Genetic and Epigenetic Profile of Patients With Alcoholic Liver Disease

Luigi Boccuto,* Ludovico Abenavoli**

* Greenwood Genetic Center, Greenwood, SC, USA. Clemson University School of Health Research, Clemson, SC, USA.
** Department of Health Sciences, University "Magna Græcia", Catanzaro, Italy.

ABSTRACT

Alcoholic liver disease (ALD) is a definition encompassing a spectrum of disorders ranging from simple steatosis to cirrhosis and hepatocellular carcinoma. Excessive alcohol consumption triggers a series of metabolic reactions that affect the liver by inducing lipogenesis, increasing oxidative stress, and causing abnormal inflammatory responses. The metabolic pathways regulating lipids, reactive oxygen species (ROS), and immune system are closely related and in some cases cross-regulate each other. Therefore, it must be taken into account that major genetic and epigenetic abnormalities affecting enzymes involved in one of such pathways can play a pivotal role in ALD pathogenesis. However, recent studies have pointed out how a significant predisposition can also be determined by minor variants, such as relatively common polymorphisms, epigenetic modifications, and microRNA abnormalities. Genetic and epigenetic factors can also affect the progression of liver diseases, promoting fibrogenesis, cirrhosis, and ultimately hepatocellular carcinoma. It is noteworthy that some of these factors, such as some of the cytokines involved in the abnormal inflammatory responses, are shared with non-alcoholic liver disease, while other factors are unique to ALD. The study of the genetic and epigenetic components involved in the liver damages caused by alcohol is crucial to identify individuals with high risk of developing ALD, design personalized protocols for prevention and/or treatment, and select the best molecular targets for new therapies.

Key words. Steatosis. Inflammation. Epigenetics. Translational research. microRNAs

INTRODUCTION

Alcohol consumption is responsible for 3.8% of global mortality and 4.6% of disability-adjusted life-years (DALYs) lost due to premature death.[1] Although the definition of a clear risk threshold for alcohol consumption is still debated, a meta-analysis found increased risks of mortality from liver cirrhosis among men and women drinking 12-24 g of ethanol per day.[2] The excessive consumption of alcoholic beverages exposes the liver cells to elevated levels of ethanol, causing a series of damages ultimately leading to a class of clinical conditions identified as alcoholic liver disease (ALD).[3]

ALD represents a wide spectrum of disorders comprising simple steatosis, alcoholic steatohepatitis (ASH), progressive fibrosis, cirrhosis and the development of hepatocellular carcinoma (HCC).[3] The pathogenesis of the liver damage follows a progressive course embracing all these disorders, but exceptions are possible, with patients eventually skipping one of the clinical stages. Up to 90% of heavy drinkers develop steatosis, but only a minority of those with steatosis progress to ASH and 10-20% eventually develop cirrhosis.[3,4] There is a dose-response relationship between the volume of alcohol consumed with the risk of ALD, and a population study has proved how subjects who consumed more than 120 g/day had the highest risk of cirrhosis, with a prevalence of 13.5%,[5] although it must be considered that the risk of cirrhosis is related to the length of time over which an individual has drunk regularly and not simply to the usual amount consumed.[6] However, it should be noted that no study has ever been able to distinguish between the effects of daily consumption from the effects of "binge"' (or compulsive) drinking.

In 2010, the burden of ALD resulted in 493,300 deaths and 14,544,000 DALYs, accounting for 0.9% of all deaths and 0.6% of all DALYs in that year.[1,7] These num-

bers highlight how ALD is the most prevalent cause of advanced liver disease and the leading cause of death among adults with excessive alcohol consumption.[1,3] The study of ALD incidence in different populations revealed several biases. In European cohorts, the deaths attributable to alcohol account for 11.0% and 1.8% of men and women, respectively.[1,6] The young account for a disproportionate amount of this disease burden, with an alcohol-associated mortality over 10% and 25% of female and male youth, respectively.[8] Another important difference was reported by a study on American populations, that showed how individuals of Hispanic origin have a greater risk of developing chronic liver disease than their European and African American counterparts.[9]

The pathogenic mechanisms underlying ALD involve multiple pathways and seem to converge into determining an inflammatory status in the liver (Figure 1). An increased susceptibility in women as compared to men has been noticed, particularly considering that it requires a lower amount of ethanol in female drinkers to determine liver damage, and suggests that other metabolic and molecular factors, yet to be identified, might play a role in the onset of ALD.[10] The information collected by epidemiologic studies indicates how individuals of different ethnicities, exposed to similar environmental contexts, show different ALD incidence, suggesting a potential role for genetic factors. Such role has been confirmed by twin studies, which have revealed a threefold higher disease concordance between monozygotic and dizygotic twins.[11,12]

GENETIC AND EPIGENETIC MECHANISMS IN ALD

Relevant genetic factors are usually copy number variants (CNVs) or single nucleotide polymorphisms (SNPs), that affect the function of one or more genes in a way that affects the probability of an individual to develop a certain trait, either increasing or decreasing it. Recently, it has been proven how epigenetic factors, can also affect the expression and function of several genes, including several ones involved in liver metabolism and cellular response to alcohol.[13] Identification of genetic and epigenetic factors can be important not only for the screening of individuals at risk, but also for the study of the pathogenic mechanisms underlying ALD.

The first evidence supporting the influence of genetic factors on ethanol effects on liver involved the alcohol metabolizing enzymes, alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH). Sequence polymorphisms in the genes encoding these preoteins have been associated with changes in enzyme kinetics. For example, the SNP rs1229984 in the *ADH1B* gene is found in 19-91% of East Asians, but in 0-10% of other populations.[14] Similarly, the SNP rs671 in ALDH2 is found in 30-50% of East Asians and is basically absent in other populations.[15] These variants affect production and removal of the toxic metabolite acetaldehyde, resulting in the development of unpleasant symptoms, such as flushing, nausea, vomiting, tachycardia, hypotension, dyspnea and headache, that
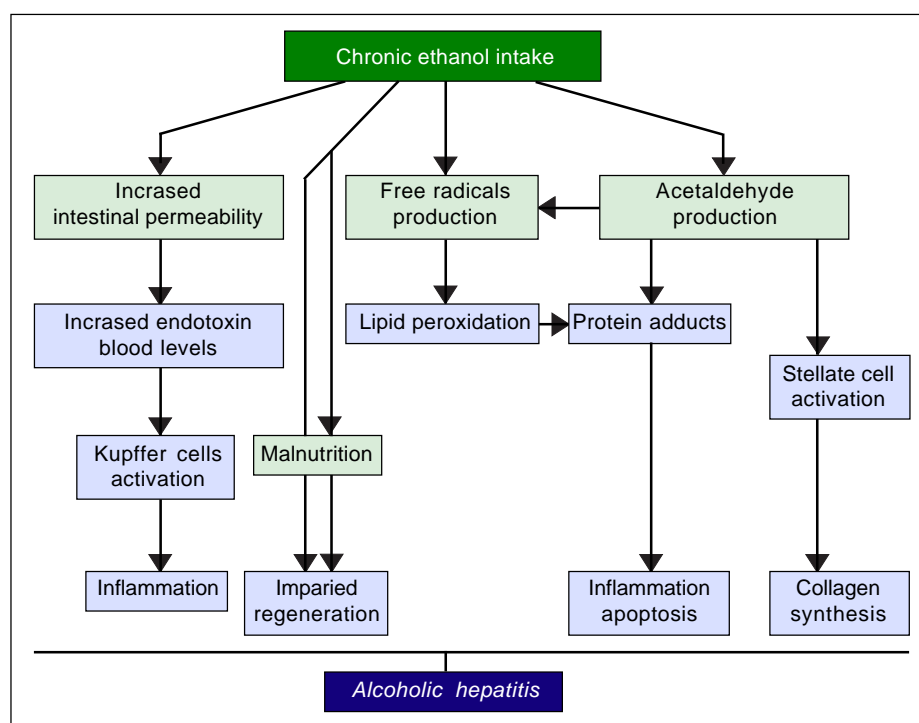

Figure 1. *Pathogenesis of ALD. The figure summarizes the main mechanisms through which chronic alcohol consumption may lead to an inflammatory status in the liver, which represents the early stage of ALD.*

492

Boccuto L, et al. Annals of Hepatology, 2017; 16 (4): 490-500

ultimately discourage the assumption of alcohol resulting in a protective effect.[16] Although to date there is still a lack of large-scale genome-wide association studies (GWAS) for ALD, some other candidate genes have emerged by several studies, such as *GABRB1*, *DRD4*, and *TH*, *PECR*, *ADH1C*, *LOC100507053*, *METAP*, and *PDLIM5*.[17-20] However, multiple studies suggest that one genetic variant in particular seems to play a pivotal role in the pathogenesis and progression of this spectrum of disorders: the rs738409 SNP in the patatin-like phospholipase domain containing 3 (*PNPLA3*) gene[21-24] (Figure 2). Interestingly, the same variant has also been associated with increased risk of nonalcoholic fatty liver disease and plasma liver enzyme levels by two independent GWAS studies.[25,26]

The *PNPLA3* gene and the metabolism of lipids

The *PNPLA3* gene (OMIM *609567) maps on chromosome 22q13.31, also know as adiponutrin, encodes a 481 amino acid protein with a molecular mass of approximately 53 kDa that in humans is mainly expressed in intracellular membrane fractions in hepatocytes, and is induced in the liver after feeding and during insulin resistance by the master regulator of lipogenesis sterol regulatory element binding protein-1c (SREBP1C).[27,28] The rs738409 SNP leads to a nucleotide transversion from a cytosine to a guanine at position 444 of the coding region (c.444C>G), in the third of the 9 exons of the gene. This change causes the substitution of the amino acid isoleucine at the
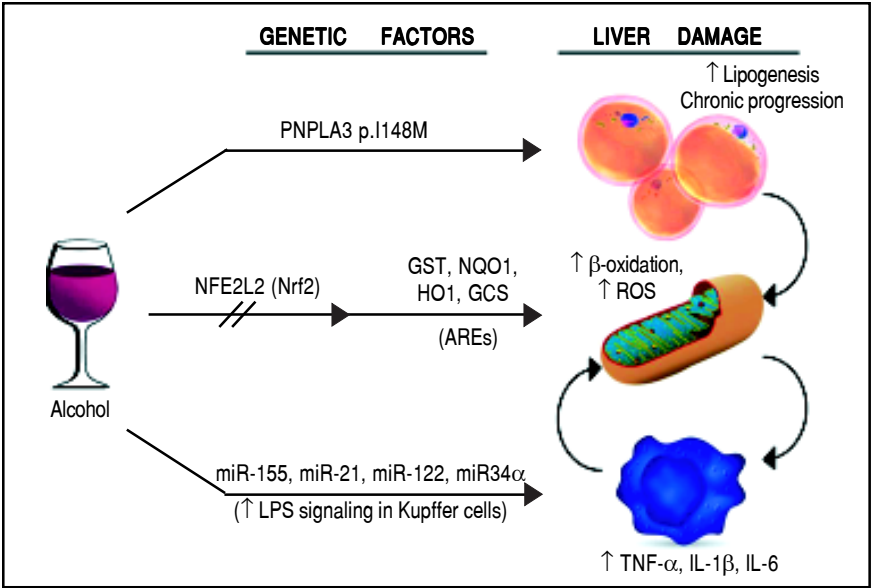
Figure 2. *Schematic representation of the genetic and epigenetic mechanisms involved in ALD. The liver damage mediated by ethanol intake, is mediated by three main pathways: lipid metabolism, oxidative stress, and inflammation. The* PNPLA3 *p.I148M SNP predisposes to increased lipogenesis and facilitates the progression of liver diseases to chronic stages. The* NFE2L2 *gene, encoding for the Nrf2, is activated by oxidative stress and regulates the activity of several antioxidant response elements (AREs). This epigenetic mechanism, based on the activation by phosphorylation of Nrf2, coordinates the cellular response to ROS. When exposed to ethanol, certain microRNAs show a positive correlation in up-regulation of the LPS signal in Kupffer cells, which in turns release the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6. This epigenetic mechanism links alcohol consumption to the inflammatory status in the liver that leads to hepatitis and steato-hepatitis.*

Table 1. Bioinformatic prediction of the effects of the *PNPLA3* p.Ile148Met variant.

Bioinformatic Website	Prediction	Website URL
CADD	deleterious	http://cadd.gs.washington.edu/
Condel	deleterious	http://bg.upf.edu/fannsdb/help/condel.html
Fathmm	tolerated	http://fathmm.biocompute.org.uk/
I-Mutant	decreased protein stability	http://folding.biofold.org/i-mutant/i-mutant2.0.html
iPTREE-STAB	destabilizing	http://210.60.98.19/IPTREEr/iptree.htm
MICO	deleterious	http://mico.ggc.org/MICO/
MUpro	deleterious	http://www.ics.uci.edu/~baldig/mutation.html
MuStab	destabilizing	http://bioinfo.ggc.org/mustab/
Mutation Assessor	medium effect	http://mutationassessor.org/
Mutation Taster	polymorphism	http://www.mutationtaster.org/
Panther	deleterious	http://www.pantherdb.org/
Provean	deleterious	http://provean.jcvi.org/index.php
PolyPhen-2	probably damaging	http://genetics.bwh.harvard.edu/pph2/index.shtml
SIFT	not tolerated - affected protein function	http://sift.bii.a-star.edu.sg/
SNPs&GO	neutral	http://snps-and-go.biocomp.unibo.it/snps-and-go/

Genetic and Epigenetic Profile of Patients With Alcoholic Liver Disease. Annals of Hepatology, 2017; 16 (4): 490-500

493

residue 148 of the protein with a methionine (p.Ile148 Met or p.I148M). Table 1 lists the predicted effects of the change on the *PNPLA3* protein, according to several bioinformatic platforms. Such platforms are designed to create computational models to calculate the impact of an amino acid change, considering parameters like evolutionary conservation of the protein residue and domain, biochemical interactions of the original and mutant amino acid, conformational stability of the protein, and frequency of the mutant allele in the general population. Overall, the vast majority of the calls (12 out of 15) indicate a potentially deleterious effect, while only 3 websites consider the p.I148M variant as benign or neutral.

Considering the impact on the protein function, it is relatively surprising how frequent the G allele is in the general population, and even more so in certain ethnicities. According to the 1,000 genomes project (http://www.internationalgenome.org/home), the G allele has a frequency of 26.2% (1.313 out of 5.008 total alleles tested), with a peak of 71.8% in the Peruvian population (122 out of 170 alleles), and in general a relatively high rate among individuals of Latin American origin (48.4%, 336/694). On the other hand, the sub-populations with the lowest rates of the minor G allele are composed by individuals of African origin (11.8%, 156/1.322 alleles), particularly the Luhya ethnicity (8.6%, 17/198). European individuals also show relatively low rates (22.6%, 227/1.006), with the lowest one detected in the Finnish population (17.2%, 34/198). The allelic frequency is naturally reflected in the genotype distribution: out of 2,508 individuals, 1424 carry the C/C genotype (56.9%), 847 are heterozygous (33.8%), and 233 are homozygous for the G allele (9.3%).

Similar frequencies emerge from other genomic databases, for example the Exome Variant Server (http://evs.gs.washington.edu/EVS/) reports 2540 G alleles out of 13.006 (19.5%), with a discrepancy between European American (22.1%) and African American individuals (14.5%). The genotype count lists 4,229 individuals with C/C (65%), 2.008 with C/G (30.9%), and 266 with G/G (4.1%). The ExAC Browser Beta database, created by Exome Aggregation Consortium (http://exac.broadinstitute.org/), reports 31.954 G alleles out of 121,386 (26.3%), with peaks of 57.2% in the Latino population (high) and 13.8% in the African one (low).

The fact that the genotype rates overlap the expected frequencies according to the Hardy-Weinberg model (54.5% for C/C, 38.7% for C/G, and 6.9% for G/G, using the 1,000 genome data) suggests that the p.I148M variant does not affect the genetic fitness. In other words, the change does not alter the chances of the carriers to reproduce, and therefore pass the mutant allele to the next generation. This could be explained with the fact that most of the conditions associated with ALD occur after the sec-

ond-third decade of life and their impact on life quality does not affect the possibility of an individual to procreate.

Wild-type (p.148I) PNPLA3 has lipolytic activity towards triglycerides.[27,28] The p.I148M mutation determines a critical aminoacidic substitution next to the catalytic domain, likely reducing the access of substrates and reducing the PNPLA3 enzymatic activity towards glycerolipids, thereby leading to the development of macrovesicular steatosis.[27,29] However, other reported a gain of lipogenic function associated with the 148M variant, which would acquire the ability to synthesize phosphatidic acid from lysophosphatidic acid.[30,31] In addition, results deriving from murine models gave contradictory results.[32-34] Human studies have also suggested a possible direct or indirect influence of *PNPLA3* genotype on adipose tissue biology, which however awaits replication.[35,36]

Other than in ALD, the lipogenic effect of the p.I148M variant appears to play a significant role in predisposing to multiple liver disorders, such as nonalcoholic fatty liver disease (NAFLD), nonalcoholic steato-hepatitis (NASH), chronic hepatitis C virus, primary sclerosing cholangitis.[37-41] It has also been shown that this substitution can promote disease progression from NAFLD to NASH, fibrosis progression in ALD, and favors hepatic carcinogenesis in several liver diseases.[22,42]

Oxidative stress and antioxidant response elements

Chronic exposure to ethanol increases the production of reactive oxygen species (ROS), lowers cellular levels of antioxidants, and enhances oxidative stress in several tissues, particularly the liver.[43] Ethanol is first metabolized in the liver by alcohol dehydrogenase, but when this system reaches saturation, like during chronic alcohol consumption, the cytochrome P450 2E1 (CYP2E1) gets involved, leading to the production of ROS, free radicals, and, most importantly, acetaldehyde, a highly toxic intermediate and carcinogen, which is in turn metabolized by aldehyde dehydrogenase to acetate.[44,45] The detoxification process of acetaldehyde is associated to an array of antioxidant mechanisms, regulated by nuclear erythroid 2-related factor 2 (Nrf2), a member of the cap-n-collar basic leucine family of transcription factors.[46] In its inactive state, Nrf2 interacts in the cytoplasm with the actin binding protein, Kelch-like ECH-associating protein 1 (Keap1), and is rapidly degraded by the ubiquitin-proteasome pathway. However, upon exposure to oxidative or electrophilic stress, phosphorylation of Nrf2 leads to its dissociation from Keap1, allowing its subsequent translocation into the nucleus.[45,47] Once there, Nrf2 binds to antioxidant response elements (AREs), which are particular genomic

sequences, that work as targets for molecular messages released by the cells in response to oxidative stress (Figure 2). The Nrf2/AREs functions in partnership with other nuclear proteins, as a strong transcriptional activator of ARE-responsive genes, encoding antioxidant proteins and enzymes such the ones (Table 2): heme oxygenase-1 (HO-1) NAD(P)H; quinone oxidoreductase 1 (NQO1); glutathione-S-transferases (GST); and group C streptococcus (GCS).[45]

It has also been proven in mouse models that the oxidative stress induced by ethanol via CYP2E1 upregulates Nrf2 activity, which in turn regulates ethanol induction of the cytochrome P450 2A5 (CYP2A5) and protects against alcohol-induced steatosis.[48] Chronic ethanol administration in *Nrf2*-knockout mice significantly increased mortality associated with liver failure.[49] The loss of Nrf2 caused a reduced ability to detoxify acetaldehyde, leading to its accumulation, marked steatosis and inflammatory response mediated by Kupffer cells, with consequent depletion of glutathione, one of the most effective antioxidants in the mitochondria. The glutathione deficiency determines an increase in the level of ROS generated by highly energetic processes occurring in the mitochondria, such as the β-oxidation of fatty acids, and ultimately leads to structural and functional changes to mitochondria.[49] In order to further prove the key role of the Nrf2/AREs pathway in

Table 2. Epigenetic mechanisms involved in ALD.

Gene/Protein	Epigenetic mechanism	Pathway	Effect upon exposure to alcohol	References
NFE2L2/Nrf2	Activation by phosphorylation elements (AREs)	Antioxidant response oxygen species (ROS)	Cellular protection from reactive	45, 46, 47, 48, 49
miR-212, miR-122	Micro-RNA regulation of gene expression	Tight junction proteins in the zonula occludens-1 (ZO-1)	Increased gut permeability to lipopolysaccharide (LPS)	52, 53
miR-155, miR-21	Micro-RNA regulation of gene expression	NF-kB pathway	Induction in macrophages and Kupffer cells of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and repression of anti-inflammatory cytokines (IL-10)	53, 54,55, 56
miR-122	Micro-RNA regulation of gene expression	Cell cycle	Down-regulation: decreased inhibition of genes promoting cell proliferation	58
miR-34	Micro-RNA regulation of gene expression	p53-mediated apoptosis	Up-regulation: reduced apoptosis	59
SIRT1 / sirtuin	Alternative mRNA splicing	Sirtuin/ SFRS10/Lipin-1 pathway	Decreased Sirtuin: increased lipin-1β/α ratio, associated with increased expression of lipogenic genes and excessive fat accumulation in the liver, activation of stellate cells resulting in collagen deposition, and increased inflammatory response mediated by the activation of inflammatory genes by the NFATC4 transcription factor	61, 62, 63, 64, 65, 66
Multiple genes	Histone acetylation	Epigenetic regulation of gene expression	Increased gene-selective levels of histone H3 acetylation at lysine 9 (H3K9): pro-inflammatory shift in immune system	67, 68, 69, 70, 71, 72, 73, 74, 75

Genetic and Epigenetic Profile of Patients With Alcoholic Liver Disease. Annals of Hepatology, 2017; 16 (4): 490-500

495

cellular response to oxidative stress, Wu, *et al*. demonstrated how activating Nrf2 through *Keap1* knockdown and hepatocyte-specific knockout mice blunted the increase in liver levels of serum triglyceride and hepatic-free fatty acid following the exposure to ethanol.[50]

Inflammation and micro-RNAs

Alcohol assumption increases gut permeability and translocation of bacterial products, such as lipopolysaccharide (LPS), also known as endotoxin, into the intestinal and, subsequently, portal circulation.[51] Such process involves several genetic and epigenetic effectors: ethanol directly induces an increase in the expression of the miR-212 microRNA, which contributes to the loss of tight junction proteins in the zonula occludens-1 (ZO-1) of the endothelial cells of the intestinal lumen.[52] The ZO-1 proteins are also targeted by another microRNA whose expression is influenced by the levels of circulating ethanol, *miR-122*, and the tight junctions are also weakened by the ethanol metabolite, acetaldehyde[53] (Table 2). Once the LPS reaches the liver, it binds to the toll-like receptor 4 (TLR4) on the surface of the Kupffer cells via lipopolysaccharide binding protein (LBP). Such binding activates the Kupffer cells via a signalling cascade that includes CD14, MyD88, MD-2, and finally results in the activation of mitogen-activated protein kinases (such as ERK1, ERK2, JNK, and p38), NF-κB, and AP-1.54 The binding of LPS to TLR4 induces also an increase in the expression of the microRNAs *miR-155* and *miR-21*, which activate the NF-κB pathway. This cascade effect promotes the production in macrophages, in particular in Kupffer cells, of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, while represses the expression of anti-inflammatory cytokines, such as IL-10[53] (Figure 2). It has been proven that microRNAs *miR-125b*, *miR-146a*, and *miR-155* can regulate the inflammatory response to the TNF-α induced by LPS in Kupffer cells, and that miR-155 contributes to alcohol-induced activation of TNF-α in macrophages from patients with ALD.[55,56] Abnormal expression levels of several microRNAs have been reported in different liver diseases, generating specific expression profiles for chronic hepatitis, liver fibrosis, and hepatocellular carcinoma.[57] If we consider ALD alone, the expression levels of microRNAs, such as *miR-155*, *miR-21*, *miR-34a*, and *miR-486*, have been reported as increased, other microRNAs, like *miR-125b*, *miR181a*, and *let-7b*, appear to be decreased, while the liver-specific microRNA *miR-122* has been reported both as increased and decreased, probably depending on the stage of the disease.[53] Two of these microRNAs, *miR-122* and *miR-34a*, produce opposite effects on cell cycle: *miR-122* inhibits genes promoting cell proliferation, while miR-34a

is a critical regulator of p53-mediated apoptosis.[53,58,59] Exposure to ethanol, in both mice and humans, induces up-regulation of *miR-34a* and down-regulation of *miR-122*, ultimately promoting the abnormal cell survival and proliferation of hepatocytes observed in ALD53 (Table 2).

One of the target genes of *miR-34a* is the silent mating type information regulation 2 homolog 1 (SIRT1), encoding the sirtuin protein.[60] Sirtuin in involved in a complex pathway regulating β-oxidation of fat acids and lipogenesis, in which the key role is played by lipin-1α. This protein functions both as an Mg^{2+}-dependent phosphatidic acid phosphohydrolase in the triglyceride synthesis pathway and as transcriptional co-activator to promote fat oxidation and suppress *de novo* lipogenesis.[61-63] Lipin-1 is encoded by the LPIN1 gene and has two major isoforms in hepatocytes, depending on an alternative mRNA splicing: lipin-1α, lacking exon 6 and located in the nucleus, and lipin-1β, containing exon 6, located in the cytoplasm, and associated with increased expression of lipogenic genes and excessive fat accumulation in the liver of animal models.[61,64-66] Sirtuin appears to regulate *LPIN1* splicing via SFRS10, a member of the SR-like protein family of splicing factors, whose expression is decreased in livers of both mice and humans exposed to high-fat diet.[66] Reduced levels of sirtuin have been reported in patients with ALD and the resulting effects have been investigated by Yin, *et al*. in Sirt1-KO mouse models:[61] the decreased expression of SFRS10 causes an increase in the ration between the β and α isoforms of lipin-1, leading to triglyceride accumulation, activation of stellate cells resulting in collagen deposition, and increased inflammatory response mediated by the activation of inflammatory genes by the NFATC4 transcription factor (Table 2). The overall result resembles the histopathological presentation of steatohepatitis in humans, with fatty liver, mild inflammation, and fibrosis. The authors also noted that deletion of *Sirt1* exacerbates the effects of chronic-binge ethanol assumption on mouse liver and the oxidative stress in hepatocytes.[61]

Other than on microRNAs, ethanol has been proven to have several deleterious effects on epigenetic regulation: increased gene-selective levels of histone H3 acetylation at lysine 9 (H3K9) in liver, lungs, spleen, and testes, a phenomenon that seems to be associated with chronic but not acute consumption, increased levels of enzymes mediating histone acetylation, and a generalized increase in DNA methylation.[67-73] Once again, these epigenetic-mediated effects of ethanol consumption seem to point primarily to an exacerbation of the inflammatory response, especially considering that a key pro-inflammatory cytokine, such as TNF-α, is silenced by H3K9 methylation and activated by H3K9 acetylation.[74] The epigenetic-mediated consequences of chronic alcohol abuse on the immune system affect more than just liver macrophages and Kupffer cells and

496

Boccuto L, et al. ANNALS of Hepatology, 2017; 16 (4): 490-500

include effects specific to certain tissues and certain immune cell-types, such as influencing cell recruitment to infected or inflamed tissue, altering cytokine and chemokine production and secretion, skewing differentiation towards a particular cell fate or preventing cell replication, impairing antigen presentation, interfering with phagocytosis and granulopoiesis, or inducing apoptosis.[74,75] The ultimate results of ethanol-induced immune dysregulation are increased susceptibility to infection, excessive innate immune response, elevated oxidative stress, and exacerbated or prolonged inflammation due to a skewed macrophage polarization toward the pro-inflammatory M1 phenotype determined by increased production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IFNγ, impairment of histone methylation/acetylation, and promotion of the Th2 lineage specification of the T-helper population of lymphocytes[74,75] (Table 2).

In an effort to better characterize the role of innate immune signalling in liver disorders, Petrasek et al. were able to identify differences in ASH versus NASH, as well as pathogenic features shared by both conditions.[76] The common traits include a central role played by Kupffer cells, an increase of Gram-negative bacteria in the intestinal lumen, and the beneficial effect of intestinal sterilization on decreasing LPS levels, liver inflammation and fibrosis. In patients with ASH the TLR-4 signalling is mediated via the TRIF/IRF3-dependent and MyD88-independent pathway, while in NASH cases is mediated by the MyD88-dependent pathway.[76] Moreover, while in ASH IL-1β plays a critical role, the activation of the inflammasome represents an early event and its activation is specific to bone marrow-derived Kupffer cells, in NASH inactivation of IL-1β protects from steatosis but not from liver damage, the inflammasome is activated later than in ASH and includes hepatocytes in addition to Kupffer cells.[76]

OVERVIEW

The study of the pathogenic mechanisms in ALD highlights three main pathways in which genetic and/or epigenetic variants can affect an individual's response to ethanol consumption: lipid metabolism, oxidative stress, and immune system. Even if these pathways seem to be quite diverse and distinct for functions, tissues, and substrates, they are connected by an intricate network of tight interactions, as proven in several experimental models.[54,77] Further studies will be necessary to validate in humans many of the pathogenic mechanisms proved in such experimental models, and it is highly probable that other pathways will be linked to ALD pathogenesis in the future. However, the evidence collected so far is sufficient to justify a prominent role for lipid metabolism, oxidative

stress, and immune system in the onset and progression of ALD. Some effects of alcohol consumption are systemic, such as the promotion of the inflammatory response,[64] some others are tissue-specific, like the reduced β-oxidation of fatty acids and increased lipogenesis in the liver.[21,22,54] Effects occur also in a relatively short time span, like the increased gut permeability to LPS, some others require a multiple-step apparatus and have a later onset, such as the collagen deposition mediated by the activated stellate cells.[51] However, it would be a mistake to evaluate each effect individually, even if it affects apparently independent pathways: ethanol operates at the same time on its multiple targets and its effects are often amplified by the cascade effects linking the involved pathways (Figure 2). Both the abnormal lipid metabolism and the pro-inflammatory switch in the immune system increase the production of ROS, which overload mitochondrial activity inducing oxidative stress, leading to tissue damage and ultimately promoting the inflammatory status and the chronic progression to liver disorders from steatosis to steatohepatitis and eventually cirrhosis and fibrosis.[22,54,77]

Genetic/epigenetic factors can play a role in each of the pathways involved in ALD pathogenesis either by protecting against ethanol effects or exacerbating them. Examples of the first option are the Nrf2/AREs system, regulating the antioxidant response to acetaldehyde, or the SIRT1/SFRS10/Lipin-1 pathway, controlling the expression of lipogenic genes and preventing excessive lipid accumulation in the liver. Constitutive genetic variants can affect proteins involved in these pathways by either decreasing their expression levels and/or disrupting their function. Although there has not been any report yet of patients with ALD carrying loss-of-function mutations in these pathways, it cannot be ruled out the gene dosage effect of multiple variants that are individually considered of unknown significance. Several studies corroborate this hypothesis showing how removing these protective systems in mouse models produce more severe effects after exposure to alcohol.[44,49,61] On the other hand, the best documented genetic factor to ALD, the *PNPLA3* p.Ile148Met change, represents the typical example of gain-of function variant, in which a carrier does not lack a protective system, but rather possesses a congenital predisposition to amplify the metabolic damage triggered by ethanol.[21-24,36] In both cases, in order to better understand the genotype/phenotype correlation in ALD, it is mandatory to improve our knowledge of these pathways and learn how they interact with other cellular functions.

FUTURE PERSPECTIVES

In the era of personalized medicine, the identification of genetic factors predisposing to ALD will have a tre-

mendous impact on the management of this disorder. The most immediate and direct application would be providing biomarkers for the early screening of individuals at risk in the general population to be enrolled in monitoring programs, based on dietary surveillance and/or prophylactic treatment. Genetic screening for the pIle148Met variant in *PNPLA3*, for example, would be a relative quick and inexpensive service to provide to individuals with positive family history for ALD, and would orient significantly their future clinical management, since this variant can affect not only the onset of the disorder but also its progression.

Another potential application is represented by the identification of potential therapeutic targets. Currently, the treatment of ALD is based on alcohol abstinence, medical treatment (e.g. corticosteroids, prednisolone, pentoxifylline) in the presence of severe liver damage, and liver transplantation in presence of advanced liver disease and/or liver insufficiency.[3,78] New approaches have been explored recently and have brought several clinical trials for targeted treatment of alcoholic hepatitis and alcoholic cirrhosis.[44] However, a more detailed evaluation of the genetic background will allow the physicians to design the best therapeutic approach for each individual with ALD. For example, targeting the microRNAs involved in the pro-inflammatory switch in macrophages and Kupffer cells will be more efficient and than a steroid-based treatment and will bare less side effects. Moreover, it has to be considered that epigenetic factors can be manipulated by environmental factors, such as diet and medications, and therefore represent an ideal target for genetic therapy.

Including the genetic and epigenetic factors in the design of tailored therapeutic protocols will not only guarantee better results in the treatment of ALD, but will also provide new and more powerful tools for prophylaxis and prevention, resulting in an improvement of both quality and quantity of life for many people.

ABBREVIATIONS

- **ADH:** alcohol dehydrogenase.
- **ALD:** alcoholic liver disease.
- **ALDH:** acetaldehyde dehydrogenase.
- **AREs:** antioxidant response elements.
- **ASH:** alcoholic steatohepatitis.
- **CNVs:** copy number variants.
- **CYP2A5:** cytochrome P450 2A5.
- **CYP2E1:** cytochrome P450 2E1.
- **DALYs:** disability-adjusted life-years.
- **GCS:** group C streptococcus.
- **GST:** glutathione-S-transferases.
- **GWAS:** genome-wide association studies.

- **H3K9:** histone H3 acetylation at lysine 9.
- **HCC:** epatocellular carcinoma.
- **IL:** interleukin.
- **Keap1:** Kelch-like ECH-associating protein 1.
- **LBP:** lipopolysaccharide binding protein.
- **LPS:** lipopolysaccharide.
- **NAD(P)H:** heme oxygenase-1 (HO-1).
- **NAFLD:** nonalcoholic fatty liver disease.
- **NASH:** nonalcoholic steato-hepatitis.
- **NQO1:** quinone oxidoreductase 1.
- **Nrf2:** nuclear erythroid 2-related factor 2.
- **PNPLA3:** patatin-like phospholipase domain containing 3.
- **ROS:** reactive oxygen species.
- **SIRT1:** silent mating type information regulation 2 homolog 1.
- **SNPs:** single nucleotide polymorphisms.
- **SREBP1C:** sterol regulatory element binding protein-1c.
- **TLR4:** toll-like receptor 4.
- **TNF-α:** tumor necrosis factor-α.
- **ZO-1:** zonula occludens 1.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

REFERENCES

1. Masarone M, Rosato V, Dallio M, Abenavoli L, Federico A, Loguercio C, Persico M. Epidemiology and natural history of alcoholic liver disease. *Rev Recent Clin Trials* 2016; 11: 167-74.
2. Roerecke M, Nanau R, Rehm J, Neuman M. Ethnicity matters: A Systematic Review and Meta-Analysis of the Non-Linear Relationship Between Alcohol Consumption and Prevalence and Incidence of Hepatic Steatosis. *EBioMedicine* 2016; 8: 317-30.
3. Abenavoli L, Masarone M, Federico A, Rosato V, Dallio M, Loguercio C, Persico M. Alcoholic hepatitis: pathogenesis, diagnosis and treatment. *Rev Recent Clin Trials* 2016; 11: 159-66.
4. Mathurin P, Bataller R. Trends in the management and burden of alcoholic liver disease. *J Hepatol* 2015; 62: S38-46.
5. Bellentani S, Saccoccio G, Costa G, Tiribelli C, Manenti F, Sodde M, Crocè L, et al. Drinking habits as cofactors of risk for alcohol induced liver damage. The Dionysos Study Group. *Gut* 1997; 41: 845-50.
6. European Association for the Study of Liver. EASL clinical practical guidelines: management of alcoholic liver disease. *J Hepatol* 2012; 57: 399-420.

7. Rehm J, Samokhvalov AV, Shield KD. Global burden of alcoholic liver diseases. *J Hepatol* 2013; 59: 160-8.
8. Dwyer-Lindgren L, Flaxman AD, Ng M, Hansen GM, Murray CJ, Mokdad AH. Drinking patterns in US Countries from 2002 to 2012. *Am J Public Health* 2015; 105: 1120-7.
9. Flores YN, Yee HF Jr, Leng M, Escarce JJ, Bastani R, Salmerón J, Morales LS. Risk factors for chronic liver disease in Blacks, Mexican Americans, and Whites in the United States: results from NHANES IV, 1999-2004. *Am J Gastroenterol* 2008; 103; 2231-8.
10. Stokkeland K, Hilm G, Spak F, Franck J, Hultcrantz R. Different drinking patterns for women and men with alcohol dependence with and without alcoholic cirrhosis. *Alcohol Alcohol* 2008; 43: 39-45.
11. Stickel F, Hampe J. Genetic determinants of alcoholic liver disease. Gut 2012; 61: 150-9.
12. Stickel F, Moreno C, Hampe J, Morgan MY. The genetics of alcohol dependence and alcohol-related liver disease. *J Hepatol* 2017; 66: 195-211.
13. Anstee QM, Seth D, Day CP. Genetic Factors That Affect Risk of Alcoholic and Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2016; 150: 1728-44.
14. Goedde HW, Agarwal DP, Fritze G, Meier-Tackmann D, Singh S, Beckmann G, et al. Distribution of ADH2 and ALDH2 genotypes in different populations. *Hum Genet* 1992; 88: 344-6.
15. Eng MY, Luczak SE, Wall TL. ALDH2, ADH1B, and ADH1C genotypes in Asians: a literature review. *Alcohol Res Health* 2007; 30: 22-7.
16. Hurley TD, Edenberg HJ. Genes encoding enzymes involved in ethanol metabolism. *Alcohol Res* 2012; 34: 339-44.
17. Stickel F, Moreno C, Hampe J, Morgan MY. The genetics of alcohol dependence and alcohol-related liver disease. *J Hepatol* 2017; 66: 195-211.
18. Long JC, Knowler WC, Hanson RL, Robin RW, Urbanek M, Moore E, et al. Evidence for genetic linkage to alcohol dependence on chromosomes 4 and 11 from an autosome-wide scan in an American Indian population. *Am J Med Genet* 1998; 81: 216-21.
19. Treutlein J, Cichon S, Ridinger M, Wodarz N, Soyka M, Zill P, Maier W, et al. Genomewide association study of alcohol dependence. *Arch Gen Psychiatry;* 2009; 66: 773-84.
20. Gelernter J, Kranzler HR, Sherva R, Almasy L, Koesterer R, Smith AH, Anton R, et al. Genome-wide association study of alcohol dependence: significant findings in African-and European-Americans including novel risk loci. *Mol Psychiatry* 2014; 19: 41-9.
21. Tian C, Stokowski RP, Kershenobich D, Ballinger DG, Hinds DA. Variant in PNPLA3 is associated with alcoholic liver disease. *Nat Genet* 2010; 42: 21-3.
22. 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: 6969-78.
23. 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.
24. Trepo E, Gustot T, Degre D, Lemmers A, Verset L, Demetter P, Ouziel R, et al. Common polymorphism in the PNPLA3/adiponutrin gene confers higher risk of cirrhosis and liver damage in alcoholic liver disease. *J Hepatol* 2011; 55: 906-12.
25. 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: 1461-5.
26. 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: 520-8.
27. He S, McPhaul C, Li JZ, Garuti R, Kinch L, Grishin NV, Cohen JC, et al. A sequence variation (I148M) in PNPLA3 associated with nonalcoholic fatty liver disease disrupts triglyceride hydrolysis. *J Biol Chem* 2010; 285: 6706-15.
28. Huang Y, He S, Li JZ, Seo YK, Osborne TF, Cohen JC, Hobbs HH. A feed-forward loop amplifies nutritional regulation of PNPLA3. *Proc Natl Acad Sci USA* 2010; 107: 7892-7.
29. Pirazzi C, Adiels M, Burza MA, Mancina RM, Levin M, Ståhlman M, Taskinen MR, et al. Patatin-like phospholipase domain-containing 3 (PNPLA3) I148M (rs738409) affects hepatic VLDL secretion in humans and in vitro. *J Hepatol* 2012; 57: 1276-82.
30. 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.
31. Basantani MK, Sitnick MT, Cai L, Brenner DS, Gardner NP, Li JZ, Schoiswohl G, et al. Pnpla3/Adiponutrin deficiency in mice does not contribute to fatty liver disease or metabolic syndrome. *J Lipid Res* 2011; 52: 318-29.
32. Li JZ, Huang Y, Karaman R, Ivanova PT, Brown HA, Roddy T, Castro-Perez J, et al. Chronic overexpression of PNPLA3I148M in mouse liver causes hepatic steatosis. *J Clin Invest* 2012; 122: 4130-44.
33. Kumashiro N, Yoshimura T, Cantley JL, Majumdar SK, Guebre-Egziabher F, Kursawer R, Vatner DF, et al. Role of patatin-like phospholipase domain-containing 3 on lipid-induced hepatic steatosis and insulin resistance in rats. *Hepatology* 2013; 57: 1763-72.
34. Chen W, Chang B, Li L, Chan L. Patatin-like phospholipase domain-containing 3/adiponutrin deficiency in mice is not associated with fatty liver disease. *Hepatology* 2010; 52: 1134-42.
35. Santoro N, Kursawe R, D'Adamo E, Dykas DJ, Zhang CK, Bale AE, Calì AM, et al. A common variant in the patatin-like phospholipase 3 gene (PNPLA3) is associated with fatty liver disease in obese children and adolescents. *Hepatology* 2010; 52: 1281-90.
36. Valenti L, Rametta R, Ruscica M, Dongiovanni P, Steffani L, Motta BM, Canevesi E, et al. The I148M PNPLA3 polymorphism influences serum adiponectin in patients with fatty liver and healthy controls. *BMC Gastroenterol* 2012; 12: 111.
37. Ravi Kanth VV, Sasikala M, Sharma M, Rao PN, Reddy DN. Genetics of non-alcoholic fatty liver disease: From susceptibility and nutrient interactions to management. *World J Hepatol* 2016; 8: 827-37.
38. Valenti L, Al-Serri A, Daly AK, Galmozzi E, Rametta R, Dongiovanni P, Nobili V, et al. Homozygosity for the patatin-like phospholipase-3/adiponutrin I148M polymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease. *Hepatology* 2010; 51: 1209-17.
39. 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: 791-9.
40. Rembeck K, Maglio C, Lagging M, Christensen PB, Färkkilä M, Langeland N, Buhl MR, et al. PNPLA 3 I148M genetic variant associates with insulin resistance and baseline viral load in HCV genotype 2 but not in genotype 3 infection. *BMC Med Genet* 2012; 13: 82.

41. Friedrich K, Rupp C, Hov JR, Steinebrunner N, Weiss KH, Stiehl A, Brune M, et al. A frequent PNPLA3 variant is a sex specific disease modifier in PSC patients with bile duct stenosis. *PloS One* 2013; 8: e58734.

42. Fujii H, Kawada N. Fibrogenesis in alcoholic liver disease. *World J Gastroenterol* 2014; 20: 8048-54.

43. Wu D, Cederbaum AI. Oxidative stress and alcoholic liver disease. *Semin Liver Dis* 2009; 29: 141-54.

44. Shin SM, Yang JH, Ki SH. Role of the Nrf2-ARE pathway in liver diseases. *Oxid Med Cell Longev* 2013; 2013: 763257.

45. Tang W, Jiang YF, Ponnusamy M, Diallo M. Role of Nrf2 in chronic liver disease. *World J Gastroenterol* 2014; 20: 13079-87.

46. Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc Natl Acad Sci USA* 1994; 91: 9926-30.

47. Li W, Kong AN. Molecular mechanisms of Nrf2-mediated antioxidant response. *Mol Carcinog* 2009; 48: 91-104.

48. Lu Y, Zhang XH, Cederbaum AI. Ethanol induction of CYP2A5: role of CYP2E1-ROS-Nrf2 pathway. *Toxicol Sci* 2012; 128: 427-38.

49. Lamle J, Marhenke S, Borlak J, von Wasielewski R, Eriksson CJ, Geffers R, Manns MP, et al. Nuclear factor-eythroid 2-related factor 2 prevents alcohol-induced fulminant liver injury. *Gastroenterology* 2008; 134: 1159-68.

50. Wu KC, Liu J, Klaassen CD. Role of Nrf2 in preventing ethanol-induced oxidative stress and lipid accumulation. *Toxicol Appl Pharmacol* 2012; 262: 321-9.

51. Scarpellini E, Forlino M, Lupo M, Rasetti C, Fava G, Abenavoli L, De Santis A. Gut microbiota and alcoholic liver disease. *Rev Recent Clin Trials* 2016; 11: 213-19.

52. Bala S, Szabo G. MicroRNA signature in alcoholic liver disease. *Int J Hepatol* 2012; 2012: 498232.

53. McDaniel K, Herrera L, Zhou T, Francis H, Han Y, Levine P, Lin E, et al. The functional role of microRNAs in alcoholic liver injury. *J Cell Mol Med* 2014; 18: 197-2.

54. Louvet A, Mathurin P. Alcoholic liver disease: mechanisms of injury and targeted treatment. *Nat Rev Gastroenterol Hepatol* 2015; 12: 231-42.

55. Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD. MicroRNAs: new regulators of immune cell development and function. *Nat Immunol* 2008; 9: 839-45.

56. Bala S, Marcos M, Kodys K, Csak T, Catalano D, Mandrekar P, Szabo G. Up-regulation of microRNA-155 in macrophages contributes to increased tumor necrosis factor {alpha} (TNF{alpha}) production via increased mRNA half-life in alcoholic liver disease. *J Biol Chem* 2011; 286: 1436-44.

57. Wang XW, Heegaard NH, Orum H. MicroRNAs in liver disease. *Gastroenterology* 2012; 142: 1431-3.

58. Girard M, Jacquemin E, Munnich A, Lyonnet S, Henrion-Caude A. miR-122, a paradigm for the role of microRNAs in the liver. *J Hepatol* 2008; 8: 648-56.

59. Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N, Bentwich Z, et al. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell* 2007; 26: 731-43.

60. Lee J, Padhye A, Sharma A, Song G, Miao J, Mo YY, Wang L, et al. A pathway involving farnesoid X receptor and small heterodimer partner positively regulates hepatic sirtuin 1 levels via microRNA-34a inhibition. *J Biol Chem* 2010; 285: 12604-11.

61. Yin H, Hu M, Liang X, Ajmo JM, Li X, Bataller R, Odena G, et al. Deletion of SIRT1 from hepatocytes in mice disrupts lipin-1 signaling and aggravates alcoholic fatty liver. *Gastroenterology* 2014; 146: 801-11.

62. Harris TE, Finck BN. Dual function lipin proteins and glycerolipid metabolism. *Trends Endocrinol Metab* 2011; 22: 226-33.

63. Peterfy M, Phan J, Xu P, Reue K. Lipodystrophy in the fld mouse results from mutation of a new gene encoding a nuclear protein, lipin. *Nat Genet* 2001; 27: 121-4.

64. Yin H, Hu M, Zhang R, Shen Z, Flatow L, You M. MicroRNA-217 promotes ethanol-induced fat accumulation in hepatocytes by down-regulating SIRT1. *J Biol Chem* 2012; 287: 9817-26.

65. Bou Khalil M, Sundaram M, Zhang HY, Links PH, Raven JF, Manmontri B, Sariahmetoglu M, et al. The level and compartmentalization of phosphatidate phosphatase-1 (lipin-1) control the assembly and secretion of hepatic VLDL. *J Lipid Res* 2009; 50: 47-58.

66. Pihlajamaki J, Lerin C, Itkonen P, Boes T, Floss T, Schroeder J, Dearie F, et al. Expression of the splicing factor gene SFRS10 is reduced in human obesity and contributes to enhanced lipogenesis. *Cell Metab* 2011; 14: 208-18.

67. Zahs A, Curtis BJ, Waldschmidt TJ, Brown LA, Gauthier TW, Choudhry MA, Kovacs EJ, et al. Alcohol and epigenetic changes: summary of the 2011 Alcohol and Immunology Research Interest Group (AIRIG) meeting. *Alcohol* 2012; 46: 783-7.

68. Park PH, Lim RW, Shukla SD. Gene-selective histone H3 acetylation in the absence of increase in global histone acetylation in liver of rats chronically fed alcohol. *Alcohol Alcohol* 2012; 47: 233-9.

69. Park PH, Lim RW, Shukla SD. Involvement of histone acetyltransferase (HAT) in ethanol-induced acetylation of histone H3 in hepatocytes: potential mechanism for gene expression. *Am J Physiol Gastrointest Liver Physiol* 2005; 289: G1124-36.

70. Shukla SD, Lim RW. Epigenetic effects of ethanol on the liver and gastrointestinal system. *Alcohol Res* 2013; 35: 47-55.

71. Shukla SD, Restrepo R, Fish P, Lim RW, Ibdah JA. Different mechanisms for histone acetylation by ethanol and its metabolite acetate in rat primary hepatocytes. *J Pharmacol Exp Ther* 2015; 354: 18-23.

72. James TT, Aroor AR, Lim RW, Shukla SD. Histone H3 phosphorylation (Ser10, Ser28) and phosphoacetylation (K9S10) are differentially associated with gene expression in liver of rats treated in vivo with acute ethanol. *J Pharmacol Exp Ther* 2012; 340: 237-47.

73. Choudhury M, Pandey RS, Clemens DL, Davis JW, Lim RW, Shukla SD. Knock down of GCN5 histone acetyltransferase by siRNA decreases ethanol-induced histone acetylation and affects differential expression of genes in human hepatoma cells. *Alcohol* 2011; 45: 311-24.

74. Nagy LE. The role of innate immunity in alcoholic liver disease. *Alcohol Res* 2015; 37: 237-50.

75. Curtis BJ, Zahs A, Kovacs EJ. Epigenetic targets for reversing immune defects caused by alcohol exposure. *Alcohol Res* 2013; 35: 97-113.

76. Petrasek J, Csak T, Ganz M, Szabo G. Differences in innate immune signaling between alcoholic and non-alcoholic steatohepatitis. *J Gastroenterol Hepatol* 2013; Suppl 1: 93-8.

77. Orman ES, Odena G, Bataller R. Alcoholic liver disease: pathogenesis, management, and novel targets for therapy. *J Gastroenterol Hepatol;* 2013; Suppl 1: 77-84.

78. Abenavoli L, Milic N, Rouabhia S, Addolorato G. Pharmacotherapy of acute alcoholic hepatitis in clinical practice. *World J Gastroenterol* 2014; 7: 2159-67.

Correspondence and reprint request:
Ludovico Abenavoli, M.D., Ph.D.
Department of Health Sciences, University Magna Graecia of
Catanzaro, Italy.
Te.: +39 0961 3694387 Fax: +39 0961 754220
E-mail: l.abenavoli@unicz.it