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Liver Transcriptome Analysis in NAFLD Patients

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CHAPTER 1

Non-Alcoholic Fatty Liver Disease

Non-Alcoholic Fatty Liver Disease (NAFLD) is associated with metabolic and cardiovascular disorders, such as obesity, Insulin Resistance (IR), hypertension, dyslipidaemia and type 2 diabetes. [1] It is frequently recognized as the hepatic manifestation of metabolic syndrome [2] and constitutes the most frequent liver pathological condition worldwide. [3,4]

NAFL is characterized by increased liver fat content (>5% of the liver's weight) in the absence of significant alcohol consumption (< 30g/day for men and 20g/day for women) or other secondary causes of steatosis. [5,6] The term NAFLD encompasses a wide spectrum of conditions from simple steatosis to Non-Alcoholic Steatohepatitis (NASH), characterized by lobular inflammation and hepatocyte ballooning with or without perisinusoidal fibrosis, [7] which can progress to cirrhosis and Hepatocellular Carcinoma (HCC). [8]

Since NAFLD is a metabolic disease, it has been recently renamed Metabolic-Associated Fatty Liver Disease (MAFLD). The term MAFLD acknowledges the condition as a distinct disease entity and removes the requirement for the absence of excessive alcohol consumption from its definition.[9]

The incidence and prevalence of NAFLD is increasing worldwide. Statistically, the global prevalence of NAFLD is around 25–29%, with the lowest rate in Africa (13%) and the highest in Southeast Asia (42%).[10] In Europe, the prevalence of NAFLD is approximately 24%, with notoriously higher rates in Southern than Northern Europe. [11] NAFLD incidence is higher in men than in women (37% vs. 23%) and more common in older people than in younger populations (32% age > 45 years vs. 27% age < 45 years). [10]

Although NAFLD is strongly associated with obesity, it is found even in lean subjects, with prevalence rates around 16%. [12]

1.2 Genetic risk factors in NAFLD pathogenesis

Understanding the intricate mechanisms that underlie the development and progression of NAFLD is of utmost importance, even though its pathophysiology is complex and not fully understood.

Unhealthy dietary patterns, including the consumption of excessive calories, high levels of fructose, and lack of physical activity, stand as prominent risk factors for NAFLD. Moreover, an individual's susceptibility to developing NAFLD may, in part, find its explanation in inherited factors, specifically, Single Nucleotide Polymorphisms (SNPs) within genes that govern the handling of lipids in the liver.

Several genetic variants associated with NAFLD and NASH were identified by Genome-Wide Association Studies (GWAS) and the relative genes could represent candidate for precision medicine approaches to treatment.

The first genetic variant associated with NAFLD was the rs738409 (C >G) in the Patatin-like Phospholipase Domain-containing 3 (PNPLA3) gene, also known as adiponutrin. [13]

The C to G substitution at nucleotide position 444 of *PNPLA3* leads to isoleucine/ methionine substitution at amino acid position 148 of PNPLA3 protein; this I148M PNPLA3 genetic variant is associated with hepatic steatosis, steatohepatitis, elevated levels of plasma liver enzyme, hepatic fibrosis and cirrhosis. [13–17]

Carriers of the G at-risk alleles are more prevalent in Hispanics (0.49) than in Europeans (0.23) and less frequent in African-Americans (0.17), explaining in part the inter-ethnic difference in NAFLD susceptibility.[13] Most interestingly, the effect of PNPLA3 on hepatic fat accumulation was independent from IR and lipid concentration. *In vitro* studies, indicated that PNPLA3, localised to the surface of Lipid Droplets (LDs), [18] has triglyceride lipase activity[19,20] and is involved in lipid remodelling and hepatic retention of polyunsaturated fatty acids (Figure 1).[21,22] Furthermore, PNPLA3 has retinyl-palmitate lipase activity *in vitro*, and it is involved in retinol release by Hepatic Stellate Cells (HSCs).[23]

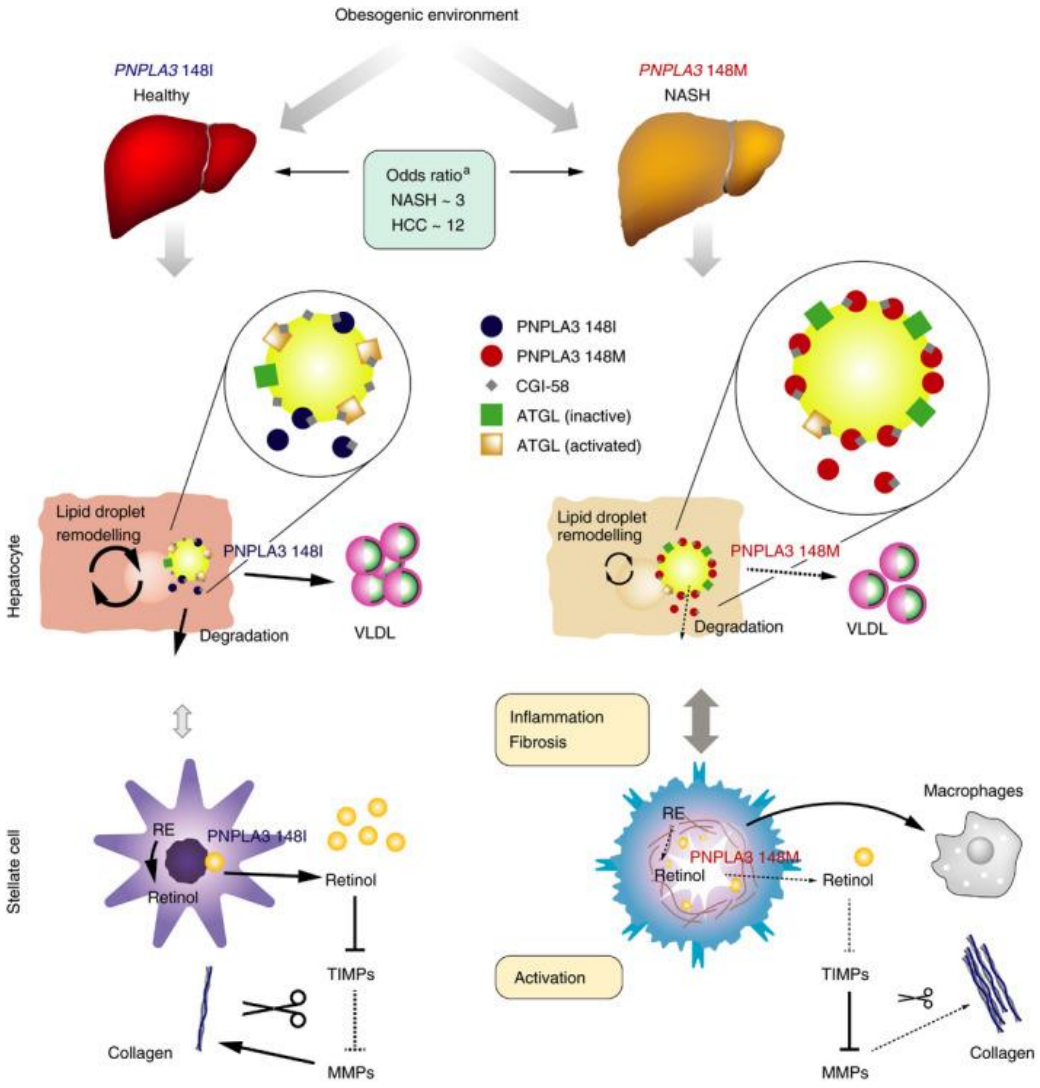

Figure 1. *Role of PNPLA3 in the pathophysiology of NAFLD. Abbreviations: ATGL, adipose triglyceride lipase; HCC, hepatocellular carcinoma; MMP, matrix metalloproteinase; NASH, non-alcoholic steatohepatitis; RE, retinol esters; TIMP, tissue inhibitor of metalloproteinase; VLDL, very low-density lipoprotein.*[24]

The SNP rs738409 and the consequent missense variation I148M disrupts the enzyme's phospholipase activity, thereby interfering with lipid catabolism. [18,20]

However, the deletion of PNPLA3 in mice did not cause hepatic steatosis. [25]Thus, elevated hepatic fat storage, induced by PNLPA3^{I148M}, is not a result of PNPLA3's functional loss; rather, it is, probably, attributed to a modification in its function. Two independent studies demonstrated that overexpression of PNPLA3^{I148M} in the liver of mice induced hepatic steatosis, whereas mice overexpressing the wild-type PNPLA3 had normal hepatic triglyceride content. Furthermore, PNPLA3 protein levels, on the surface of LDs, are higher for 148M than 148I in both the PNPLA3-overexpressing and knock-in mouse models. [26,27] Increased PNPLA3 levels, on LDs, appear to reduce hepatic lipolysis via sequestration of a lipase cofactor, CGI-58; enzymatically inactive PNPLA3 148M is still able to bind CGI-58,

preventing its activity on other lipases present on LDs (Figure 1).[28] PNPLA3 I148M may also disrupt retinol release by HSCs, potentially leading to fibrosis (Figure 1). Impaired retinoid production may lead to reduced secretion of matrix metalloproteinases and tissue inhibitors of metalloproteinase, resulting in extracellular matrix deposition. [17,23,29]The effects of PNPLA3 I148M on lipid droplet remodelling in hepatocytes and on retinol production by HSCs (Figure 1), suggest that inhibitors of PNPLA3 could provide therapeutic benefits to I148M-carrying patients with NASH, in the context of precision medicine.

In our contemporary knowledge, PNPLA3 stands out as a key genetic factor in the development of NAFLD. Nevertheless, it's worth acknowledging that numerous other genetic variants have been unveiled, which contribute significantly to the susceptibility and the progression of this condition.

In a human exome-wide association study, the rs58542926 (C>T; E167K) variant of Transmembrane 6 Superfamily Member 2 (TM6SF2) was associated with increased hepatic triglyceride content and higher risk of advanced fibrosis in NAFLD patients,[30,31] but paradoxically associated with a lower concentration of hepatic-derived triglyceride-rich lipoproteins.[32] Therefore, despite the increased risk of NAFLD, carriers of TM6SF2 E167K have a lower risk of cardiovascular disease. TM6SF2 plays a role in the pathway for hepatic Very-Low-Density Lipoproteins (VLDL) secretion: elective knockdown of TM6SF2 protein expression in mice led to a threefold increase in liver triglyceride content and a 50% decrease in VLDL secretion, indicating that TM6SF2 normally promotes VLDL secretion.[30] Prill et al. generated and characterized a 3D spheroid model from primary human hepatocytes, obtained from individual donors, either wild-type or heterozygous for the TM6SF2 ^{E167K} allele, demonstrating that the genetic variant induced elevated fat storage in hepatocytes by reducing secretion of apolipoprotein B 100 (apoB100) and promoting *De Novo Lipogenesis* (DNL).[33] Variations in the Glucokinase Regulator (GCKR) gene were associated with histological NAFLD, showing a modest effect on the risk of NAFLD development.[34]

In particular, it has been identified the SNP rs1260326 C>T in *GCKR* locus, encoding a proline to leucine substitution at amino acid position 446 (P446L).[35] *GCKR* P446L is a loss of-function variant that increases DNL by inducing glycolysis.[36] This variant was associated with increased susceptibility to NAFLD, NASH and NASH-derived HCC.[37–39]

Membrane-bound O-acyltransferase domain-containing 7 (MBOAT7) is a membrane-anchored enzyme with six transmembrane domains involved in remodelling endomembrane phospholipid acyl chains. MBOAT7 genetic variant rs641738 C>T may predispose to NAFLD and NASH by changing the acyl remodelling of phospholipids in the liver.[40]

This variant, characterized by downregulation of MBOAT7 at mRNA and protein level, [34] was associated with fibrosis in the absence of lobular inflammation.

Murine NASH-inducing diet model, with hepatocyte-specific knockout of MBOAT7, showed increased hepatic fibrosis without induction of inflammation as shown by the decrease in monocytes and unchanged levels of inflammatory mediators.[41]

The rs72613567 (T>TA) frameshift variant in the HSD17B13 gene leads to the synthesis of a truncated enzyme,[42] and protects against advanced NAFLD, NASH, ballooning degeneration, lobular inflammation, fibrosis [43] and HCC. [44] HSD17B13 has been identified as a hepatic lipid droplet associated protein, with retinol dehydrogenase activity. The loss-of-function variant, rs72613567, significantly showed a reduced or absent enzymatic capacity to catalyze the oxidation of retinol, mediating anti-fibrotic and anti-inflammatory effects.[45] Interestingly, this variant is sufficient to mitigate the risk of liver injury among PNPLA3^{I148M} allele carriers, a population genetically predisposed to NAFLD. This effect was associated with a decrease in PNPLA3 mRNA in an allele dose-dependent manner.[42]

In addition to the most reliable fatty liver genes PNPLA3, TM6SF2, GCKR, MBOAT7 and HSD17B13, several other genetic determinants of NAFLD have been identified, that appear to be specific for only one ethnic population or have been confirmed by few studies.

Fares et al. have demonstrated that the Uncoupling Protein 2 (UCP2) -866 G>A polymorphism (rs695366), associated with increased hepatic UCP2 expression, reduced risk of NASH in subjects with NAFLD or severe obesity.[46] UCP2 regulates mitochondrial lipid fluxes and Reactive Oxygen Species (ROS) production by the respiratory chain. It is involved in the pathogenesis of liver damage progression in NASH, where mitochondria play a key role, as the main cellular site of fatty acid oxidation, ATP synthesis and ROS production.

Another genetic variant rs4374383 G>A, in the Myeloid-Epithelial-Reproductive Tyrosine Kinase (MERTK) locus, showed a protective role from fibrotic phenotype in NAFLD. The mechanism is, probably, mediated by decreased expression of MERTK in AA genotype. [47]

Role of polymorphism in Ectoenzyme Nucleotide Pyrophosphate Phosphodiesterase 1(*ENPP1* or *PC1*) and in Insulin Receptor Substrate-1 (*IRS1*) genes, which are related to IR, have also been described in NAFLD patients.[48]

In summary, several genetic variants are associated with NAFLD and have been identified in GWAS. Despite these findings, a substantial portion of heritability remains unexplained, indicating the existence of unidentified genetic factors that await discovery.

1.3 Mechanisms of NAFLD pathogenesis: the multiple-hit

The underlying mechanism for NAFLD development and progression is complex and incompletely understood. Different theories have been formulated, leading initially to the 'two-hit hypothesis', which in recent years is considered outdated, because it fails to adequately explain the numerous molecular and metabolic changes occurring in NAFLD. Instead, the 'multiple hit' hypothesis has emerged as a more accurate explanation for NAFLD pathogenesis.[2]

According to this hypothesis, NAFLD is a multifactorial, non-communicable disease, resulting from a complex interaction of multiple environmental and metabolic 'hits' with a predisposing genetic background.

The initial 'hit' involves the accumulation of fat in the liver, triggered by increased fat synthesis and uptake (from diet, DNL and adipose tissue lipolysis), reduced fat export (in the form of VLDLs) and diminished fat oxidation.[49]

The progression of hepatic steatosis to more advanced stages is attributed to subsequent 'hits,' including mitochondrial dysfunction, proinflammatory cytokines (such as IL-1, TNF-α, IL-37 and IL-6) and adipokines (such as adiponectin) production,[50] endoplasmic reticulum (ER) stress,[51] and the influx of bacterial endotoxins from the gut. [52]

In this context, IR is one of the key drivers in the development of steatosis, increasing DNL and impairing inhibition of adipose tissue lipolysis, with consequent increased flux of Free Fatty Acids (FFAs) into the liver. [53] IR also promotes adipose tissue dysfunction, leading to altered production and secretion of adipokines and inflammatory cytokines. [54] Fat accumulates in the liver as Triglycerides (TGs), forming LDs, and this happens contemporarily with increased lipotoxicity from high levels of FFAs, free cholesterol and other lipid metabolites. Consequently, mitochondrial dysfunction with oxidative stress and production of ROS and ER stress associated mechanisms, are activated.[55] Furthermore, inflammation and ROS stimulate the activation of Kupffer Cells (KCs), which produce inflammatory cytokines exasperating hepatocellular injury and lobular inflammation. The inflammatory environment causes the advance of steatosis in NASH, a condition characterized by liver inflammation and hepatocyte apoptosis (Figure 2).[56] The persistence of inflammation and cellular damage, caused by the combination of these insults, leads to the development of fibrosis.[57]As fibrosis advances, the liver architecture changes, marked by hepatocellular injury manifesting as ballooning, apoptotic body formation and lytic necrosis. The loss of cells triggers liver regeneration and fibrogenesis, stimulating the activation of HSCs, the production of extracellular matrix (ECM) and increasing collagen,

thereby accelerating the progression of liver fibrosis (Figure 2).[58] Advanced fibrosis significantly heightens the probability of developing cirrhosis, and ultimately, it may progress to hepatocellular cancer and liver failure.[58–60]

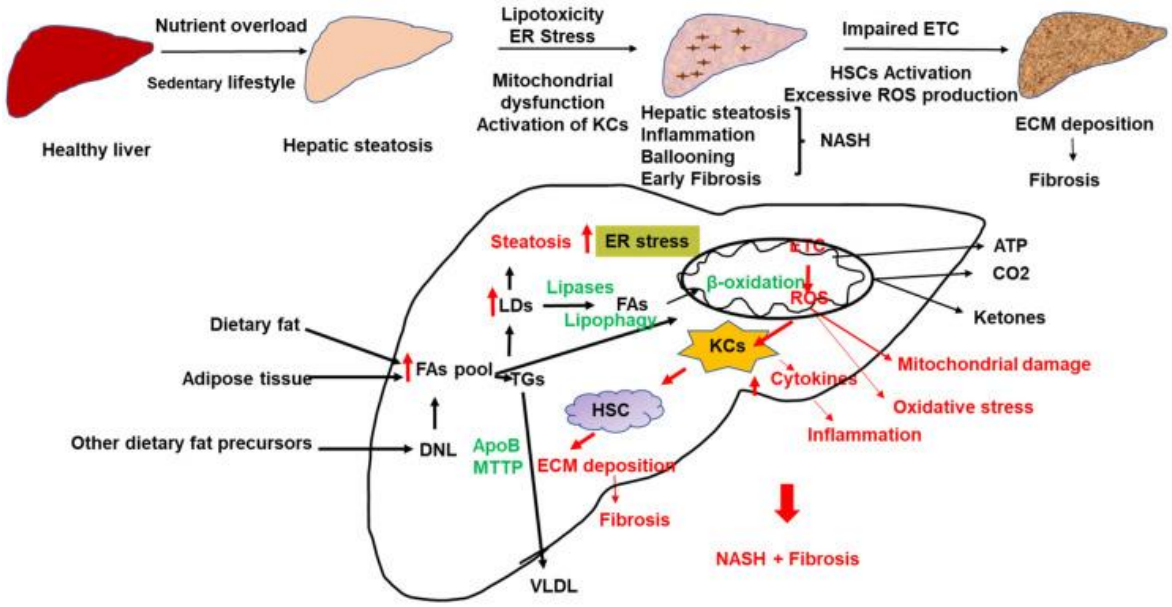

Figure 2. *Schematic representing the progression of NAFLD. Top panel: Hepatic steatosis results from nutrient overload and a sedentary lifestyle. Multiple factors lead to inflammation, NASH, and the progression to fibrosis. Bottom panel: mechanisms for NAFLD development and progression.*[61]

1.3.1 De Novo Lipogenesis

DNL plays a substantial role in the pathogenesis of NAFLD, accounting for 26 % of hepatic triglycerides in human subjects. [62] Hepatic DNL is a metabolic pathway for the conversion of carbohydrates, commonly glucose, into FFAs. The DNL of fatty acids, in the liver, involves a complex cytosolic process in which glucose is converted to acetyl-CoA through glycolysis and pyruvate oxidation. Then, acetyl-CoA is converted to malonyl-CoA by Acetyl-CoA Carboxylase (ACC). Fatty Acid Synthase (FAS) converted malonyl-CoA to palmitic acid, the first product in DNL. New fatty acid may then, undergo a range of desaturation, elongation, and esterification steps before ultimately being stored as triglycerides or exported as VLDL particles. Thus, increased DNL can cause hepatic steatosis and/or hypertriglyceridemia, but it may also cause inflammation and apoptosis, due to palmitate accumulation, leading to steatohepatitis.[63] Multiple transcription factors control the expression of enzymes directly involved in DNL, including the Sterol Regulatory

Element-Binding Protein-1c (SREBP-1c), which is activated by insulin and Liver X Receptors (LXRs), and Carbohydrate Responsive Element-Binding Protein (ChREBP), manly activated by carbohydrates. [64]

Insulin resistance drives DNL in NAFLD: in conditions of insulin resistance, Insulin Receptor Substrate 2 (IRS-2) is downregulated, leading to the overexpression of SREBP-1c and the subsequent upregulation of DNL.[65] SREBP1 expression is enhanced in patients with NAFLD[66] and is considered one of the predominant regulators of DNL, upregulating genes coding for ACC and FAS.[67]

1.3.2 Liver fatty acid uptake

IR, in obesity and NAFLD, increases adipose tissue lipolysis and the release of circulating FFAs, which are taken by fatty acid transporters within the liver. The transport is predominately mediated by Fatty Acid Transport Proteins (FATP), FA translocase CD36, Fatty Acid Binding Proteins (FABPs) and caveolins in the hepatocyte plasma membrane.[68] Between the six mammalian FATP isoforms, FATP2 and FATP5 are found primarily in the liver.[69] In mice, FATP2 or FATP5 knockout decreased FA uptake in the liver [70], whereas the overexpression of FATP2 increases FA uptake in human hepatoma cells.[71] The level of FATP5 correlated inversely with histological features of NASH, including ballooning and fibrosis. Studies have shown that FATP5 expression is elevated in patients with less severe steatohepatitis but is reduced during advanced NASH.[72]

CD36 is closely associated with the development of NAFLD, its expression increased in animal models and humans with NAFLD;[73–75] upregulation of CD36 increases FA uptake in the liver, suggesting a role for the protein in pathogenic conditions. [71,76]

The translocation of CD36 protein from the cytoplasm to membrane of hepatocyte may be a triggering event in NAFLD progression. [77] In addition to its role in FFA uptake, CD36 might play other intracellular roles in lipid processing, such as VLDL secretion.[73]

FABP1 is the highly expressed FABP in the liver. FABP1 facilitates the transportation, storage, and utilization of fatty acids and their acyl-CoA derivatives. It may exert a protective effect against lipotoxicity by binding otherwise cytotoxic free fatty acids and facilitating their oxidation or incorporation into triglycerides.[78]

Interestingly, FABP1 protein levels are upregulated in obese patients with steatosis but decrease in NASH, with a further decrease in advanced fibrosis.[79] Thus, increased FABP1 in the earlier stages of NAFLD may enhance lipid flux as a compensatory mechanism to limit lipotoxicity. As the disease progresses, diminishing levels of FABP1, potentially ensues

lipotoxicity, promoting disease progression by damaging essential organelles and cells in the liver.

The caveolins comprise a family of three membrane proteins contributing to lipid trafficking and lipid droplets formation. Caveolin 1 was increased in the liver of mice with NAFLD, and located mainly in the centrilobular zone 3, where the steatosis was most severe. [80]

The pool of FFAs from the different pathways is then directed to LDs for storage as TGs, incorporated into lipoproteins for secretion, used in β-oxidation, or used for post-translational modifications (Figure 2).

1.3.3 Lipid droplets formation

LDs are dynamic and metabolically active organelles that consist of a hydrophobic core of neutral lipids (predominantly TGs and cholesterol esters) enveloped by a phospholipid monolayer. Liver LDs accumulation is an adaptive response to the increased flow of FFAs from the diet, adipose tissue, and *de novo lipogenesis* in hepatocytes. Under physiological conditions, the liver stores less than 5% of lipids in the form of TGs in cytoplasmic LDs. Dysregulation of LDs biogenesis and degradation can increase intracellular lipid accumulation and promote the activation of pathogenetic mechanisms, leading to steatosis, hepatocellular inflammation and fibrosis.

The release of FFAs from TGs is regulated by cytosolic lipases, particularly Adipose Triglyceride Lipase (ATGL), or through autophagy of LDs. Deletion of ATGL in mice promoted hepatic steatosis.[81] ATGL has been shown to function as an inducer of autophagy/lipophagy.[82] Lipophagy is a specific form of autophagy where LDs are engulfed by the autophagosomes and then degraded via lysosomes.[83] Inhibition of lipophagy in the liver promotes LDs accumulation and attenuates β-oxidation of the released FFAs.[84]

Chaperone-mediated autophagy has been shown to contribute to hepatic LDs catabolism via its degradation of the LD protein perilipin 2 (PLIN2). [85] The degradation of this protein allows ATGL to gain access to LDs and facilitate lipolysis.

In NAFLD, PLIN2 is upregulated and is associated with the hepatic accumulation of ceramides.[86] While ATGL loss was associated with large LDs, the accumulation of small LDs has been suggested to arise from a defect in lipophagy.[87]

1.3.4 Lipoprotein secretion

The export of TGs contributes to reducing the hepatic lipid content. Fatty acids, due to their hydrophobic nature, can only be exported from the liver after being packed into water-soluble VLDL. VLDL particles are formed in the ER, where apoB100 is lipidated in a

process catalysed by the enzyme Microsomal Triglyceride Transfer Protein (MTTP). The nascent VLDL particle is then transferred to the Golgi apparatus, and during this process, the particle is further lipidated until a mature VLDL particle is formed.[88]

The transport of VLDL from the ER to the Golgi appears to be mediated by specialized vesicles, called VLDL transport vesicles, containing Coat Protein II (COPII) components, such as the transmembrane 6 superfamily 2 (TM6SF2), the cargo receptor surfeit 4 (SURF4), the secretion associated Ras related GTPase 1B (SAR1B) and meningioma-expressed antigen 6 (Mea6). [89,90] Liver-specific deletion of TM6SF2 in mice impaired VLDL secretion and promoted hepatic steatosis, fibrosis, and HCC. [91]

Defective VLDL assembly and secretion is one of the key contributing factors in the pathogenesis of NAFLD. Moderate exposure to fatty acids increased apoB100 secretion, but prolonged exposure leads to ER degradation of apoB100. In particular, *in vivo* and *in vitro* experiments have shown that apoB100 inhibition is linked with ER stress and NAFLD progression.[92,93]

ApoB and MTTP genetic mutations are associated with progressive liver disease. [94,95]

Specific deletion of MTTP in the liver results in hepatic steatosis and complete inhibition of VLDL and apoB secretion.[96,97] Insulin reduces hepatic lipid export by inducing apoB100 degradation and suppressing MTTP synthesis.[93] Interestingly, in the first stage of NAFLD, selective hepatic insulin resistance triggers DNL without reducing VLDL production.[98]

VLDL secretion is increased in patients with NAFLD, and liver triglyceride content is directly associated with VLDL-TG secretion rates.[99] Nonetheless, once hepatic fat content surpasses the 10% mark, VLDL-TG secretion reaches a plateau.[100] ApoB synthesis is lower in individuals with NASH compared to those who are lean or obese without NASH. [100]

Consequently, the liver's capacity to maintain a delicate equilibrium between lipid storage and VLDL secretion assumes paramount importance in determining the outcome of NAFLD.

1.3.5 Oxidation of fatty acids and mitochondrial dysfunction in NAFLD progression

The steady state balance of hepatic triglycerides is also controlled by the consumption of fatty acids by mitochondrial β-oxidation, which is critical for the production of both ATP and ketone bodies.

Fatty acyl-CoAs, the activated form of fatty acids, traverse mitochondrial membranes in a carnitine-dependent manner. Inside the mitochondria, acyl-CoA is sequentially degraded by the β-oxidation cycle into acetyl-CoA.[101] Acetyl-CoA is further oxidized in the tricarboxylic acid (TCA) cycle where NADH and FADH2 are generated for ATP synthesis by the electron transport chain.

When present in excess, acetyl-CoAs are processed to ketone bodies by a series of reactions for which 3-hydroxy-3- methylglutaryl-CoA (HMG-CoA) synthase is the rate limiting enzyme. While most of the fatty acid oxidation occurs in the mitochondria, peroxisomes and cytochromes also play a role in specific conditions. When mitochondria lack the ability to oxidize very long chain fatty acids, these are preferably metabolized via peroxisomal β-oxidation.[102] In case of lipid overload, such as in NAFLD, ω-oxidation in the cytochromes also contributes.[103] Nevertheless, these processes produce substantial quantities of ROS, leading to oxidative stress development and toxic dicarboxylic acids production. This can potentially exacerbate inflammation and contribute to the progression of disease. The hepatic expression of Peroxisome Proliferator-Activated Receptor α (PPARα) is crucial for glucagon-mediated fatty acid oxidation.

The activation of PPARα leads to the transcription of various genes associated with fatty acid oxidation in the mitochondria, peroxisomes, and cytochromes, ultimately resulting in the reduction of hepatic lipid content. [104] Knockout of PPARα in ob/ob mice results in hepatic steatosis, emphasizing the critical role of PPARα in promoting fatty acid oxidation and preventing hepatic lipid accumulation.[105] It was observed that PPARα was downregulated in patients with NASH when compared to individuals with steatosis and healthy controls. Furthermore, the expression of PPARα exhibited a decline that correlated with an increase in NAFLD activity scores and fibrosis stage.[106] Decreased PPARα in NASH also enhanced the DNA-binding capacity of c-Jun N-terminal Kinase 1 (JNK1) and Nuclear Factor kappa-light-chain enhancer of activated B cells (NF-κB) leading to increased hepatic inflammation.[107] Thus, PPARα expression may be related to several aspects of NASH progression, modulating not only lipid homeostasis, but inflammation as well.

In fatty liver, considerable FFAs flux and chronic production of acetylCoA can uncouple the TCA cycle function from mitochondrial respiration leading to increased ROS generation.[108,109] Excessive ROS production contributes to lipid peroxidation and several proinflammatory cytokines synthesis (TNFα, IL-1β, IL-6 and IL-8), which culminate in hepatocytes apoptosis and necrosis.[110]

When hepatocyte mitochondria are damaged, they release various Damage-Associated Molecular Patterns (DAMPs) that trigger the activation of inflammasomes, including NLRP3, and other components of the innate immune system.[111,112]

The convergence of processes involving ROS-associated lipid peroxidation, the release of mitochondrial DAMPs, and the activation of caspases sets the stage for chronic liver injury. This intricate interplay fuels a cascade leading to the infiltration of inflammatory cells, perpetuating the ongoing damage and inflammation within the liver.[113,114]

Studies in High Fat Diet (HFD)-fed mice showed that mitochondrial DNA released by injured hepatocytes activate Toll Like Receptor 9 (TLR9) on KCs and HSCs, stimulating innate immunity as well as fibrogenic responses. [114]

In summary, the transition from NAFL to NASH is accompanied by the decrease in mitochondrial plasticity, resulting in the decline of ketogenesis, TCA turnover, Oxidative Phosphorylation System (OXPHOS) capacity, antioxidant activity and ATP production. Dysfunctional mitochondria lead to the activation of inflammasomes and provide a chronic inflammatory milieu, which is responsible for the development of steatohepatitis and fibrosis. [115]

Moreover, lipotoxicity, inflammation, oxidative stress, mitochondrial dysfunction, and ER stress are factors that contribute to generating the ideal environment for tumor promotion.[116]

Increased ROS levels can activate Nrf2 signaling pathways. While Nrf2 serves as a crucial transcription factor safeguarding the liver against oxidative stress in the early phases of NAFLD, it is paradoxically regarded as a promoter of HCC development in later stages.[117,118]

The accumulation of damaged mitochondria drives the metabolic reprogramming of neoplastic cells. This reprogramming is characterized by a shift toward the Warburg effect, mutagenesis, epithelial-mesenchymal transition (EMT), and evasion of apoptosis, stimulating compensatory proliferation and HCC onset. [119,120]

1.4 Role of Sirtuins in NAFLD

Sirtuins (SIRTs) are a family of enzymes that exert a pivotal role in post-translation modification of target protein via NAD^+- dependent deacylation of lysine residues.[121]

In mammals, seven distinct sirtuins (SIRT1–7) are described, and they are strategically located within different compartments of cells: SIRT1, SIRT6 and SIRT7 are nuclear proteins; SIRT2 is localized in the cytoplasm, while SIRT3, SIRT4 and SIRT5 in the mitochondria.[122]

Nuclear sirtuins catalyze modifications of both histone and nonhistone proteins, including many transcription factors and coactivators, and play an important role in the control of transcriptional regulation. Instead, the extranuclear sirtuins, in the mitochondria and cytosol, target enzymes involved in various metabolic pathways such as inflammation, endoplasmic reticulum stress, insulin resistance, fatty acid oxidation, steatosis and antioxidative, protective defense mechanisms. [123,124]

All sirtuins have a conserved NAD^+ binding domain [125]and they can act as energy sensors by being sensitive to the NAD^+/NADH ratio.[126] Therefore, a change in the NAD^+ level in

response to different energetic challenges such as fasting or overfeeding, will affect the sirtuin activity. [127,128]

Dysregulation of sirtuins by metabolic stress, induced by excessive caloric intake and unhealthy diet, can be anticipated to contribute to the pathogenesis of metabolic diseases.

In this context, recent research has unveiled their pivotal involvement in NAFLD development. [129–131]

SIRT1 and SIRT3 are the most studied sirtuins in NAFLD. SIRT1 regulates, via deacetylation of transcription factors and proteins, multiple metabolic pathways in the liver, including FA synthesis and oxidation, oxidative phosphorylation, inflammation, mitochondrial biogenesis, and autophagy.[132–135] SIRT1 is downregulated in humans with NAFLD, which was associated with increased expression of lipogenic proteins, such as SREBP1, ACC, and FAS (Figure 3). [136]

Liver-specific deletion of SIRT1 resulted in fatty liver, inflammation, and endoplasmic reticulum stress, due to impaired PPARα/PGC1α (peroxisome proliferator-activated receptor α/ peroxisome proliferator-activated receptor γ coactivator-1α) pathway. [137,138]

Reduced SIRT1 activity decreases the expression of antioxidant enzymes, consequently elevating cellular ROS levels, [139] and activates the transcription factor NF-κB, leading to inflammatory cytokines production (Figure 3). [140]

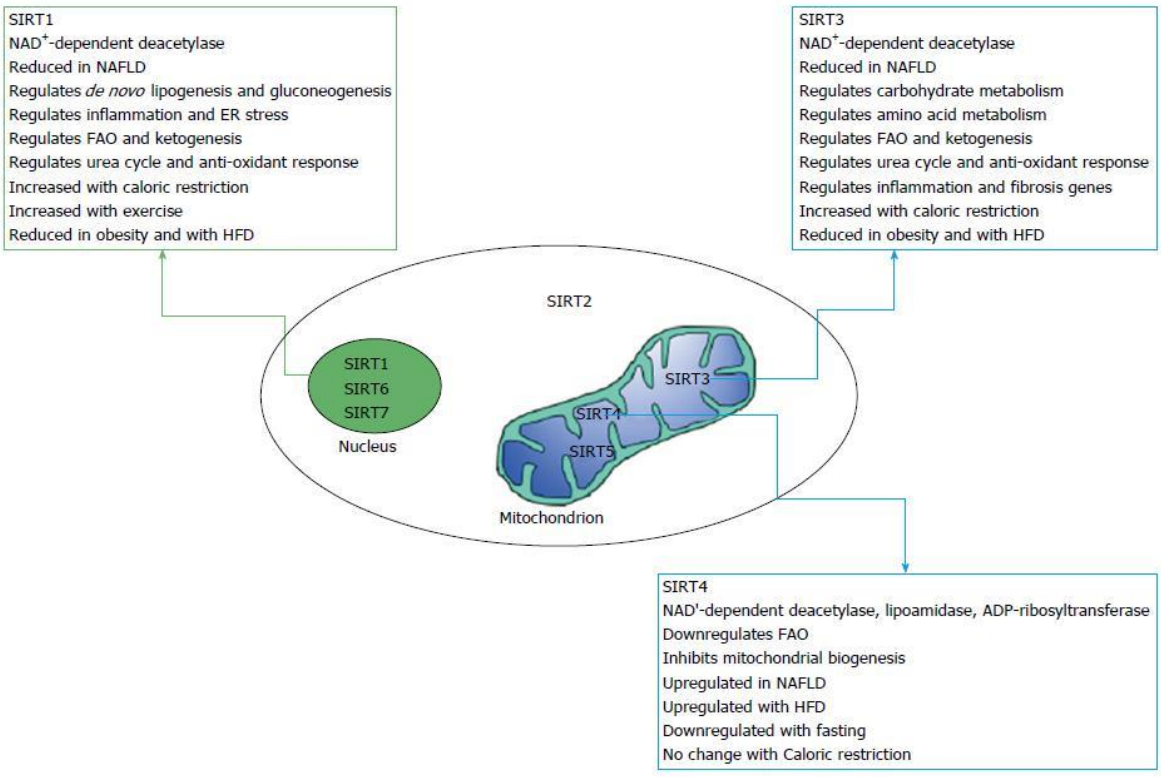

Figure 3. *Representation of various sirtuins with summary findings for SIRT1, SIRT3, and SIRT4. NAFLD: Non-alcoholic fatty liver diseases; FAO: Fatty acid oxidation; HFD: High fat diet.*[135]

SIRT3 is localized in the mitochondrial matrix where, acts as global regulator of mitochondrial protein acetylation and function, coordinating cellular responses to nutrient status and energy homeostasis. [141–143]

SIRT3 is the highly expressed sirtuin in mouse liver[141] and has been shown to improve mitochondrial function and NAFLD by regulating β oxidation, ketogenesis, mitophagy and the antioxidant response system.[143–149]

The expression of SIRT3 is activated during fasting and calorie restriction, while chronic HFD and obesity reduce SIRT3 activity.[150,151] In human and mouse model of NAFLD, SIRT3 is downregulated (Figure 3).[152]

In the absence of SIRT3, mitochondrial proteins become hyperacetylated, impairing mitochondrial function and leading to NAFLD development. [153,154]

SIRT3 knockout mice, with HFD induced NAFLD, exhibit decreased activity in respiratory complexes III and IV, accompanied by an increase in ROS levels.[155,156]

HFD feeding in mice lacking SIRT3 exacerbated obesity, insulin resistance, hyperlipidemia, hepatic steatosis and inflammation.[146,156] In addition to its effect on the mitochondria, SIRT3 deficiency in the liver aggravates hepatic steatosis through upregulation of proteins involved in FFAs uptake, such as CD36 and VLDL receptor.[157] Moreover, SIRT3 deletion aggravates hepatic steatosis, inflammation and fibrogenesis by reducing the antioxidant defense system.[158]

It has been reported that the expression of SIRT1, SIRT3, SIRT5, and SIRT6 in NAFLD is downregulated, while the expression of SIRT4 is upregulated. [152]

The activity of SIRT4 increases under conditions of adequate nutrition, inhibiting fatty acids oxidation and promoting fat synthesis and metabolism (Figure 3).

In terms of mechanism, SIRT4 may suppress fatty acid oxidation in hepatocytes by inhibiting the transcriptional activity of PPARα. [159]

Unlike other sirtuins, SIRT5 has very weak deacetylase activity, but efficiently removes succinyl, malonyl, and glutaryl groups from lysine residues of its target proteins.[160]

In the last years, SIRT5 has emerged as a master regulator of metabolic homeostasis, controlling different metabolic processes including glycolysis, TCA cycle, fatty acid oxidation, electron transport chain, ketone body formation, and ROS detoxification. [161]

SIRT5 was described as a potential promoter of metastasis in HCC: it was observed that SIRT5 regulated the expression of E2F1, a connector and coordinator between cell proliferation and metabolic pathways in mitochondria.[162]

Recently, the rs12216101 T>G non-coding SNP at the SIRT5 gene locus, was reported to be associated with ultrasonographically detected carotid plaques in a genome-wide association study. [163]These data suggested that *SIRT5* gene variation may modulate the susceptibility to cardiometabolic diseases.

However, so far, no data are available regarding the impact of *SIRT5* genotypes on metabolic liver damage in candidate gene studies**.**

In summary, human sirtuins have captivated the attention of both academic researchers and industry experts, emerging as promising therapeutic targets for a wide spectrum of diseases, including cancer, neurodegenerative disorders, and NAFLD. Over the past two decades, the scientific community has experienced a surge in the discovery of small molecule sirtuin regulators. Despite this influx, the number of compounds specifically designed to target human sirtuins in clinical trials remains relatively limited.[164,165]

1.5 Immunity as an emerging player in the progression of NAFLD

Lately, there has been significant research interest in understanding the intricate involvement of both the innate and adaptive immune responses in NAFLD.

Scientists have been focusing on unravelling how immune cells are activated and mobilized within the liver. This activation can occur due to signals originating locally within the liver or from external sources such as adipose tissue or gut. These signals play a pivotal role in triggering an inflammatory response, which in turn leads to hepatocyte damage and death.

Robust evidence highlights the pivotal role of the immune response in promoting NASH, the inflammatory progressive stage of NAFLD.

In the context of NASH, the crucial players in the hepatic immune cell spectrum encompass various innate and adaptive immune effectors. These include natural killer T cells, macrophages, mucosal-associated invariant T cells, γδ T cells, as well as conventional CD8+ T cells and distinct subsets of CD4+ T cells such as TH1, TH2, TH17, and regulatory T (Treg) cells. [166]

It has been demonstrated that dysregulation of immune cells promoted by pathological metabolic changes is directly involved in the tissue damage process and in the progression to HCC.[167] Stressed or dying hepatocytes, during lipotoxicity, release specific intracellular molecules, DAMPs, that can act on various immune cells in the liver, triggering a homeostatic response designed to heal and repair tissue injuries.[168,169] However, the persistence of these signals can induce an exuberant response, that results in a full

inflammatory response with tissue inflammation and excessive scarring, leading to advanced fibrosis and ultimately to cirrhosis and HCC.[170]

Metabolic disturbances, oxidative stress and translocated bacterial products were shown to activate KCs via TLRs, resulting in increased NF-κB signalling and proinflammatory cytokine and chemokines production [TNF-α, IL-1β, IL-12 and C-C motif ligand 2 and 5 (CCL2 and CCL5)].[171] This is a key step in triggering local inflammation and to promote additional hepatocyte cell injury, leading to the release of DAMPs. DAMPs in turn further activate KCs, via TLRs-signalling pathways, thus creating a vicious inflammatory circle. Moreover, some of the above-mentioned cytokines (i.e. CCL2 and CCL5) have overlapping pro-inflammatory and pro-fibrotic properties, contributing to the chemotaxis of inflammatory cells and the activation of HSC. [172]

Expansion of KCs and their activation in M1 phenotype, is an early inflammatory phenomenon and precedes the recruitment of other immune cells: neutrophils and monocytes.[173] Neutrophils may contribute to liver inflammation and damage by secreting elastase, myeloperoxidase and Neutrophil Extracellular Traps (NETs) and engaging to monocytes recruitment.[174–176] In the injured liver, monocytes can differentiate into inflammatory, angiogenic, and fibrogenic macrophages (M1) which release mediators, such as TGF-β1 and PDGF, to activate HSC or other precursor cells, involved in NASH progression to fibrosis.[177] This persistent chronic inflammatory environment leads the activation of adaptative immune response, inducing the increasing of T reg and cytotoxic CD8+ T lymphocytes.[178]

Continuous regenerative response leads to chronic activation of HSCs, collagen deposition, fibrosis, and subsequently cirrhosis predisposing to the switch of fibrotic phenotype to pro-carcinogenic one.[179] Even though chronic inflammation occurs along the entire spectrum of NAFLD, it is mainly involved in the advanced stages of the disease, mostly in cirrhosis and during the transition to HCC. [170,180]

In this context of chronic inflammation, different regulatory pathways try to counterbalance immune system activation, such as the Programmed cell Death protein- 1 (PD-1), a membrane receptor expressed on T cells, that counteracts lymphocytes activity by engaging its ligand, Programmed cell Death Ligand- 1 (PDL-1). PD-1 plays a critical role in regulating the immune system and maintaining peripheral immune tolerance, but, on the other hand, promotes the immune escape from cancer. [181]

1.5.1 PD-1/PDL-1 axis in NAFLD

PD-1 pathway is under scrutiny for its role in tumour immunosuppression via T-cell, but it probably exerts other immune functions. PD-1 is upregulated, during T cell activation,

mainly in CD8+ T cells and in other cell types, such as B cells, macrophages and natural killer cells. [181] PD-1 is activated by PDL-1, a ligand constitutively expressed on B cells, dendritic cells, macrophages and cultured bone marrow-derived mast cells.[182] In addition, PDL-1 is not constitutively expressed by tumour cells, but its expression may represent an adaptive response to escape immunological antitumor activity. PD-1 acts by limiting the over activation of immune responses, engaging with T cell receptors, inducing inhibition of T-cell proliferation, cytokine production and impairing T cell survival.[183] In addition, the PD-1/PDL-1 pathway promotes inflammatory resolution and immune homeostasis restoration. In fatty liver disease setting, metabolic lipotoxicity and cellular stress induce prolonged stimulation of lymphocytes leading to continuous activation, resulting in an exhausted phenotype acquisition, characterized by the overexpression of the co-inhibitory receptor PD-1. Recent data in experimental models of NASH mice, suggest that PD-1 is expressed by a subset of $CD8^{+}$ T cells, which exhibit an exhausted phenotype and accumulated during the progression of NASH. These exhausted T cells, inducing auto-aggressive hepatocyte killing, in an MHC-class-I-independent manner, facilitated the onset of inflammation, leading to disease progression and promoting hepatic carcinogenesis.[184]
The gradual accumulation of exhausted CD8+PD-1+ T cells in the liver affected by NASH is confirmed by Pfister et al. They demonstrated that, in preclinical models of NASH-induced HCC, immunotherapy targeting PD-1, expanded activated CD8+PD-1+ T cells within tumours. However, this expansion did not result in tumour regression, indicating impaired tumour immune surveillance and promoting tumour escape.[185] The increasing number and size of HCC cells, triggered by anti-PD-1 treatment, was curbed by depleting CD8+ T cells or neutralizing TNF, suggesting that CD8+ T cells may be implicated in the induction of NASH–HCC, rather than enhancing or executing immune surveillance functions. Notably, patients with NASH-driven HCC, which received anti-PD-1 or anti-PDL-1 treatment exhibited overall reduced survival compared to those with other underlying causes.[185] These results collectively suggest that non-viral HCC, particularly NASH–HCC, may be less responsive to immunotherapy, due to NASH-induced aberrant T cell activation causing tissue damage and impairing immune surveillance. In summary, in metabolic fatty liver disease, the dysregulation of immune cells and the exhausted phenotype of cytotoxic lymphocytes, inhibit immune responses against HCC and promote tumour escape. In keeping with these findings, PDCD1 genetic variation was associated with an increased risk of NASH-HCC,[186] thus suggesting that the impact of genetic variants can modify the hepatic immunological milieu during HCC, but the impact on liver damage remains uncertain.

Objectives

Currently, there is no established consensus regarding the optimal therapeutic approach for individuals with NAFLD. The primary strategy for managing NAFLD continues to revolve around lifestyle interventions, which emphasize the importance of adopting healthy dietary habits, engaging in regular physical activity, and achieving weight loss.

However, it has been demonstrated, as highlighted by Dongiovanni et al. in 2017, that individuals exhibit varying responses to dietary due to their unique genetic background.[187]

In this context, it becomes of utmost significance to investigate the genetic susceptibility to NAFLD, discover novel genetic variants, and elucidate the molecular mechanisms that drive the onset and progression of this disease.

Analyzing the patterns of hepatic gene expression during NAFLD progression could provide novel insights into disease mechanisms and may help identify tractable therapeutic targets.

In a recent study, Baselli et al. demonstrated that carriage of the PNPLA3 I148M variant is one of the major determinants of liver transcriptome variability. [188]

The authors found a higher association between circulating IL32 and hepatic mRNA levels in patients with severe NAFLD, identifying IL32 as a candidate for a non-invasive biomarker and a therapeutic target.[188]

Govaere et al., adopting an integrative transcriptomic approach, identify a 25 gene-set 'signature' associated with steatohepatitis and fibrosis. About these 25 genes, AKR1B10 and GDF15 are candidate serum markers of advanced NAFLD. [189]

These detailed studies provide novel insights into the pathophysiology of progressive fibrosing-steatohepatitis, as well as proof of principle that transcriptomic changes represent potentially tractable and clinically relevant disease biomarkers.

In this context, my research project utilizes the RNA sequencing technology.

The primary goal is to analyze how specific genetic variants, identified as predictive of the outcome of metabolic liver disease, can influence the hepatic transcriptome of patients at various stages of the pathology. Identifying specific signatures associated with NAFLD progression could clarify the molecular mechanisms involved in the onset and progression of NAFLD. It might have a potential to discover new biomarkers and therapeutic targets for the approach to NAFLD patient in the era of personalized medicine.

CHAPTER 2

SIRT5 rs12216101 T>G variant is associated with liver damage and mitochondrial dysfunction in patients with non-alcoholic fatty liver disease

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SIRT5 rs12216101 T>G variant is associated with liver damage and mitochondrial dysfunction in patients with non-alcoholic fatty liver disease

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2.1 SIRT5 rs12216101 T>G variant is associated with disease severity in NAFLD patients

We started our study by assessing the impact of SIRT5 rs12216101 T>G variant in multicenter cohort of 2,606 NAFLD patients. The frequency distribution of *SIRT5* rs12216101 TT, TG and GG genotypes was 47.0%, 42.3% and 10.7% respectively.

Prevalence of moderate-severe steatosis, as well as prevalence of grade 2-3 lobular inflammation did not vary across rs12216101 genotypes (Figure 4A and 4B). Similarly, the rs12216101 T>G variant showed no association with moderate-severe steatosis or inflammation in the multivariate logistic regression analysis (OR 1.04, 95% C.I. 0.92-1.19, p=0.49 for steatosis grade 2-3; OR 1.10, 95% C.I. 0.96-1.30, p=0.23 for lobular inflammation grade 2-3).

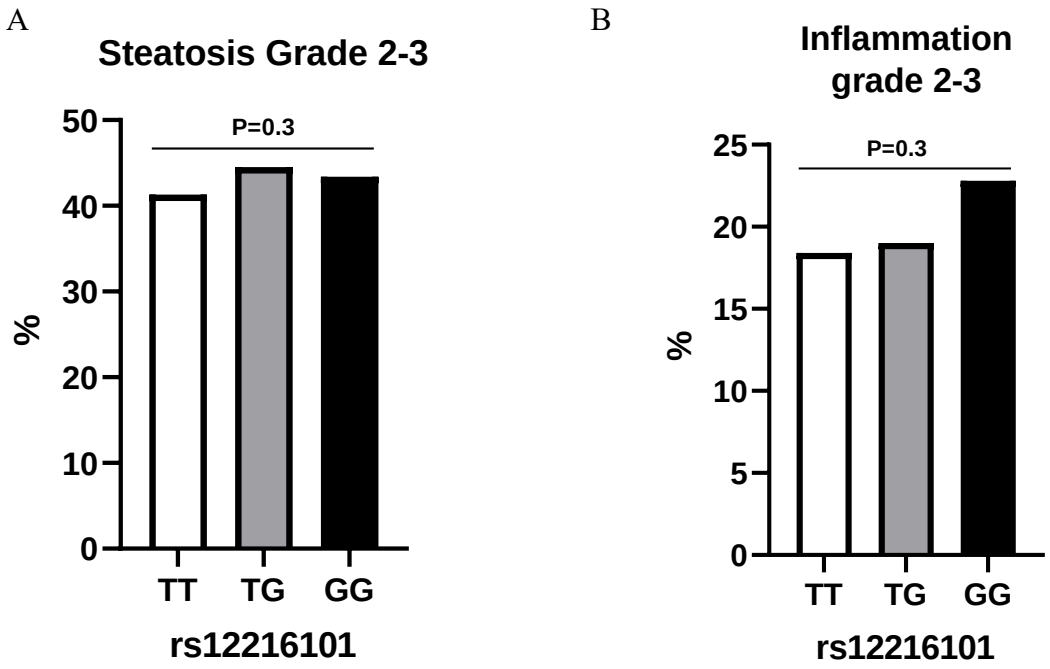

Figure 4*. Prevalence of moderate-severe steatosis (A), moderate-severe inflammation (B) in the whole of multicentric NAFLD cohort. P were calculated by chi-square.*

However, the prevalence of ballooning and NASH was higher in carriers of the G allele in homozygosis (Figure 5A and 5B). At multivariate logistic regression, *SIRT5* rs12216101 T>G variant was associated with presence of ballooning (OR 1.20, 95% C.I. 1.04-1.39; p=0.01) and NASH (OR 1.20, 95% C.I. 1.03-1.40; p=0.01).

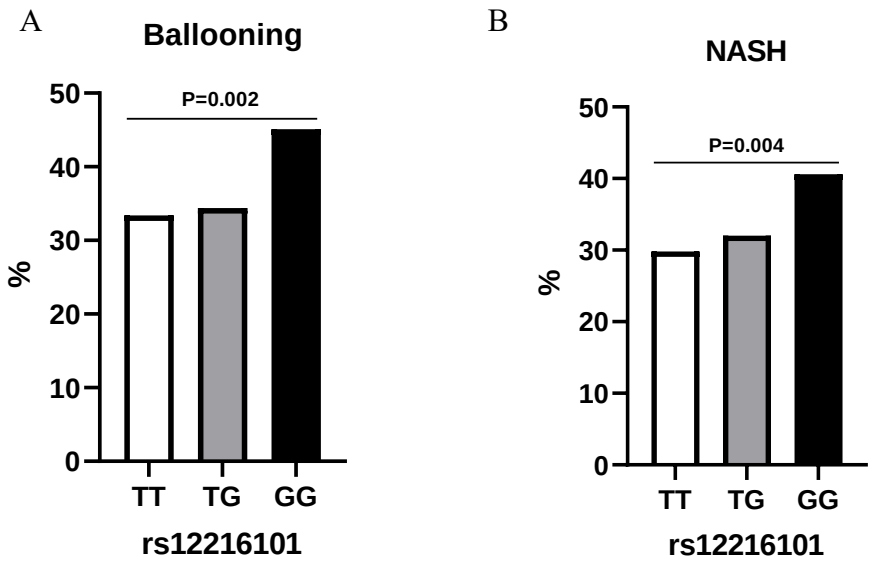

Figure 5. *Prevalence of ballooning (A) and NASH (B) in the whole of multicentric NAFLD cohort. P were calculated by chi-square.*

Moreover, individuals carrying the GG genotype exhibited higher prevalence of clinically significant fibrosis, stage F2-F4 (Figure 6) which was confirmed at multivariate logistic regression analysis (OR 1.18, 95% C.I. 1.00-1.37; p=0.04) after adjusting for gender, age>50 years, diabetes, and *PNPLA3* rs738409 genotype.

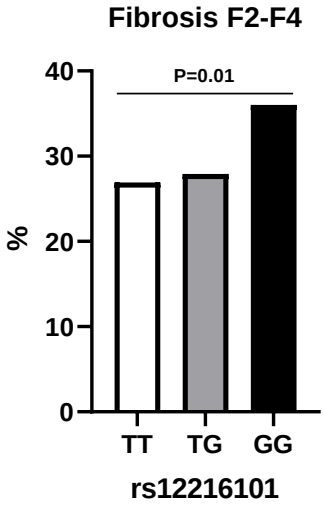

Figure 6. *Prevalence of F2-F4 fibrosis in the whole of multicentric NAFLD cohort. P were calculated by chi-square.*

2.2 SIRT5 rs12216101 T>G impact on liver transcriptome

We next analyzed the impact of *SIRT5* genotype on the liver transcriptome, for better understanding the molecular mechanisms involved in liver damage development observed in GG carriers.

The transcriptome analysis was performed in subset of 112 bariatric patients (TT = 54, TG=43, GG = 15) with available liver biopsies. We found that the expression of 1618 genes were associated with *SIRT5* genotype (unadjusted p-value<0.05); between them, 566 were upregulated in carriers of the rs12216101 T>G variant, whereas 1052 were downregulated. Afterward, we conducted a Gene Set Enrichment Analysis (GSEA) using the 1618 differentially expressed genes to indagate the pathways influenced by SIRT5 variation (Figure 7A).

We found that the SIRT5 rs12216101 T>G variant is linked to an enrichment of genes involved in oxidative phosphorylation, fatty acid metabolism, adipogenesis, glycolysis, and pathways induced by the Myc oncogene (Figure 7A).

Figure 7. *Pathways enriched in genes differentially expressed in dependence of SIRT5 rs12216101 T>G variant genotype in the "transcriptomic" cohort, a genotyped cohort of 112 patients (TT = 54, TG = 43, GG = 15) with liver samples available for transcriptomics (A). Consistent results have been obtained analyzing the pathways co-regulated with hepatic SIRT5 expression in the cohort (B). In both the analysis a p-value < 0.05 was considered for each differential expressed gene negative binomial generalized linear model was employed. Only data presenting a corrected p-value for false discovery rate < 0.05 are considered and showed in panel A and B.*

When we compared these results with the list of transcripts that were coregulated with SIRT5, we found that oxidative phosphorylation, adipogenesis, glycolysis, and MTORC1 signaling were upregulated both with overall SIRT5 transcript levels and in carriers of the rs12216101 T>G variant (Figure 7B). These data suggest that the *SIRT5* haplotype carrying the rs12216101 T>G risk variant confers an overall gain of function on SIRT5 activity, accompanied by the upregulation of various metabolic pathways. Notably, OXPHOS emerged as the second most enriched pathway linked to the G allele (Figure 7A) and ranked at the top of the list of upregulated pathways co-regulated with SIRT5 transcript (Figure 7 B).

2.3 SIRT5 rs12216101 T>G variant and SIRT5 isoforms expression in NAFLD patients

Given that rs12216101 T>G variant is not in linkage with any variant influencing SIRT5 protein sequence, and that it was not associated with overall SIRT5 mRNA levels in the transcriptomic cohort, to delve deeper into understanding how the variant influences liver biology, we investigated its potential connection with altering the expression patterns of specific alternative SIRT5 transcripts. In the transcriptomic cohort, bioinformatic analysis showed that the expression of SIRT5 isoform 4 increases with the rs12216101 T>G allele dosage ($p<0.01$, adjusted for age and sex) (Figure 8). These results indicate that the rs12216101 T>G variant could impact alternative splicing, potentially serving as an expression quantitative trait locus (eQTL) specifically for the SIRT5 isoform 4 gene product.

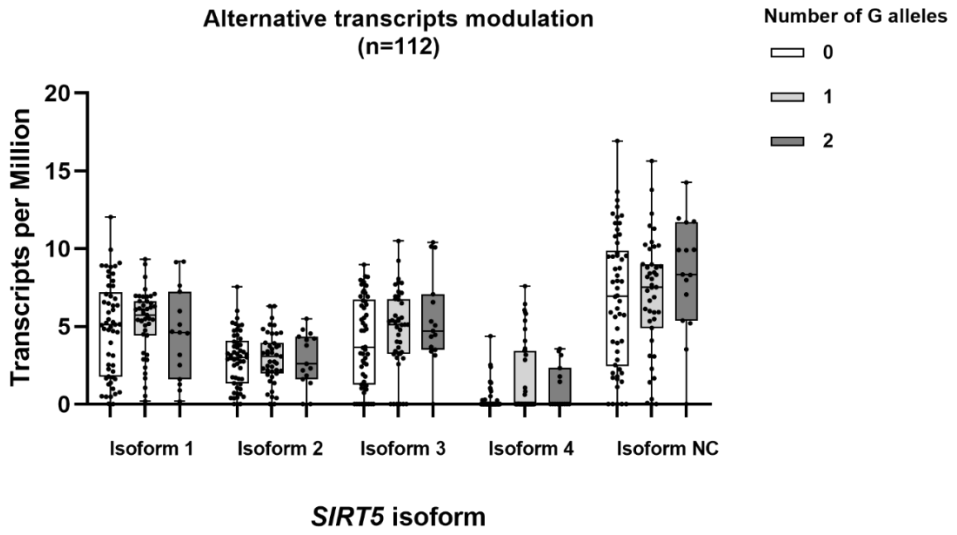

Figure 8. *Alternative transcript modulation in terms of transcript per million depending on SIRT5 genotype. Expression of SIRT5 isoform 4 increases with the rs12216101 T>G allele dosage, thus SIRT5 rs12216101 T>G variant is an eQTL for the expression of isoform 4. Genotypes: TT=54 (white=0), TG=43 (grey=1), GG=15 (dark grey=2). **p<0.01 adjusted for age and sex in a generalized linear model. NC, non-coding isoforms. TPM, transcript per million.*

CHAPTER 3

Programmed cell death 1 genetic variant and liver damage in nonalcoholic fatty liver disease

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ORIGINAL ARTICLE

Programmed cell death 1 genetic variant and liver damage in nonalcoholic fatty liver disease

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3.1 PDCD1 rs13023138 G>C common variant was associated with HCC in UKBB

We initiated our study by investigating 231 common genetic variants located within 20 Kb of PDCD1 flanking regions, each with a minor allele frequency >1%. Our analysis focused on their potential connection with hepatocellular carcinoma (HCC) in a sample of general population of 363.393 unrelated European participants from the UK Biobank (HCC cases=314).

Among the genetic variants explored, one specific variant stood out significantly. The PDCD1 rs13023138 G>C emerged as the primary variant in the region, showing a notable association with HCC (FDR <0.05, p = 5.28E-4, OR = 1.32, 95% CI = [1.1,1.5]) (Figure 9). Notably, rs13023138 is an intronic variant located in a proximal enhancer-like signature in the PDCD1 locus.

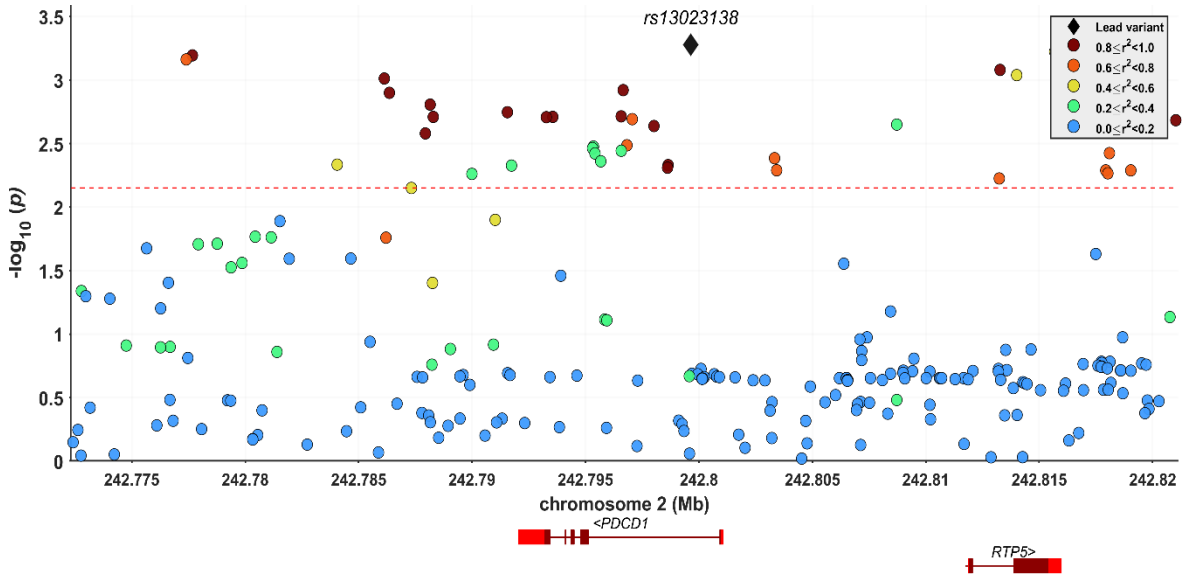


Figure 9. *Regional plot for the association of PDCD1 and HCC in UKBB. The plot encompassed a range of ±20 Kb on either side of the gene, incorporating 231 common variants (MAF>1). The lead variant was denoted by a square diamond marker. Red dashed line delineates the false discovery rate (FDR) threshold, determined using the Benjamini-Hochberg method. The analysis involved a substantial cohort of 337,000 unrelated white-British participants from the UK Biobank, and the LD (linkage disequilibrium) structure within this sample was considered during the analysis.*

3.2 PDCD1 rs13023138 G>C common variant was associated with NAFLD severity and progression

Subsequently, our investigation extended to examine the relationship between the rs13023138 variant and the severity of liver disease in NAFLD patients. This analysis encompassed a cohort comprising 2,889 individuals, all at risk for liver-related conditions, and each having undergone a liver biopsy.

Among these individuals, the distribution of PDCD1 rs13023138 genotypes revealed that 38.5% carried the CC genotype, 45% the CG genotype, and 16.5% the GG genotype. Importantly, these genetic frequencies are in Hardy-Weinberg equilibrium. The G allele was significantly associated with higher serum cholesterol levels (Table 1)

	CC (n=1113)	**CG (n=1300)**	**GG (n=476)**	**Pvalue**
Age (*)	48.6±12.2	49±12.2	48.9±13	0.73
Age>50 years (#)	46.2%	73.2%	29.8%	0.73
Male (#)	46.7%	45.9%	50.4%	0.23
BMI > 30 kg/m^2 (#)	66.2%	62.5%	57.7%	0.005
Diabetes (#)	46.1%	73.22%	29.96%	0.83
Glucose – mg/dl (§)	95 (87-109)	95 (87-107)	98 (88-113)	0.97
Insulin - µU/mL (§)	16 (10.7-23.2)	16 (10.8-22.98)	16.55 (11.7-23.92)	0.44
HOMA Index	3.79 (2.49-5.8)	3.78 (2.43-5.56)	4 (2.61-5.91)	0.42
Total cholesterol - mg/dl (§)	180 (152.1-210.6)	184 (157-211.25)	188 (159.9-218)	0.02
LDL cholesterol - mg/dl (§)	109.2 (83.8-135.1)	112 (88.8-136)	117 (92-145)	0.002
HDL cholesterol - mg/dl (§)	46 (38-55)	47 (38.22-56)	47 (39-56)	0.13
Tryglicerides – mg/dl (§)	120.1 (89-168.8)	118 (86-164)	120 (86-172)	0.63
PLT – mmc (§)	243 (198.7-286.2)	235 (184-286)	237 (183-289)	0.79
AST – U/L (§)	28 (21-40)	29 (21-41.5)	28 (22-46)	0.78
ALT – U/L (§)	37 (24-59)	37 (23-60)	40 (24-64.5)	0.20
Albumin – g/dl (*)	4.34±0.44	4.33±0.43	4.34±0.42	0.12

Table 1. *Baseline demographic, laboratory, and metabolic features of 2889 NAFLD patients, stratified by PDCD1 rs13023138 genotype. Abbreviations: BMI, body mass index; HOMA, homeostatic model assessment; LDL, low density lipoprotein; HDL, high density lipoprotein; PLT, platelets; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Data are given as: (*) mean ± standard deviations, (§) median and interquartile range, or (#) percentage of cases (%). P-values of the differences were adjusted by using the Benjamini-Hochberg correction for multiple tests.*

In Figure 10, the data illustrates the incidence of severe steatosis based on the PDCD1 rs13023138 genotype (p = 0.01). Through a multivariate logistic regression analysis, it was found that the presence of the G allele was significantly correlated with grade 3 steatosis (OR 1.17, 95% C.I. 1.02-1.34; p=0.01), even after adjusting for age, gender, BMI, diabetes, *PNPLA3* rs738409 as well as variations between different enrolment centres.

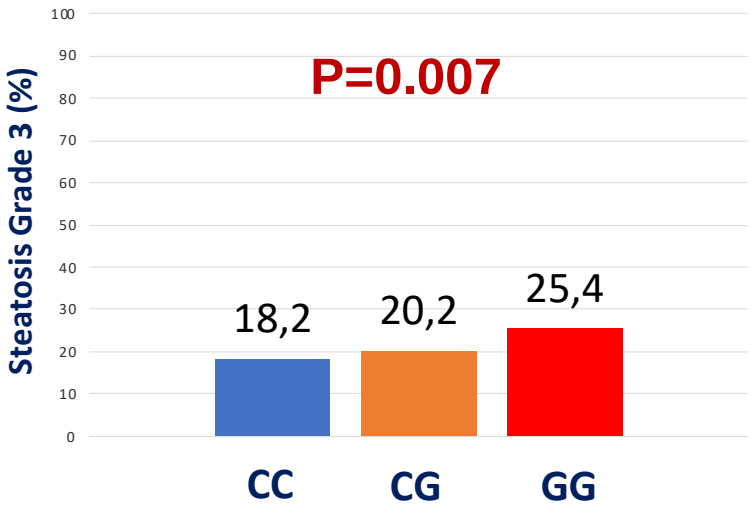


Figure 10. *Association between PDCD1 rs13023138 genotype and incidence of severe steatosis*

Similarly, the PDCD1 rs13023138 G allele was associated with severity of lobular inflammation (Figure 11) and with the presence of ballooning (Figure 12). These relationships were validated through multivariate analyses, where adjustments were made for confounding variables.

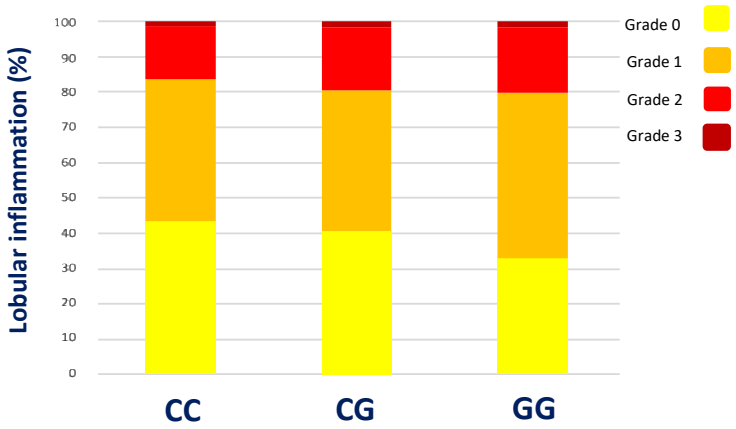


Figure 11. *Association between PDCD1 rs13023138 genotype and severity of lobular inflammation (OR 1.17, 95% C.I. 1.05-1.29, p = 0.002).*

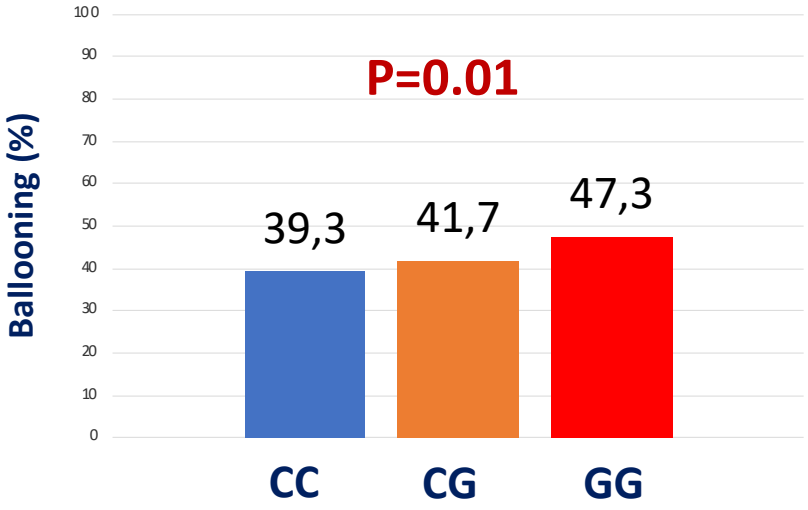


***Figure 12**. Association between PDCD1 rs13023138 genotype and ballooning (OR 1.17, 9% C.U. 1.04-1.32, p = 0.008)*

Furthermore, the presence of the PDCD1 rs13023138 G allele exhibited a significant association with an increased prevalence of NASH, as depicted in Figure 13 ($p < 0.001$). This association was robustly confirmed through multivariate logistic regression analysis (OR 1.22, 95% C.I. 1.09-1.37, $p < 0.001$). Remarkably, this correlation remained robust even after rigorous adjustments for various confounding factors, including centres, demographics, metabolic parameters, and genetic variables.

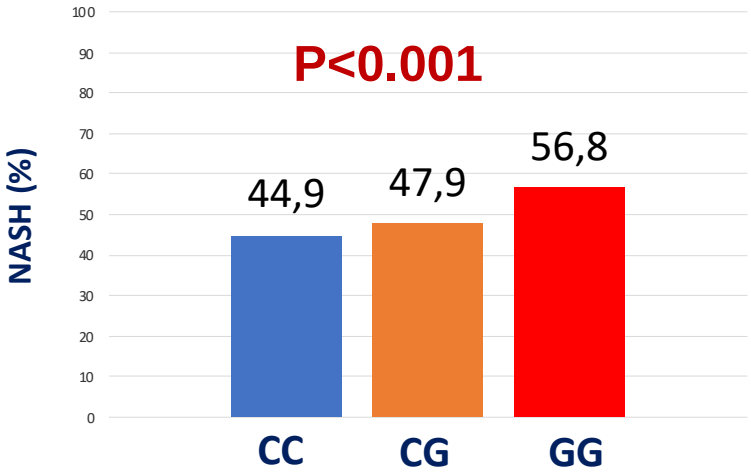


***Figure 13**. Association between PDCD1 rs13023138 genotype and presence of NASH.*

Finally, the *PDCD1* rs13023138 G allele was associated with a higher risk of advanced liver fibrosis (Figure 14; $p = 0.002$); these data being confirmed at multivariate logistic regression analysis (OR 1.26, 95% C.I. 1.06-1.50, $p = 0.007$) after adjusting for gender, age > 50 years, diabetes, centres and *PNPLA3* rs738409.

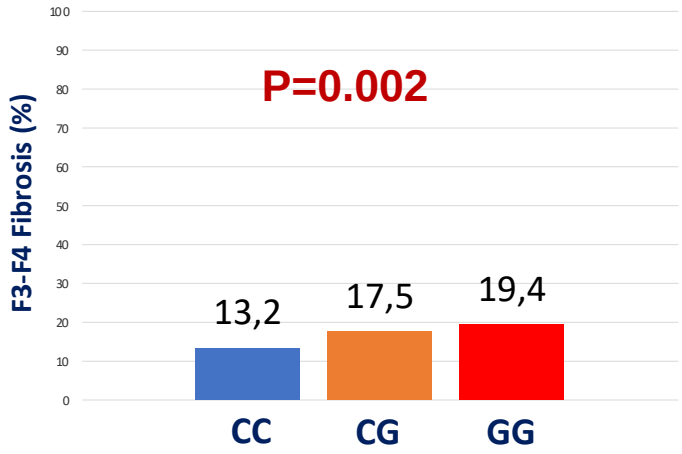
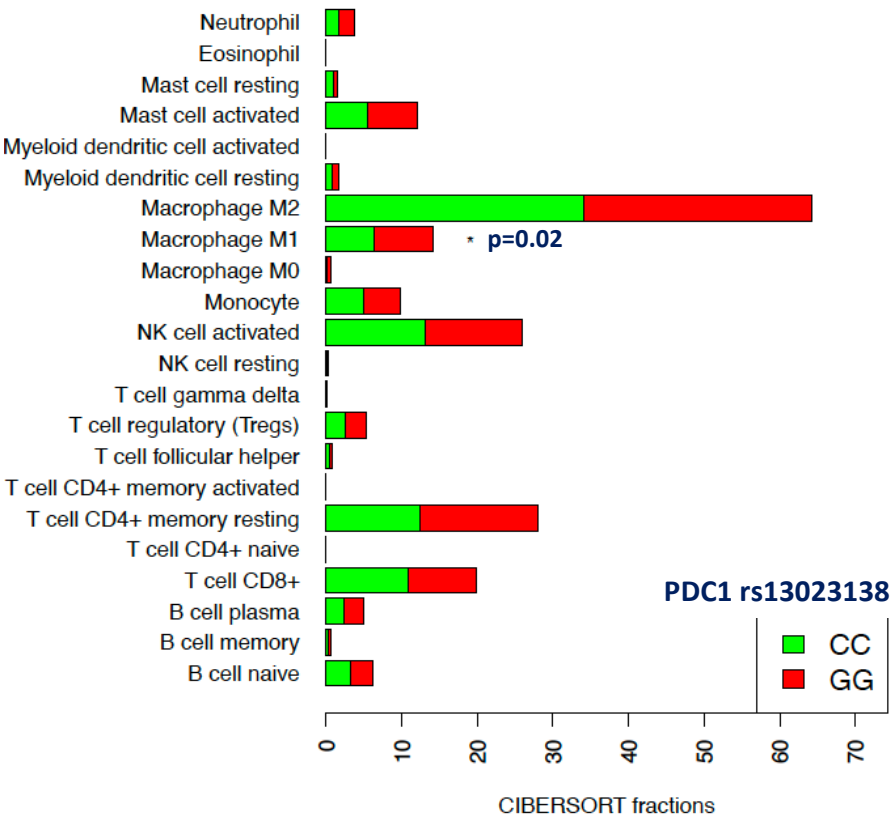


Figure 14. *Association between PDCD1 rs13023138 genotype and presence of severe fibrosis.*

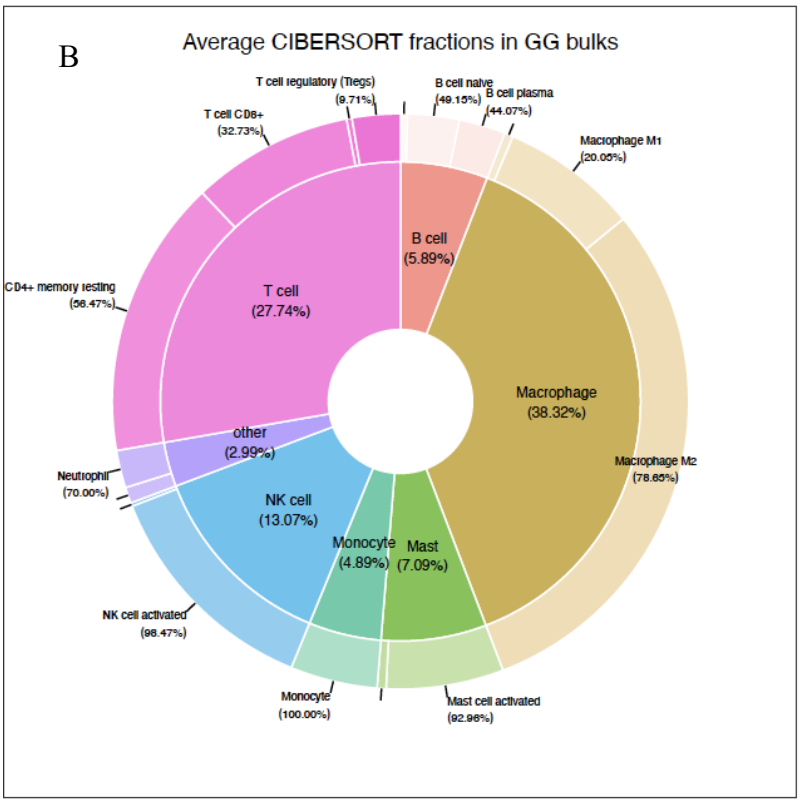
3.3 PDCD1 rs13023138 genotype correlates with specific changes in liver immune cell types

We then proceeded to investigate the hypothesis that the influence of PDCD1 rs13023138 on liver damage might coincide with alterations in the composition of liver immune cell types. To explore this hypothesis, we examined a subset of 121 bariatric patients (47 CC, 56 CG, 18 GG) with accessible liver transcriptomics data. Utilizing bulk RNA-seq data from patient's liver, we conducted deconvolution analyses that integrated PDCD1 rs13023138 genotypes and immune cell-type proportions. We noted that the samples, distinguished by different PDCD1 genotypes, exhibited a relatively uniform immune microenvironment composition. Notably, there were no distinct clusters observed, except for the fractions of macrophage M1 pro-inflammatory and pro-fibrotic cells. These fractions were more prevalent in the livers of patients with the GG genotype (20,05%) compared to those with the CC genotype (15,72%) (log2-fold change = 0.27; Wilcoxon-Mann-Whitney p-value = 0.024) (Figure 15).

A


Neutrophil
Eosinophil
Mast cell resting
Mast cell activated
Myeloid dendritic cell activated
Myeloid dendritic cell resting
Macrophage M2
Macrophage M1
Macrophage M0
Monocyte
NK cell activated
NK cell resting
T cell gamma delta
T cell regulatory (Tregs)
T cell follicular helper
T cell CD4+ memory activated
T cell CD4+ memory resting
T cell CD4+ naive
T cell CD8+
B cell plasma
B cell memory
B cell naive
* p=0.02
PDC1 rs13023138
CC
GG
0
10
20
30
40
50
60
70
CIBERSORT fractions

B


Average CIBERSORT fractions in GG bulks
T cell regulatory (Tregs) (9.71%)
B cell naive (49.15%)
B cell plasma (44.07%)
T cell CD8+ (32.73%)
Macrophage M1 (20.05%)
B cell (5.89%)
T cell (27.74%)
CD4+ memory resting (56.47%)
Macrophage (38.32%)
other (2.99%)
Macrophage M2 (78.65%)
Neutrophil (70.00%)
NK cell (13.07%)
Monocyte (4.89%)
Mast (7.09%)
NK cell activated (98.47%)
Monocyte (100.00%)
Mast cell activated (92.96%)

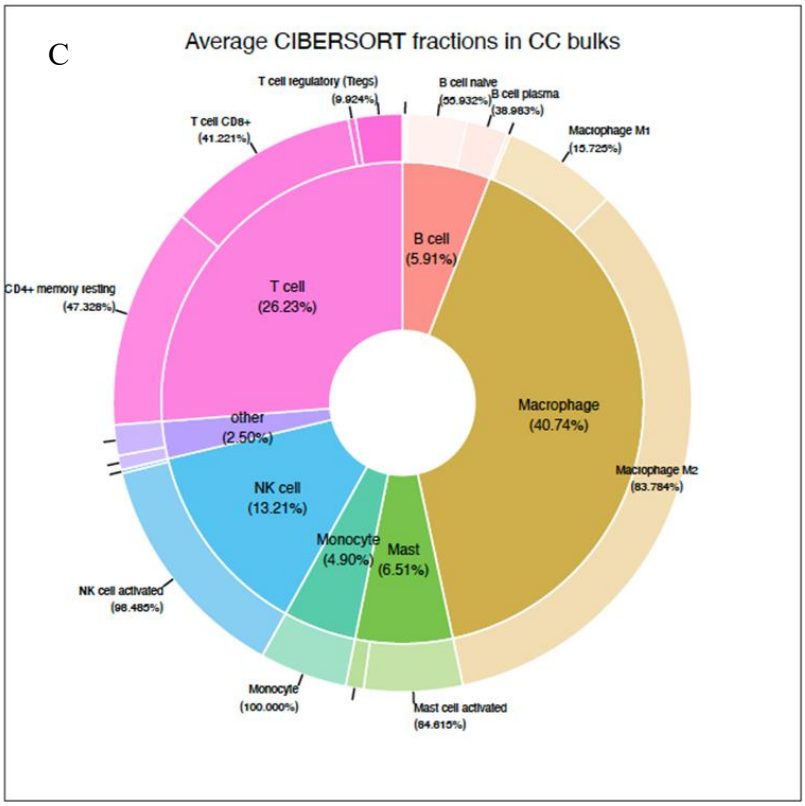


Figure 15. *(A) Average CIBERSORT fractions of cell types over CC and GG PDCD1 categories. Wilcoxon-Mann- Whitney test has been applied to compare fraction distributions groups. Pie chart representation of the average CIBERSORT fractions among PDCD1 GG (B) and CC categories (C).*

3.4 Impact of the PDCD1 rs13023138 G variant on liver transcriptome

In the same subset of 121 bariatric patients (47 CC, 56 CG, 18 GG) with available liver transcriptomics data, we tested the impact of the rs13023138 G variant on PDCD1 gene expression. Surprisingly, our results revealed no association between the PDCD1 rs13023138 G genotype and PDCD1 expression levels for all the models we tested (additive, dominant and regressive models) (Figure 16)

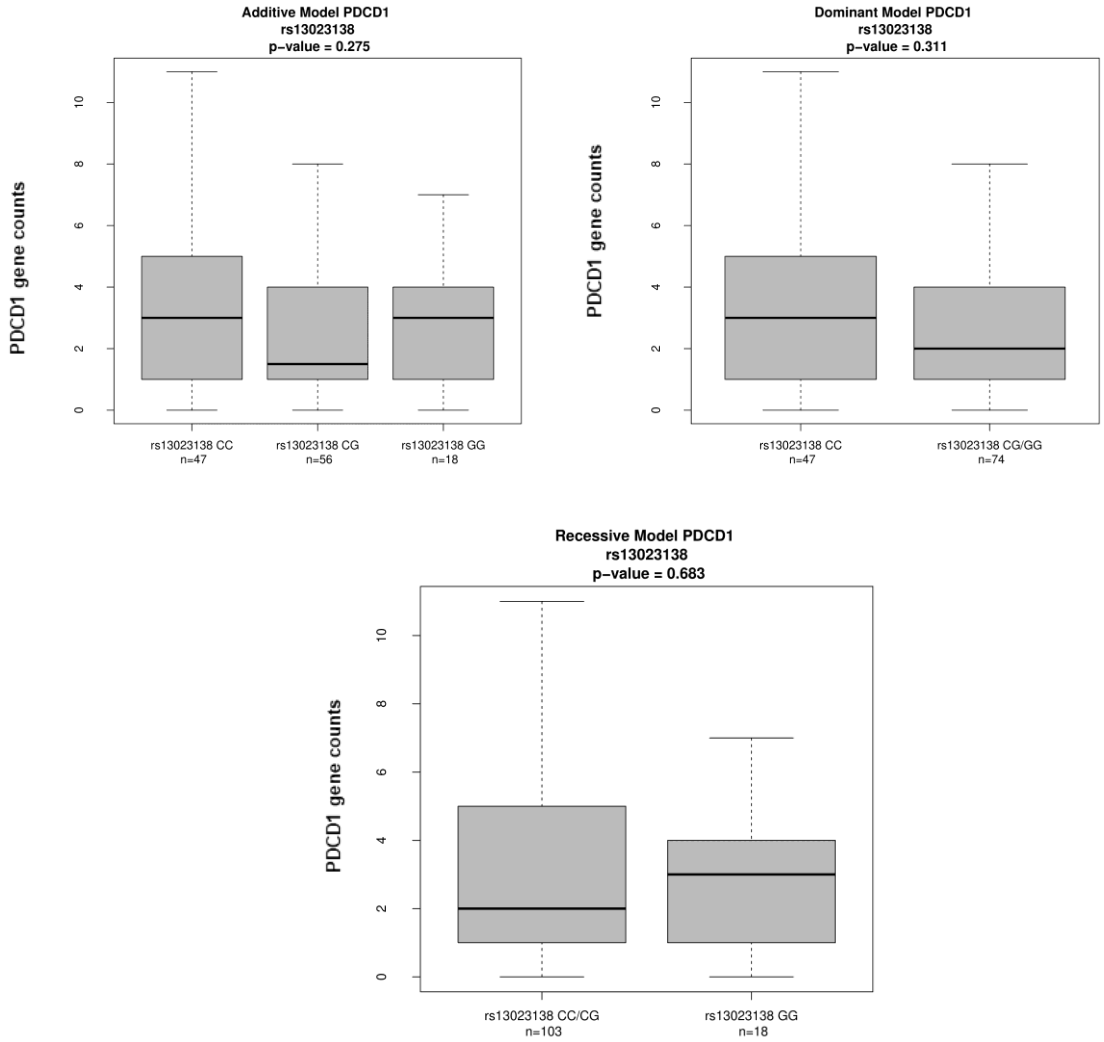


Figure 16. *Association between PDCD1 rs13023138 genotype and PDCD1 expression*

However, our transcriptomic analysis reveals that 3067 genes exhibit distinct expression profiles correlated with the dosage of the PDCD1 rs13023138 G allele. Between them, 847 genes were upregulated, while 2220 were downregulated.

Gene Set Enrichment Analysis revealed that the G allele was significantly associated with overexpression of pathways related to inflammation and carcinogenesis. Conversely, it was linked to the downregulation of crucial metabolic pathways, Myc targets, oxidative phosphorylation, and DNA repair control/mechanisms as shown in Figure 17.

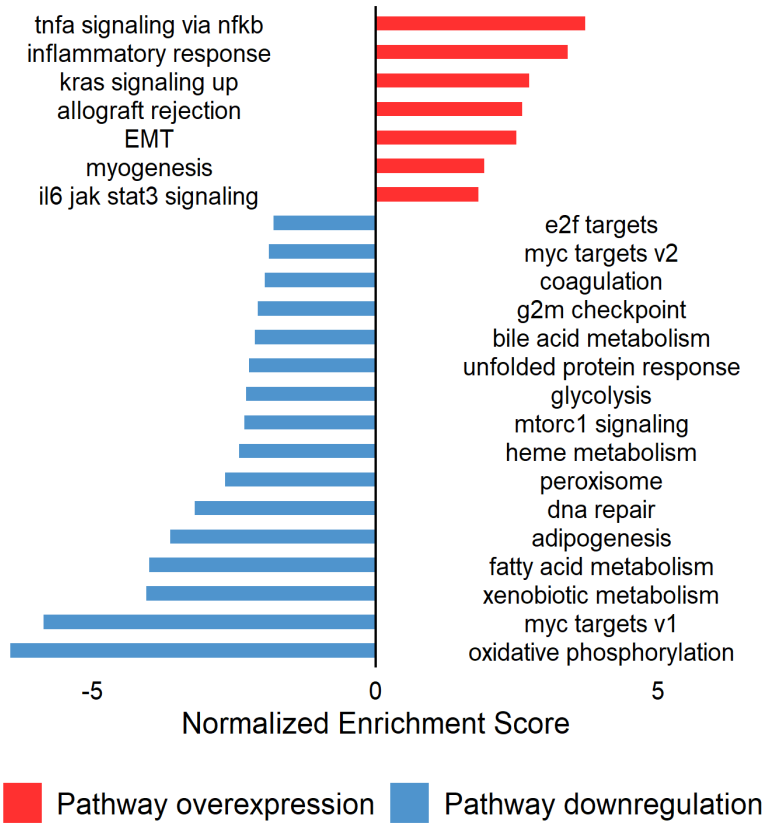


Figure 17*. Pathways enriched in genes differentially expressed (p < 0.05) in dependence of PDCD1 rs13023138 C>G variant genotype in n = 121 individuals in whom liver cohort transcriptomic was available. Only genes presenting a multiple test adjusted p-value for false discovery rate <0.05 are considered.*

Collectively, these results are in line with the increase in liver inflammation and cell proliferation, and with the reduction in lipid metabolism/turnover and in DNA repair mechanisms in individuals carrying the PDCD1 rs13023138 G variant.

3.5 PDCD1 rs13023138 variant affects CXCR6 gene expression in the liver

For better elucidate the different cellular signalling that are activated by G *PDCD1* rs13023138 variant, we delved into the NCATS BioPlanet dataset, focusing specifically on genes that showed significant differential expression after adjustment for FDR. The results revealed enrichment in two distinct pathways: "T cell receptor regulation of apoptosis" ($p = 0.005$) and "Binding of chemokines to chemokine receptors" ($p = 0.006$), both emerging as significant gene sets after correction for multiple tests. Notably, our investigation spotlighted that the CXCR6 gene, which encodes a chemokine receptor crucial for recruiting effector T-cells to inflammatory site, [190] exhibited differential expression, being upregulated depending on *PDCD1* rs13023138 G allele dosage in the model corrected for age, sex, batch factor and *PNPLA3* rs738409 genotype (Figure 18). Intriguingly, CXCR6 is expressed in a subset of hepatic $CXCR6^{+}PD1^{high}$ $CD8^{+}$ T cells, that shown an exhausted phenotype and are linked with NASH progression and HCC. [185] This result enhances our comprehension of the intricate PDCD1 genetic mechanism.

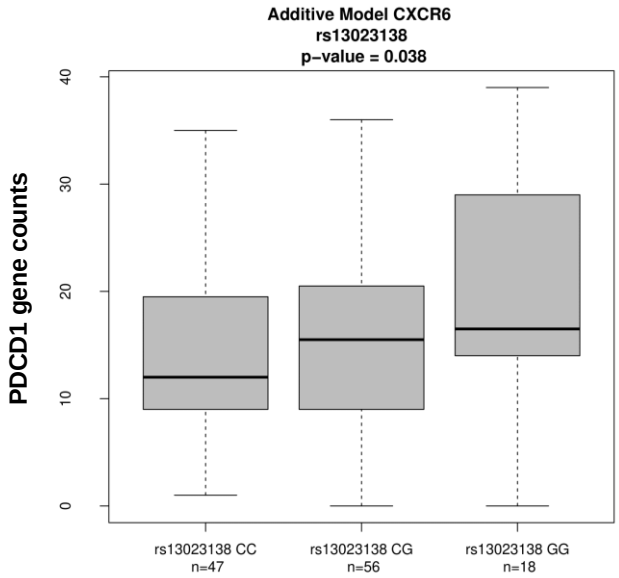


Figure 18. *Association between PDCD1 rs13023138 genotype and CXCR6 expression*

CHAPTER 4

MATERIALS AND METHODS

4.1 UKBB

The UK Biobank (UKBB) is a large-scale study including more than 500 000 individuals aged 40-69 years and recruited between 2006 and 2010 across the UK (ukbiobank.ac.uk). [191] The data utilized in this research were obtained from the UKBB under Application Number 37142. In this investigation, we only focused on unrelated European individual, totally 363,393 participants. This group of individuals with no familial relationships (no third degree or closer relatives) is identified thought the calculation of pairwise kinship coefficients [192].

International Classification of Diseases, 10th Revision (ICD-10) code was used to define hepatocellular carcinoma (HCC, C22.0) using UK cancer registry (data-field 40 006), hospitalization records (data-field41 270) and death registries (data-fields, 40 006, 41 270, 40 001 and 40 001). Finally, all individuals with chronic viral hepatitis were excluded from the analysis (B18-B19).

4.2 Liver Biopsy study cohort

We analysed data from 2,606 /2,889 patients prospectively recruited for suspected NASH, of whom blood samples and genetic analyses were available. The study cohort included individuals from: 1) the Metabolic Liver Diseases outpatient service, Fondazione IRCCS Ca' Granda and Ospedale Maggiore Policlinico, Milan, Italy; 2) the Division of Gastroenterology and Hepatology of Palermo University Hospital, Palermo, Italy; 3) the Department of Medicine, University of Eastern Finland and Kuopio University Hospital, Finland; and 4) Campus Bio-Medico University Hospital, Rome, Italy. Individuals with other causes of liver disease were excluded from this study (alcohol intake >20 g/day, viral hepatitis, autoimmune hepatitis, hereditary hemochromatosis and alpha-1 antitrypsin deficiency).

4.3 Clinical and laboratory assessment

Clinical and anthropometric data were collected at the time of liver biopsy. Body mass index (BMI) was calculated on the basis of weight in kilograms and height in meters. Obesity was defined as BMI ≥30 Kg/m2. The diagnosis of type 2 diabetes was based on the revised criteria of the American Diabetes Association, using a value of fasting blood glucose ≥126 mg/dL. [193]In patients with a previous diagnosis of type 2 diabetes, current therapy with insulin or oral hypoglycaemic agents was documented. A 12-h overnight fasting blood sample was collected at the time of biopsy to determine serum levels of ALT, total cholesterol, HDL-cholesterol, triglycerides and plasma glucose concentrations.

4.4 Genetic analyses

DNA was purified using the QIAmp blood Mini Kit (Qiagen, Mainz, Germany) and DNA samples were quantified using spectrophotometric determination. Genotyping for *PNPLA3* (*rs738409 C>G*), *PDCD1 (rs13023138 C>G)* and *SIRT5* (*rs12216101 T>G*) was carried out using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems). Commercial genotyping assays were available (*PDCD1 rs13023138*. catalogue number 4351379, C_57931315_10; PNPLA3 rs738409. catalogue number 4351379, C_7241_10. SIRT5 rs12216101. catalogue number 4351379, C_27074924_10 (ThermoFisher, Italy).
Genotypes were called by the SDS software v.2.3 (StepOne Plus, Applied Biosystems).
The genotype frequencies of *rs13023138* evaluated in each cohort and adjusted by using the Bonferroni correction to account for multiple comparisons, P values considered significant if p <0.008, were in Hardy-Weinberg proportion.

4.5 Assessment of histology

Slides were coded and read at each clinical centre by one expert pathologist who was unaware of the patients' identity and history. A minimum 15 mm length of the biopsy specimen or the presence of at least 10 complete portal tracts was required.
Steatosis was assessed as the percentage of hepatocytes containing fat droplets (minimum 5%). Kleiner classification [194] was used to compute steatosis, ballooning and lobular inflammation, and to stage fibrosis from 0 to 4.
Steatohepatitis was considered to be present when steatosis, lobular inflammation and ballooning were concomitantly present.

4.6 Transcriptomic and bioinformatic analysis

Total RNA was isolated using RNeasy mini-kit (Qiagen, Hulsterweg). RNA was sequenced in paired-end mode (read length 150 nt) using the Illumina HiSeq 4000 (Novogene). Reads were mapped by a custom pipeline[195] encompassing reads quality check[196] low-quality reads trimming[197] and mapping on GRCh37 reference genome[198]by STAR mapper.[199]

Samples with insufficient mapping quality (<10 million mapped reads, uniquely mapped <60% mapped reads) were excluded. Gene reads were counted according to the ENSEMBL human transcript reference assembly version 75 exploiting RSEM[200] package. Raw counts normalization and differential gene expression analysis were performed by exploiting DESeq2[201] package according to the standard workflow. To identify differentially expressed pathways, pre-ranked gene set enrichment analysis (GSEA) [202,203] was performed on differentially expressed or correlated genes. After performing the Transcript Per Million normalization (TPM) of gene expression data, CIBERSORT cell fractions have been estimated using the TIMER2.0 web tool[204] and average cell fractions have been calculated across *PDCD1 rs13023138* G>C genotypes (i.e. n = 47 CC, n = 56 GC and n = 18 GG).

Differences among CIBERSORT cell fraction distributions over *PDCD1* categories have been evaluated through Kruskal-Wallis and Wilcoxon-Mann- Whitney tests. Pie plots have been obtained using the PieDonut function of the webr R package.

4.7 Statistical analysis

Continuous variables were summarized as mean ± standard deviation and categorical variables as frequency and percentage. The t test, ANOVA and the chi-square test were used, when appropriate. Univariate and multivariate ordinal regression models were used to assess the factors independently associated with severity of lobular inflammation (in PDCD1 study), while univariate and multivariate logistic regression models were used to assess factors associated with presence of severe steatosis, ballooning, NASH and advanced fibrosis (in PDCD1 and SIRT5 study). As candidate risk factors, we selected gender, age > 50 years (median age of the studied population), obesity, the presence of diabetes, *PNPLA3* rs738409 genotype (additive model) *PDCD1 rs13023138* genotype (additive model) and *SIRT5 rs12216101* (additive model). All models were also adjusted for enrolling centre. $p <$ 0.05 was considered to be statistically significant. Liver biopsy cohort regression analyses were performed using SPSS (SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.). In UK Biobank, the association between common genetic variants (minor allele frequency >1%) in a window of 20 kb on each side of *PDCD1*

gene and HCC was examined using a binary logistic regression analysis adjusted for age, sex, BMI, first 10 PCs of ancestry and genotyping array. In the "transcriptomic cohort, after the pre-ranked GSEA, p-values have been corrected for multiplicity by Benjamini-Hochberg false discovery rate method. Only adjusted *p*-values <0.05 were considered statistically significant. Statistical analyses in UKBB and for transcriptomic analysis were carried out using the R software V.3.6.0.

Discussion

NAFLD is one of the most insidious liver disorders due to the lack of non-invasive biomarkers, especially for NASH, the diagnosis of which still relies on liver biopsy.

Currently, despite several clinical trials, no pharmacological treatment has been approved for the condition. Therefore, scientific efforts, in recent years, have been focused on researching non-invasive liver damage biomarkers, applicable on a large scale, and genetic polymorphisms associated with the onset and progression of NAFLD.

The aim is to accurately guide screening and follow-up programs and identify new therapeutic targets in the context of precision medicine.

In this scenario, my PhD project analysed the impact of two different genetic variants, *PDCD1* rs13023138 and *SIRT5* rs12216101, on the liver severity of NAFLD. My study was aimed at identifying, using hepatic transcriptome analysis approach, the correlation between polymorphic status of *SIRT5* and *PDCD1* and their differential gene expression.

We found that the rs12216101 T>G variant, located at a flanking region of SIRT5, was associated with a higher risk of steatohepatitis and clinically significant fibrosis.

Our findings demonstrated that the rs12216101 T>G variant is linked to the upregulation of genes related to mitochondrial energetic pathways within the liver, especially those involved in the oxidative phosphorylation process. Transcriptomic analysis indicated the upregulation of OXPHOS in G allele carriers. A result later confirmed, by our biochemistry colleagues, through western blotting, revealing elevated expressions of complex III, IV, and V in GG carriers. The colorimetric assay showed that the upregulation of OXPHOS complexes coincided with decreased ATP levels and increased ROS, nitrogen free radicals (RNS) and malondialdehyde levels, in liver samples of the transcriptomic cohort (data not shown). Consequently, our findings propose that the heightened OXPHOS activity, in GG carriers, results in elevated levels of mitochondria-generated ROS and RNS, molecules able to trigger inflammation and fibrogenesis.[205]

Additionally, considering that SIRT5 operates as NAD^+- dependent enzyme, the increased activity of SIRT5, in GG carriers, might deplete the cellular NAD^+ pool. This observation appears significance because NAD^+ plays a vital role in maintaining metabolic homeostasis and its depletion is known to occur in conditions of lipotoxicity, as seen in NAFLD.[206]

The increased activity of SIRT5, contribute to the pathological NASH-fibrosing phenotype, through dysregulation of mitochondrial activity, particularly in OXPHOS. This evidence led

us to hypothesize that inhibiting SIRT5 could positively influence mitochondrial oxidative metabolism representing a potential therapeutic strategy in NAFLD context.

Consistent with this hypothesis, in HepG2 steatosis *in vitro* model, our colleagues demonstrated that both, the specific pharmacological inhibitor MC3482 and siRNA of SIRT5, led to the restoration of key parameters of mitochondrial energetics (data not shown).

Regarding the mechanism linking the rs12216101 variant T>G with SIRT5 activity, it was observed that this variant is not in linkage disequilibrium with any variant influencing SIRT5 protein sequence and does not operate exclusively as an eQTL. Given these findings, we explored the possibility of the modulation of SIRT5 alternative splicing. Through bioinformatic prediction, it was found a significant increase in the regulatory isoform 4 in the livers of obese individuals carrying the GG genotype. Although in this study we could not precisely determine the effect of the rs12216101 T>G variant on SIRT5 enzymatic activity, we provided evidence that isoform 4 overexpression in HepG2 may impair mitochondrial energetics, leading to lower GSH levels and higher ROS production. This is coherent with higher disease severity, mitochondrial dysfunction and oxidative stress observed in patients carrying the GG genotype (data not shown).

In summary, our study is the first to establish a link between the rs12216101 T>G SIRT5 variant and severe liver disease in NAFLD patients. Significantly, this risk variant correlates with SIRT5 gain-of-function, resulting in the upregulation of mitochondrial OXPHOS and increased oxidative stress. These findings provide compelling evidence for the involvement of SIRT5 in NAFLD progression.

During my PhD course, I applied the transcriptomic approach to the evaluation of the involvement of immune system in NAFLD progression. Recently, the immunotherapy, particularly the use of immune checkpoint inhibitors,[207] has revolutionized cancer treatment, including HCC.[208] One key target of immunotherapy is PD-1 receptor and PD-1 inhibitors are used to block the interaction between PD-1 on T cells and PDL-1 on cancer cells. By doing so, these inhibitors unleash the immune system, allowing T cells to recognize and attack cancer cells effectively. Research in the field of immunotherapy, especially targeting the PD-1 pathway, continues to advance. Scientists are exploring biomarkers to predict response, combination therapies, and ways to minimize side effects, with the goal of improving the outcomes and quality of life for cancer patients.

Considering this perspective, our investigation delved into prevalent PDCD1 gene variants linked to hepatocellular carcinoma (HCC) onset within the UK Biobank cohort, culminating in the identification of the rs13023138 C>G variant as the most significant genetic marker for HCC. Aligning with these findings and recognizing the growing importance of adaptive

immunity in NASH development,[167,184,209] we proceeded to explore the hypothesis that this association with HCC susceptibility might be attributable to an inclination toward the development of more severe liver disease, potentially through the facilitation of inflammation pathways. Consistently, we found that in our large clinical cohort, carriers of the *PDCD1* rs13023138 C>G variant, and especially those homozygotes for the minor allele (GG), had a higher risk of the entire spectrum of liver disease severity, in terms of lobular inflammation, ballooning, NASH and advanced fibrosis.

PD-1 plays a pivotal role in immune regulation, being expressed in various T cell subsets as well as in B cells, macrophages, and natural killer (NK) cells.[181] Our findings indicate that the PDCD1 rs13023138 C>G variant is linked to an increased presence of M1 polarized macrophages in the liver. These macrophages promote NASH and fibrosis by releasing pro-inflammatory factors like CXCL2, IL-1β, TNF-alpha, CXCL10, and IL-6. [210,211]

This suggests a potential mechanism through which the evidenced genetic variant influences the progression of liver diseases.

In line with these findings, our data indicate that the PDCD1 rs13023138 C>G variant is associated with an increased expression of transcripts involved in inflammation mediated by TNF-alpha signaling via NFKB and IL6 JAK STAT3 signaling pathways.[212]

Notably, these pathways play a crucial role in driving inflammation and fibrosis progression in NAFLD and are also implicated in mechanisms leading to M1 polarization of macrophages.[211]

Additionally, carriers of the PDCD1 rs13023138 C>G variant exhibited a downregulation of metabolic pathways related to glycolysis, adipogenesis, oxidative phosphorylation, as well as peroxisome and fatty acid metabolism. Interestingly, it is worth noting that the PDCD1 rs13023138 G variant was not associated with altered PDCD1 expression. This suggests that the polymorphic status of PDCD1 affects differential gene expression without exerting a direct impact on PDCD1 expression itself. The PDCD1 rs13023138 C>G is an intronic variant, is not believed to directly impact liver PD-1 expression or the mRNA levels of genes within the 2q37.3 locus. However, it remains plausible that the observed phenotypic effects could be ascribable to other genetic variants in linkage disequilibrium with the leading ones, although such variants have not yet been identified.

Moreover, because it is located in a proximal enhancer-like signature of the *PDCD1* locus, we hypothesized that, in the context of dysfunctional metabolic *milieu*, it may stimulate overexpression of key genes activating the immune response.

The main limitation of this study is the lack of single-cell RNA sequencing data that further affects the interpretation of the results because our whole liver transcriptomic analyses do

not discriminate among specific subsets of liver immune cells associated with liver disease severity. Therefore, we were unable to assess the impact of the rs13023138 C>G variant on PD-1 mRNA expression in specific subpopulations of immune cells (e.g. $CD8^+$ T lymphocytes). Nevertheless, we discovered the upregulation of CXCR6 associated with the rs13023138 G risk allele. This recent discovery is fascinating, considering that a specific immune subset of auto-aggressive CD8+ T lymphocytes displaying an exhausted phenotype, identified by the markers $PDCD1^+$ $CXCR6^+$, has recently been shown to contribute to tissue damage and inflammation, ultimately leading to fibrosis and HCC in both experimental models and NAFLD patients.[184,185]

In summary, we observed that the PDCD1 rs13023138 C>G variant is independently associated with HCC development in the general population and with liver disease severity in NAFLD patients at high risk of progression. This connection appears to be linked to the modulation of the immune response, creating a proinflammatory and profibrogenic environment. These results imply a potential influence of individual variations in immune tolerance induction among NAFLD patients, opening new avenues for research in the pathogenesis and treatment of NASH.

Finally, during the research conducted throughout my PhD studies, two new potential risk factors for the progression of NAFLD have emerged: the rs12216101 T>G variant of SIRT5, which appears to play a crucial role in oxidative stress induction, and the rs13023138 C>G variant of PDCD1, which seems to be significant in inflammation induction through immune system involvement. These findings pave the way for new avenues in understanding NAFLD and could be pivotal for screening individuals at risk of developing severe liver conditions, as well as for the development of targeted and preventive therapies in the future.

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