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FUNCTIONAL CHARACTERIZATION OF A NOVEL GENETIC VARIANT PREDISPOSING TO ADVANCED FIBROSIS AND HEPATOCELLULAR CARCINOMA DEVELOPMENT IN NONALCOHOLIC FATTY LIVER DISEASE.

Settore scientifico disciplinare MED/09

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Summary

INTRODUCTION

Nonalcoholic Fatty Liver Disease (NAFLD)

NAFLD definition and epidemiology

Nonalcoholic fatty liver disease (NAFLD) is rapidly becoming the most common liver disease worldwide. Due to obesity epidemic, represents a clinical issue of growing importance in the Western countries. NAFLD is defined as a liver fat content >5%, as assessed both directly by liver histology or indirectly using non-invasive methods as imaging by magnetic resonance (1-4), in absence of other liver injury etiologies as drug abuse and excess alcohol intake (<30 g/die in men and <20 g/die in women) (4-6). Today ≈25% of the worldwide population is estimated to be affected by NAFLD and the prevalence of the disease is growing dramatically. Furthermore, due to the obesity epidemic, NAFLD is very common in some Western countries (7, 8) (Figure 1). Indeed, NAFLD most frequently represents the hepatic feature of the metabolic syndrome as its relationship with metabolic disorders, as obesity, insulin resistance (IR), type 2 diabetes mellitus (T2M), and metabolic syndrome (MetS) is today well established (6, 9-11). The umbrella term "NAFLD" refers to a wide spectrum of liver disorders ranging from isolated steatosis to a Nonalcoholic steatohepatitis (NASH). NASH is characterized by the establishment of a chronic inflammatory response in the liver, hepatocyte degeneration (Hepatocellular ballooning), characteristic histological lesions as Mallory-Denk bodies with or without presence of liver fibrosis. While steatosis is considered a benign condition and not necessarily evolve into a progressive disease, NASH is a chronic and progressive liver disease that can lead to severe complications. About one-third of NAFLD patients develop NASH, and NASH patients are estimated to represent about the 5% of the UK population.

Furthermore, 20% of all NASH patients progress to advanced fibrosis (bridging fibrosis or cirrhosis) (12, 13). Both NASH and cirrhosis are severe risk factors for the development of hepatocellular carcinoma (HCC), the third most common cause of cancer death (14). Disease progression is summarized in Figure 1.

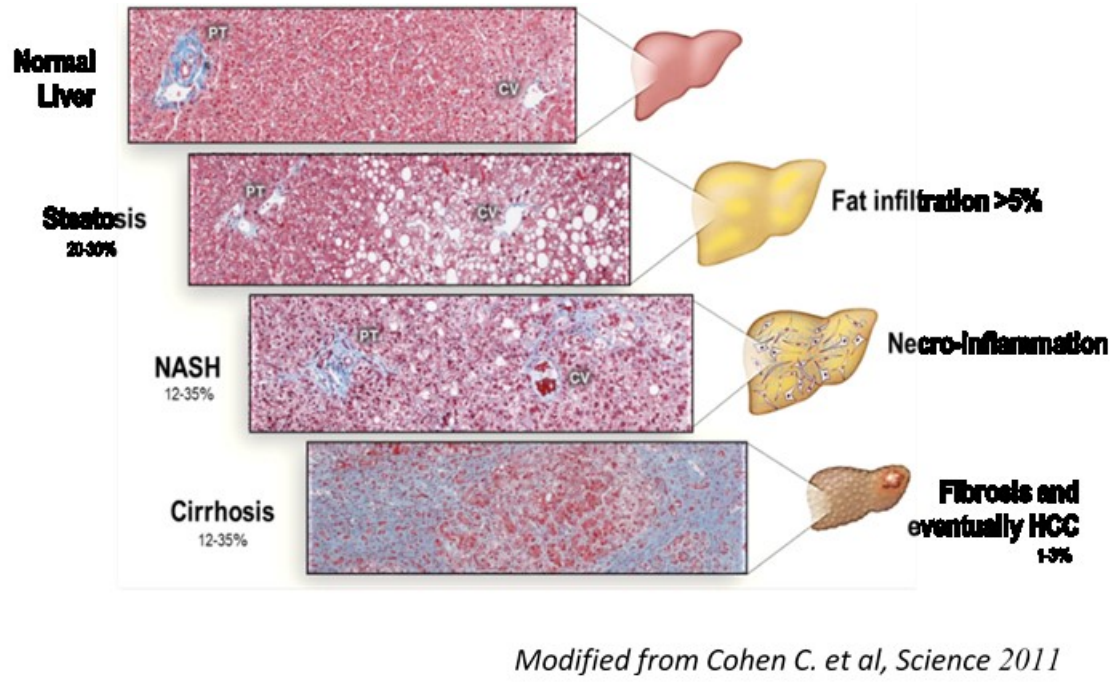

Figure 1 - Spectrum of Nonalcoholic Fatty Liver Disease

Pathogenesis of NASH

The "two hits" model

Molecular mechanisms beyond NAFLD progression toward NASH and fibrosis are still object of active research. A pathogenetic model, the so called "two hits model", was proposed by Day and Jones 1998. In this paradigm, dyslipidemia, hyperinsulinemia, and excessive dietary intake of lipids promote a "first hit" represented by steatosis development. Fat accumulation in the liver represents

a risk factor for the development of a chronic inflammatory response in the liver due to different "second hits" (15). Initially, Day and Jones identified a key role for reactive oxygen species (ROS) produced in the P450 cytochromes (e.g. *CYP2E1*) dependent lipid peroxidation pathway. Indeed, is today well established the role of these molecular species in promoting oxidative stress, cell damage and tissue inflammation. Furthermore, other possible causes of tissue inflammation, as gut-derived endotoxins and FFAs, were also identified (15, 16).

The "Multiple parallel hits" model

The contribution of liver inflammation to steatosis and insulin resistance development and the observation that not always steatosis precedes NASH were not accounted in the "two-hits" hypothesis. For this reason, more complex models, as the "multiple parallel hits model" (summarized in Figure 2), were proposed. In this pathogenetic model, steatosis and inflammation are seen as correlated factors, but different hits can induce liver inflammation in parallel. Liver fat accumulation can be the "first hit" sensitizing the tissue to the action of other stressors, but can be, in some cases, a consequence of other insults (17).

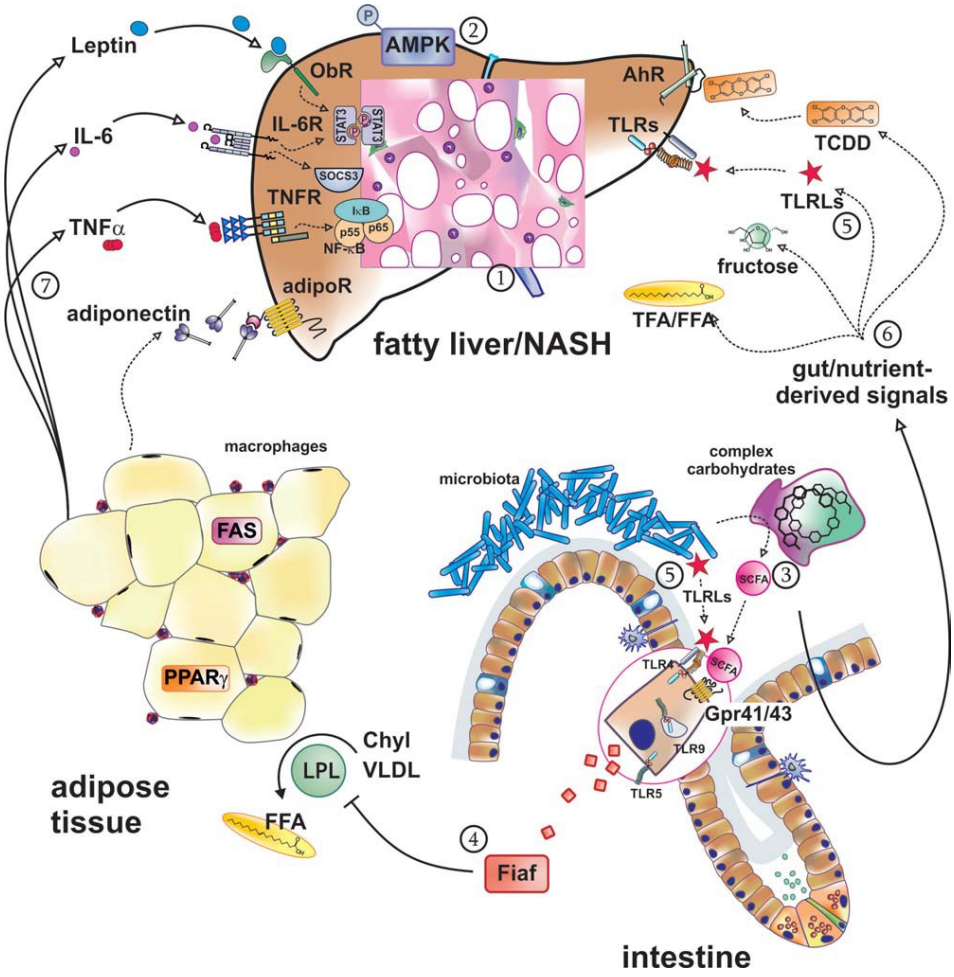

Figure 2 - The multiple parallel hits model. Lipotoxicity: (1) A liver loaded with lipids consisting primarily of triglycerides might reflect a benign condition. Furthermore, hyperleptinemia leads to peroxidation of hepatic lipids, thereby also protecting this organ from lipotoxicity. When the capacity of peripheral and central organs of detoxifying ''aggressive lipids'' fails, lipotoxic attack of the liver might begin. Inflammation may precede steatosis in NASH. Gut-derived signals: Many signals beyond endotoxin might affect hepatic steatosis and inflammation. Several pathways have been identified how the gut microbiota might influence host energy metabolism: (2) Absence of the microbiota in germ-free mice correlates with increased activity of phosphorylated AMPK in the liver and the muscle (not shown). (3) Some of the breakdown products of polysaccharides are metabolized to short chain fatty acids (SCFAs). SCFAs such as propionate and acetate are ligands for the G protein–coupled receptors Gpr41 and Gpr43. Shortage of SCFAs might allow the evolution of

systemic inflammatory events. (4) The microbiota decreases epithelial expression of fasting-induced adipocyte factor (Fiaf), which functions as a circulating lipoprotein lipase (LPL) inhibitor and therefore is an important regulator of peripheral fat storage. (5) Several TLRs, such as TLR5 or TLR9, are not only able to affect microbiota but also to regulate metabolism, systemic inflammation, and insulin resistance, thus highlighting the role of the innate immune system in metabolic inflammation as observed in NASH. (6) Various nutrients such as trans fatty acids (TFAs), fructose or aryl hydrocarbon receptor (AhR) ligands such as 2,3,7,8-tetrachlorodibenzodioxin (TCDD) may directly lead to steatosis/liver inflammation. Adipose tissue–derived signals: Signals derived from the adipose tissue beyond toxic lipids might play a central role in NAFLD/NASH. (7) Here, adipokines such as adiponectin and leptin, certain proinflammatory cytokines such as TNF-α or IL-6 and others (the death receptor Fas, PPARc) are of key relevance. The cytokine/adipokine milieu might be critical because ob/ob-adiponectin TG mice, although becoming severely obese, are not insulin-resistant. This suggests that in the hierarchy of processes soluble mediators play the central role. Adipose-derived mediators might indeed affect target organs such as the liver, because JNK1 adipose-deficient mice are protected from diet-induced obesity, and experiments have demonstrated that this effect is mediated mainly by IL-6 (a cytokine), which is of key importance in human obesity (17).

Hepatic steatosis as a trigger of liver inflammation

Lipids accumulating in the liver can derive form different sources. Dietary lipids are taken up and stored by the liver via chylomicron remnants uptake. The major contribution comes from fatty acids released from the adipose tissue. During systemic inflammation and adipose insulin resistance typical of metabolic syndrome (MetS), impaired of insulin signaling in the adipose induces lipolysis leading to an aberrant and continuous efflux of free fatty acids (FFAs). Hepatic tissue uptake and re-esterify FFAs producing triglycerides (TGs). In turn, TGs are stored into the lipid droplets (17, 18). Furthermore, due to the so called "selective insulin resistance" occurring in the liver, hyperinsulinemia induces hyperactivation of the sterol receptor elements binding protein 1c (Srebp-1c) promoting also hepatic *de-novo* lipogenesis and further TGs accumulation (19).

FFAs esterification into TGs seems to protects the tissue against lipotoxicity and inflammatory response activation. Indeed, mice NASH models defective for TGs synthesis pathways due to deletion of diacylglycerol acyl transferase 2 (DGAT2), the last enzyme of the TG synthesis pathway were protected from steatosis development but more susceptible to liver damage progression. Today, the role of "aggressive lipids" as cholesterol and free fatty acids in inducing oxidative stress, inflammation and fibrosis is well established (1, 20).

Inflammation promotes hepatic steatosis

Steatosis grade is highly variable among NASH patients. Indeed, NASH patients can also be affected by low or moderate steatosis (21). This process can be partly modulated by myeloid population role in promoting lipid deposition. Furthermore, anti TNF-α antibodies (infliximab) are associated to steatosis improvement in by alcoholic steatohepatitis (ASH) patients (22). These evidences, together

with the key role of liver inflammation in inducing insulin resistance in insulin sensitive tissues, point out role for inflammatory response in further promoting fat accumulation in the liver (17).

Endotoxins and gut microbiota in obesity

Gut microbiota derived endotoxins, as lipopolysaccharide (LPS), are known triggers of innate immune responses and were considered one important "second hit" in the earlier NASH pathogenetic model (15). Indeed, they are recognized as danger signals "DAMPs" by cells via activation of different Toll-like receptors triggering an innate immune response (23). Impairment of the gut barrier determining an increased efflux of endotoxins towards the liver often occurs in NAFLD patients and is associated with liver steatosis (24). However, its association with liver inflammation is still on debate A clear association between steatosis and increased gut permeability was identified. However, increased gut permeability was not associated with liver inflammation appears to be less evident and is still on debate (24).

To date, the role of gut microbiota composition in modifying NAFLD onset and progression is widely recognized. Indeed, obesity and NAFLD are often associated with alternation in the microbiota composition and different bacterial species have been pointed out as disease modifiers (1, 23, 25, 26). This association is partly explained by the ability of different bacterial species in metabolizing several nutrients producing bioactive molecules as ethanol and short chain fatty acids (SCFAs) (17). However, molecular mechanisms beyond this association are still debated (1, 27).

Role of adipose tissue and adipokines

Adipose tissue is widely recognized as an endocrine tissue producing several mediators active on target tissues. Among anti-inflammatory adipokines Adiponectin is one of the most studied as is known to play an important role in NASH (28, 29). Studies on leptin-deficient genetically obese mice (ob/ob mice), together with clinical observations, demonstrated adiponectin ability in improving insulin resistance, liver steatosis and inflammation (30, 31).

Leptin is a hormone produced by adipose tissue that promotes fatty acids mobilization in adipocytes through an autocrine/paracrine mechanism. Increased leptin production by the hypertrophic adipose tissue seems to contribute to dyslipidemia in obesity, thus promoting liver steatosis and inflammation due to toxicity of FFAs (17, 31).

In obesity, adipose tissue is reported to produce high levels of interleukin-6 (IL-6) and TNF-α significantly promoting systemic inflammation (32). These cytokines are reported to promote insulin resistance in liver in a JNK-IKKB-NF-kB dependent manner thus promoting liver steatosis, hyperinsulinemia, and hyperglycemia (17). It has also been demonstrated that, in a mouse model of obesity induced HCC, IL-6 and TNF-α play a central role in the induction of inflammation and tumor development. Indeed IL-6 or TNFR1 (TNF receptor 1) deficient mice are protected against obesity induced tumorigenesis (33). Role of IL-6, anyway, is not completely clear: IL-$6^{-/-}$ mice are in fact prone to obesity (34). Adipose tissue inflammation is a common event in morbid obesity, and this tissue may be the major cytokines source in obesity. Adipose tissue–derived mediators might attack the liver, thus promoting liver inflammation (17).

Genetics of NAFLD

As previously stated, NAFLD is an "umbrella term" referring to a spectrum of liver pathophysiological alterations ranging from isolated steatosis toward a progressive hepatitis leading to liver fibrosis and HCC. Notably, NAFLD is characterized by a strong interindividual variability in all aspects of its natural history. Only ~40% of patients with NAFLD have progressive liver fibrosis. The same, only in the 20% of individuals in this group fibrosis progress rapidly toward cirrhosis (35, 36). This strong inter-individual variability is, at least in part, explained by the contribution of hereditary factors as demonstrated in different studies with complementary approaches including genetic and epidemiological studies (37). Family studies demonstrated that first degree relatives of NAFLD patients have an increased disease susceptibility independently of body mass index (BMI) (38, 39). These results were confirmed by twin studies conducted in different populations (40, 41). Recent studies exploiting non-invasive and highly accurate liver fat and liver fibrosis assessment by magnetic resonance imaging, confirmed a 52% heritability of NAFLD in general population. Also, 50% of liver fibrosis variability was explained by hereditary traits. Importantly steatosis and fibrosis shared largely (78%) their heritable component (42). Overall, these data points out NAFLD as a complex disease influenced both by environmental factors and heritable traits. In the last decades many studies including genome-wide association studies (GWAS) and candidate gene studies, identified different genetic modifiers of NAFLD. A growing number of variants in genes involved in the hepatic lipid metabolism have been associated with NAFLD onset and progression (Table 1) (43).

Variant (MAF in Italy)	Function	Class (Impact)	Phenotype
PNPLA3 rs738409 C>G (0.27)	Lipid droplets remodeling	Coding (LOF)	↑ NAFLD, NASH, fibrosis, HCC
TM6SF2 rs58542926 C>T (0.07)	VLDL secretion	Coding (LOF)	↑ NAFLD, NASH, fibrosis
MBOAT7 rs641738 C>T (0.44)	Phosphatidylinositol metabolism	eQTL (LOF)	↑ NAFLD, fibrosis, HCC
GCKR rs1260326 C>T (0.29)	*de novo* lipogenesis	Coding (LOF)	↑ NAFLD, NASH, fibrosis
GCKR rs780094 A>G (0.30)	*de novo* lipogenesis	Intronic (-)	↑ NAFLD, NASH, fibrosis
LYPLAL1 rs12137855 C>T (0.16)	TG catabolism	Intronic (-)	↑ NAFLD
APOB Several (<0.001)	VLDL secretion	Coding (LOF)	↑ NAFLD, NASH, fibrosis, HCC
MTTP Several (<0.001)	VLDL secretion	Coding (LOF)	↑ NAFLD
LPIN1 rs13412852 C>T (0.21)	Lipid metabolism	Intronic (-)	↓ NASH, fibrosis
SOD2 rs4880 C>T (0.41)	Antioxidant system	Coding (LOF)	↑ fibrosis
UCP2 rs695366 G>A (0.26)	Lipid metabolism	eQTL (LOF)	↓ NASH
ENPP1 rs1044498 A>C (0.34)	Insulin signalling	Coding (GOF)	↑ fibrosis
IRS1 rs1801278 A>C (0.05)	Insulin signalling	Coding (GOF)	↑ fibrosis
IFNL3 rs12979860 C>T (0.36)	Innate immunity	eQTL (LOF)	↓ fibrosis
KLF6 rs3750861 G>A (0.07)	*de novo* lipogenesis fibrogenesis	Splice (LOF)	↓ fibrosis
MERTK rs4374383 G>A (0.36)	Innate immunity fibrogenesis	Intronic (LOF)	↓ fibrosis
FNDC5 rs3480 A>G (0.41)	Fibrogenesis	eQTL (-)	↓ fibrosis
HSD17B13 rs72613567 T>TA (0.22)	Lipid metabolism	Splice (LOF)	↓ NASH, fibrosis, HCC

Table 1 – Genetic variations associated with NAFLD onset and/or progression. MAF: Minor allele frequency; eQTL: expression quantitative trait locus; LOF: loss of function; GOF: gain of function.

Main genetic determinants of NAFLD

Patatin-like phospholipase domain-containing protein 3

The rs738409 C>G encoding for an isoleucine to methionine substitution at position 148 in the *Patatin-like phospholipase domain-containing protein 3* (*PNPLA3* I148M) aminoacidic sequence represents the major validated genetic determinant of NAFLD (44-46). Carriage of this variant in homozygosis is associated with an almost 9 fold increased risk of NAFLD-HCC development among general population of European descent (47). *PNPLA3* plays a key role in lipid droplets remodeling and fat mobilization in hepatocytes and hepatic stellate cells due to its triglycerides and retinyl esters hydrolase activity (48-50). The I148M substitution is associated with a reduced *PNPLA3* enzymatic activity resulting in an entrapment of triglycerides and retinyl esters in lipid droplets thus inducing liver steatosis (48-50). Furthermore, I148M substitution impairs proteasomal degradation of PNPLA3. Defective PNPLA3 accumulates on lipid droplets inhibiting activity of other lipases as PNPLA2 in hepatocytes (51). Indeed, Pnpla3 deletion do not results in steatosis in mice upon excess intake of carbohydrate, while overexpression of mutant Pnpla3 does (51). Furthermore, the rs2294918 variant, that encodes the E434K *PNPLA3* variant but also behave as an expression quantitative trait locus (eQTL) affecting gene expression level, shows an interaction effect with rs738409 further supporting this mechanism (52). Indeed, *PNPLA3* downregulation by antisense oligonucleotides have been proposed a therapeutic strategy for NAFLD therapy in patients carrying the 148M allele (53).

Transmembrane 6 superfamily member 2

The rs58542926 C>T polymorphism encodes for an E to K function-loss substitution at position 167 of the Transmembrane 6 superfamily member 2 (TM6SF2). TM6SF2 mediates the transfer of triglycerides to the apolipoprotein B100, thus representing a key factor in the very low-density lipoprotein secretory pathway. Carriage of the rs58542926 T allele results in a reduction of circulating lipoproteins together with an increased risk of NAFLD onset and progression. Interestingly, carriers are protected from cardiovascular events (54-57). Recent studies suggest also a TM6SF2 role in regulating also lipid biosynthesis and the number of secreted lipoprotein particles (58, 59).

Membrane bound O-acyltransferase domain-containing 7

Membrane bound O-acyltransferase domain-containing 7 (MBOAT7) is involved in the cell membrane remodeling catalyzing arachidonic acid transfer to lysophosphatidylinositol within the so called "Lands cycle". The rs641738 C>T in the 3' untranslated region (UTR) of MBOAT7 mRNA is a common eQTL variant associated with reduction of MBOAT7 expression both at mRNA and protein levels. MBOAT7 impairment results in reduced levels of arachidonic acid containing phosphatidylinositol in hepatocytes and the circulation (60, 61) and was recently associated with increased risk of liver steatosis, inflammation, fibrosis and cancer (60, 62).

Glucokinase regulator

Genetic variants in the glucokinase regulator (GCKR) gene, that is involved in the hepatocytes *de novo* lipogenesis by controlling glucose influx, have also been associated with NAFLD (63-66). The

common GCKR P446L variant (rs1260326) seems to be the causal variant beyond the gene to phenotype association (67). Indeed, the P446L substitution impairs GCKR ability to inhibit glucokinase in response to fructose-6-phosphate resulting in aberrant glucose uptake in hepatocytes (66). This leads to decreased glycemia and insulin levels, but induces aberrant *de novo* lipogenesis, malonyl-CoA accumulation and subsequent blockade of fatty acids beta-oxidation in hepatocytes favoring NAFLD onset and progression.

Other genes involved in NAFLD

A large number of other genetic determinants of NAFLD have been identified in case control and cross-sectional studies. Different variants in genes involved in lipid metabolism, inflammation, insulin signaling, oxidative stress and fibrogenesis were associated to NAFLD progression (37).

Lipid metabolism

Other variants in lipid metabolism have been associated in NAFLD. Recent study from our group highlighted the possible role Apolipoprotein B (APOB) gene variations in determining lipoprotein export impairment resulting in lower circulating triglycerides and susceptibility to NAFLD-HCC, with a mechanism similar to that of the TM6SF2 E167K variant (68).

Recently an insertion affecting splicing (rs72613567 T>TA) of the 17β-Hydroxysteroid dehydrogenase type 13 (HSD17B13) mRNA, has also been pointed out as a strong genetic modifier of both alcoholic and non-alcoholic liver disease. HSD17B13 protein has been reported to be highly expressed on hepatocyte lipid droplets and seems to be involved in retinol metabolism. However, the molecular mechanism behind this association are still object of active research (69).

Transcriptional regulators

Transcription factors involved in regulation of lipid metabolism as those of the peroxisome proliferator-activated nuclear receptors (PPAR) family play a key role in NAFLD pathogenesis. In particular, PPAR-α activation regulates fatty acids catabolism inducing mitochondrial uptake and lipids β-oxidation. PPAR-γ is instead associated with an insulin sensitizing and anti-inflammatory activity. Variation in these receptors were associated to NAFLD progression, however results are still inconsistent and requires further validations (70, 71). LPIN1 is a phosphatidate phosphatase highly expressed in the liver and adipose tissue and involved in phospholipids and triglycerides biosynthesis. Furthermore, a role in regulating lipid metabolism through its transcriptional co-activator activity has been identified for this protein (72). The rs13412852 TT allele in the LPIN1 *locus*, has been associated with increased LPIN1 expression levels and protection against NAFLD (73).

Oxidative stress

Mitochondrial fatty acids oxidation represents a key pathway in NAFLD. Indeed, activation of this pathway results in protection against liver steatosis. On the other hand, due to the high amount of ROS generated by this catabolic pathway, its activation is associated with progression to NASH (74). The Uncoupling protein 2 (UCP2) , plays a key role in regulating mitochondrial redox potential and excess energy dissipation (75).The UCP2 promoter region rs695366 G>A minor allele is associated with UCP2 overexpression and protection from NASH (76). Another example is represented by the rs4880 in the superoxide-dismutase 2 (SOD2) gene. This common polymorphism encodes for A to V change at position 16 of SOD2 altering mitochondrial targeting consensus sequence, facilitating

mitochondrial import, and protecting against oxidative stress (75). Indeed, this variant has been linked with reduced fibrosis in NASH patients (77, 78). Of particular importance are variants involved in iron metabolism. Different variants associated with hepatic iron overload are involved in liver damage progression mainly due to iron ability in catalyzing ROS production through the Fenton reaction (79-81).

Insulin resistance

The role for insulin resistance in NAFLD pathogenesis is well established. Different variants involved in insulin signaling have been pointed out as disease modifiers. In example rs1044498 single nucleotide polymorphism (SNP) associated with a gain-of-function effect of the insulin-signaling negative regulator Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (ENPP1), as well as the rs1801278 variant determining a loss-of-function of the insulin receptor substrate 1 (IRS1) protein were both associated with more sustained fibrosis in NAFLD patients (82).

Innate immunity and inflammation

Chronic inflammation represents a key process in NAFLD progression (83). Genetic variation at the interferon (IFN)-λ3 and 4 locus are associated with NAFLD (84, 85). Of particular importance, rs12979860 CC allele in the INFL3 locus is associated with a higher risk in developing liver inflammation and fibrosis (86, 87). Furthermore, variations in the MER proto-oncogene tyrosine kinase (MERTK), involved in immune cells activation, behave as disease progression modifiers. The rs4374383 G>A SNP, associated reduction of MERTK expression, protects against fibrosis development both in NAFLD and chronic hepatitis C infection (88-90).

Remarks

In conclusion, as previously underlined, NAFLD is a highly prevalent condition with a large heritable component. A few genetic modifiers of this disease have been identified to date by genome-wide association and targeted studies. Common variants with a large effect on the disease have been identified, being those in PNPLA3, TM6SF2, MBOAT7 and GCKR the most validated. A schematic representation of the major pathways implicated by genetic studies in the pathogenesis of NAFLD development and progression, and the gene loci whose involvement has been most robustly validated are depicted in Figure 3. Genetic associations described in almost all processes involved in NASH pathogenesis represents another proof of the importance of genetic background in the disease. Today novel sequencing technologies allow researcher to evaluate the possible role of rare genetic variants exploiting hypothesis-free studies. These data will provide important information to improve risk stratification and to further clarify NAFLD pathogenesis leading to the identification of new molecular targets for the treatment of this disease.

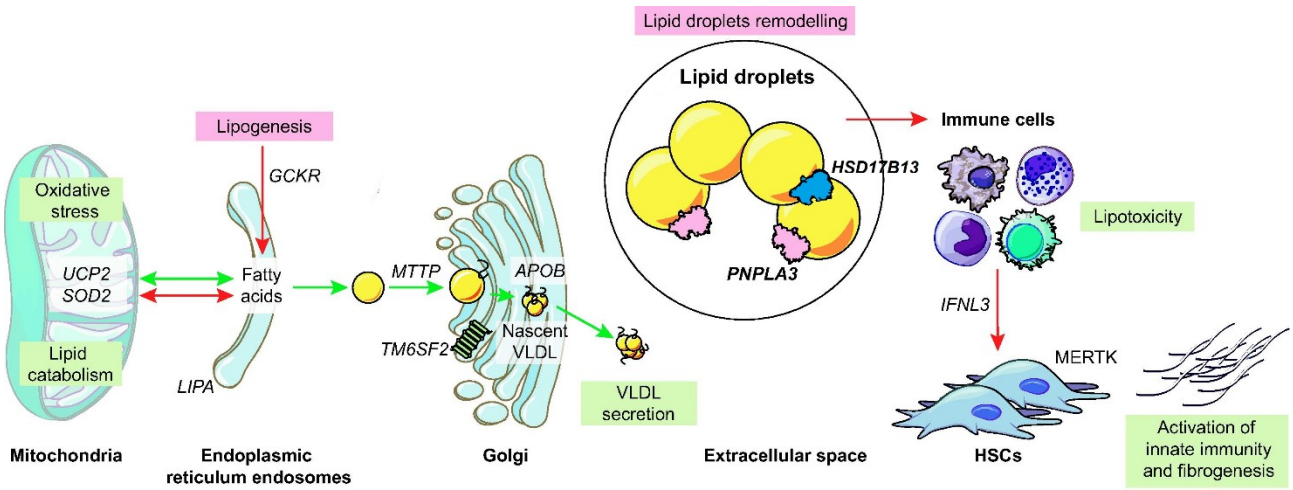

Figure 3 - Major pathways involved in the susceptibility towards NAFLD and gene loci robustly associated with development and progression of the disease. Modified from (91).

NAFLD as a risk factor for HCC

Epidemiology and risk factors

Liver cancer is the fifth most frequent cancer and the second cause of cancer-associated mortality (92). Hepatocellular carcinoma (HCC) accounts for 70-85% of liver cancers representing the most frequent histological type (92). In the past, due to NAFLD misdiagnosis and lack of targeted studies, the importance of NASH and NAFLD as risk factors for HCC was not specifically addressed (93). Furthermore, the lower prevalence of metabolic risk factors as compared to chronic viral hepatitis reduced the impact of NAFLD on the burden of liver disease. On the contrary, NAFLD is now emerging as one of the leading causes of HCC particularly in the Western countries. A first study highlighting NASH as an HCC risk factor was conducted by Powell et al. in 1990 (94). A later study confirmed these observation reporting a 2,6% HCC incidence among NAFLD-cirrhosis patients (95). Despite this incidence was lower to that observed in chronic C hepatitis patients with cirrhosis (4%), these data raised concerning due to the rising prevalence of NAFLD in the population. A large cohort study by Sanyal and colleagues (96) in the US population, identified NAFLD as the most frequent risk factor for HCC being present in the 59% of HCC cases. In keeping, HCC due to NAFLD was pointed out as an emerging cause for liver transplantation in the USA (97). The association between NAFLD and HCC was recently confirmed in a large cohort study from the Veterans Health Administration (98). Indeed, NAFLD patients had a higher incidence of HCC than those without (0.21/1000 vs

0.02/1000 person-years, HR = 7.62, 95% CI 5.76–10.1). Furthermore, NAFLD cirrhosis was confirmed to be a key risk factor for HCC development as HCC incidence among NAFLD-cirrhosis patients was higher (10.6 per 1000 person-years). However, 20% of NAFLD-HCC patients in this cohort did not have evidence of cirrhosis, thus confirming previous reports that NAFLD represents a risk factor for HCC also independently of liver fibrosis (99) Indeed, NAFLD along with obesity, insulin resistance and T2M are considered established risk factors for HCC (100-102). However, due to the well-known relationship between these risk factors, the leading cause NAFLD to HCC transition remains an open question (103).

Molecular mechanisms involved in pathogenesis of HCC in NAFLD

Both chronic inflammation, metabolic alterations, and liver regeneration in response to damage occurring in NASH can favor hepatic tumorigenesis (104, 105). Despite the precise molecular mechanisms driving carcinogenesis related to hepatic fat overload are still largely unknown, a contribution of different molecular pathways has been pointed out. These include cell proliferation, DNA damage response (DDR), oxidative stress, immune responses, autophagy and the contribution of the gut microbiome (106).

Cell Proliferation

Activation of oncogenes involved in cell proliferation was demonstrated to be the leading event of tumorigenesis in mouse models of diethylnitrosamine (DEN) induced HCC (107). Furthermore, the Ras oncogene – MAP kinases (MAPK) axis is known to be a master regulator of cell proliferative responses and was dysregulated by multiple mechanisms in HCC related with different etiologies (108). Studies in mouse models of HCC induced by both chemicals and high fat diet (HFD),

demonstrated a link between carcinogenesis and the dysregulation of micro RNA (miRNA) of key importance in the regulation of different proliferative pathways including the Wnt/β-catenin, MAPK, mTOR, and EGF signaling cascades (109). Further studies exploiting pure NASH-HCC models will be of key importance to clarify the exact contribution of proliferative pathways in the context of NAFLD related carcinogenesis.

Oxidative stress

Response to DNA damage due to oxidative stress plays a key role in HCC development in NAFLD. In NAFLD patients, high levels of DNA damage due to oxidative stress and aberrant epigenetic modifications of key tumor suppressor genes have been reported (110). Importantly, oxidative DNA damage is even more sustained in NASH patients developing HCC compared with individuals not developing tumors (111). As previously discussed, ROS are produced in hepatocytes due to fatty acids metabolism through the mitochondrial fatty acid β-oxidation pathway. In NASH, due to liver fat overload, disruption of fatty acids homeostasis with hyperactivation of β-oxidation and lipid biosynthesis along with impairment of cell anti-oxidant system always occurs, thus leading to excess ROS production and cell stress (112, 113). Establishment of a chronic inflammatory response also contributes to oxidative stress in NASH. Indeed, activated effectors of both innate and adaptive immune response represent major sources of reactive oxygen and nitrogen species. Furthermore, oxidative stress associated with immune response is a key factor in the pathogenesis of different tumors (114, 115). ROS are mainly generated by phagocytes as a defense mechanism against pathogens. In NASH, ROS produced by neutrophils and macrophages recruited to the liver induce DNA damage playing a key role in HCC pathogenesis (116, 117).

DNA damage response in NAFLD

DNA damage response (DDR) is a network of cellular pathways that sense, signal and repair DNA lesions. Surveillance proteins that monitor DNA integrity can activate cell cycle checkpoints, DNA repair pathways, and apoptosis in response to DNA damage, to prevent the generation of potentially deleterious mutations. Impairment of DDR seems to be strongly associated with the progression of NASH and the development of HCC however its contribution has not been fully understood.

ATM kinase is a master regulator of DDR being activated in response to double strand DNA breaks or ROS leading to DNA repair or apoptosis (118). In rodent models of NAFLD the activation of the ATM kinase was associated to liver damage progression in NASH by promoting liver apoptosis and fibrosis (119). However, patients with defects in this kinase affected by ataxia-telangiectasia syndrome show genomic instability, due to impaired in DNA repair (120). Overall, the effect of the ATM pathway activation may represent a risk factor in NASH, leading to increased liver damage in response to ROS, but also may represent a protection mechanism against HCC development favoring DNA repair or apoptosis in response to DNA damage and so counteracting genomic instability (121).

The DNA dependent protein kinase (DNA-PK) is nuclear kinase encoded by the PRKDC gene regulating response to DNA double strand breaks mediating repair by the non homologous end joining (NHEJ) pathway. NHEJ pathway mediates DNA repair without the need for a repair template as the homology directed repair (HDR) pathway. However, the NHEJ pathway always introduces random mutation at the repaired breakpoint potentially leading to genomic instability (122). Genes involved in the DNA-PK dependent NHEJ pathway are overexpressed in HCC suggesting an important

role for this pathway in tumorigenesis (123) (124). Indeed, copy-number-variations (CNVs) in the PRKDC locus resulting in increased DNA-PK expression occurs in >50% of human HCCs (123). Furthermore, DNA-PK complex overexpression in tumor is associated with reduced survival in HCC patients (123). Inhibition of DNA-PK activity is in study as a novel therapeutic approach for HCC (125).

Autophagy and HCC

Autophagy is a catabolic process mediating lysosome dependent degradation of macromolecules and entire organelles in response to metabolic stress to maintain cell energetic homeostasis (126). Autophagy related (ATG) proteins are key players in this process that, of course, occurs in a selective manner by tagging autophagy targets with a ubiquitination-like process (127). NAFLD is associated with autophagy impairment due to fatty acids overload resulting in increased oxidative stress (128). In response to oxidative stress cells activate the Sequestosome 1 (SQSTM1) - Kelch-like ECH-associated protein 1 (KEAP1) - Nuclear factor erythroid 2-related factor 2 (NRF2) axis (129). NRF2 acts as a transcription factor inducing the expression of key protein of the antioxidant system as gluthatione-S-transferase and thioredoxin reductase 1 to counter high levels of ROS (130). When the antioxidant system is not sufficient to balance the high ROS levels anymore cells undergo DNA damage and eventually death (131, 132). However, the constitutive activation of the SQSTM1 axis, due to autophagy impairment, seems to be a major contributor to the HCC pathogenesis. Indeed, activating mutations in the NRF2 and KEAP1 genes are frequently found in human HCCs (133, 134). Also, studies on rodents HCC models confirmed this association (135). NRF2 activation is also associated to increased cell proliferation and overexpression of the biliary/hepatic progenitor

marker cytokeratin 19 (CK19) (135). CK19 expression in HCC is associated with invasion, metastasis, chemoresistance, early recurrence and reduced overall survival (106, 136). Furthermore, SQSTM1 can also activate MYC oncogene thus further inducing cell proliferation (106).

Gut microbiome and bile acids signalling.

As previously discussed, a growing number of studies underlined importance of the gut microbiota as a NAFLD natural history modifier. Importantly, composition of the gut microbiota regulates the composition of bile acids (137, 138) through induction of both hepatic and gastrointestinal Farnesoid X Receptor (FXR) signaling (139). FXR is a transcription factor regulating bile acids metabolism mainly inducing expression of fibroblast growth factor 19 (FGF19) in the ileum and small heterodimer partner (SHP) in the liver. Both these molecules suppress cholesterol 7α-hydroxylase (CYP7A1), a key enzyme of the hepatic bile acid synthesis (140). Furthermore, FXR is also involved in glucose metabolism regulation, insulin signaling, and the immune response (106). Dysregulation of bile acids metabolism has been associated with HCC both in patients and mouse models (141, 142). Indeed, higher hepatic levels of certain species, such as deoxycholic acid (DCA) and lithocholic acid (LCA), is associated with DNA damage, cell death and activation of the inflammatory response, thus promoting hepatic carcinogenesis (141, 143). Moreover, deletion of FXR in mice is associated with spontaneous tumor development (144), intestinal FXR activation was sufficient to restore homeostasis in these mice (145). Role of gastrointestinal FXR transition remains a field of active research potentially providing key information about in NASH and NASH to HCC transition.

The immune response in NAFLD

Today chronic inflammation relationship with carcinogenesis is well established and representing a field of in intensive research in oncology (106). In the context of NAFLD-HCC this correlation hase been extensively studied (33, 146). Studies in rodent HCC models have demonstrated the role of local intrahepatic chronic inflammatory responses in hepatocarcinogenesis in the context of NASH (106). In the context of NAFLD-HCC this correlation has been extensively studied (33, 146). However, the overall contribution of the different inflammatory pathways, soluble mediators of inflammation and immune cell populations is still to be clarified.

Inflammatory pathways

Tumor necrosis factor alpha (TNFα) is one of the cytokines most studied in cancer and activates both the NF-κB and Janus Kinases (JNK) signaling pathways (147, 148). In rodent models of NAFLD carcinogenesis (HFD DEN mice) knockdown of TNF receptor type I (TNFRI) resulted in protection from tumor development (33, 149, 150). Nf-KB role in carcinogenesis remains elusive. Inflammation mediated by NF-κB is known to promote tumorigenesis (149-151). Indeed, in different models of genetically induced HCC, NF-κB inhibition was reported to suppress carcinogenesis. Conversely, an opposite effect was observed in chemically induced HCC models (DEN mice) (152). Interleukin 6 (IL6)-Signal Transducer and Activator of Transcription 3 (STAT3) axis is known to play a pivotal role in liver repair and hepatocyte replication, promoting hepatocarcinogenesis (147). Indeed, increased IL-6 levels and overactivated STAT3 have been observed in HCC patients (153) and activation of the IL6-STAT3 axis promoted DEN induced carcinogenesis DEN in mice (154, 155). The IL6-STAT3 axis may be of important in NAFLD HCC pathogenesis especially in patients carrying the PNPLA3 I148M

mutation. Indeed, HCC cells with carrying this mutation showed increased proliferation via the IL6-STAT3 pathway upon exposure of low dose FFA *in vitro* and in an HFD-fed xenograft HCC model *in vivo* (156). Innate immune receptors as Toll-Like Receptor 4 (TLR4) role in carcinogenesis is to date well established (154, 157). Indeed, knockout of TLR4 reduced HCC risk in PTEN$^{\Delta hep}$ mice, developing NASH-like HCC. Although, this reduction was dependent on gut microbiota depletion by orally administering nonabsorbable antibiotics (158). These findings suggest that TLR4 and gut-derived LPS make an essential contribution to HCC development. Another report demonstrated that hepatic stellate cell (HSC) derived tenascin C induces TLR4 signaling cascade activation in hepatocyte and Kupffer cells promoting HCC development in diet induced obesity mice models (159).

Immune cell populations

The complex contribution of the different cell population in HCC pathogenesis is summarized in Figure 4. Both Kupffer cells (KC), resident liver macrophages, and liver infiltrating macrophages have been shown to promote hepato-carcinogenesis in mice (160). Nevertheless, macrophages may have an even more important role in HCC progression (160). Today the role of tumor associated macrophages (TAM) modulating inflammatory microenvironment promoting tumor progression is extensively studied in different neoplasms (161). However, in the context of HCC the overall contribution of these cells remains to be determined (106). Amon innate immune cells, natural killers (NK) play a pivotal role in protection from tumorigenesis. Indeed, myeloid derived suppressor cell (MDSC) dependent impairment of NK cells function in patients with HCC have been reported to impaired clearance of tumor cells (162). Also, cells involved in adaptive immunity plays a pivotal role in HCC onset and progression in NAFLD. Activation of CD8+ T lymphocytes and natural killer T (NKT)

cells has been shown to drive the NASH to HCC transition in rodents(146). Conversely, CD4+ T lymphocytes seems to have a protective role in both onset and progression of HCC being key effectors of the senescence surveillance of pre- malignant hepatocytes (163). On the other side, tumor- infiltrating CD4+ regulatory T (Treg) cells have been associated with poor prognosis in patients undergoing HCC surgical resection (164).

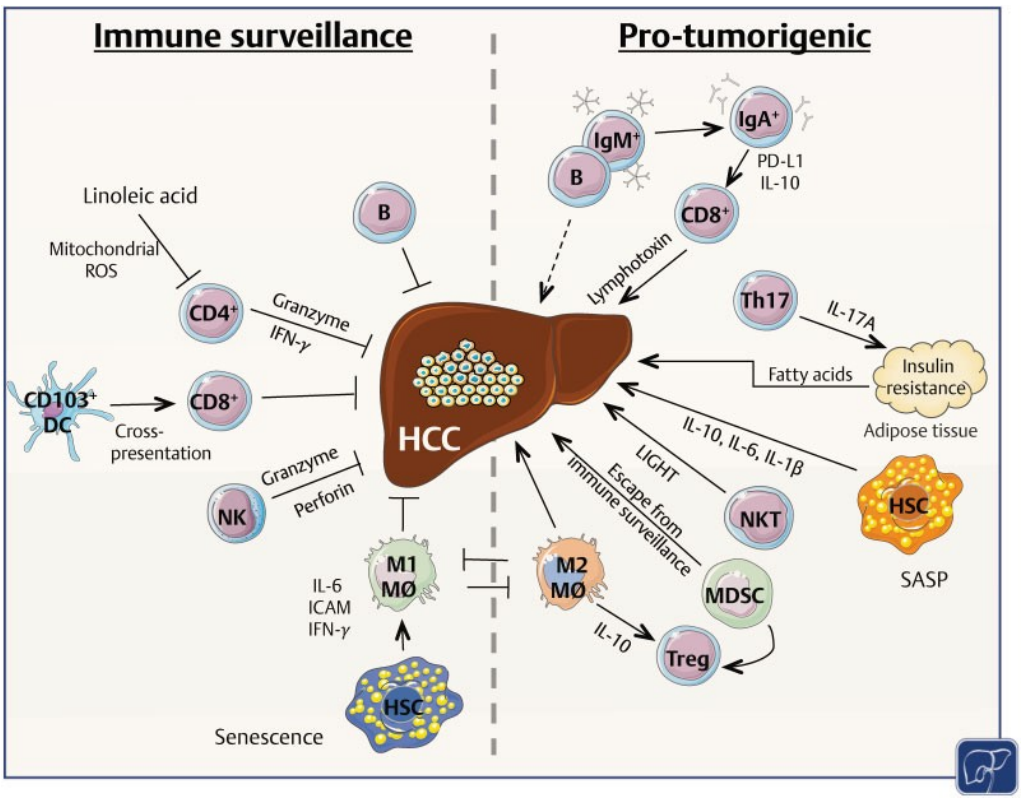

Figure 4 - Role of the immune cell network in hepatocellular carcinoma (HCC) development. Immune surveillance by natural killer (NK) cells, CD4þ, CD8þ T cells, and B cells suppresses HCC development. However, the expansion of CD8 T cells, immunoglobulinA (IgA)-producing plasmacells, Th17-cells, NKT cells, regulatory T cells, myeloid-derived suppressor cells (MDSCs), and M2 macrophages promotes HCC development. Hepatic Stellate Cells (HSCs) senescence has been proposed to play both pro- and antitumorigenic roles by inducing the senescence-associated secretory phenotype (SASP) and by promoting M1 macrophages polarization (165).

Genetic determinants of NAFLD HCC

As previously discussed, familial, epidemiologic and twin studies suggest a major role of heritable traits in susceptibility of NAFLD (27). At the population level, occurrence of HCC in a NAFLD context can be considered a rare condition. Among inherited variants modifying NAFLD natural history, the PNPLA3 I148M SNP represents the one with the most established effect on hepatic carcinogenesis. Also, contribution of rare variability seems to play a role in determining HCC risk (44). Study of the rare variability associated with HCC in large cohorts will provide important knowledge to improve the clinical management of this disease.

Common variants: Patatin-like phospholipase domain-containing 3

As previously discussed, the rs738409 C>G SNP encoding for the I148M amino-acidic change in the *PNPLA3* protein sequence represents the most relevant heritable modifier of the NAFLD natural history (44, 166). Importantly, the PNPLA3 variant has also been associated with increased HCC risk independently of its effect on liver fibrosis (167). This association was identified for the first time in a retrospective cohort of patients affected by chronic hepatitis C virus (HCV) infection (168) and confirmed by other studies in both HCV and alcoholic liver disease patients (27). Importantly, this SNP was associated with HCC both in the context of obesity and NAFLD (47, 169). The *PNPLA3* I148M variant was also reported to be a modifier of the natural history of HCC influencing both histopathological features of the tumors and the response to treatment as evidenced in context of NAFLD or ALD in Italian patients and further confirmed by studies conducted in US (170, 171). Overall, evidence highlight the role for rs738409 as an independent modifier of also HCC onset and progression in different pathological backgrounds.

Rare variants

Hypobetalipoproteinemia (HBL) represents a rare disorder of lipoprotein metabolism characterized by reduced plasma levels of total cholesterol, low-density lipoprotein-cholesterol (LDL-C) and apolipoprotein B (APOB). Familial HBL is a monogenic disease due to presence of loss of function variants in the *APOB* locus. In these patients, impaired lipoprotein export results in liver fat accumulation and truncating mutations in *APOB* were anecdotally reported in cases of HCC without cirrhosis as well as cryptogenic cirrhosis (172, 173). Associations between rare loss of function variants in APOB and HCC were also confirmed in a familial study by Cefalù et al (174) and in a recent next generation sequencing based study by our group (68). This evidence underpins the importance of rare *APOB* variants in influencing liver fat content and HCC risk.

Rare mutations in the human *telomerase reverse transcriptase* (*TERT*) gene have been also associated with NAFLD HCC risk, though via a different mechanism. This gene encodes for the catalytic subunit of telomerase involved in maintenance of telomeres length (175). Despite increased telomerase activity, mainly due to *TERT* overexpression, represents a hallmark of carcinogenesis (176-178), defects in telomerase activity lead to premature stem cell senescence, tissue fibrosis, chromosomal instability, and increased cancer risk (178). Indeed, *TERT* mutations are associated with a spectrum of familial hepatic liver diseases characterized by steatosis and iron overload due to hepatocellular senescence (178). Mutations in the *TERT* gene are relatively more frequent (3-8%) in cirrhosis patients (179). In rodents, telomere shortening due to *Tert* KO was associated also with impaired liver response to acute and chronic damage resulting in steatosis and fibrosis (180). Different studies form our group associated different loss of function mutations in

TERT (181, 182) and other proteins involved in telomere metabolism (Regulator Of Telomere Elongation Helicase 1, *RTEL1*) with HCC development in NAFLD patients (68).

Interferon regulatory factor 3

Interferon regulatory factor family

Proteins of the interferon regulatory factor (IRF) family are key mediators of the innate immune response especially in the context of viral and bacterial infections (183, 184). These transcription factors are effectors of signaling cascades activated by different pattern recognition receptors (PRR) as transmembrane protein of the toll-like receptor family (TLR) and a broad range of cytosolic sensors as the stimulator of interferon genes (STING). Once activated they controls target gene expression by binding specific consensus on genomic DNA trough their amino-terminal binding domain (185).

IRF3

Interferon regulatory factor 3 (IRF3) represent a key regulator of innate immune response being the final effector of different signaling cascades and have been extensively studied in the context of viral infections (184). This ubiquitously expressed transcription factor have both a nuclear export signal and a nuclear localization signal (NES and NLS, respectively) and keeps shuttling between the nucleus and the cytoplasm (186). As IRF3 NES effect is dominant, in the steady state it localizes preferentially to the cytoplasm. Furthermore, it possesses an amino (N)-terminal DNA-binding domain (DBD) composed by a series of conserved tryptophan-rich repeats forming an helix-turn-helix domain allowing interaction with specific consensus sequences on genomic DNA termed Interferon-sensitive response element (ISRE) (187). IRF3 also displays a C-terminal IRF association domain (IAD) mediating phosphorylation-dependent oligomerization and interaction with transcriptional coactivators. Also, functional analysis of IRF-3 revealed an autoinhibitory mechanism, mediated by the IAD flanking sequences (188). It is activated in response to different

pathogen or danger associated molecular patterns (PAMPs or DAMPs respectively) by signaling cascades of different sensors converging on the IKK-related kinases, TANK-binding kinase (TBK1) and IκB kinase ε (IKK ε) (189, 190). Phosphorylation of serine residues in the Carboxy-terminal regulatory region of IRF3 results in IRF3 homo-dimerization, nuclear translocation, association with transcriptional coactivators, including CREB-binding protein (CBP) and p300, and association of complex with genomic DNA (191). Importantly, IRF3 also possesses a BH3 domain allowing phosphorylated IRF3 to directly interact with Bax, a pro-apoptotic protein of the Bcl-2 family. IRF3-Bax complexes translocate to the mitochondria activating apoptosis through the intrinsic apoptotic pathway (192).

IRF3 activation pathways

Different pathways, including RIG-I-like and Toll-like receptors (RLRs and TLRs), have IRF3 as a final effector. In particular, IRF3 is one of the most important effectors of innate response against cytosolic nucleic acids. Presence of single/double-stranded RNA (ss/dsRNA) or double-stranded DNA (dsDNA) in the cytosol results in IRF3 activation through specific pattern recognition systems (184, 193). Exogenous dsRNA molecules are recognized by cytoplasmic DExD/H box helicases as the retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation-associated gene 5 (MDA-5) (194, 195). RIG-I is involved in the recognition of antisense (−) ssRNA viruses or sense (+) ssRNA/dsRNA viruses. Conversely, MDA5 recognizes RNA structures of higher containing long dsRNA and ssRNA that are typically produced during replication of picornaviruses (196). Both RIG-I and MDA-5 activate a specific molecular adaptor (IFN β promoter stimulator 1; IPS-1, also known as MAVS) inducing recruitment IRF3 and TBK1 resulting in IRF3 phosphorylation and activation (197-

199). Presence of dsRNA in the endosomal compartment is recognized by TLR3, a PRR highly expressed in leukocytes, epithelial cells, and central nervous system resident cells (200). TLR3 activates IRF3 through a specific molecular adaptor: the TIR domain adaptor-inducing IFNβ (TRIF). In turn, TRIF activates IRF3 pathway in a TNFR-associated factor (TRAF)3-dependent fashion (201). Notably, in addition to dsRNA generated during viral replication, TLR3 also recognizes endogenous self mRNA that is released during necrosis (202). Importantly, endotoxins sensing by TLR4, also triggers TRIF-dependent signaling within the so called "MyD88 independent TLR4 signaling pathway". This requires internalization of the receptor in the endosomal compartment in a CD14 and phosphatidylinositol 3 kinase (PI(3)K)-dependent fashion (203, 204).Cytosolic DNA activates an IRF3 dependent response (205). Accumulation of cytosolic dsDNA happens in the context of infections with intracellular pathogens but also, importantly, in case of insufficient control of autologous DNA products, which may happen during oxidative damage and carcinogenesis (206). Different highly redundant sensors are involved in recognition of cytosolic DNA (207-209). However, virtually all of them requires the adaptor protein stimulator of interferon genes (STING) that appears to be necessary for IRF3 induction in response to cytosolic dsDNA (210). In response to cytosolic dsDNA, formation of an active STING dimer provides a scaffold inducing STING-TBK1-IRF3 complexes directing TBK1 dependent phosphorylation of IRF3 (211). Furthermore, STING is also a direct sensor of cyclic dinucleotides (CDN), a second messenger produced by bacteria and archaea (212). Importantly, cytosolic DNA also induces synthesis of cyclic di-GMP-AMP (cGAMP) by the cyclase cGAMP synthetase (cGAS). These second messengers are structurally related with CDNs and are able in induce STING dependent IRF3 activation (213). Furthermore, they can be transferred to neighboring cells through gap junctions, thereby spreading the antiviral state (214). Thus, STING

could function both as an adapter for DNA sensing and a receptor for cyclic dinucleotides (206). As it will be discussed in the "IRF3 and liver disease" section, the STING-IRF3 pathway is object of active research due to its implications in NAFLD, ALD and related complications (215-217). Major IRF3 activation pathways are summarized in Figure 5 .

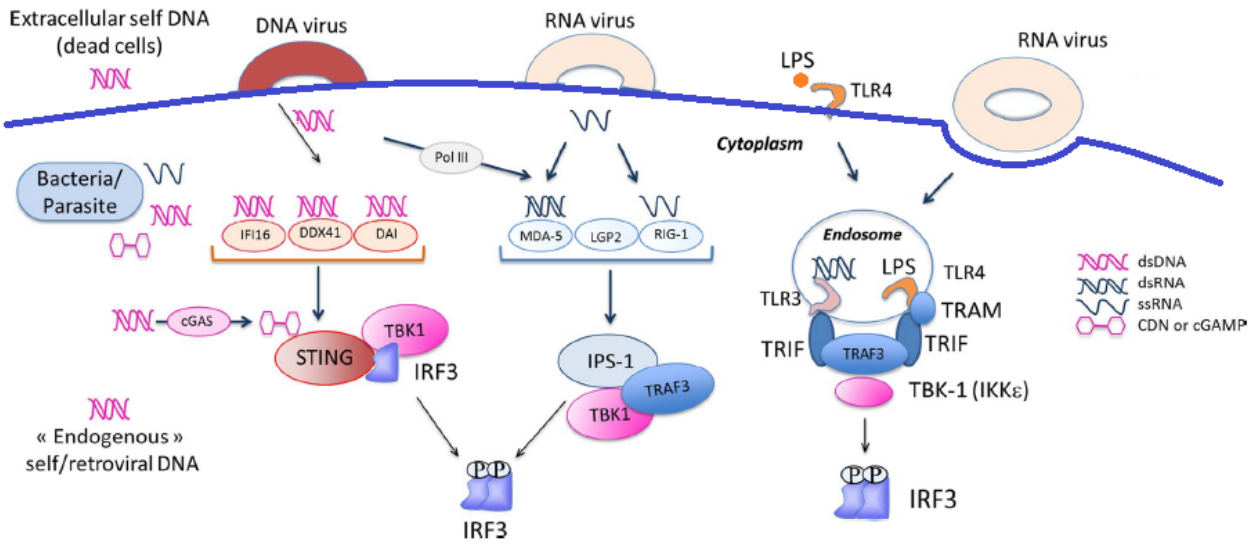

Figure 5 – Activation of TLR3 (by dsRNA) in the endosomal compartment leads to TBK1 activation in a TRIF-dependent fashion. Similarly, TLR4 activation results in internalization in the endosmal compartment and TRIF dependent IRF3 activation. The presence of single/double-stranded RNA (ss/dsRNA) or double-stranded DNA (dsDNA) in the cytosol triggers TBK1 activation through specific cytosolic pattern-recognition systems. Cytoplasmic DNA from different sources induces the production of cGAMP by the cyclase enzyme cGAS and directly activate IRF3 through the STING adaptor molecule. Viral ss/dsRNA in cytosol is sensed by RLR helicases, such as RIGI or MDA-5, that trigger IRF3 activation through the IPS-1 (MAVS) adaptor molecule. (184)

IRF3 regulatory isoforms

IRF3 pathway is highly regulated also through different *IRF3* splicing isoforms inhibiting main protein activity. The first was characterized in 2001 (218) and named IRF3a (or IRF3-nirs3). This isoform act as a decoy having a large deletion in the DBD and was found to counteract IRF3 activation both in response to viral infections and in the context of HCC (219). Another splicing isoform of *IRF3* was characterized in 2010 and termed IRF3-CL (220). The transcript encoding for IRF3-CL share a common mRNA precursor with IRF3 and results from alternative splicing. This isoform uses a different splicing acceptor on exon VII that is 16 nucleotides longer compared with that of IRF3. Thus, from exon VII, IRF3-CL use a different ORF and displays a different C-terminal region compared with that of the main isoform. (Figure 6). Indeed, this splice variant does not display the consensus sequence for TBK1/IKKε and seems to be ubiquitously expressed. Overexpression experiments the revealed ability of IRF3-CL to antagonize IRF3 activation upon Sendai virus infection, by sequestering IRF3 in the cytoplasm, thereby highlighting a possibly regulatory role for this splice variant (220).

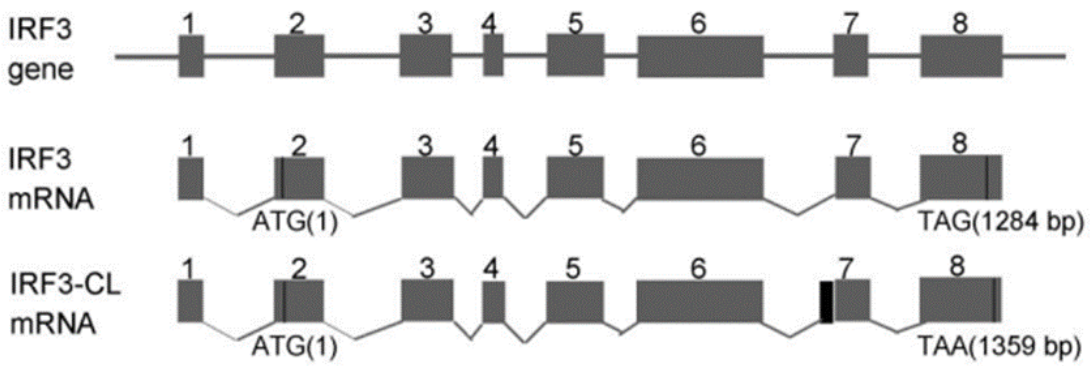

Figure 6 – Interferon regulatory factor 3 main isoform and CL isoform splicing scheme (220)

IRF3 in liver disease

Due IRF3 central activity in immune response to exogenous nucleic acids, its importance in the context of viral hepatitis is to date well established as reviewed in (221) and (222). However, growing evidence supports a key role for IRF3 also in the context of both ALD and NAFLD as well as the systemic inflammation characterizing the metabolic syndrome. Concerning ALD, in 2008 Zhao and collaborators reported a crucial role of the TRIF-IRF3 axis in inducing a TNFα response upon chronic ethanol administration in mouse models. Indeed, they demonstrated that murine macrophages isolated from Trif-/- mice do not overexpress Tnfα upon Et-OH stimulation *in vitro* resulting in protection from EtOH-induced steatosis and Tnfα dysregulation *in vivo (223)*. Furthermore, Petrasek et al in 2013, highlighted a role for the STING-IRF3 pathways in mediating liver damage induced by oxidative stress upon acute Et-OH administration. Their studies on rodent ALD models, identified the Irf3 ability in inducing mitochondrial Caspase 3 and 8 dependent apoptosis as necessary to mediate liver injury upon acute Et-OH administration (216). In a recent work, Sanz-Garcia and collaborators underlined the importance of both hepatocyte and leukocytes IRF3 non transcriptional activity in modifying liver disease progression by modulating liver immune cells environment and pointed out a pleiotropic and cell-specific contribution of IRF3 (224). Growing evidence also links IRF3 with NAFLD pathogenesis. Our results suggest that the STING-IRF3 pathway promotes hepatocyte injury and dysfunction by inducing inflammation and apoptosis and by disturbing lipid metabolism. This pathway may be a novel therapeutic target for preventing NAFLD development and progression. A recent study by Qiao et al (217) reported Irf3 overexpression in NAFLD in rodents. Knockdown of either Sting or Irf3 resulted in a marked improvement of steatosis, hepatic FFA-induced inflammation, and hepatocellular apoptosis also due to enhanced glycogen

storage, glycolysis and lipid catabolism. Importantly, another recent work by Reilly et al. demonstrated improvement of steatosis and adipose inflammation in rodents upon treatment with amlexanox, a TBK1/IKKε inhibitor already approved for the treatment of asthma, recurrent aphthous ulcers and other inflammatory conditions. In mice fed high-fat diet, amlexanox treatment resulted in imporved insulin sensitivity, reduced adipose inflammation and abrogated liver steatosis and was proposed as a new therapeutic strategy against NAFLD (225). The first Amlexanox clinical trial resulted in improvement of insulin signaling and liver steatosis also in a subset of patients with T2D (226). A further trial (NCT01842282) is currently ongoing to further study the utility of this chemical for the treatment of metabolic syndrome complications.

IRF3 in cancer

IRF3 overall contribution to carcinogenesis is still elusive. Due to its proinflammatory role and its ability to induce ER stress and mitochondrial instability driven apoptosis, it has been considered a tumor suppressor. Consistently, overexpression of DNA binding domain defective IRF3 is reported to induce transformation in *in vitro* models (227). Indeed, fatty acids overload is associated with mitochondrial dysfunction, damage, and release of mitochondrial DNA in cell cytosol resulting in STING dependent IRF3 activation, triggering in anti-tumor immune response and apoptosis (228). Furthermore, genomic instability associated with tumorigenesis leads to defects of segregation during cell mitosis resulting in genomic DNA release in cytoplasm activating STINGIRF3 axis representing a link between genomic instability and anti-tumor immune surveillance activation (229, 230). Importantly, overexpression of TLR3 and of other components of the TLR signaling cascade in the tumor tissue was associated with improved prognosis and survival in a cohort of 85

patients with HCC on different pathological backgrounds (231). On the other hand, IRF3 was reported to cross-talk with different pathways involved in control of cell proliferation as the Hippo pathway and the Wnt-β catenin axis (232, 233). Consistently a direct anti-tumor activity for amlexanox was reported in different context including gastric cancer, breast cancer, and glioblastoma (232, 234-236). Thus, the overall contribution of IRF3 in carcinogenesis, especially in the context of HCC remains elusive and further studies are required to evaluate amlexanox potential in NAFLD and NAFLD-HCC treatment.

AIMS

All stages of NAFLD are deeply influenced by a strong heritable component. In the past decades both targeted studies and GWAS highlighted different common variants as modifier of both onset and progression of this multifactorial disease. However, NAFLD heritable component remains largely unexplained. Furthermore, a large number of rare variants is considered to have a large effect on the natural course of the disease (27) but, to date, there is only little information about the contribution of rare genetic variability. Discovery of novel genetic traits influencing NAFLD progression may provide important information both to improve clinical management in a perspective of personalized medicine and to identify novel therapeutic target for the treatment of NAFLD.

To this purpose, the aim of this study wad to discover novel genetic risk factors for advanced NAFLD by evaluating rare variants altering protein sequence enriched in a cohort of Italian patients with NAFLD HCC as compared to the general population. We next validated these association in independent cohorts of Italian patients with NAFLD HCC or advanced fibrosis.

As we identified a variant affecting the regulatory *IRF3* isoform IRF3-CL (rs141490768) resulting in a gain of function of the transcription factor IRF3, we then examined the specific regulation of IRF3 transcripts in NAFLD progression, studies IRF3 and IRF3-CL role in hepatocyte biology *in vitro*, also mimicking IRF3-CL loss of function effect in hepatocyte by developing specific IRF3-CL+/- models exploiting CRISPR-Cas9 technology. Finally, we evaluated the activity of amlexanox, a IKKε/TBK1-

IRF3 axis inhibitor already approved for some inflammatory conditions, to reduce proliferation in hepatoma cells.

METHODS

Whole exome sequencing study cohorts and design

The discovery NAFLD-HCC Discovery cohort included 72 Italian patients, who were enrolled between January 2010 and 2016. All were of Caucasian ancestry. The diagnosis of HCC was based on the EASL-EORTC clinical practice guidelines for management of hepatocellular carcinoma (237). Secondary causes of steatosis were excluded based on medical history, including at risk alcohol drinking (≥ 30/20 g/day in M/F) and the use of drugs known to precipitate steatosis. Viral and autoimmune hepatitis, hereditary hemochromatosis, Wilson's disease, overt alpha-1-antitrypsin deficiency and present or previous infection with HBV (HBsAg) and HCV were ruled out using standard clinical and laboratory evaluation as well as liver biopsy features. HCC validation included 105 Italian NAFLD HCC patients with the same inclusion criteria used for NAFLD-HCC Discovery cohort. Advanced fibrosis validation cohort was represented by a set of 211 patients with advanced fibrosis due to NAFLD (histological stage F3-F4 or clinically overt cirrhosis) recruited at the Italian institutions during the same period. Discovery control population was represented by a local ethnically matched control population of comparable sex distribution including 50 Italian healthy blood donors without clinical and biochemical evidences of liver disease, NAFLD, metabolic abnormalities and no alcohol abuse (182), and the Non-Finnish Europeans (NFE) included in the Exome Aggregation Consortium (ExAC) database (ExAC-NFE, N=33,370). Validation control population was represented by other 270 ethnically matched patients without clinical or biochemical evidence of advanced liver fibrosis or HCC and the NFE included in the genome aggregation consortium (238) (gnomAD NFE, N=64603). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, was

approved by the Ethical committee of the involved Institutions and was performed according to the recommendations of the hospitals involved. Informed consent was obtained from each patient or responsible guardian. Clinical features of patients stratified by liver disease severity are described in Table 2. Patients are part of the EPIDEMIC-NAFLD (Exome sequencing for the Identification of Inherited Variants Involved in HCC development in NAFLD) project, further studies about genetic predisposition to NAFLD HCC in these patients are reported in our previous publication (68).

	Normal liver/Early fibrosis (N=320)	**Advanced fibrosis (N=211)**	**HCC (N=177)**	**p value**
Age	46±13	62±10	68±11	<0.001
Sex, F	213 (77)	106 (56)	137 (80)	<0.001
BMI, Kg/m2	25±3	30±5	30±5	<0.001
Diabetes, Yes	6 (2)	108 (61)	96 (58)	<0.001
ALT, U/L	22 {17-30}	39{29-60}	38 {27-61}	<0.001
AST, U/L	22 {18-25}	34 {27-49}	41 {27-67}	<0.001
Cholesterol, mg/dl	194±34	177±43	163±37	<0.001
LDL, mg/dl	123±35	119±73	96±34	0.003
HDL, mg/dl	56±13	50±18	47±19	<0.001
Triglycerides, mg/dl*	85 {60-123}	122 {92-174}	107 {66-138}	<0.001
PNPLA3 I148M				
I/I	145 (53)	40 (23)	33 (22)	<0.001
I/M	105 (39)	80 (45)	64 (42)	
M/M	22 (8)	56 (32)	54 (36)	

Table 2 – Clinical features of patients in study. Data are presented as meansd, (): % values, {}: interquartile range BMI: body max index; NAFLD non-alcoholic fatty liver disease; LDL low-density lipoprotein; HDL high-density lipoprotein; PNPLA3: Patatin-like phospholipase domain-containing protein 3.

Study Design

Variants Discovery

Variants discovery workflow is summarized in Figure 7. Whole-exome sequencing was performed in NAFLD HCC Discovery cohort (N=72) and in local control population (N=50). To discover rare variants predisposing to NAFLD-HCC risk alleles, coding functional variants with a minor allele frequency (MAF) < 0.005 in ExAC NFE were selected (N= 51,226 variants). To exclude false positives due to ethnical differences between cases and control population, all variants with a MAF > 0.01 in our local ethnically matched control were excluded from the analyses. Variants enrichment in NAFLD-HCC group vs control population was evaluated by Fisher Exact test corrected for multiplicity by Bonferroni method, significance threshold was thus set at $9.8*10^{-7}$.

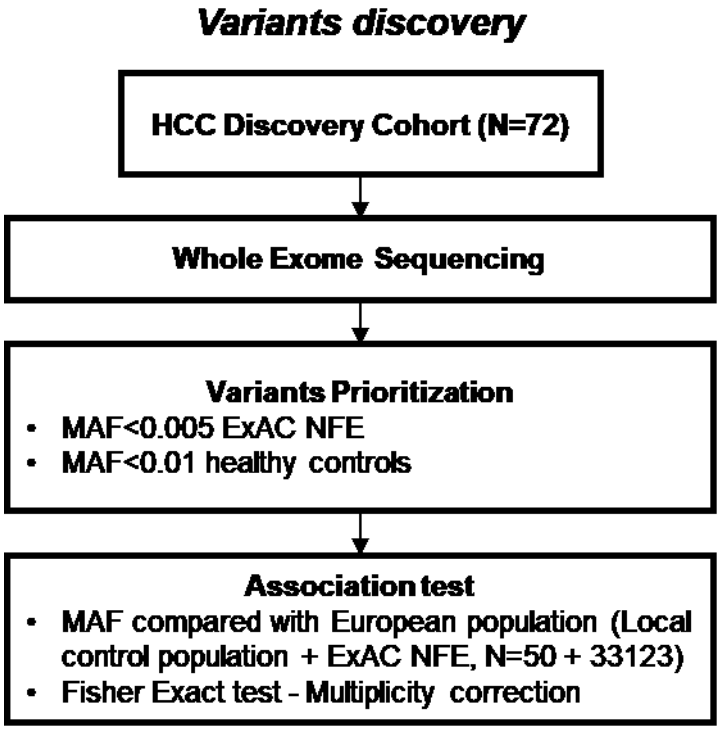

Figure 7 – Variants discovery workflow

IRF3 rs141490768 validation

Validation workflow is summarized in Figure 8. *IRF3* rs141490768 genotype was assessed by TaqMan SNP genotyping assay (Life Technologies, Carlsbad, CA) in the HCC validation cohort (N=105) and in the Advanced Fibrosis validation cohort (N=211) either by TaqMan assay (N=59) or WES (N=152). *IRF3* rs141490768 frequency was compared with control population including 270 ethnically matched individuals where rs141490768 genotype was assessed TaqMan assay by and NFE population of the gnomAD database (N=64,390). P<0.05 Frequency comparison was performed by Fisher Exact test, p<0.05 were considered statistically significant.

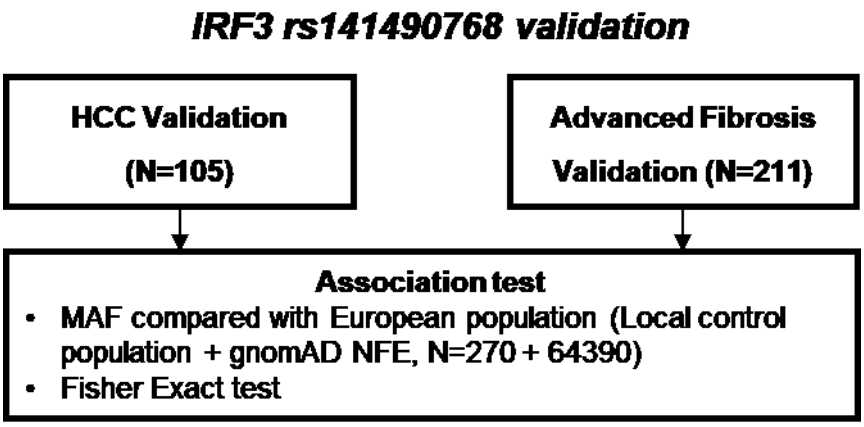

Figure 8 - IRF3 rs141490768 validation workflow

IRF3-CL Burden test

Burden test workflow is summarized in Figure 9. Enrichment in variants affecting IRF3-CL isoform but not *IRF3* main isoform was evaluated by cohort allelic sum test (CAST) (239). Briefly, we selected all variants in cases and controls affecting IRF3-CL but not principal *IRF3* isoform exploiting VEP annotation. Variants prioritization was performed to select all rare variants (ExAC NFE MAF <0.05) with a very high likelihood to impair protein activity based on *in silico* prediction (240) with a Combined Annotation Dependent Depletion (CADD) phred score > 20 thus being within the top 0.1% of most deleterious variants in genome as the CADD model. Enrichment in our discovery HCC cohort (N=72) was compared to that of our ethnically matched control population (N=50) and NFE of the gnomAD database. Association was validated in patients of the advanced fibrosis group with available WES data (N=112). $p<0.05$ were considered statistically significant.

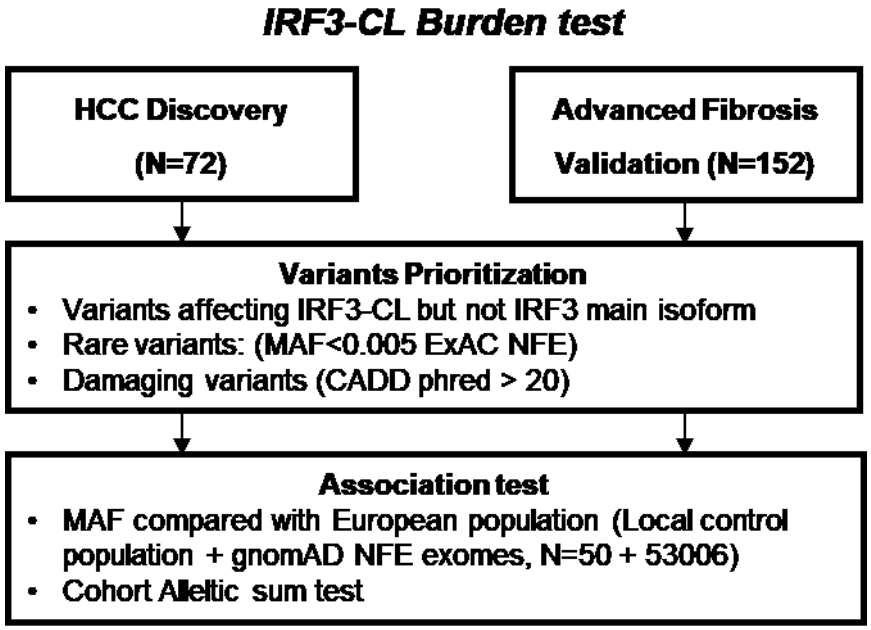

Figure 9 - Burden test workflow

Whole exome sequencing, variants identification, annotation and prioritization

Briefly, DNA was extracted from peripheral blood mononuclear cells. DNA amount was assessed using a Qubit 2.0 analyzer and Qubit dsDNA BR Assay Kit (Thermo-Fisher Scientific, Waltham, MA, USA) following Manufacturer's instructions. Samples purity was evaluated using a Nanodrop 1000 spectrophotometer (Thermo-Fisher, Waltham, MA, USA) and integrity was assessed by gel electrophoresis. The DNA library was enriched for exome sequencing by SureSelect Human All Exon v5 (Agilent, Cernusco sul Naviglio, Milan, Italy). Sequencing was subsequently performed on the HiSeq 4000 platform (Illumina). Raw reads quality control was performed using FastQC software (Brabaham bioinformatics, Cambridge, UK). Reads mapping on human GRCh37 genome was performed using MEM algorithm of Burrows Wheeler Aligner (BWA) version 0.7.10 (241). Reads with low quality alignments and duplicate reads were filtered out using Samtools (242) to generate high quality bam files. Mapping quality control was performed using Picard-tools (http://broadinstitute.github.io/picard) and Bedtools (243). Sequencing mean depth was of 73x, and no samples exhibit a mean depth lower than 50x. Sequencing resulted in a good target coverage: almost all samples exhibited more than 90% coverage of the target at 20x depth. Importantly, sequencing statistics in terms of input reads, high quality mapped reads, mean depth and coverage, did not show variations among the different cohorts. Variant calling was performed following GATK best practices (244). Briefly, indel local realignment, base quality recalibration and variants calling (Haplotypecaller algorithm) were performed using GATK version 3.3.0 (245). GVCF joint and variants filtering using variant quality score recalibration (VQSR) method were performed. Variants quality score log-odds (VQSLOD) above 99% tranche were considered true positives. To avoid the possibility of calling somatic variants due to the presence of circulating tumor DNA,

variants present in <20% of total reads were discarded. Indel left normalization was performed using BCFtools software (246). Variants annotation was performed using both variant effect predictor (VEP) (247) and ANNOVAR (248) tools. Variants filtering was performed using VCFtools (249) to exclude variants over VQSLOD threshold and variants which were called in less than 95% of samples. All intronic and synonymous variants according to VEP prediction were excluded from the analyses. ExAC NFE variant call format file was obtained by ExAC consortium (http://exac.broadinstitute.org). Variant called were prioritized and annotated using the same pipeline described for EPIDEMIC samples.

TaqMan SNP genotyping assay patients and methods

Genotyping has been performed in duplicate by TaqMan 5'-nuclease assays (Life Technologies, Carlsbad, CA) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) at the Metabolic Liver Disease lab center, at the University of Milan (Life Technologies, Carlsbad, CA).

Transcriptomic analysis patients

The study was conducted in 125 severely obese individuals (Transcriptomic cohort) who underwent to percutaneous liver biopsy performed during bariatric surgery, and for whom sufficient material for extraction of high-quality RNA was available. Informed consent was obtained from each patient and the study protocol was approved by the Ethical Committee of the Fondazione IRCCS Ca' Granda, Milan and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Individuals with increased alcohol intake (men: >30 g/day; women: >20 g/day), viral and autoimmune hepatitis or other causes of liver disease were excluded. Steatosis was graded based on the percentage of

affected hepatocytes as 0: 0-4%, 1: 5-32%, 2: 33-65%, and 3: 66-100%. Disease activity was assessed according to the NAFLD Activity Score (NAS) with systematic evaluation of hepatocellular ballooning and necroinflammation; fibrosis was also staged according to the recommendations of the NAFLD clinical research network (250). The scoring of liver biopsies was performed by an expert pathologist unaware of patients' status and genotype (56, 251). We arbitrarily defined "Severe NAFLD" as the presence of NASH, and/or NAS ≥4, and/or fibrosis stage F2 or higher. Patients clinical features are showed in Table 3. Evaluation pf the transcriptomic profile in these patients was part of another study (Appendix B, Baselli, Dongiovanni et al., Gut, under revision).

	Normal liver/isolated steatosis (N=94)	**Severe NAFLD (N=31)**	**p value**
Age	43.3±10.6	44.5±10.3	0.59
Sex, F	86 (91)	21 (68)	0.003
BMI, Kg/m^2	39.7±6.7	43.5±8.3	0.013
Diabetes, Yes	10 (11)	5 (17)	0.60
ALT, U/L	18 {14-24}	31 {24- 44}	<0.001
AST, U/L	17 {15-20}	23 {19-31}	<0.001
Cholesterol, mg/dl	212±44	197±51	0.19
LDL, mg/dl	136±33	120±47	0.098
HDL, mg/dl	55±13	52±17	0.32
Triglycerides, mg/dl*	120 {90-161}	114 {85-175}	0.61
PNPLA3 I148M			
I/I	48 (51)	12 (38)	0.47
I/M	40 (43)	16 (52)	
M/M	6 (6)	3 (10)	

Table 3 – Transcriptomic cohort clinical features

Transcriptomic analysis

Total RNA was isolated using miRNeasy mini-kit (Qiagen, Hulsterweg, Germany), according to the manufacturer's instructions. RNA quality was assessed through Agilent 2100 Bioanalyzer and samples with RNA integrity numbers (RIN) greater than or equal to 7 were used for library preparation. RNA sequencing was performed in paired-end mode with a read length of 150nt using the Illumina HiSeq 4000 (Novogene, Hong Kong, China). Raw reads were mapped against the Human Genome (252) using a custom pipeline based on the standard primary analysis procedure. The pipeline performed the primary analysis step including reads quality check (FastQC software, Babraham Bioinformatics, Cambridge, UK), low-quality reads trimming and the mapping on GRCh37 reference genome using STAR mapper (253). Samples characterized by low coverage (<10 million mapped reads) or insufficient mapping specificity (uniquely mapped < 60% mapped reads) were excluded from the analysis. To quantify gene level expression, reads count (ENSEMBL human transcript reference assembly version 75) was performed using RSEM package (254). Reads counts normalization and variance stabilization was performed exploiting DESeq2 package (255). To quantify isoform expression, a per transcript reads count and normalization in transcript per million was performed exploiting RSEM package for transcript variance stabilization a square root transformation was applied. Comparison among IRF3 transcripts of interest was performed by on e-way ANOVA followed by post-hoc Tukey test. Correlation between transcripts expression levels and liver disease stage was performed by ordinal logistic regression. For co-expression analysis variance stabilized expression of transcripts of interest were regressed to that of all genes quantified. Main technical confounding covariates as the library size and the batch factors were included in the model. Low expressed genes (mean count ≤ 5) were excluded. To identify co-expressed pathways,

pre-ranked gene set enrichment analysis (GSEA) was performed on nominally significant genes (256), correlation estimate was used as ranking metrics. The “hallmark” gene sets from the Molecular Signature Database (MSigDB) version 6.1 were employed (257).

Western blot analysis

Total protein lysates were extracted from cell cultures through RIPA lysis buffer (Hepes 10mM, EDTA 1mM, KCl 60 mM and Nonidet P-40 2%) supplemented with protease inhibitor cocktail and Phospho-STOP (Roche, Basal, Switzerland). Pierce 660nm Protein Assay (ThermoFisher scientific, Waltham U.S.A) is a ready-to-use method to quantify protein concentrations. Bolt system (ThermoFisher scientific, Waltham U.S.A) was employed for SDS-PAGE and western blotting. For protein detection anti P-IRF3 (Cell signaling #29047), IRF3 (Abcam #ab68481), β-actin (Abcam #ab6276) primary antibodies, anti-mouse and rabbit (Cell signalling #7074 and #7076) secondary antibodies, and ECL substrate (Bio-Rad, Hercules, U.S.A) were employed according to manufacturer’s instructions. Chemidoc XRS (Bio-Rad, Hercules, U.S.A) imager ImageJ software was employed for bands intensity quantification.

Immunohistochemistry

Immunohistochemistry was performed in a cohort of 18 samples stratified by liver disease severity. Liver biopsies were fixed in 10% PBS buffered formalin and then embedded in paraffin within 24 hrs of formalin fixation. We performed immunohistochemistry analysis to evaluate p(S396)-IRF3 and total IRF3 expression in liver biopsies from patients with normal livers, NAFLD, NASH or HCC. Briefly, de-paraffined sections were re-hydrated in alcohol and endogenous peroxidase activity was blocked

with methanol/10% hydrogen peroxide. After unmasking with the proper antigen retrieval and treating with blocking solutions, the slides were incubated overnight at 4°C with anti - p(S396)-IRF3 or anti - IRF3 antibodies (Cell Signaling, 29047 and Abcam, ab68481 - respectively) 1:50. After rinsing, slides were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody and developed with 3-3-diaminobenzidine (DAB). The slides were analysed with the Leica DMD108 microscope (Leica) connected to a digital camera (Leica). p(S396)-IRF3 and total IRF3 quantification was performed in 10 random non-overlapping fields per slide (200X magnification) by manually counting the number of positive cells with the software ImageJ. The results are expressed as percentage of positive cells per field. Nuclei were counterstained by Hematoxylin.

Cells culture and treatment

HepG2 cells (ATCC, Manassas, U.S.A) was cultured at 5% CO2 and humidified atmosphere in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin (all from ThermoFisher, Waltham, US). Before treatment, cells were starved for 24h using a quiescence medium containing 0,5% FBS. When specified, HepG2 cells or CRISPR Cas9 derived clones were exposed to a mixture of oleic (OA) and palmitic (PA) acids, respectively given in ratio 2:1 at 0.5mM or 10% FBS for 24 hours. Moreover, cells were treated with both Amlexanox (Sigma-Aldrich, Sant Luis, U.S.A.) at 50-100 μM and Sorafenib (Santa-Cruz biotech, Dallas, U.S.A.) 2.5 μM alone or in combination.

CRISPR-Cas9 genome editing

To evaluate the effect of IRF3-CL and noncoding transcripts knockout a somatic variant in the IRF3-CL and ENST00000599680 specific exon 7 splicing acceptor was introduced. Briefly, a Doxycycline (Therm-Fisher, Waltham, US) inducible Cas9 expressing HepG2 cell line was produced by lentiviral infection exploiting was produced exploiting Edit-R Inducible Lentiviral Cas9 Nuclease vectors (Dharmacon, Lafayette, U.S.A.) according to manufacturer's instructions. Specific guide RNA (3'-CCCCGGGTCCTCGGATCAGT-5') was designed using free online CRISPR design tool (http://crispr.mit.edu/) and cloned into a pGL3-U6-sgRNA-PGK-puromycin empty backbone (Addgene #51133) (258). After Cas9 induction cells were transfected with the expression vector, selected with 1ug/mL puromycin (Thermo-Fisher, Waltham, US) for two weeks. Single cell derived populations were obtained by limiting diluition method and presence of mutations in the specified locus was investigated by T7 nuclease assay (New England Biolabs, Ipswich, US) and further confirmed by Sanger sequencing using BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo-Fisher, Waltham, US), on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA). CRISPR/Cas9 workflow is summarized in Figure 10.

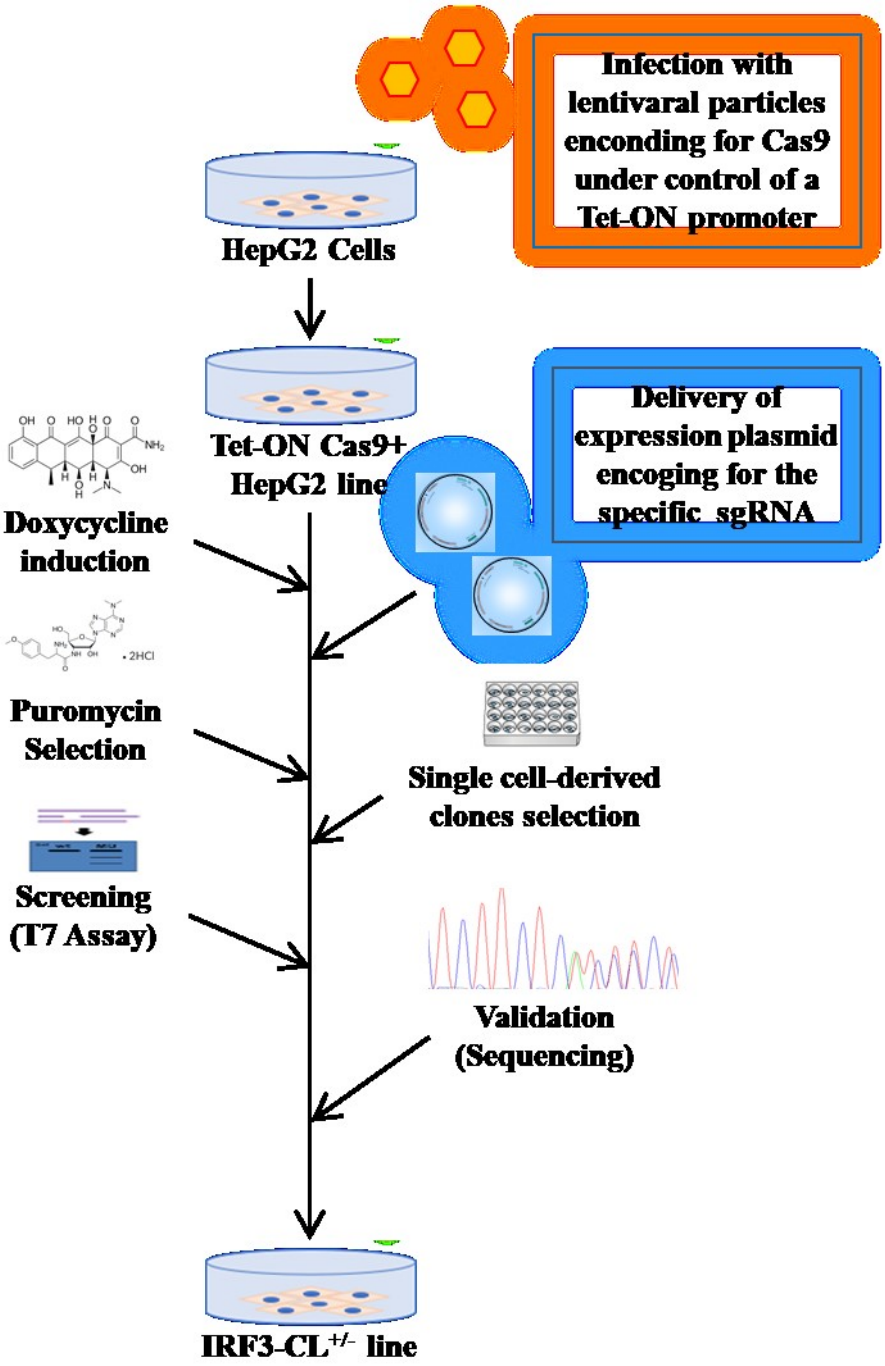

Figure 10 - CRISPR-Cas9 precise genome editing workflow

RNA extraction and RT-qPCR.

Cell lysis and RNA extraction was performed using Trizol reagent. RNA was reverse transcribed using SuperScript VILO cDNA Synthesis Kit. Gene expression levels were measured by Real time quantitative polymerase chain reaction (RT-qPCR) exploiting the SYBR green chemistry (Fast SYBR Green Master Mix) on ABI 7500 fast thermocycler (all from ThermoFisher, Waltham, US). All reactions were performed in triplicate. Gene expression levels were normalized using the $2^{-\Delta Ct}$ method. β-Actin was used as housekeeping gene exploiting 3'-GGCATCCTCACCCTGAAGTA-5' and 3'-GGGGTGTTGAAGGTCTCAAA-5' oligos (all oligos from Sigma-Aldrich, Sant Luis, U.S.A.) as forward and reverse primers, respectively. IRF3-CL was quantified using 3'-GCCCAGGAGCCTACAATGAA-5' as forward and 3'-GGGAAGAGTGGGAGTTCGAG-5' as reverse primers. For whole IRF3 mRNA levels assessment we used 3'-CGTGATGGTCAAGGTTGTGC-5' and 3'-GTTGAGGTGGTGGGGAACA-5' oligos as forward and reverse primers, respectively.

MTS Cell Proliferation Assay

To evaluate cell proliferation rate assay 2.500 cells/well on 96-well in triplicate and kept them in humidified atmosphere 5% CO_2 at 37°C for 24 hours. Growing medium and treatments were given fresh daily in all experiments. CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, U.S.A) was employed according to the manufacturer's instructions. Absorbance at 450 nm and 650 nm was measured using a Tecan microplates reader (Tecan Group, Switzerland).

Statistical analysis

For descriptive statistics, continuous variables were shown as mean and standard deviation. Highly skewed biological variables were reported as median and interquartile range and were log-transformed before analyses. Allele frequency were compared between groups using Fisher Exact test, multiplicity correction according to Bonferroni's method was used when appropriate. Adjusted p values <0.05 were considered statistically significant. Categorical variables were tested by chi-squared test and are presented as number and proportion. In all transcriptome-wide unsupervised analysis and gene set enrichment analysis, p values were corrected for multiplicity by Benjamini-Hochberg false discovery rate (FDR) method, and adjusted p-values <0.1 were considered statistically significant. Differences between groups were evaluated by two-tailed Student's t-test or analysis of variance followed by post-hoc Tukey's honest significance test, when appropriate. Statistical analyses were carried out using the R software version 3.5.0 (http://www.R-project.org/).

RESULTS

IRF3 rs141490768 is enriched in NAFLD HCC samples

In the current project we evaluated contribution of rare variants determining an alteration of protein sequence in determining the predisposition to develop NAFLD HCC with a hypothesis-free approach. As specified in the method section, association with HCC of all rare nonsynonymous or splice variants retrieved was performed by Fisher exact test followed by Bonferroni's multiplicity correction. As shown in Figure 11 and Table 4, analysis revealed a strong enrichment (OR=38.4; 95% CI= 12-97; $p=3.85*10^{-7}$; Table 4), for *Interferon regulatory factor 3* (*IRF3*) rs141490768 variant in HCC (N=72) group compared with European population (N=33173). Moreover, no patients of our local healthy donors group carrying the rs141490768 was identified. To validate the association, rs141490768 genotype was evaluated in 105 further NAFLD HCC patients (HCC validation cohort), 211 NAFLD F3-F4 fibrosis patients (Advanced fibrosis validation cohort) and further 270 Italian patients without evidence of advanced liver disease. As reported in Table 4, we couldn't identify other NAFLD HCC patients carrying the rs141490768. However, we detected a significant increased MAF in the Advanced fibrosis validation cohort (p=0.049; OR=5.8; 95% CI = 0.7-21; Table 4). Overall, variants were associated in NAFLD HCC/Advanced fibrosis patients compared with the European population (OR=11; 95% CI= 4-24; $p=6.16*10^{-6}$; Table 4) or our ethnically matched control population alone.

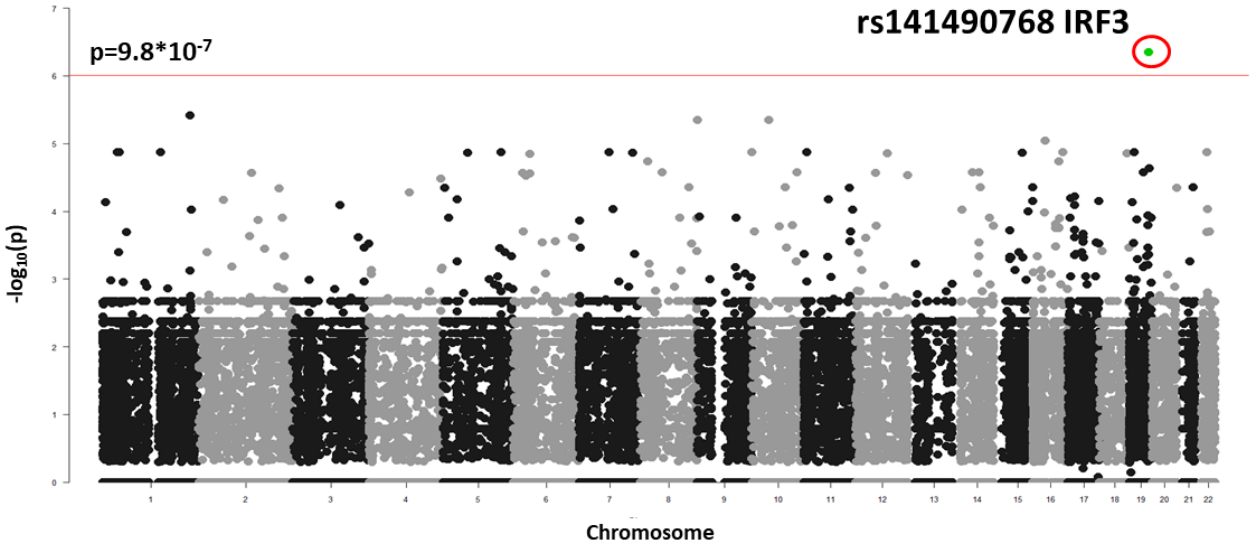

Figure 11 - Manhattan plot of variants tested in the HCC Discovery cohort.

	MAF Cases	MAF Controls	N Cases	N Controls	P	OR	95% CI
Discovery Cohort	0.035	0.0009	72	33223	**$3.85*10^{-7}$**	38.4	12-97
HCC Validation Cohort	0	0.0008	105	64660	1	0	0-22
Advanced Fibrosis Validation Cohort	0.005	0.0008	211	64660	**0.049**	5.8	0.7-21
Overall	0.009	0.0008	388	64710	**$6.16*10^{-6}$**	11	4-24

Table 4 - rs141490768 enrichment statistics in the WES and the expanded cohorts

IRF3 rs141490768 affects the IRF3-CL regulatory isoform

Interestingly, rs141490768 does not affect IRF3 main isoform protein sequence, but results in a A418T change in the regulatory isoform IRF3-CL and was predicted *in silico* with a very high likelihood to alter protein activity (CADD Phred score (240)= 24.1). Moreover, rs141490768 also determined a single nucleotide change in two noncoding transcripts (NC) described in the ENSEMBL database as ENST00000596644 and ENST00000599680, hereafter referred to as IRF3-NC1 and IRF3-NC2 (Figure 12).

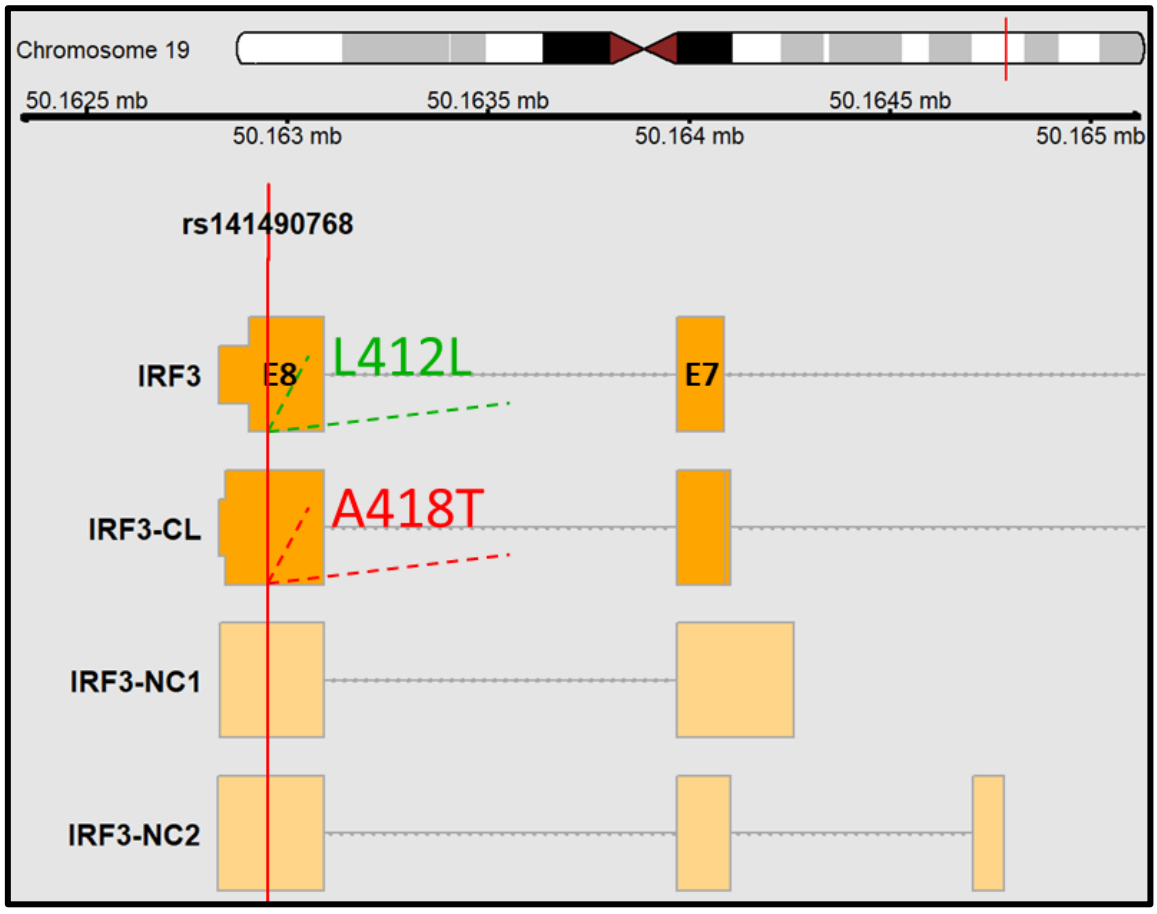

Figure 12 - IRF3 transcripts overlapping the rs141490768 locus

Rare IRF3-CL variants are enriched in patients with advanced NAFLD

IRF3 enrichment in variants with the same features of the rs141490768 in Italian patients with advanced NAFLD vs control samples and the gnomAD NFE population (N=53006) was then evaluated by CAST burden test. We thus considered all rare (MAF≤0.005 ExAC NFE) variants affecting IRF3-CL isoform sequence but not that of IRF3 with a high likelihood of impair protein function (CADD phred score > 20) and compared mutational burden in our discovery HCC cohort (N=72) and NAFLD F3-F4 fibrosis patients with available WES data (N=152). CAST test (Table 2) revealed significant association in both the discovery ($p=5.69*10^{-7}$; OR= 35.5; 95% CI = 11-90, Table 5) and the validation cohorts (OR=6.4; 95% CI= 0.8-24; p=0.04; Table 5). Overall, rare variants affecting specifically IRF3-CL isoform were more frequent in severe NAFLD patients compared to the general population ($p=7.55*10^{-7}$, OR= 15, 95% CI = 6-33). Importantly, the lone variant affecting specifically IRF3-CL in patients was the rs141490768.

	Cases (Mut/Total)	Controls (Mut/Total)	N variants	p	OR	95% CI
Discovery Cohort	5/72	111/53006	24	**$5.69*10^{-7}$**	35.5	11-90
Advanced fibrosis Validation Cohort	2/152	111/53006	24	**0.04**	6.4	0.8-24
Overall	7/224	111/53006	24	**$7.55*10^{-7}$**	15	6-33

Table 5 - IRF3 Burden test analysis results

IRF3-CL and IRF3-NC1 are highly expressed in the Liver

We therefore exploited the transcriptomic cohort to examine the expression and regulation of IRF3 and its pathway during liver damage development. As shown in Figure 13, the canonical IRF3 isoform transcripts were the most expressed in the liver, but we also found high expression levels of the IRF3-noncoding 1 (IRF3-NC1) transcript. IRF3-CL was expressed at a lower levels, but it was detectable in almost all patients, while IRF3-noncoding 2 (IRF3-NC2) was the isoform with the lowest expression, being detectable only in a subset of patients (Anova $p < 2*10^{-16}$, $p < 10^{-10}$ for all comparisons).

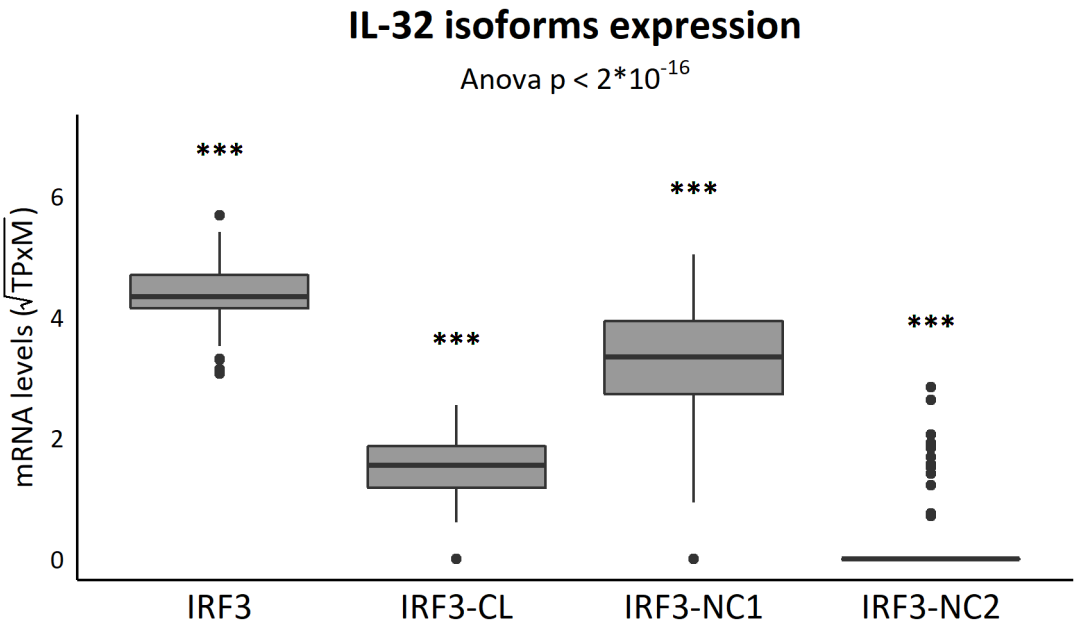

*Figure 13 - Liver expression of IRF3 isoforms of interest. *** p<0.001 vs all other isoforms, post-hoc Tukey HSD test.*

IRF3-CL and IRF3-NC1 mRNA levels correlated with liver disease severity

We could not detect any difference in the overall expression of the IRF3 gene main isoform (figure 14 A) according to liver disease severity. However, we identified a positive correlation between IRF3-CL isoform mRNA levels and disease severity (figure 14B, β= 1.31, p=0.02). Conversely, IRF3-NC1 expression was inversely correlated with liver disease stage (figure 14C, β=-0.76, p=0.02). Furthermore, we identified a positive correlation between expression levels of IRF3 main transcript and IRF3-NC1 (β=0.3, p<0.05, Figure 14D) but not with those of IRF3 (figure 14D).

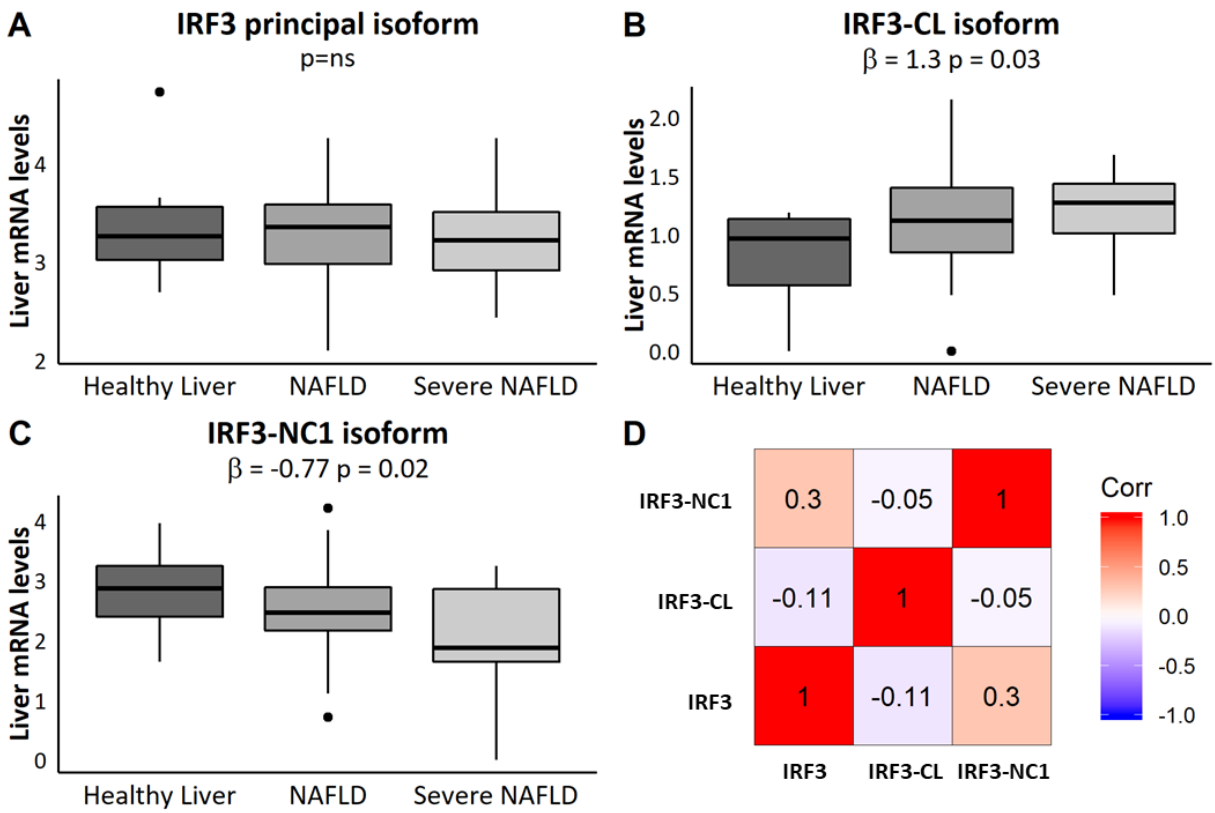

Figure 14 – A, B, and C: Liver mRNA levels of IRF3 main isoform (A), IRF3-CL isoform (B), IRF3-NC1 transcript (C) assessed in the Transcriptomic cohort according to liver disease stage. D: IRF3 transcripts expression correlation matrix.

IRF3, IRF3-CL and noncoding transcripts are associated with IRF3 pathway activation

To explore the functions of the different IRF3 transcripts, co-expression analysis was performed. As shown in Figure 15A, gene set enrichment analysis (GSEA) revealed a positive correlation between the IRF3 main isoform with pathways involved in inflammation (e.g. TNFα, IFNα, IFNγ signaling pathways), fibrogenesis (epithelial to mesenchymal transition, myogenesis), apoptosis and response to DNA damage (e.g. P53 pathway, apoptosis), and cell proliferation (Wnt-β catenin pathway, mitotic spindle). Interestingly, pathways inversely correlated with IRF3 were mainly involved in lipid metabolism (e.g. Oxidative phosphorylation, peroxisomes, fatty acids metabolism). On the other hand, pathways correlated with IRF3-CL (Figure 15B) were involved in both lipid metabolism (e.g. Oxidative phosphorylation, fatty acids metabolism), PI3K-AKT-mTOR axis, inflammation (e.g. IFNα signaling), apoptosis and cell response to stress (e.g. apoptosis, P53 pathway, ROS pathway). Consistently, a negative correlation with genes involved in reduction of UV response was evidenced. Concerning the noncoding transcripts, IRF3-NC1 (Figure 15C) was positively correlated with genes involved in DNA repair and Wnt-β catenin pathway. Interestingly, IRF3-NC1 was negatively correlated with genes mainly involved in metabolism (e.g. oxidative phosphorylation, PI3K-AKT signaling, fatty acids, bile acids and heme metabolism), cell stress response (UV respon se down) and inflammation (IL2, IL6 signaling pathways).

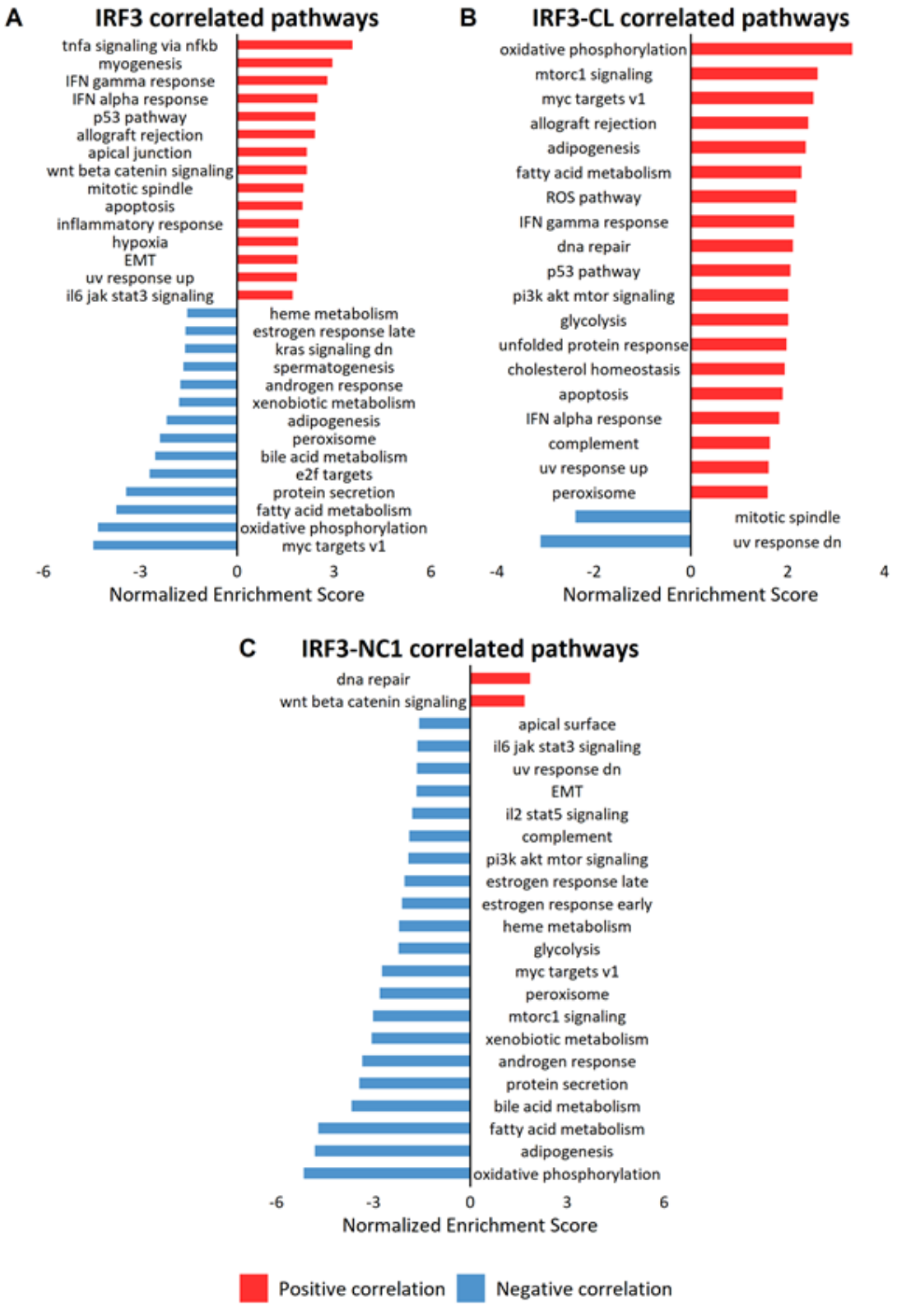

Figure 15 - Co-expression analysis of IRF3 main isoform (A), IRF3-CL (B), and IRF3-NC1 (C).

Expression of IRF3 protein and nuclear localization are increased with liver damage severity and transition to cancer

To further investigate IRF3 role in NAFLD progression, IRF3 protein levels and its transcriptionally active form P-Ser396 IRF3 were evaluated in a cohort of samples (N=18) stratified by severity of liver disease (Figure 16A). In NASH patients, we detected an increase in IRF3 ($p<0.05$) and P-IRF3 protein levels, as compared to isolated steatosis (Figure 16B, $p<0.01$) and normal liver. In NAFLD-HCC, we detected increased levels of both IRF3 and P-IRF3 compared as compared to the extra-tumoral tissue (Figure 16C), suggesting that IRF3 is upregulated during hepatic carcinogenesis and transcriptionally competent. In keeping, IRF3 mRNA levels were evaluated in HCC and control tissue of 20 patients of the cancer genome atlas project (TCGA), who were selected because they developed HCC not related to chronic viral hepatitis or alcohol abuse (Figure 17, $p<0.05$).

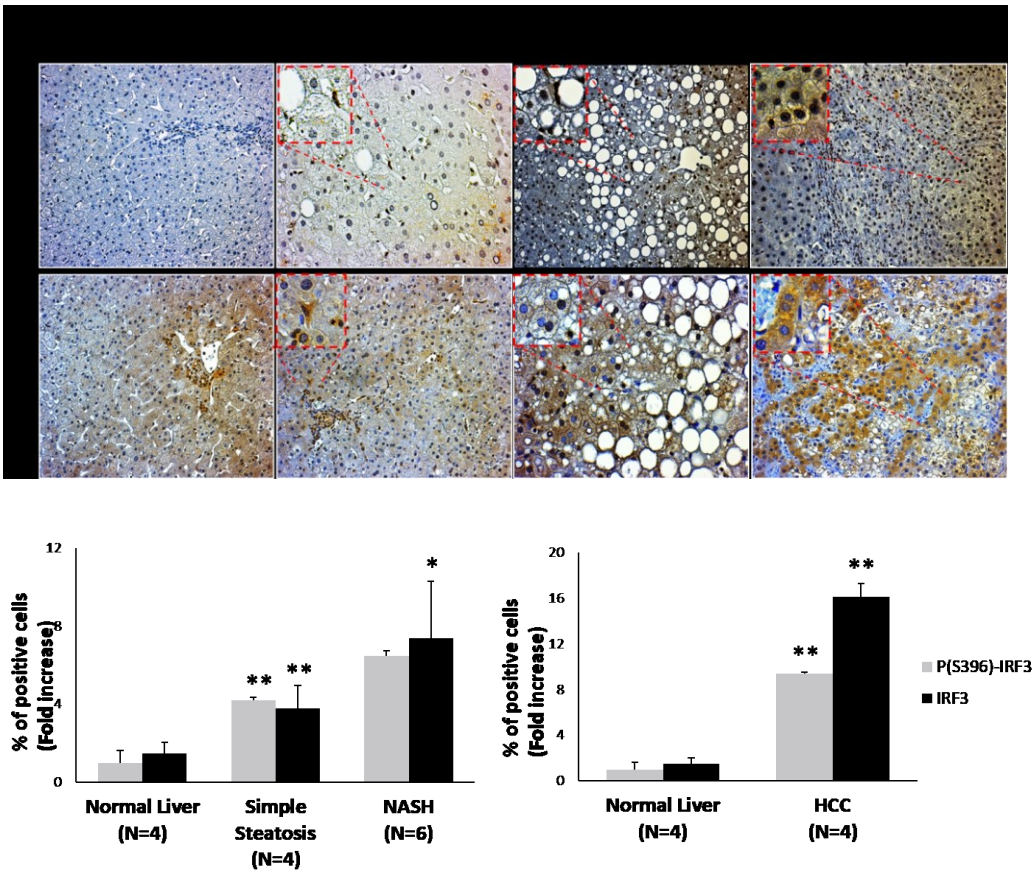

*Figure 16 - IHC Staining(A) and quantification (B and C) of IRF3 and PSer396-IRF3. * p<0.05 vs Normal liver, ** p<0.01 vs Normal liver, Student's T test*

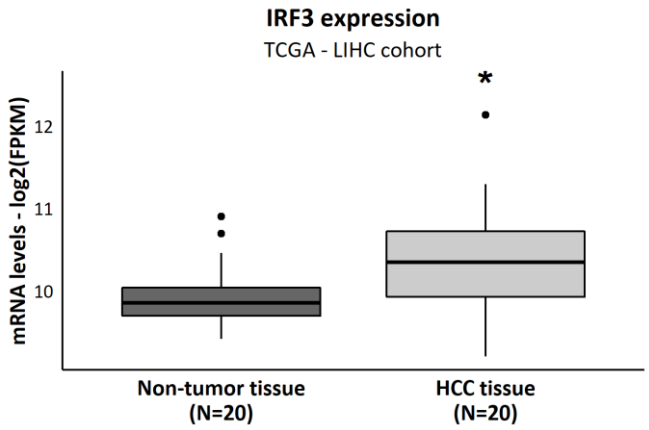

*Figure 17 – Expression levels of IRF3 in tumor healthy tissue of patients of the TCGA-LIHC cohort. * p<0.05 Student's T test*

Free fatty acids and proliferation stimuli activates IRF3

To investigate the mechanisms leading to IRF3 activation during NAFLD progression, we next tested whether increased free fatty acids exposure, which is the hallmark of NAFLD, can trigger IRF3 activation in HepG2 hepatoma cells, a widely used *in vitro* model, characterized by relatively preserved regulation of lipid metabolism. HepG2 exposure to a mixture of saturated (palmitic acid) and mono-unsaturated (oleic-acids) determined an increase in Ser396 phosphorylation of IRF3 (Figure 18A and 18B, $p<0.05$). These data are consistent with the hypothesis that free fatty acids play an active role in activating IRF3. Moreover, given the relationship between the expression of IRF3 principal isoform and pathways involved in cell proliferation, we asked whether IRF3 could be responsive also to proliferation stimuli. In HepG2 cells exposed to a growth medium supplemented with 10% fetal bovine serum we detected strongly increased IRF3 phosphorylation levels (Figure 18C and 18D, $p<0.05$).

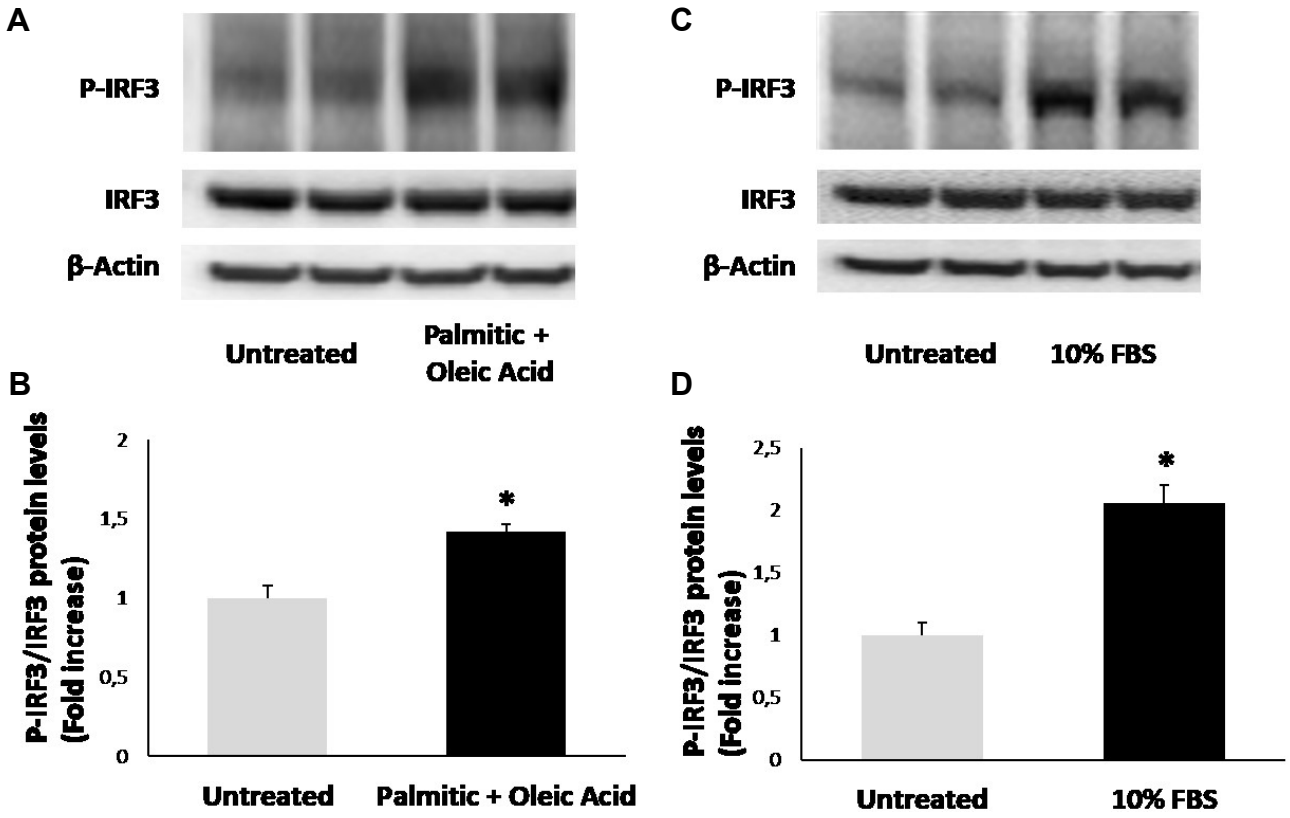

*Figure 18 - Western blot analysis of IRF3 activation upon Free fatty acids (A and B) and Fetal Bovine Serum (C and D). Graphs (B and D) are representative of three independent experiments, * p<0.05 Student's T test*

Amlexanox treatment inhibit FBS induced IRF3 activation

Amlexanox is a selective inhibitor of IKKε/TBK1, a kinase cascade pathway that is responsible to activation of cellular stress responses during lipotoxicity and other triggers of cell stress. We first confirmed that 100µM amlexanox is capable of impairing of IRF3 activation in response to 10% FBS in HepG2. As shown in Figure 19, the upregulation of of P(Ser396)-IRF3 upon FBS stimulation was prevented by amlexanox pre-treatment.

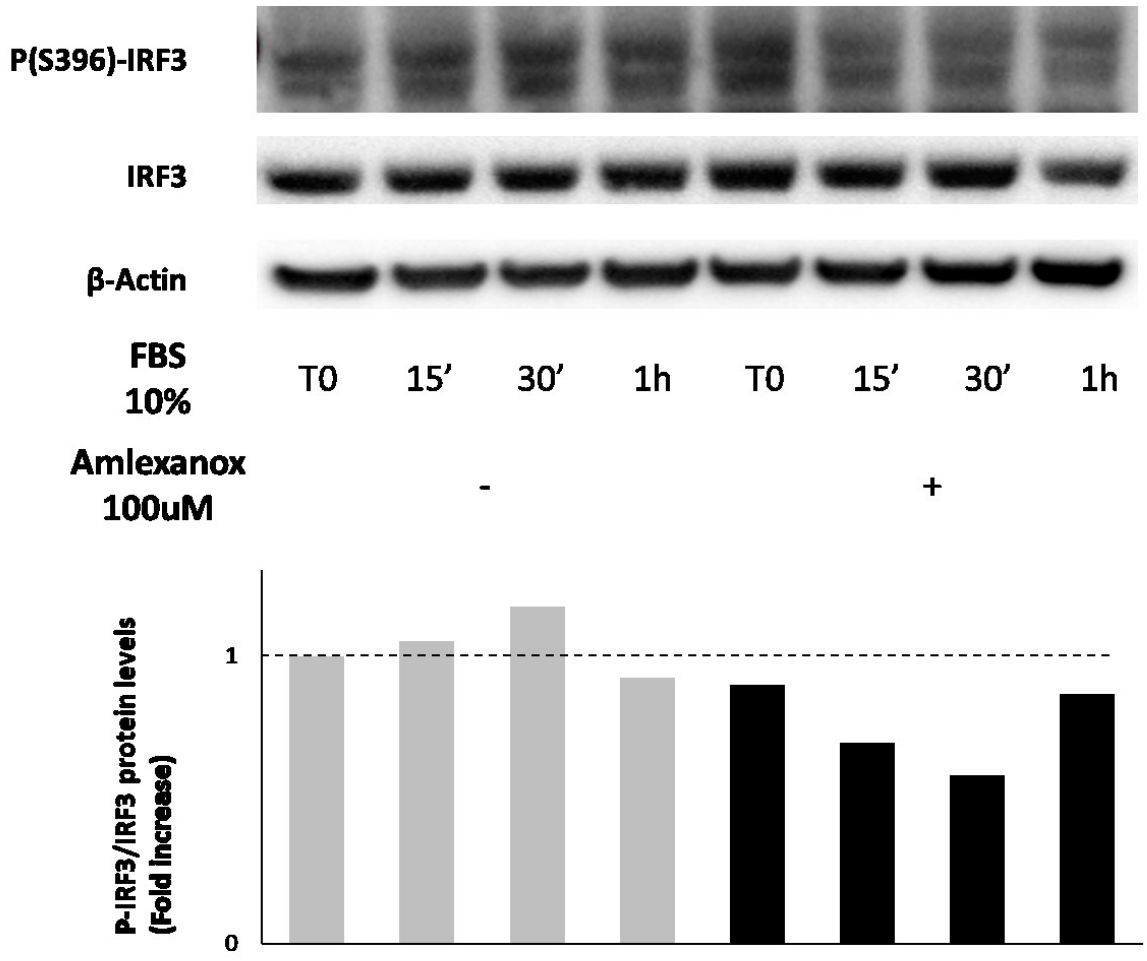

Figure 19 – Activation levels of IRF3 in HepG2 Cells upon FBS stimulation pre-treated or not with 100µM amlexanox.

Amlexanox treatment impairs HepG2 cells proliferation

To further investigate the relationship between IRF3 activation and cell proliferation, we next evaluated the effect of Amlexanox, on cells growth rate. We found that 100µM Amlexanox hampered cell number increased both when administered alone or in combination with 2.5 µM Sorafenib (Figure 20A and B, $p<0.05$). These data suggest that IRF3 activation favors the proliferation of hepatocytes, while inhibition contrast cell growth or favor apoptosis.

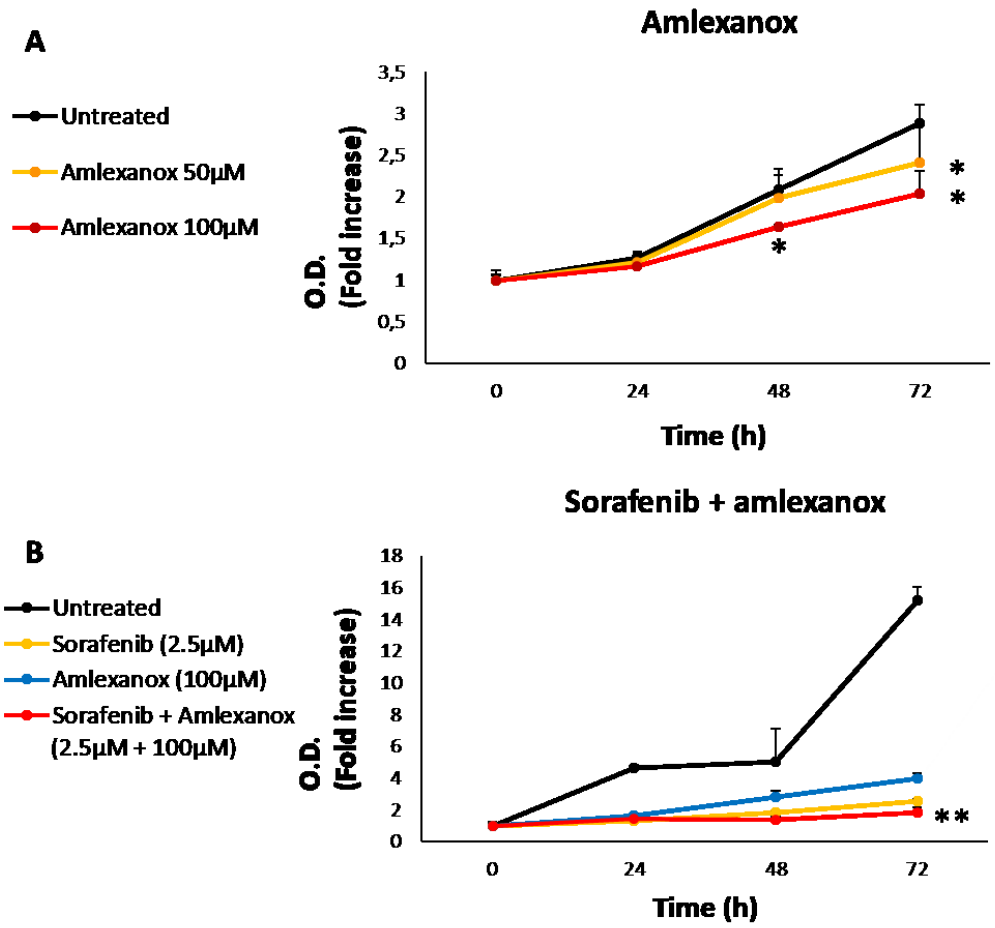

*Figure 20 - HepG2 treatment with Amlexanox (A) and Sorafenib + Amlexanox (B). Graphs are representative of three independent experiments, * $p<0.05$ vs Control ** $p<0.05$ vs Sorafenib, Student's T test*

Heterozygous disruption of IRF3-CL splicing consensus results in impaired IRF3-CL expression

Following exposure of Cas9+ cells with specific sgRNA expressing plasmids after the induction of the enzyme, we could isolate a clone carrying insertion/deletion in the specific exon VII consensus sequence of IRF3-CL without affecting IRF3 canonical exon VII splicing consensus in heterozygosity (Figure 21). As specified in the method section recombination event was assessed by T7 nuclease heteroduplex analysis and confirmed by direct sequencing wit Sanger method. Importantly, in keeping with the predicted impact of this genetic alteration, we detected an 50% reduction of IRF3-CL mRNA in IRF3-CL+/- cells ($p<0.05$, Figure 22).

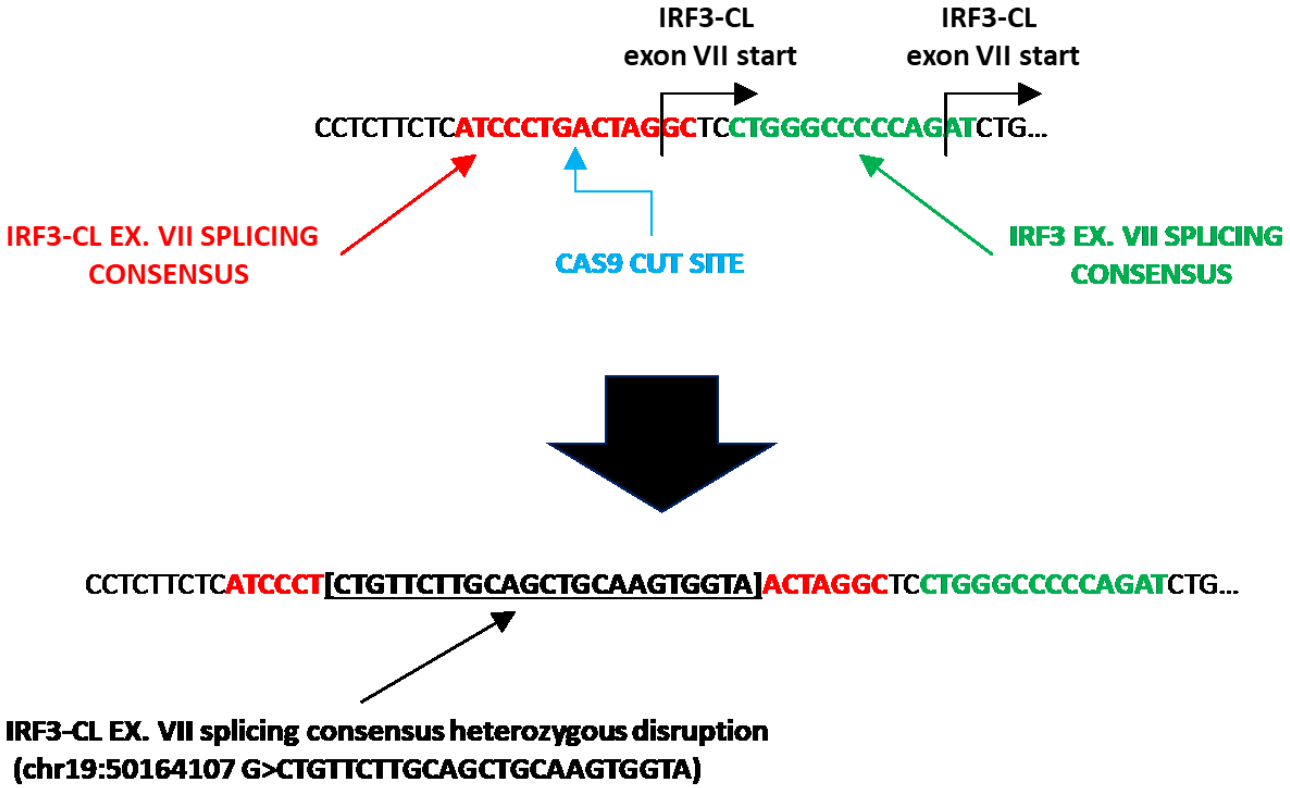

Figure 21 – Schematic representation of the mutation induced in HepG2 cells.

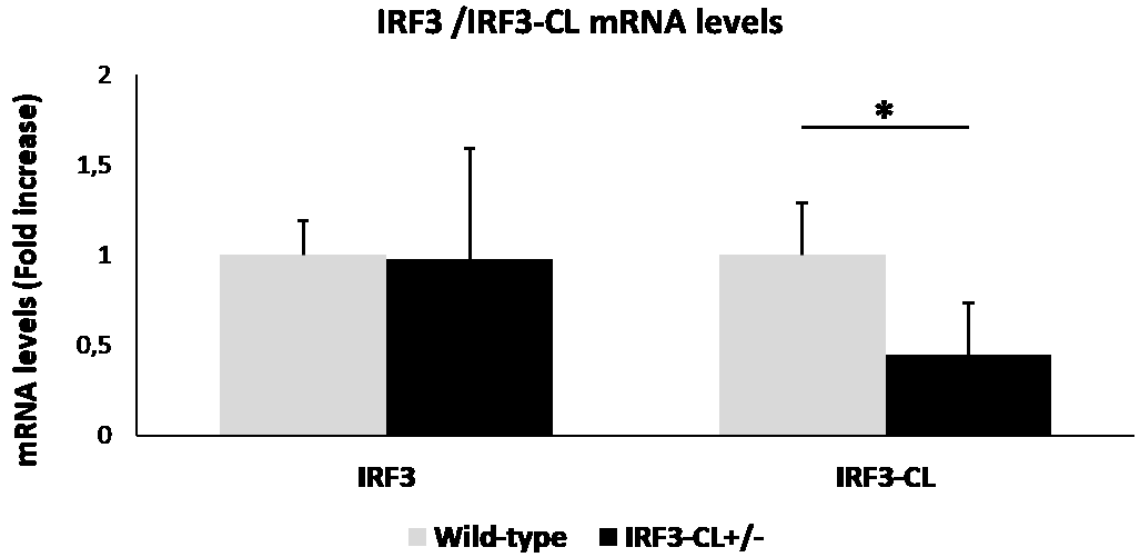

*Figure 22 – Expression levels of the whole IRF3 gene products (left) and the specific IRF3-CL mRNA. Graphs are representative of three independent experiments, *p<0.05 Student's T test.*

Knockout of IRF3-CL results in IRF3 increased activity

IRF3+- cells displayed higher pSer396-IRF3 levels upon exposure to 10% FBS, thus supporting an inhibitory role for IRF3-CL or noncoding transcripts on IRF3 activity (Figure 23A and B).

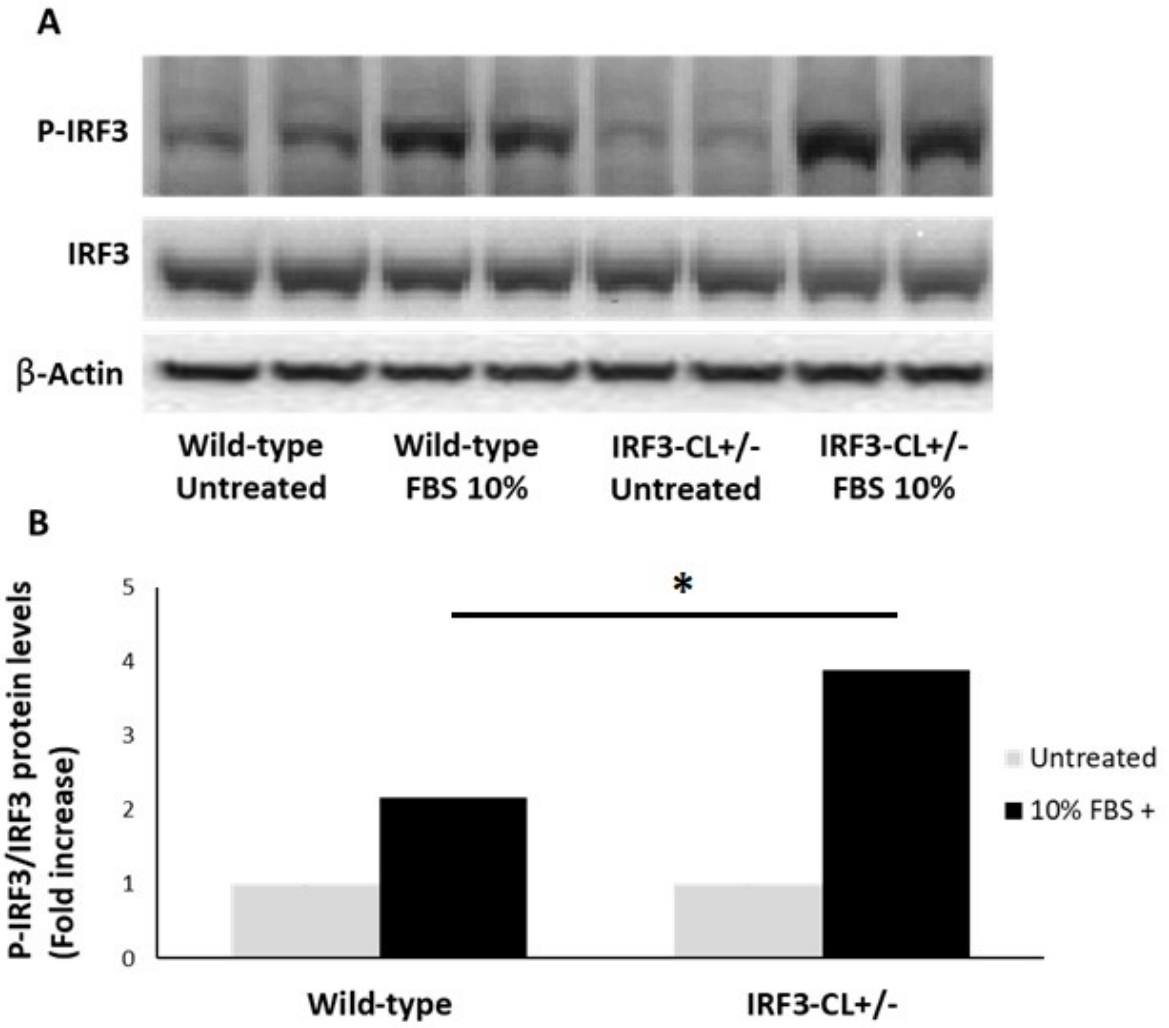

*Figure 23 - Western blot analysis of IRF3 activation upon Fetal Bovine Serum (A and B) in wild-type or IRF3-CL+/- cells. Graphs (B) is representative of three independent experiments, * p<0.05 Student's T test*

IRF3-CL+/- cells are more susceptible to amlexanox-induced inhibition of growth

We then tested if IRF3-CL+/- could have an enhanced cell proliferation and a more sustained response to amlexanox. IRF3-CL+/- HepG2 and *wild-type* littermates were then cultured in presence or not of amlexanox 100µM, sorafenib 2.5µM or both the drugs. Cell growth was assessed at the baseline and after 24, 48, and 72 hours of treatment by MTS assay. We couldn't identify an increased growth rate in IRF3-CL+/- cells compared with *wild types* in standard growing conditions. However, heterozygous knockdown of IRF3-CL resulted in an increased sensitivity to amlexanox treatment (Figure 24A, $p<0.05$). This effect was detectable also in presence of sorafenib (Figure 24B, $p<0.05$). These data suggest that inhibition of IRF3 pathway may increase the response to anti neoplastic treatments in immortalized hepatocytes.

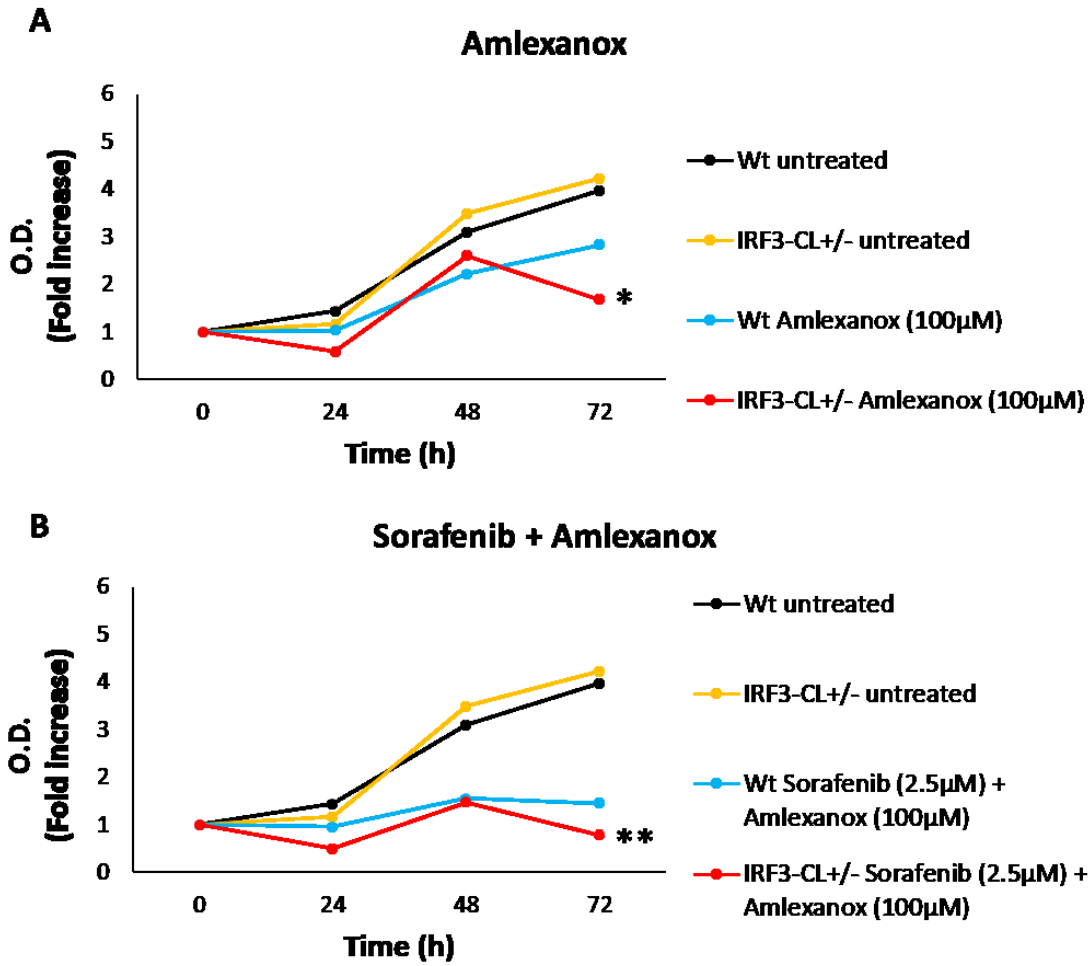

*Figure 24 - HepG2 and IRF3-CL+/- clones treatment with Amlexanox (A) and Sorafenib + Amlexanox (B) Graphs are representative of three independent experiments; * p<0.05 vs Control ** p<0.05 vs Sorafenib, Student's T test*

DISCUSSION

In the first part of the study we identified a novel genetic variant, IRF3 rs141490768, as associated with a strongly increased risk for the development of advanced NAFLD at exome-wide level. The lack of a statistically significant association of this variant with the risk of NAFLD-HCC in our validation cohort alone represent a limitation of the current study. However, we could validate the association in an independent cohort of patients with F3-F4 fibrosis. Furthermore, the association remained strongly significant considering all patients. Moreover, IRF3 gene resulted enriched in rare variants affecting specifically the previously described inhibitory isoform IRF3-CL, overlapping with non-coding transcripts (220), in both our WES discovery and validation cohort, supporting a role for mutations in this section of the gene in the progression of liver disease. Moreover, ENSEMBL database reports the presence of two non-coding transcripts altered by these two variants here referred as IRF3-NC1 and IRF3-NC2 of unknown functionIRF3 is a transcription factor involved in innate immunity response to exogenous nucleic acids as dsRNA or cytosolic dsDNA (193, 259). Its activation results mainly in a Type-I interferons inflammatory response activation and apoptosis (193, 259). Recently, IRF3 activity was linked also to cell proliferation due to crosstalk with WNT-B catenin and YAP-TAZ pathways (228, 232, 260). Moreover, other stimuli for its activation, e.g. ER stress and free fatty acids exposure (215), were identified. Importantly, a function of IRF3 in pathogenesis of both alcoholic hepatitis and NAFLD have been already identified (216, 217, 224, 261). However, IRF3 activity in NAFLD along with the role of IRF3-CL and of the two noncoding transcripts remains largely elusive. Interestingly, transcriptome analysis revealed high expression levels for IRF3-NC1, also IRF3-CL was detectable in all patients. Moreover, both IRF3-CL and IRF3-

NC1 expression levels were correlated to the liver disease stage suggesting an involvement in NAFLD pathogenesis. Co-expression analysis confirmed a possible role of IRF3 in orchestrating inflammatory response, cell proliferation and apoptosis, as it was coregulated with these pathways. Moreover, the analysis highlighted a strong relationship between IRF3 and metabolism. IRF3-CL effect on lipid metabolism was opposite to that of IRF3, while it was correlated with the inflammatory response, suggesting it may mediate a negative-feedback mechanism. Conversely IRF3-NC1 mRNA levels were negatively correlated with those of genes involved in inflammatory pathways. In keeping with the proposed role of IRF3 in promoting NASH and with our transcriptomic analyses, IRF3 was found to be progressively induced at the protein and localized to the nucleus during NAFLD progression level and particularly overexpressed in NAFLD HCC. Moreover, in HepG2 cells IRF3 was responsive to free fatty acids and proliferation triggers, further supporting its role in orchestrating hepatocytes metabolism and proliferation. Taken together, these data supports an active role for IRF3 in NAFLD progression toward fibrosis and HCC. Indeed amlexanox, an inhibitor of the IKK/TBK1-IRF3 axis, is currently in study for the treatment of NAFLD and obesity linked disfunctions (225, 226). Importantly, amlexanox treatment reduced the in vitro growth of hepatoma cells, alone or in combination with sorafenib, further supporting a role for this transcription factors in modulating cell proliferation. Considering IRF3-CL, heterozygous IRF3-CL KO resulted in an increased activation of IRF3 in response to FBS confirming IRF3-CL activity as an IRF3 negative regulator. This suggest that the rs141490768 mutation results in increased IRF3 activity, promoting by this mechanism NAFLD progression and cancer development. Moreover, HepG2 IRF3-CL+/- clones were more sensitive to amlexanox treatment supporting that cell proliferation reduction observed in HepG2 cells were due to IRF3 activity impairment. These data suggest that amlexanox

treatment may be particularly useful for HCC showing upregulation of IRF3 or in patients carrying IRF3-CL loss-of-function mutations.

In conclusion, the rs141490768 *IRF3* variant is associated with an increased risk to develop advanced NAFLD. Moreover, IRF3 was found to be upregulated during liver disease progression, in parallel with the inflammatory response, altered lipid metabolism, and cell proliferation. Even if further mechanistic studies are required to clarify the complex network of interactions among IRF3, IRF3-CL and the two noncoding transcripts considered, our in vitro experiments confirm that IRF3-CL exerts an inhibitory effect on the activation of the main IRF3 isoform, which promotes cell proliferation. Altogether, data suggest that the mechanism underpinning the association of the rs141490768 variant with NAFLD progression to severe fibrosis and HCC is related to facilitation of IRF3 pathway activation. Finally, results support the necessity of further studies to examine the possible role of amlexanox (or other Ike inhibitors) in NAFLD treatment and points out this chemical as good candidate to be further evaluated also in the treatment of NAFLD HCC in combination with Sorafenib.

LIMITATIONS AND FUTURE DIRECTIONS

Association between rs141490768 and endpoint NAFLD and burden test results have to be further confirmed in larger cohorts. Our study together with literature confirm IRF3 involvement in NAFLD transition to severe fibrosis and NAFLD HCC. However, further insights about the contribution of this transcription factor to hepatocytes biology as well as further information about IRF3-CL activity are required. To this purpose, further studies on IRF3-CL+/- models are currently ongoing to determine variations in terms of cell cycle stage, metabolism and apoptosis. Furthermore, the generation and characterization of *IRF3*-full KO HepG2 hepatocytes is ongoing. Considering the two noncoding transcripts, we could provide only limited insights about function of IRF3-NC1 representing another limitation of the current study. Finally, rs141490768 effect on IRF3-CL activity is based on an *in silico* prediction (240). Due to its extremely low allele frequency we could not perform any further genotype-phenotype association providing insights about the overall effect of this variant. Moreover, rs141490768 impact on the functions of IRF3-NC1 and IRF3-NC2 activity remains unexplored. To address these limitations, we are now developing an HepG2 cell line carrying this variant (knock-in line) exploiting CRISPR/Cas9 precise genome editing method (262). Studies on this model will be of great value to confirm the link between rs141490768 and cancer and to clarify the molecular mechanisms beyond this association.

REFERENCES

1. Arab JP, Arrese M, Trauner M. Recent Insights into the Pathogenesis of Nonalcoholic Fatty Liver Disease. Annu Rev Pathol 2018;13:321-350.

2. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-1321.

3. Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology 2018;67:328-357.

4. (EASL) EAftSotL, (EASD) EAftSoD, (EASO) EAftSoO. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol 2016;64:1388-1402.

5. Anstee QM, McPherson S, Day CP. How big a problem is non-alcoholic fatty liver disease? BMJ 2011;343:d3897.

6. Lonardo A, Nascimbeni F, Mantovani A, Targher G. Hypertension, diabetes, atherosclerosis and NASH: Cause or consequence? J Hepatol 2018;68:335-352.

7. Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nat Rev Gastroenterol Hepatol 2018;15:11-20.

8. Diehl AM, Day C. Cause, Pathogenesis, and Treatment of Nonalcoholic Steatohepatitis. N Engl J Med 2017;377:2063-2072.

9. Tilg H, Moschen AR. Insulin resistance, inflammation, and non-alcoholic fatty liver disease. Trends Endocrinol Metab 2008;19:371-379.

10. Tilg H, Moschen AR, Roden M. NAFLD and diabetes mellitus. Nat Rev Gastroenterol Hepatol 2017;14:32-42.

11. Yki-Järvinen H. Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. Lancet Diabetes Endocrinol 2014;2:901-910.

12. Takahashi Y, Fukusato T. Histopathology of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World J Gastroenterol 2014;20:15539-15548.

13. Estes C, Razavi H, Loomba R, Younossi Z, Sanyal AJ. Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease. Hepatology 2018;67:123-133.

14. Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. Hepatology 2010;51:1820-1832.

15. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998;114:842-845.

16. Day CP, Saksena S. Non-alcoholic steatohepatitis: definitions and pathogenesis. J Gastroenterol Hepatol 2002;17 Suppl 3:S377-384.

17. Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology 2010;52:1836-1846.

18. Frayn KN, Coppack SW. Insulin resistance, adipose tissue and coronary heart disease. Clin Sci (Lond) 1992;82:1-8.

19. Otero YF, Stafford JM, McGuinness OP. Pathway-selective insulin resistance and metabolic disease: the importance of nutrient flux. J Biol Chem 2014;289:20462-20469.

20. Malhi H, Gores GJ. Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. Semin Liver Dis 2008;28:360-369.

21. Tiniakos DG, Vos MB, Brunt EM. Nonalcoholic fatty liver disease: pathology and pathogenesis. Annu Rev Pathol 2010;5:145-171.

22. Tilg H, Jalan R, Kaser A, Davies NA, Offner FA, Hodges SJ, Ludwiczek O, et al. Anti-tumor necrosis factor-alpha monoclonal antibody therapy in severe alcoholic hepatitis. J Hepatol 2003;38:419-425.

23. Dumas ME, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, Fearnside J, et al. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. Proc Natl Acad Sci U S A 2006;103:12511-12516.

24. Amar J, Burcelin R, Ruidavets JB, Cani PD, Fauvel J, Alessi MC, Chamontin B, et al. Energy intake is associated with endotoxemia in apparently healthy men. Am J Clin Nutr 2008;87:1219-1223.

25. Bigorgne AE, John B, Ebrahimkhani MR, Shimizu-Albergine M, Campbell JS, Crispe IN. TLR4-Dependent Secretion by Hepatic Stellate Cells of the Neutrophil-Chemoattractant CXCL1 Mediates Liver Response to Gut Microbiota. PLoS One 2016;11:e0151063.

26. Targher G, Lonardo A, Byrne CD. Nonalcoholic fatty liver disease and chronic vascular complications of diabetes mellitus. Nat Rev Endocrinol 2018;14:99-114.

27. Dongiovanni P, Romeo S, Valenti L. Hepatocellular carcinoma in nonalcoholic fatty liver: role of environmental and genetic factors. World J Gastroenterol 2014;20:12945-12955.

28. Asano T, Watanabe K, Kubota N, Gunji T, Omata M, Kadowaki T, Ohnishi S. Adiponectin knockout mice on high fat diet develop fibrosing steatohepatitis. J Gastroenterol Hepatol 2009;24:1669-1676.

29. Yamauchi T, Nio Y, Maki T, Kobayashi M, Takazawa T, Iwabu M, Okada-Iwabu M, et al. Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. Nat Med 2007;13:332-339.

30. Kim JY, van de Wall E, Laplante M, Azzara A, Trujillo ME, Hofmann SM, Schraw T, et al. Obesity-associated improvements in metabolic profile through expansion of adipose tissue. J Clin Invest 2007;117:2621-2637.

31. Tilg H, Hotamisligil GS. Nonalcoholic fatty liver disease: Cytokine-adipokine interplay and regulation of insulin resistance. Gastroenterology 2006;131:934-945.

32. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 1993;259:87-91.

33. Park EJ, Lee JH, Yu GY, He G, Ali SR, Holzer RG, Osterreicher CH, et al. Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. Cell 2010;140:197-208.

34. Wallenius V, Wallenius K, Ahren B, Rudling M, Carlsten H, Dickson SL, Ohlsson C, et al. Interleukin-6-deficient mice develop mature-onset obesity. Nat Med 2002;8:75-79.

35. Rinella M, Charlton M. The globalization of nonalcoholic fatty liver disease: Prevalence and impact on world health. Hepatology 2016;64:19-22.

36. Serfaty L, Lemoine M. Definition and natural history of metabolic steatosis: clinical aspects of NAFLD, NASH and cirrhosis. Diabetes Metab 2008;34:634-637.

37. Dongiovanni P, Anstee QM, Valenti L. Genetic predisposition in NAFLD and NASH: impact on severity of liver disease and response to treatment. Curr Pharm Des 2013;19:5219-5238.

38. Schwimmer JB, Celedon MA, Lavine JE, Salem R, Campbell N, Schork NJ, Shiehmorteza M, et al. Heritability of nonalcoholic fatty liver disease. Gastroenterology 2009;136:1585-1592.

39. Willner IR, Waters B, Patil SR, Reuben A, Morelli J, Riely CA. Ninety patients with nonalcoholic steatohepatitis: insulin resistance, familial tendency, and severity of disease. Am J Gastroenterol 2001;96:2957-2961.

40. Makkonen J, Pietiläinen KH, Rissanen A, Kaprio J, Yki-Järvinen H. Genetic factors contribute to variation in serum alanine aminotransferase activity independent of obesity and alcohol: a study in monozygotic and dizygotic twins. J Hepatol 2009;50:1035-1042.

41. Loomba R, Rao F, Zhang L, Khandrika S, Ziegler MG, Brenner DA, O'Connor DT. Genetic covariance between gamma-glutamyl transpeptidase and fatty liver risk factors: role of beta2-adrenergic receptor genetic variation in twins. Gastroenterology 2010;139:836-845, 845.e831.

42. Loomba R, Schork N, Chen CH, Bettencourt R, Bhatt A, Ang B, Nguyen P, et al. Heritability of Hepatic Fibrosis and Steatosis Based on a Prospective Twin Study. Gastroenterology 2015;149:1784-1793.

43. Dongiovanni P, Romeo S, Valenti L. Genetic Factors in the Pathogenesis of Nonalcoholic Fatty Liver and Steatohepatitis. Biomed Res Int 2015;2015:460190.

44. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, Boerwinkle E, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2008;40:1461-1465.

45. Dongiovanni P, Donati B, Fares R, Lombardi R, Mancina RM, Romeo S, Valenti L. PNPLA3 I148M polymorphism and progressive liver disease. World J Gastroenterol 2013;19:6969-6978.

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

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

48. Huang Y, Cohen JC, Hobbs HH. Expression and characterization of a PNPLA3 protein isoform (I148M) associated with nonalcoholic fatty liver disease. J Biol Chem 2011;286:37085-37093.

49. Pingitore P, Pirazzi C, Mancina RM, Motta BM, Indiveri C, Pujia A, Montalcini T, et al. Recombinant PNPLA3 protein shows triglyceride hydrolase activity and its I148M mutation results in loss of function. Biochim Biophys Acta 2014;1841:574-580.

50. Pirazzi C, Valenti L, Motta BM, Pingitore P, Hedfalk K, Mancina RM, Burza MA, et al. PNPLA3 has retinyl-palmitate lipase activity in human hepatic stellate cells. Hum Mol Genet 2014;23:4077-4085.

51. BasuRay S, Smagris E, Cohen JC, Hobbs HH. The PNPLA3 variant associated with fatty liver disease (I148M) accumulates on lipid droplets by evading ubiquitylation. Hepatology 2017;66:1111-1124.

52. Donati B, Motta BM, Pingitore P, Meroni M, Pietrelli A, Alisi A, Petta S, et al. The rs2294918 E434K variant modulates patatin-like phospholipase domain-containing 3 expression and liver damage. Hepatology 2016;63:787-798.

53. Valenti L, Dongiovanni P. Mutant PNPLA3 I148M protein as pharmacological target for liver disease. Hepatology 2017;66:1026-1028.

54. Kozlitina J, Smagris E, Stender S, Nordestgaard BG, Zhou HH, Tybjærg-Hansen A, Vogt TF, et al. Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2014;46:352-356.

55. Holmen OL, Zhang H, Fan Y, Hovelson DH, Schmidt EM, Zhou W, Guo Y, et al. Systematic evaluation of coding variation identifies a candidate causal variant in TM6SF2 influencing total cholesterol and myocardial infarction risk. Nat Genet 2014;46:345-351.

56. Dongiovanni P, Petta S, Maglio C, Fracanzani AL, Pipitone R, Mozzi E, Motta BM, et al. Transmembrane 6 superfamily member 2 gene variant disentangles nonalcoholic steatohepatitis from cardiovascular disease. Hepatology 2015;61:506-514.

57. Liu YL, Reeves HL, Burt AD, Tiniakos D, McPherson S, Leathart JB, Allison ME, et al. TM6SF2 rs58542926 influences hepatic fibrosis progression in patients with non-alcoholic fatty liver disease. Nat Commun 2014;5:4309.

58. Luukkonen PK, Zhou Y, Nidhina Haridas PA, Dwivedi OP, Hyötyläinen T, Ali A, Juuti A, et al. Impaired hepatic lipid synthesis from polyunsaturated fatty acids in TM6SF2 E167K variant carriers with NAFLD. J Hepatol 2017;67:128-136.

59. Kim DS, Jackson AU, Li YK, Stringham HM, Kuusisto J, Kangas AJ, Soininen P, et al. Novel association of. J Lipid Res 2017;58:1471-1481.

60. Mancina RM, Dongiovanni P, Petta S, Pingitore P, Meroni M, Rametta R, Borén J, et al. The MBOAT7-TMC4 Variant rs641738 Increases Risk of Nonalcoholic Fatty Liver Disease in Individuals of European Descent. Gastroenterology 2016;150:1219-1230.e1216.

61. Luukkonen PK, Zhou Y, Hyötyläinen T, Leivonen M, Arola J, Orho-Melander M, Orešič M, et al. The MBOAT7 variant rs641738 alters hepatic phosphatidylinositols and increases severity of non-alcoholic fatty liver disease in humans. J Hepatol 2016;65:1263-1265.

62. Donati B, Dongiovanni P, Romeo S, Meroni M, McCain M, Miele L, Petta S, et al. MBOAT7 rs641738 variant and hepatocellular carcinoma in non-cirrhotic individuals. Sci Rep 2017;7:4492.

63. Speliotes EK, Yerges-Armstrong LM, Wu J, Hernaez R, Kim LJ, Palmer CD, Gudnason V, et al. Genome-wide association analysis identifies variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits. PLoS Genet 2011;7:e1001324.

64. Santoro N, Zhang CK, Zhao H, Pakstis AJ, Kim G, Kursawe R, Dykas DJ, et al. Variant in the glucokinase regulatory protein (GCKR) gene is associated with fatty liver in obese children and adolescents. Hepatology 2012;55:781-789.

65. Petta S, Miele L, Bugianesi E, Cammà C, Rosso C, Boccia S, Cabibi D, et al. Glucokinase regulatory protein gene polymorphism affects liver fibrosis in non-alcoholic fatty liver disease. PLoS One 2014;9:e87523.

66. Valenti L, Alisi A, Nobili V. Unraveling the genetics of fatty liver in obese children: additive effect of P446L GCKR and I148M PNPLA3 polymorphisms. Hepatology 2012;55:661-663.

67. Beer NL, Tribble ND, McCulloch LJ, Roos C, Johnson PR, Orho-Melander M, Gloyn AL. The P446L variant in GCKR associated with fasting plasma glucose and triglyceride levels exerts its effect through increased glucokinase activity in liver. Hum Mol Genet 2009;18:4081-4088.

68. Pelusi S, Baselli G, Pietrelli A, Dongiovanni P, Donati B, McCain MV, Meroni M, et al. Rare Pathogenic Variants Predispose to Hepatocellular Carcinoma in Nonalcoholic Fatty Liver Disease. Sci Rep 2019;9:3682.

69. Abul-Husn NS, Cheng X, Li AH, Xin Y, Schurmann C, Stevis P, Liu Y, et al. A Protein-Truncating HSD17B13 Variant and Protection from Chronic Liver Disease. N Engl J Med 2018;378:1096-1106.

70. Dongiovanni P, Valenti L. Peroxisome proliferator-activated receptor genetic polymorphisms and nonalcoholic Fatty liver disease: any role in disease susceptibility? PPAR Res 2013;2013:452061.

71. Dongiovanni P, Rametta R, Fracanzani AL, Benedan L, Borroni V, Maggioni P, Maggioni M, et al. Lack of association between peroxisome proliferator-activated receptors alpha and gamma2 polymorphisms and progressive liver damage in patients with non-alcoholic fatty liver disease: a case control study. BMC Gastroenterol 2010;10:102.

72. Kumari M, Schoiswohl G, Chitraju C, Paar M, Cornaciu I, Rangrez AY, Wongsiriroj N, et al. Adiponutrin functions as a nutritionally regulated lysophosphatidic acid acyltransferase. Cell Metab 2012;15:691-702.

73. Valenti L, Motta BM, Alisi A, Sartorelli R, Buonaiuto G, Dongiovanni P, Rametta R, et al. LPIN1 rs13412852 polymorphism in pediatric nonalcoholic fatty liver disease. J Pediatr Gastroenterol Nutr 2012;54:588-593.

74. Caldwell SH, Swerdlow RH, Khan EM, Iezzoni JC, Hespenheide EE, Parks JK, Parker WD. Mitochondrial abnormalities in non-alcoholic steatohepatitis. J Hepatol 1999;31:430-434.

75. Berardi MJ, Chou JJ. Fatty acid flippase activity of UCP2 is essential for its proton transport in mitochondria. Cell Metab 2014;20:541-552.

76. Fares R, Petta S, Lombardi R, Grimaudo S, Dongiovanni P, Pipitone R, Rametta R, et al. The UCP2 -866 G>A promoter region polymorphism is associated with nonalcoholic steatohepatitis. Liver Int 2015;35:1574-1580.

77. Al-Serri A, Anstee QM, Valenti L, Nobili V, Leathart JB, Dongiovanni P, Patch J, et al. The SOD2 C47T polymorphism influences NAFLD fibrosis severity: evidence from case-control and intra-familial allele association studies. J Hepatol 2012;56:448-454.

78. Namikawa C, Shu-Ping Z, Vyselaar JR, Nozaki Y, Nemoto Y, Ono M, Akisawa N, et al. Polymorphisms of microsomal triglyceride transfer protein gene and manganese superoxide dismutase gene in non-alcoholic steatohepatitis. J Hepatol 2004;40:781-786.

79. Valenti L, Fracanzani AL, Bugianesi E, Dongiovanni P, Galmozzi E, Vanni E, Canavesi E, et al. HFE genotype, parenchymal iron accumulation, and liver fibrosis in patients with nonalcoholic fatty liver disease. Gastroenterology 2010;138:905-912.

80. Valenti L, Canavesi E, Galmozzi E, Dongiovanni P, Rametta R, Maggioni P, Maggioni M, et al. Beta-globin mutations are associated with parenchymal siderosis and fibrosis in patients with non-alcoholic fatty liver disease. J Hepatol 2010;53:927-933.

81. Valenti L, Rametta R, Dongiovanni P, Motta BM, Canavesi E, Pelusi S, Pulixi EA, et al. The A736V TMPRSS6 polymorphism influences hepatic iron overload in nonalcoholic fatty liver disease. PLoS One 2012;7:e48804.

82. Dongiovanni P, Valenti L, Rametta R, Daly AK, Nobili V, Mozzi E, Leathart JB, et al. Genetic variants regulating insulin receptor signalling are associated with the severity of liver damage in patients with non-alcoholic fatty liver disease. Gut 2010;59:267-273.

83. Miele L, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, Masciana R, et al. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. Hepatology 2009;49:1877-1887.

84. Eslam M, McLeod D, Kelaeng KS, Mangia A, Berg T, Thabet K, Irving WL, et al. IFN-λ3, not IFN-λ4, likely mediates IFNL3-IFNL4 haplotype-dependent hepatic inflammation and fibrosis. Nat Genet 2017;49:795-800.

85. Petta S, Valenti L, Tuttolomondo A, Dongiovanni P, Pipitone RM, Cammà C, Cabibi D, et al. Interferon lambda 4 rs368234815 TT>δG variant is associated with liver damage in patients with nonalcoholic fatty liver disease. Hepatology 2017;66:1885-1893.

86. Eslam M, Hashem AM, Leung R, Romero-Gomez M, Berg T, Dore GJ, Chan HL, et al. Interferon-λ rs12979860 genotype and liver fibrosis in viral and non-viral chronic liver disease. Nat Commun 2015;6:6422.

87. Petta S, Grimaudo S, Cammà C, Cabibi D, Di Marco V, Licata G, Pipitone RM, et al. IL28B and PNPLA3 polymorphisms affect histological liver damage in patients with non-alcoholic fatty liver disease. J Hepatol 2012;56:1356-1362.

88. Petta S, Valenti L, Marra F, Grimaudo S, Tripodo C, Bugianesi E, Cammà C, et al. MERTK rs4374383 polymorphism affects the severity of fibrosis in non-alcoholic fatty liver disease. J Hepatol 2016;64:682-690.

89. Rüeger S, Bochud PY, Dufour JF, Müllhaupt B, Semela D, Heim MH, Moradpour D, et al. Impact of common risk factors of fibrosis progression in chronic hepatitis C. Gut 2015;64:1605-1615.

90. Musso G, Cassader M, De Michieli F, Paschetta E, Pinach S, Saba F, Bongiovanni D, et al. MERTK rs4374383 variant predicts incident nonalcoholic fatty liver disease and diabetes: role of mononuclear cell activation and adipokine response to dietary fat. Hum Mol Genet 2017;26:1747-1758.

91. Eslam M, George J. Genetic and epigenetic mechanisms of NASH. Hepatol Int 2016;10:394-406.

92. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69-90.

93. Seyda Seydel G, Kucukoglu O, Altinbasv A, Demir OO, Yilmaz S, Akkiz H, Otan E, et al. Economic growth leads to increase of obesity and associated hepatocellular carcinoma in developing countries. Ann Hepatol 2016;15:662-672.

94. Powell EE, Cooksley WG, Hanson R, Searle J, Halliday JW, Powell LW. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. Hepatology 1990;11:74-80.

95. Ascha MS, Hanouneh IA, Lopez R, Tamimi TA, Feldstein AF, Zein NN. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. Hepatology 2010;51:1972-1978.

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

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

98. Wong RJ, Cheung R, Ahmed A. Nonalcoholic steatohepatitis is the most rapidly growing indication for liver transplantation in patients with hepatocellular carcinoma in the U.S. Hepatology 2014;59:2188-2195.

99. Kim GA, Lee HC, Choe J, Kim MJ, Lee MJ, Chang HS, Bae IY, et al. Association between non-alcoholic fatty liver disease and cancer incidence rate. J Hepatol 2017.

100. Reeves HL, Zaki MY, Day CP. Hepatocellular Carcinoma in Obesity, Type 2 Diabetes, and NAFLD. Dig Dis Sci 2016;61:1234-1245.

101. Mantovani A, Targher G. Type 2 diabetes mellitus and risk of hepatocellular carcinoma: spotlight on nonalcoholic fatty liver disease. Ann Transl Med 2017;5:270.

102. Baffy G. Hepatocellular Carcinoma in Obesity: Finding a Needle in the Haystack? Adv Exp Med Biol 2018;1061:63-77.

103. Streba LA, Vere CC, Rogoveanu I, Streba CT. Nonalcoholic fatty liver disease, metabolic risk factors, and hepatocellular carcinoma: an open question. World J Gastroenterol 2015;21:4103-4110.

104. Sunny NE, Bril F, Cusi K. Mitochondrial Adaptation in Nonalcoholic Fatty Liver Disease: Novel Mechanisms and Treatment Strategies. Trends Endocrinol Metab 2017;28:250-260.

105. Nakagawa H, Hayata Y, Kawamura S, Yamada T, Fujiwara N, Koike K. Lipid Metabolic Reprogramming in Hepatocellular Carcinoma. Cancers (Basel) 2018;10.

106. Anstee QM, Reeves HL, Kotsiliti E, Govaere O, Heikenwalder M. From NASH to HCC: current concepts and future challenges. Nat Rev Gastroenterol Hepatol 2019;16:411-428.

107. Teoh NC, Dan YY, Swisshelm K, Lehman S, Wright JH, Haque J, Gu Y, et al. Defective DNA strand break repair causes chromosomal instability and accelerates liver carcinogenesis in mice. Hepatology 2008;47:2078-2088.

108. Delire B, Stärkel P. The Ras/MAPK pathway and hepatocarcinoma: pathogenesis and therapeutic implications. Eur J Clin Invest 2015;45:609-623.

109. de Conti A, Ortega JF, Tryndyak V, Dreval K, Moreno FS, Rusyn I, Beland FA, et al. MicroRNA deregulation in nonalcoholic steatohepatitis-associated liver carcinogenesis. Oncotarget 2017;8:88517-88528.

110. Nishida N, Yada N, Hagiwara S, Sakurai T, Kitano M, Kudo M. Unique features associated with hepatic oxidative DNA damage and DNA methylation in non-alcoholic fatty liver disease. J Gastroenterol Hepatol 2016;31:1646-1653.

111. Tanaka S, Miyanishi K, Kobune M, Kawano Y, Hoki T, Kubo T, Hayashi T, et al. Increased hepatic oxidative DNA damage in patients with nonalcoholic steatohepatitis who develop hepatocellular carcinoma. J Gastroenterol 2013;48:1249-1258.

112. Begriche K, Massart J, Robin MA, Bonnet F, Fromenty B. Mitochondrial adaptations and dysfunctions in nonalcoholic fatty liver disease. Hepatology 2013;58:1497-1507.

113. Masarone M, Rosato V, Dallio M, Gravina AG, Aglitti A, Loguercio C, Federico A, et al. Role of Oxidative Stress in Pathophysiology of Nonalcoholic Fatty Liver Disease. Oxid Med Cell Longev 2018;2018:9547613.

114. Wiseman H, Halliwell B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. Biochem J 1996;313 (Pt 1):17-29.

115. Kuper H, Adami HO, Trichopoulos D. Infections as a major preventable cause of human cancer. J Intern Med 2000;248:171-183.

116. Wilson CL, Jurk D, Fullard N, Banks P, Page A, Luli S, Elsharkawy AM, et al. NFκB1 is a suppressor of neutrophil-driven hepatocellular carcinoma. Nat Commun 2015;6:6818.

117. Canli Ö, Nicolas AM, Gupta J, Finkelmeier F, Goncharova O, Pesic M, Neumann T, et al. Myeloid Cell-Derived Reactive Oxygen Species Induce Epithelial Mutagenesis. Cancer Cell 2017;32:869-883.e865.

118. Guo Z, Kozlov S, Lavin MF, Person MD, Paull TT. ATM activation by oxidative stress. Science 2010;330:517-521.

119. Daugherity EK, Balmus G, Al Saei A, Moore ES, Abi Abdallah D, Rogers AB, Weiss RS, et al. The DNA damage checkpoint protein ATM promotes hepatocellular apoptosis and fibrosis in a mouse model of non-alcoholic fatty liver disease. Cell Cycle 2012;11:1918-1928.

120. McKinnon PJ. ATM and the molecular pathogenesis of ataxia telangiectasia. Annu Rev Pathol 2012;7:303-321.

121. Gao D, Wei C, Chen L, Huang J, Yang S, Diehl AM. Oxidative DNA damage and DNA repair enzyme expression are inversely related in murine models of fatty liver disease. Am J Physiol Gastrointest Liver Physiol 2004;287:G1070-1077.

122. Collis SJ, DeWeese TL, Jeggo PA, Parker AR. The life and death of DNA-PK. Oncogene 2005;24:949-961.

123. Cornell L, Munck JM, Alsinet C, Villanueva A, Ogle L, Willoughby CE, Televantou D, et al. DNA-PK-A candidate driver of hepatocarcinogenesis and tissue biomarker that predicts response to treatment and survival. Clin Cancer Res 2015;21:925-933.

124. Evert M, Frau M, Tomasi ML, Latte G, Simile MM, Seddaiu MA, Zimmermann A, et al. Deregulation of DNA-dependent protein kinase catalytic subunit contributes to human hepatocarcinogenesis development and has a putative prognostic value. Br J Cancer 2013;109:2654-2664.

125. Pascale RM, Joseph C, Latte G, Evert M, Feo F, Calvisi DF. DNA-PKcs: A promising therapeutic target in human hepatocellular carcinoma? DNA Repair (Amst) 2016;47:12-20.

126. Pfeifer U. Inverted diurnal rhythm of cellular autophagy in liver cells of rats fed a single daily meal. Virchows Arch B Cell Pathol 1972;10:1-3.

127. Khaminets A, Behl C, Dikic I. Ubiquitin-Dependent And Independent Signals In Selective Autophagy. Trends Cell Biol 2016;26:6-16.

128. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 2011;13:132-141.

129. Taniguchi K, Yamachika S, He F, Karin M. p62/SQSTM1-Dr. Jekyll and Mr. Hyde that prevents oxidative stress but promotes liver cancer. FEBS Lett 2016;590:2375-2397.

130. Taguchi K, Motohashi H, Yamamoto M. Molecular mechanisms of the Keap1–Nrf2 pathway in stress response and cancer evolution. Genes Cells 2011;16:123-140.

131. Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, Tanaka K, et al. Autophagy regulates lipid metabolism. Nature 2009;458:1131-1135.

132. Tanaka S, Hikita H, Tatsumi T, Sakamori R, Nozaki Y, Sakane S, Shiode Y, et al. Rubicon inhibits autophagy and accelerates hepatocyte apoptosis and lipid accumulation in nonalcoholic fatty liver disease in mice. Hepatology 2016;64:1994-2014.

133. Totoki Y, Tatsuno K, Covington KR, Ueda H, Creighton CJ, Kato M, Tsuji S, et al. Trans-ancestry mutational landscape of hepatocellular carcinoma genomes. Nat Genet 2014;46:1267-1273.

134. Schulze K, Imbeaud S, Letouzé E, Alexandrov LB, Calderaro J, Rebouissou S, Couchy G, et al. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. Nat Genet 2015;47:505-511.

135. Petrelli A, Perra A, Cora D, Sulas P, Menegon S, Manca C, Migliore C, et al. MicroRNA/gene profiling unveils early molecular changes and nuclear factor erythroid related factor 2 (NRF2) activation in a rat model recapitulating human hepatocellular carcinoma (HCC). Hepatology 2014;59:228-241.

136. Govaere O, Wouters J, Petz M, Vandewynckel YP, Van den Eynde K, Van den Broeck A, Verhulst S, et al. Laminin-332 sustains chemoresistance and quiescence as part of the human hepatic cancer stem cell niche. J Hepatol 2016;64:609-617.

137. Swann JR, Want EJ, Geier FM, Spagou K, Wilson ID, Sidaway JE, Nicholson JK, et al. Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4523-4530.

138. Sayin SI, Wahlström A, Felin J, Jäntti S, Marschall HU, Bamberg K, Angelin B, et al. Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. Cell Metab 2013;17:225-235.

139. Parséus A, Sommer N, Sommer F, Caesar R, Molinaro A, Ståhlman M, Greiner TU, et al. Microbiota-induced obesity requires farnesoid X receptor. Gut 2017;66:429-437.

140. Wahlström A, Kovatcheva-Datchary P, Ståhlman M, Khan MT, Bäckhed F, Marschall HU. Induction of farnesoid X receptor signaling in germ-free mice colonized with a human microbiota. J Lipid Res 2017;58:412-419.

141. Wang X, Fu X, Van Ness C, Meng Z, Ma X, Huang W. Bile Acid Receptors and Liver Cancer. Curr Pathobiol Rep 2013;1:29-35.

142. Zhang J, Dinh TN, Kappeler K, Tsaprailis G, Chen QM. La autoantigen mediates oxidant induced de novo Nrf2 protein translation. Mol Cell Proteomics 2012;11:M111.015032.

143. Jansen PL. Endogenous bile acids as carcinogens. J Hepatol 2007;47:434-435.

144. Yang F, Huang X, Yi T, Yen Y, Moore DD, Huang W. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. Cancer Res 2007;67:863-867.

145. Degirolamo C, Modica S, Vacca M, Di Tullio G, Morgano A, D'Orazio A, Kannisto K, et al. Prevention of spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice by intestinal-specific farnesoid X receptor reactivation. Hepatology 2015;61:161-170.

146. Wolf MJ, Adili A, Piotrowitz K, Abdullah Z, Boege Y, Stemmer K, Ringelhan M, et al. Metabolic activation of intrahepatic CD8+ T cells and NKT cells causes nonalcoholic steatohepatitis and liver cancer via cross-talk with hepatocytes. Cancer Cell 2014;26:549-564.

147. Ringelhan M, Pfister D, O'Connor T, Pikarsky E, Heikenwalder M. The immunology of hepatocellular carcinoma. Nat Immunol 2018;19:222-232.

148. Taniguchi K, Karin M. NF-κB, inflammation, immunity and cancer: coming of age. Nat Rev Immunol 2018;18:309-324.

149. Song IJ, Yang YM, Inokuchi-Shimizu S, Roh YS, Yang L, Seki E. The contribution of toll-like receptor signaling to the development of liver fibrosis and cancer in hepatocyte-specific TAK1-deleted mice. Int J Cancer 2018;142:81-91.

150. Haybaeck J, Zeller N, Wolf MJ, Weber A, Wagner U, Kurrer MO, Bremer J, et al. A lymphotoxin-driven pathway to hepatocellular carcinoma. Cancer Cell 2009;16:295-308.

151. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, et al. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461-466.

152. Maeda S, Kamata H, Luo JL, Leffert H, Karin M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. Cell 2005;121:977-990.

153. Aleksandrova K, Boeing H, Nöthlings U, Jenab M, Fedirko V, Kaaks R, Lukanova A, et al. Inflammatory and metabolic biomarkers and risk of liver and biliary tract cancer. Hepatology 2014;60:858-871.

154. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, Karin M. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 2007;317:121-124.

155. He G, Dhar D, Nakagawa H, Font-Burgada J, Ogata H, Jiang Y, Shalapour S, et al. Identification of liver cancer progenitors whose malignant progression depends on autocrine IL-6 signaling. Cell 2013;155:384-396.

156. Liu Z, Chen T, Lu X, Xie H, Zhou L, Zheng S. Overexpression of variant PNPLA3 gene at I148M position causes malignant transformation of hepatocytes via IL-6-JAK2/STAT3 pathway in low dose free fatty acid exposure: a laboratory investigation in vitro and in vivo. Am J Transl Res 2016;8:1319-1338.

157. He G, Yu GY, Temkin V, Ogata H, Kuntzen C, Sakurai T, Sieghart W, et al. Hepatocyte IKKbeta/NF-kappaB inhibits tumor promotion and progression by preventing oxidative stress-driven STAT3 activation. Cancer Cell 2010;17:286-297.

158. Miura K, Ishioka M, Minami S, Horie Y, Ohshima S, Goto T, Ohnishi H. Toll-like Receptor 4 on Macrophage Promotes the Development of Steatohepatitis-related Hepatocellular Carcinoma in Mice. J Biol Chem 2016;291:11504-11517.

159. Benbow JH, Thompson KJ, Cope HL, Brandon-Warner E, Culberson CR, Bossi KL, Li T, et al. Diet-Induced Obesity Enhances Progression of Hepatocellular Carcinoma through Tenascin-C/Toll-Like Receptor 4 Signaling. Am J Pathol 2016;186:145-158.

160. Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. Cell Mol Immunol 2016;13:316-327.

161. Nielsen SR, Schmid MC. Macrophages as Key Drivers of Cancer Progression and Metastasis. Mediators Inflamm 2017;2017:9624760.

162. Hoechst B, Voigtlaender T, Ormandy L, Gamrekelashvili J, Zhao F, Wedemeyer H, Lehner F, et al. Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor. Hepatology 2009;50:799-807.

163. Kang TW, Yevsa T, Woller N, Hoenicke L, Wuestefeld T, Dauch D, Hohmeyer A, et al. Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. Nature 2011;479:547-551.

164. Fu J, Xu D, Liu Z, Shi M, Zhao P, Fu B, Zhang Z, et al. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. Gastroenterology 2007;132:2328-2339.

165. Yang YM, Kim SY, Seki E. Inflammation and Liver Cancer: Molecular Mechanisms and Therapeutic Targets. Semin Liver Dis 2019;39:26-42.

166. Yuan X, Waterworth D, Perry JR, Lim N, Song K, Chambers JC, Zhang W, et al. Population-based genome-wide association studies reveal six loci influencing plasma levels of liver enzymes. Am J Hum Genet 2008;83:520-528.

167. Corradini SG, Burza MA, Molinaro A, Romeo S. Patatin-like phospholipase domain containing 3 sequence variant and hepatocellular carcinoma. Hepatology 2011;53:1776; author reply 1777.

168. Valenti L, Rumi M, Galmozzi E, Aghemo A, Del Menico B, De Nicola S, Dongiovanni P, et al. Patatin-like phospholipase domain-containing 3 I148M polymorphism, steatosis, and liver damage in chronic hepatitis C. Hepatology 2011;53:791-799.

169. Burza MA, Pirazzi C, Maglio C, Sjöholm K, Mancina RM, Svensson PA, Jacobson P, et al. PNPLA3 I148M (rs738409) genetic variant is associated with hepatocellular carcinoma in obese individuals. Dig Liver Dis 2012;44:1037-1041.

170. Hassan MM, Kaseb A, Etzel CJ, El-Serag H, Spitz MR, Chang P, Hale KS, et al. Genetic variation in the PNPLA3 gene and hepatocellular carcinoma in USA: risk and prognosis prediction. Mol Carcinog 2013;52 Suppl 1:E139-147.

171. Valenti L, Motta BM, Soardo G, Iavarone M, Donati B, Sangiovanni A, Carnelutti A, et al. PNPLA3 I148M polymorphism, clinical presentation, and survival in patients with hepatocellular carcinoma. PLoS One 2013;8:e75982.

172. Lonardo A, Tarugi P, Ballarini G, Bagni A. Familial heterozygous hypobetalipoproteinemia, extrahepatic primary malignancy, and hepatocellular carcinoma. Dig Dis Sci 1998;43:2489-2492.

173. Bonnefont-Rousselot D, Condat B, Sassolas A, Chebel S, Bittar R, Federspiel MC, Cazals-Hatem D, et al. Cryptogenic cirrhosis in a patient with familial hypocholesterolemia due to a new truncated form of apolipoprotein B. Eur J Gastroenterol Hepatol 2009;21:104-108.

174. Cefalù AB, Pirruccello JP, Noto D, Gabriel S, Valenti V, Gupta N, Spina R, et al. A novel APOB mutation identified by exome sequencing cosegregates with steatosis, liver cancer, and hypocholesterolemia. Arterioscler Thromb Vasc Biol 2013;33:2021-2025.

175. Dwyer J, Li H, Xu D, Liu JP. Transcriptional regulation of telomerase activity: roles of the the Ets transcription factor family. Ann N Y Acad Sci 2007;1114:36-47.

176. Daniel M, Peek GW, Tollefsbol TO. Regulation of the human catalytic subunit of telomerase (hTERT). Gene 2012;498:135-146.

177. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-674.

178. Chaiteerakij R, Roberts LR. Telomerase mutation: a genetic risk factor for cirrhosis. Hepatology 2011;53:1430-1432.

179. Calado RT, Brudno J, Mehta P, Kovacs JJ, Wu C, Zago MA, Chanock SJ, et al. Constitutional telomerase mutations are genetic risk factors for cirrhosis. Hepatology 2011;53:1600-1607.

180. Rudolph KL, Chang S, Millard M, Schreiber-Agus N, DePinho RA. Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. Science 2000;287:1253-1258.

181. Valenti L, Dongiovanni P, Maggioni M, Motta BM, Rametta R, Milano M, Fargion S, et al. Liver transplantation for hepatocellular carcinoma in a patient with a novel telomerase mutation and steatosis. J Hepatol 2013;58:399-401.

182. Donati B, Pietrelli A, Pingitore P, Dongiovanni P, Caddeo A, Walker L, Baselli G, et al. Telomerase reverse transcriptase germline mutations and hepatocellular carcinoma in patients with nonalcoholic fatty liver disease. Cancer Med 2017;6:1930-1940.

183. Yanai H, Negishi H, Taniguchi T. The IRF family of transcription factors: Inception, impact and implications in oncogenesis. Oncoimmunology 2012;1:1376-1386.

184. Ysebrant de Lendonck L, Martinet V, Goriely S. Interferon regulatory factor 3 in adaptive immune responses. Cell Mol Life Sci 2014;71:3873-3883.

185. Taniguchi T, Takaoka A. A weak signal for strong responses: interferon-alpha/beta revisited. Nat Rev Mol Cell Biol 2001;2:378-386.

186. Kumar M, Liu H, Rice AP. Regulation of interferon-β by MAGI-1 and its interaction with influenza A virus NS1 protein with ESEV PBM. PLoS One 2012;7:e41251.

187. Honda K, Taniguchi T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol 2006;6:644-658.

188. Lin R, Mamane Y, Hiscott J. Structural and functional analysis of interferon regulatory factor 3: localization of the transactivation and autoinhibitory domains. Mol Cell Biol 1999;19:2465-2474.

189. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, et al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003;4:491-496.

190. McWhirter SM, Fitzgerald KA, Rosains J, Rowe DC, Golenbock DT, Maniatis T. IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. Proc Natl Acad Sci U S A 2004;101:233-238.

191. Servant MJ, Grandvaux N, Hiscott J. Multiple signaling pathways leading to the activation of interferon regulatory factor 3. Biochem Pharmacol 2002;64:985-992.

192. Chattopadhyay S, Marques JT, Yamashita M, Peters KL, Smith K, Desai A, Williams BR, et al. Viral apoptosis is induced by IRF-3-mediated activation of Bax. EMBO J 2010;29:1762-1773.

193. Dhanwani R, Takahashi M, Sharma S. Cytosolic sensing of immuno-stimulatory DNA, the enemy within. Curr Opin Immunol 2018;50:82-87.

194. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 2004;5:730-737.

195. Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, et al. Cell type-specific involvement of RIG-I in antiviral response. Immunity 2005;23:19-28.

196. Pichlmair A, Schulz O, Tan CP, Rehwinkel J, Kato H, Takeuchi O, Akira S, et al. Activation of MDA5 requires higher-order RNA structures generated during virus infection. J Virol 2009;83:10761-10769.

197. Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, et al. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol 2005;6:981-988.

198. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 2005;437:1167-1172.

199. Seth RB, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell 2005;122:669-682.

200. Zhang SY, Herman M, Ciancanelli MJ, Pérez de Diego R, Sancho-Shimizu V, Abel L, Casanova JL. TLR3 immunity to infection in mice and humans. Curr Opin Immunol 2013;25:19-33.

201. Häcker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, Kamps MP, et al. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature 2006;439:204-207.

202. Cavassani KA, Ishii M, Wen H, Schaller MA, Lincoln PM, Lukacs NW, Hogaboam CM, et al. TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events. J Exp Med 2008;205:2609-2621.

203. Aksoy E, Taboubi S, Torres D, Delbauve S, Hachani A, Whitehead MA, Pearce WP, et al. The p110δ isoform of the kinase PI(3)K controls the subcellular compartmentalization of TLR4 signaling and protects from endotoxic shock. Nat Immunol 2012;13:1045-1054.

204. Zanoni I, Ostuni R, Marek LR, Barresi S, Barbalat R, Barton GM, Granucci F, et al. CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. Cell 2011;147:868-880.

205. Ishii KJ, Coban C, Kato H, Takahashi K, Torii Y, Takeshita F, Ludwig H, et al. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. Nat Immunol 2006;7:40-48.

206. Paludan SR, Bowie AG. Immune sensing of DNA. Immunity 2013;38:870-880.

207. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, Lu Y, et al. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature 2007;448:501-505.

208. Parvatiyar K, Zhang Z, Teles RM, Ouyang S, Jiang Y, Iyer SS, Zaver SA, et al. The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response. Nat Immunol 2012;13:1155-1161.

209. Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, Sirois CM, et al. IFI16 is an innate immune sensor for intracellular DNA. Nat Immunol 2010;11:997-1004.

210. Ishikawa H, Ma Z, Barber GN. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature 2009;461:788-792.

211. Tanaka Y, Chen ZJ. STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway. Sci Signal 2012;5:ra20.

212. Burdette DL, Monroe KM, Sotelo-Troha K, Iwig JS, Eckert B, Hyodo M, Hayakawa Y, et al. STING is a direct innate immune sensor of cyclic di-GMP. Nature 2011;478:515-518.

213. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science 2013;339:786-791.

214. Ablasser A, Schmid-Burgk JL, Hemmerling I, Horvath GL, Schmidt T, Latz E, Hornung V. Cell intrinsic immunity spreads to bystander cells via the intercellular transfer of cGAMP. Nature 2013;503:530-534.

215. Mao Y, Luo W, Zhang L, Wu W, Yuan L, Xu H, Song J, et al. STING-IRF3 Triggers Endothelial Inflammation in Response to Free Fatty Acid-Induced Mitochondrial Damage in Diet-Induced Obesity. Arterioscler Thromb Vasc Biol 2017;37:920-929.

216. Petrasek J, Iracheta-Vellve A, Csak T, Satishchandran A, Kodys K, Kurt-Jones EA, Fitzgerald KA, et al. STING-IRF3 pathway links endoplasmic reticulum stress with hepatocyte apoptosis in early alcoholic liver disease. Proc Natl Acad Sci U S A 2013;110:16544-16549.

217. Qiao JT, Cui C, Qing L, Wang LS, He TY, Yan F, Liu FQ, et al. Activation of the STING-IRF3 pathway promotes hepatocyte inflammation, apoptosis and induces metabolic disorders in nonalcoholic fatty liver disease. Metabolism 2018;81:13-24.

218. Karpova AY, Ronco LV, Howley PM. Functional characterization of interferon regulatory factor 3a (IRF-3a), an alternative splice isoform of IRF-3. Mol Cell Biol 2001;21:4169-4176.

219. Marozin S, Altomonte J, Stadler F, Thasler WE, Schmid RM, Ebert O. Inhibition of the IFN-beta response in hepatocellular carcinoma by alternative spliced isoform of IFN regulatory factor-3. Mol Ther 2008;16:1789-1797.

220. Li C, Ma L, Chen X. Interferon regulatory factor 3-CL, an isoform of IRF3, antagonizes activity of IRF3. Cell Mol Immunol 2011;8:67-74.

221. Huang M, Jiang JD, Peng Z. Recent advances in the anti-HCV mechanisms of interferon. Acta Pharm Sin B 2014;4:241-247.

222. Seth RB, Sun L, Chen ZJ. Antiviral innate immunity pathways.

223. Zhao XJ, Dong Q, Bindas J, Piganelli JD, Magill A, Reiser J, Kolls JK. TRIF and IRF-3 binding to the TNF promoter results in macrophage TNF dysregulation and steatosis induced by chronic ethanol. J Immunol 2008;181:3049-3056.

224. Sanz-Garcia C, Poulsen KL, Bellos D, Wang H, McMullen MR, Li X, Chattopadhyay S, et al. The non-transcriptional activity of IRF3 modulates hepatic immune cell populations in acute-on-chronic ethanol administration in mice. J Hepatol 2019.

225. Reilly SM, Chiang SH, Decker SJ, Chang L, Uhm M, Larsen MJ, Rubin JR, et al. An inhibitor of the protein kinases TBK1 and IKK-ε improves obesity-related metabolic dysfunctions in mice. Nat Med 2013;19:313-321.

226. Oral EA, Reilly SM, Gomez AV, Meral R, Butz L, Ajluni N, Chenevert TL, et al. Inhibition of IKKε and TBK1 Improves Glucose Control in a Subset of Patients with Type 2 Diabetes. Cell Metab 2017;26:157-170.e157.

227. Kim TY, Lee KH, Chang S, Chung C, Lee HW, Yim J, Kim TK. Oncogenic potential of a dominant negative mutant of interferon regulatory factor 3. J Biol Chem 2003;278:15272-15278.

228. Yuan L, Mao Y, Luo W, Wu W, Xu H, Wang XL, Shen YH. Palmitic acid dysregulates the Hippo-YAP pathway and inhibits angiogenesis by inducing mitochondrial damage and activating the cytosolic DNA sensor cGAS-STING-IRF3 signaling mechanism. J Biol Chem 2017;292:15002-15015.

229. Santaguida S, Richardson A, Iyer DR, M'Saad O, Zasadil L, Knouse KA, Wong YL, et al. Chromosome Mis-segregation Generates Cell-Cycle-Arrested Cells with Complex Karyotypes that Are Eliminated by the Immune System. Dev Cell 2017;41:638-651.e635.

230. Mackenzie KJ, Carroll P, Martin CA, Murina O, Fluteau A, Simpson DJ, Olova N, et al. cGAS surveillance of micronuclei links genome instability to innate immunity. Nature 2017;548:461-465.

231. Yuan MM, Xu YY, Chen L, Li XY, Qin J, Shen Y. TLR3 expression correlates with apoptosis, proliferation and angiogenesis in hepatocellular carcinoma and predicts prognosis. BMC Cancer 2015;15:245.

232. Jiao S, Guan J, Chen M, Wang W, Li C, Wang Y, Cheng Y, et al. Targeting IRF3 as a YAP agonist therapy against gastric cancer. J Exp Med 2018;215:699-718.

233. Ding C, He J, Zhao J, Li J, Chen J, Liao W, Zeng Y, et al. β-catenin regulates IRF3-mediated innate immune signalling in colorectal cancer. Cell Prolif 2018;51:e12464.

234. Bishop RT, Marino S, de Ridder D, Allen RJ, Lefley DV, Sims AH, Wang N, et al. Pharmacological inhibition of the IKKε/TBK-1 axis potentiates the anti-tumour and anti-metastatic effects of Docetaxel in mouse models of breast cancer. Cancer Lett 2019;450:76-87.

235. Cheng C, Ji Z, Sheng Y, Wang J, Sun Y, Zhao H, Li X, et al. Aphthous ulcer drug inhibits prostate tumor metastasis by targeting IKKε/TBK1/NF-κB signaling. Theranostics 2018;8:4633-4648.

236. Liu Y, Lu J, Zhang Z, Zhu L, Dong S, Guo G, Li R, et al. Amlexanox, a selective inhibitor of IKBKE, generates anti-tumoral effects by disrupting the Hippo pathway in human glioblastoma cell lines. Cell Death Dis 2017;8:e3022.

237. Liver EAFTSOT, Cancer EOFRATO. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol 2012;56:908-943.

238. Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alföldi J, Wang Q, Collins RL, et al. Variation across 141,456 human exomes and genomes reveals the spectrum of loss-of-function intolerance across human protein-coding genes. bioRxiv 2019:531210.

239. Morgenthaler S, Thilly WG. A strategy to discover genes that carry multi-allelic or mono-allelic risk for common diseases: a cohort allelic sums test (CAST). Mutat Res 2007;615:28-56.

240. Rentzsch P, Witten D, Cooper GM, Shendure J, Kircher M. CADD: predicting the deleteriousness of variants throughout the human genome. Nucleic Acids Res 2019;47:D886-D894.

241. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 2010;26:589-595.

242. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009;25:2078-2079.

243. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 2010;26:841-842.

244. Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G, Levy-Moonshine A, Jordan T, et al. From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. Curr Protoc Bioinformatics 2013;43:11.10.11-33.

245. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010;20:1297-1303.

246. Danecek P, McCarthy SA. BCFtools/csq: haplotype-aware variant consequences. Bioinformatics 2017;33:2037-2039.

247. McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GR, Thormann A, Flicek P, et al. The Ensembl Variant Effect Predictor. Genome Biol 2016;17:122.

248. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 2010;38:e164.

249. Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, Handsaker RE, et al. The variant call format and VCFtools. Bioinformatics 2011;27:2156-2158.

250. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-1321.

251. Dongiovanni P, Petta S, Mannisto V, Mancina RM, Pipitone R, Karja V, Maggioni M, et al. Statin use and non-alcoholic steatohepatitis in at risk individuals. J Hepatol 2015;63:705-712.

252. Cunningham F, Amode MR, Barrell D, Beal K, Billis K, Brent S, Carvalho-Silva D, et al. Ensembl 2015. Nucleic Acids Res 2015;43:D662-669.

253. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15-21.

254. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 2011;12:323.

255. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.

256. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545-15550.

257. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst 2015;1:417-425.

258. Shen B, Zhang W, Zhang J, Zhou J, Wang J, Chen L, Wang L, et al. Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. Nat Methods 2014;11:399-402.

259. Chen N, Xia P, Li S, Zhang T, Wang TT, Zhu J. RNA sensors of the innate immune system and their detection of pathogens. IUBMB Life 2017;69:297-304.

260. Hillesheim A, Nordhoff C, Boergeling Y, Ludwig S, Wixler V. β-catenin promotes the type I IFN synthesis and the IFN-dependent signaling response but is suppressed by influenza A virus-induced RIG-I/NF-κB signaling. Cell Commun Signal 2014;12:29.

261. Iracheta-Vellve A, Petrasek J, Gyongyosi B, Satishchandran A, Lowe P, Kodys K, Catalano D, et al. Endoplasmic Reticulum Stress-induced Hepatocellular Death Pathways Mediate Liver Injury and Fibrosis via Stimulator of Interferon Genes. J Biol Chem 2016;291:26794-26805.

262. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc 2013;8:2281-2308.

APPENDIX – MAIN OTHER PROJECTS

A - Rare pathogenic variants predispose to Hepatocellular Carcinoma in NAFLD