength of our study is that the beneficial effects of bariatric surgery are examined in a large cohort (more than 3,000 subjects) and over a long-term period. Thus, our data show that bariatric surgery has a both a short and a long-term protective effect against chronic liver damage and, by reducing the chronic damage, may prevent the onset of severe liver disease.

5.2 Interaction between environmental and genetic factors

In paper II we studied the interaction between environmental and genetic factors in the onset of chronic liver disease. In particular, we examined the effect of two main variables on the incidence of alcoholic cirrhosis: the age at onset of at-risk alcohol consumption, which has not been deeply investigated previously; a well-known genetic variant, PNPLA3 I148M, which has not been studied in a longitudinal way as regard to ALD.

We show that age at onset of at-risk alcohol consumption and PNPLA3 I148M are independent risk factors for alcoholic cirrhosis in at-risk drinkers. Moreover, the risk conferred by the genetic variant is modulated by the age at onset of at-risk alcohol consumption. Thus the interplay between these two factors determines the individual susceptibility to cirrhosis.

5.2.1 Effect of age at the exposure

We analysed the effect of the age at exposure (i.e., the age at the onset of at-risk alcohol consumption) on the incidence of alcoholic cirrhosis in at-risk drinkers. So we stratified the cohort by the median value of this variable. The incidence of cirrhosis was higher in subjects with an older (≥24 years) than in those with a younger (<24 years) age at onset of at-risk alcohol consumption. Consistent with this, the risk of cirrhosis onset was increased by 2.76-fold per each 10 years of age. When analysing the differences between the subjects with younger or older age at onset of at-risk alcohol consumption, we observed that those with older age had a more severe cirrhosis stage, despite a lower daily alcohol intake and a shorter duration of at-risk alcohol consumption.
Our data show that age of exposure to at-risk alcohol consumption is a risk factor for cirrhosis; in particular, the older the age the higher the risk. Moreover, in those who start drinking later in life, the amount of alcohol and the duration of the abuse needed to develop cirrhosis might be lower than in those who start drinking at a younger age. This suggests that a liver affected by natural aging processes might be more vulnerable to damage.

5.2.2 Effect of PNPLA3 on the incidence of alcoholic cirrhosis

We examined the effect of PNPLA3 I148M on the cumulative incidence of alcoholic cirrhosis. The incidence of cirrhosis was higher in subjects carrying the PNPLA3 148M allele. The genetic variant had an additive effect: the wild-type homozygotes (148II) had the lowest incidence, the mutant homozygotes (148MM) the highest incidence, and the heterozygotes (148IM) an intermediate incidence. Consistent with this, PNPLA3 I148M was an independent risk factor for alcoholic cirrhosis and specifically a 50% increase in the risk was observed for each 148M allele.

Our data confirm in a prospective analysis that PNPLA3 I148M is a risk factor for alcoholic cirrhosis.

5.2.3 Environmental factors may modulate the risk due to the genetic background

Next we examined if age at onset of at-risk alcohol consumption might modulate the effect due to PNPLA3 I148M. PNPLA3 148M allele carriers had the highest incidence of cirrhosis independently of the age at onset of at-risk drinking. However, given the same PNPLA3 I148M genotype, the incidence was higher in subjects who started drinking at an older age. The lowest incidence of cirrhosis was observed in subjects with lower age carrying PNPLA3 148II genotype while the highest incidence was found in subjects with higher age carrying PNPLA3 148MM genotype. The risk of cirrhosis due to the 148M allele was not the same according to the age at onset of at-risk drinking. In fact, for each 148M allele we observed a 3-fold increase of the risk in subjects who started drinking early in life, while the increase was only 1.6-fold in those who started drinking later.

These data show that the genetic background has a stronger effect early in life, whereas in older liver other factors might contribute to the onset of cirrhosis.
5.2.4 Comparison with data in the literature

We found that age at onset of at-risk alcohol consumption is a risk factor for the onset of alcoholic cirrhosis. A previous study carried out to identify risk factors for fibrosis progression showed that the starting alcohol consumption after 40 years of age was associated with an increased risk of alcoholic cirrhosis. However, that study was performed in subjects whose alcohol consumption was at least of 50 g/day during the year preceding their enrolment. No further details on alcohol consumption and no information on other risk factors (environmental or genetic causes) were available. On the other hand, other previous studies focused on age of the patients rather than the age at exposure to the liver stressor (i.e., alcohol).

Our finding is in line with previous reports that showed that age at exposure to HCV infection affect the onset of chronic liver disease and donor age affect liver transplant outcome. Specifically, older age of exposure to HCV infection or to ischemia-reperfusion injury leads to a faster progression of HCV-related fibrosis and to a worse graft function after transplantation, respectively. All these findings indicate that an old liver is more susceptible to damage. Regarding alcohol damage, older age might imply a greater susceptibility of the liver to damage, which may be ascribed to a reduced hepatic metabolism of alcohol and to cellular senescence with impaired hepatocyte function.

The novelty and the strength of this study is that we examined, for the first time, the effect of PNPLA3 I148M variant combined with the age at onset of at-risk alcohol consumption on the incidence of cirrhosis. Indeed, previous studies showed an association between PNPLA3 I148M and alcoholic cirrhosis but only in cross-sectional studies. On the contrary, this is the first study that investigated the role of this genetic variant on the incidence of cirrhosis, thus in a longitudinal way. From a clinical point of view, our findings suggest that PNPLA3 I148M genotyping might be used in a screening program to identify patients who are at risk of developing a severe chronic liver disease. This information might lead to define appropriate intervention or prevention strategies to avoid the onset of a severe chronic liver disease and its life-threatening sequelae.
5.3 Molecular genetics of PNPLA3 and its 148M variant in HSCs

In Paper III we aimed to understand the molecular mechanism underlying the association between PNPLA3 I148M and the onset of chronic liver disease. In particular, we examined whether PNPLA3 was expressed in HSCs, which are key players in liver fibrogenesis, and its function in this cell type.

We show that PNPLA3 is expressed in HSCs and has an esterase activity on retinyl palmitate. This protein is involved in retinol metabolism by regulating retinol secretion from HSCs, the main storage site. The 148M mutant protein has an impaired enzymatic activity that leads to the accumulation of retinol in HSCs.

5.3.1 Role of PNPLA3 on retinol metabolism

The gene expression analysis showed that PNPLA3 was highly expressed in liver and retina. The link between these two far away organs is retinol: the liver is the main storage site for retinol, which is specifically stored in the HSCs; in the retina, retinol is necessary for vision. So we speculated that PNPLA3 might be expressed in the HSCs and involved in retinol metabolism. Analysing specifically the PNPLA3 expression in two of the hepatic cell types, hepatocytes and HSCs, we observed that PNPLA3 was more expressed in the HSCs. These findings strengthened the hypothesis that PNPLA3 might be involved in the retinol metabolism in HSCs.

Retinol is mainly stored as retinyl palmitate in HSCs. Thus we incubated HSCs with retinol and palmitic acid up to 48 hours and we assessed the intracellular lipid content by ORO staining. We observed a progressive accumulation of retinol in the HSCs over time combined with a parallel reduction of the PNPLA3 protein amount. Similarly, when cells were incubated without retinol and palmitic acid we observed a progressive reduction of retinol in the HSCs over time combined with a parallel increase of the PNPLA3 protein amount. These data suggest that PNPLA3 is involved in the storage/release of retinol from HSCs and this function is regulated by the availability of retinol.

To further study this function, we examined the intracellular content of retinol after up-regulation of PNPLA3 by insulin and after silencing PNPLA3 by siRNA. We observed that the accumulation of retinol in the HSCs was lower in cells treated with insulin compared with those treated with insulin and PNPLA3-siRNA. These results thus suggest that PNPLA3 is specifically involved in the storage/secrection of the retinol from HSCs. Since PNPLA3 is involved in the secretion of triglycerides as VLDL in hepatocytes, we hypothesized that it was specifically involved in the secretion of retinol in HSCs. So to test this hypothesis, we compared the intracellular and
extracellular content of radiolabeled retinol in normal HSCs and HSCs treated with siRNA. We found that the intracellular retinol content was higher and the extracellular release was lower after PNPLA3 silencing.

Taken all together our data give us some hints on PNPLA3 function in HSCs: they indicate that PNPLA3 is involved in the release of retinol probably by carrying out an esterase activity on retinyl palmitate.

5.3.2 Enzymatic activity and effect of 148M variant

The next step in our project was to functionally test the hypothesis that PNPLA3 has an esterase activity on retinyl palmitate. We examined this enzymatic activity for both the wild-type (148I) and the mutant (148M) protein. We purified those two proteins in a yeast system, incubated them with radiolabeled retinyl [14C]-palmitate, and assessed the enzymatic kinetic by using the Michaelis–Menten model. After analysing the amount of the released [14C]-palmitic acid, we found that the V<sub>max</sub> and the K<sub>m</sub> were higher and lower, respectively, for the 148I than from the 148M protein. The Vmax indicates the enzymatic activity while Km indicates the enzymatic affinity (the lower the greater affinity). These data indicate that PNPLA3 148I has a greater esterase activity and a greater affinity for its substrate (i.e., retinyl palmitate) than 148M mutant protein.

So we showed that PNPLA3 has an esterase activity on retinyl palmitate, as shown by the release of radiolabeled palmitic acid, and this enzymatic function is impaired for the 148 mutant protein.

To test the effect of the I148M variant in humans, we measured the serum level of RBP4 in subjects with NAFLD and available PNPLA3 genotype. Serum RBP4 was lower in carriers of the 148MM genotype than in those of the 148I allele. Since serum RBP4 strongly correlates with retinol levels, subjects homozygous for 148M protein have lower serum retinol than those heterozygous or homozygous for the 148I protein.

Thus, from a broader point of view, our data indicate that the 148M mutant protein has a weaker enzymatic function that leads to a lower release and to the intracellular accumulation of retinol in HSCs.

5.3.3 Comparison with data in the literature

Previous studies showed that PNPLA3 has an esterase activity on triglycerides and that the 148M is a loss-of-function variant. In hepatocytes, PNPLA3 is involved in the release of triglycerides through the VLDL machinery. The 148M variant leads to an impairment of the triglyceride secretion and accumulation of lipids in the hepatocytes.
Our group proposed a model for PNPLA3 function and the effect of the 148M variant in hepatocytes to explain the association between PNPLA3 and steatosis. According to this model, PNPLA3 hydrolyse triglycerides, stored in the lipid droplets, to free fatty acids, which are redirected to the endoplasmic reticulum and Golgi where they are incorporated into VLDL. The 148M mutant protein has a lower enzymatic activity that leads to impaired hydrolysis of stored triglycerides, decreased incorporation into VLDL, and increased intracellular triglyceride content.

In this paper, we show that PNPLA3 has an esterase activity on retinyl palmitate. To date, no other studies examined the role of PNPLA3 in HSCs and previous studies did not succeed in identifying the enzyme responsible of the hydrolysis of retinyl palmitate. So an important result of this study is the identification of PNPLA3 as esterase of retinyl palmitate.

Moreover, our data on the function of PNPLA3 and the effect of the 148M variant on retinyl palmitate are in line with the previous data on the esterase activity on triglycerides\textsuperscript{133,134}. PNPLA3 hydrolyses retinyl palmitate and regulates the release of retinol from HSCs similarly to what it does on triglycerides in hepatocytes. Moreover, in both cases the 148M protein has a lower activity which leads to a reduction of the lipid secretion and its accumulation in the cell.

**5.3.4 PNPLA3, HSCs, and chronic liver disease**

The mechanism underlying the association between PNPLA3 I148M and chronic liver disease is not known. In paper III we show for the first time that PNPLA3 is a lipase responsible of the retinyl palmitate in HSCs, which are the main players in hepatic fibrogenesis.

During chronic damage of the liver, HSCs undergo activation, which is characterised by a change in the phenotype (towards a myofibroblast-like phenotype) and by the depletion of intracellular retinol. Whether the retinol depletion is a cause or a consequence of the HSCs activation is debated and available data are conflicting. However, it is known that retinoic acid is an important molecule that interacts with nuclear receptors (e.g., retinoic acid receptor or RAR, retinoid X receptor or RXR)\textsuperscript{185} to modify gene expression. We found that PNPLA3 148M leads to accumulation of retinol in the HSCs and to reduced secretion of retinol. Thus it is possible to hypothesise that retinol release during HSC activation might be a signal to other cells (e.g., immune cells) for the activation of pathways to respond to and contain liver damage. When PNPLA3 148M is present, this signal is blocked and the progression of the damage and deposition of collagen fibres lead to fibrosis and, ultimately, to cirrhosis.
6 CONCLUSION

This thesis aimed to study the susceptibility to chronic liver disease by examining environmental and genetic factors.

Obesity is a known and nowadays common risk factor for chronic liver disease. However, we show that its effective treatment by bariatric surgery may prevent chronic liver damage and subsequently the onset of severe chronic liver disease. In a previous paper from our group, we showed that the association between \textit{PNPLA3} I148M variant and transaminases is modulated by weight loss after bariatric surgery\cite{186}. Specifically, two years after bariatric surgery (that is right after the weight loss has reached its peak) the association between the 148M variant and higher transaminases is abolished. Conversely, it is still present in obese subjects whose body weight remained unchanged. We also show that the start of at-risk drinking at an older age increases by two-fold the risk of alcoholic cirrhosis conferred by \textit{PNPLA3} 148M variant in younger subjects. These first two parts of the thesis show that both environmental (e.g., obesity and alcohol) and genetic (e.g., \textit{PNPLA3}) factors are risk factors for the onset of chronic liver disease. However, the individual susceptibility to chronic liver disease depends on the interaction between environmental and genetic factors. Since we can more easily modify environmental factors, we should try to reduce or abolish them (e.g., by long-term weight loss in obese subjects) or to identify, among exposed subjects, those who are at higher risk and should be more followed-up or should benefit more from a specific treatment.

Genetic association studies help us finding new links between genes and diseases or traits. However the information that comes from those studies does not include a causality explanation. It just indicates possible new clues in the pathogenesis of a disease. So in the last part of this thesis we exploit the information coming from genetic association studies to understand and fill gaps of knowledge in the pathophysiology of chronic liver damage, which ultimately leads to fibrosis. \textit{PNPLA3}, a lipase, is involved in liver fibrosis, which cirrhosis is the most severe stage of. Hepatic fibrogenesis involves many players, but the main actors are the HSCs. These cells are the main storage sites for retinol but in case of liver damage they become activated, which means that they appear depleted of retinol and change towards a myofibroblast-like phenotype, leading to deposition of collagen fibres. Thus we tried to understand if there is the link between \textit{PNPLA3} and HSCs and, being \textit{PNPLA3} a lipase highly expressed in liver and retina (two organs that have retinol in common), if this link might be retinol metabolism. We found that \textit{PNPLA3} is the enzyme responsible for the release of retinol from HSCs and this function is impaired when the 148M mutant protein is present. We also confirmed these findings \textit{in vivo} in humans. These data thus suggest that the alteration of retinol
release from HSCs mediated by PNPLA3 may be one important step in the progression of liver damage to chronic liver disease and fibrosis.

Understanding the susceptibility to of chronic liver disease, and so its pathophysiology, is important to identify new molecular players and pathways that may help us to prevent diseases or to discover new treatment targets.
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