Chapter 12
Suppressed Fat Mobilization Due to PNPLA3 rs738409 -Associated Liver Damage in Heavy Drinkers: The Liver Damage Feedback Hypothesis

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Abstract PNPLA3 variant rs738409 has been identified as important progression factor in patients with ALD and NAFLD, the most common liver diseases worldwide. These findings point towards similarities between metabolism of alcohol and fat with regard to the PNPLA3 gene. However, despite many efforts, neither the mechanisms of PNPLA3-related liver damage nor the physiological role of PNPLA3 are fully understood. Based on a large monocentric cohort of Caucasian heavy drinkers we could recently provide evidence that PNPLA3 GG primarily correlated with signs of liver damage (steatohepatitis, ballooning) but less with steatosis. Moreover, upon alcohol withdrawal, PNPLA3 GG carriers showed a delayed inflammation-associated resolution of liver stiffness. In line with the histological findings, hepatic fat content as quantified by CAP (controlled attenuation parameter) did not depend on PNPLA3 status and decreased equally in all genotypes by ca. 30 dB/m during alcohol withdrawal. Preliminary additional analysis from this large cohort indicates that PNPLA3 GG carriers (8.2%) drink significantly less high percentage beverages (23% vs 55%, p < 0.001) but show no metabolic phenotype such as increased weight, BMI or diabetes. On the molecular level, key molecules, important for lipolysis and flow of free fatty acids to the liver were drastically reduced in G carriers. These included the liver-synthesized serum ApoA1, the LD-associated protein perilipin5 and the recently identified hepato-protective transcriptional cofactor transducin beta-like-related 1 (TBLR1). Based on these findings, we here introduce the liver damage feedback hypothesis. Accordingly,

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PNPLA3-mediated liver damage (e.g. by enhanced metabolic activity) suppresses the mobilization of fat towards the liver at various levels (reduced serum lipid flux to the liver and fat mobilization from peripheric adipose tissues, suppressed hepatocyte fat release and avoidance of high percentage alcohol beverages). Finally, the liver damage feedback hypothesis identifies a novel and central role of liver damage on systemic fat homeostasis, which has not been appreciated so far.

Keywords Liver damage · Fatty liver · Steatosis · Fat mobilization · PNPLA3 · TBLR1 · Perilipin · Lipid droplet · Lipolysis · Diabetes · Alcoholic liver disease · Fatty acids · Obesity

12.1   Introduction

Alcoholic liver disease (ALD) is the most common chronic liver disease in the Western world [1]. ALD encompasses a broad spectrum of partly overlapping disorders ranging from simple steatosis evolving in nearly all drinkers, to severe forms of liver injury, including alcoholic steatohepatitis, fibrosis and cirrhosis. Most patients will eventually die from alcoholic cirrhosis with hepatocellular carcinoma (HCC, 1–2% per year) as the most common complications of cirrhosis. Although the majority (80–90%) of heavy drinkers with an alcohol consumption >80 g per day develop steatosis, only 35% show signs of inflammation and about 8–20% progress to cirrhosis [2]. Thus, only a small number of drinkers develop severe liver disease suggesting the existence of disease modifiers, which may determine an individual's risk for disease progression while heavy alcohol consumption. The underlying mechanisms are complex and still not fully understood, but suggest interactions between polygenic backgrounds and environmental factors as well as drinking habits (pattern and amount of alcohol consumption) and other liver-related comorbidities such as adiposity or hepatitis infection [3, 4].

12.2   Health Statistics of ALD

Chronic alcohol consumption is one of the major risk factors worldwide affecting significantly both mortality and years of life loss (YLL) [5]. In 2012, nearly half of the world's population consumed alcohol with about 3.3 million deaths (5.9% of all global deaths) attributable directly to alcohol (Global Status Report on Alcohol and health, WHO, 2014). Most of the world's population displays a stable five-year trend in recorded alcohol consumption, while in the African region and the South-East Asia region an increase can be noted (WHO, 2014). Although alcohol affects many other organ systems such as the heart, nervous system, pancreas, breast, the liver remains the major target organ of alcohol consumption and more

than 80% of drinkers will ultimately die from liver-related causes. According to the Global Burden of Disease study 2010 alcohol-attributable liver cirrhosis was responsible for nearly half a million deaths (157,000 female and 336,000 male deaths) [6]. Alcohol-attributable liver cancer was responsible for over 80,000 deaths and liver cancer ranked at position 12 and 16 in the actual death statistics [7]. In 2010 ca. one million people died from liver cirrhosis with nearly 50% of those were directly attributed to alcohol. This is a considerable number when comparing for instant with coronary heart disease with about ten million deaths ranking the leading cause of mortality in the global death statistics. In Central Europe, liver cirrhosis even ranks at the fourth position of YLL and hepatocellular carcinoma (HCC) is now the most common fatal complication of patients with alcoholic liver cirrhosis. Taken together, these dramatic numbers of liver-related death due to alcohol generate high interest in the molecular mechanisms and gene-related factors that drive the disease in only a minority of individuals.

12.3   Genetic Confounders: (Role of PNPLA3)

The role of genetic confounders that render the liver sensitivity to alcohol is under-lined by various findings (ALD): First, monozygotic twins have a higher risk to develop alcoholic cirrhosis than dizygotic ones [8, 9]. Second, female drinkers are more sensitive to alcohol when exposed to the same amounts of alcohol than males [10] and third, the white men and women of Hispanic origin are at higher risk for developing alcoholic cirrhosis and have the highest mortality rate when compared to non-Hispanics [11]. In the last decades, continued research and increasing knowl-edge of genetic variations, the availability of new analytical methods and decreasing costs for genetic studies raises the possibilities to search for genetic factors influenc-ing the course of alcohol-induced liver disease progression. Various candidate genes were identified in case control studies comparing allelic and/or genotypic frequen-cies of certain genetic variants like single nucleotide polymorphisms (SNP) between individuals with a background of alcohol dependence and ALD [12]. Most human sequence variation is attributable to SNPs, which are found in 1% of the population and occur on average every 1.9 kilobases within the human genome. As a conse-quence, about 1.42 million SNPs are found in the total human genome [13, 14]. A SNP is a variation in a single nucleotide (e.g. A → C) leading to an altered base triplet potentially coding for a different amino acid, thereby eventually changing the genetic code. This is not always the case due to degeneracy of the genetic code. Variations in the coding region of genes can alter the function of the generated pro-tein; if the SNP changes the amino acid sequence of a protein this is called non-synonymous SNPs, whereas synonymous SNPs do not affect the protein sequence. However, most polymorphisms (>99%) are located in non-coding regions of the genome and have no direct known impact on the phenotype of an individual, but may still affect gene splicing or transcription factor binding [15].

## 12.4   Discovery of the PNPLA3 Polymorphism rs738409 and Its Role in ALD

Due to genome-wide association studies (GWAS) it was possible to analyze relationships between a given phenotype/disease and millions of SNPs in thousands of different individuals [16]. Using this hypothesis-free approach, a GWAS using a map of SNPs found an association between patatin-like phospholipase domain containing 3 (PNPLA3/Adiponutrin) and plasma levels of liver transaminases in 2008 [17]. Another GWAS performed in the same year by Romeo et al. identified a non-synonymous SNP out of 9229 SNPs, the rs738409 variant, that encodes for an isoleucine to methionine substitution at position 148 (I148M) in the PNPLA3 gene as genetic variant associated with hepatic triglyceride content (steatosis, P = $5.9x10^{-10}$) and enhanced inflammation (P = $3.7x10^{-4}$) in 2111 multiethnic participants of the Dallas Heart study. Individuals heterozygous for this allele had higher hepatic fat levels compared to wild-type carriers, whereas possession of two copies of the 148M allele had a multiplicative effect. This allele was most common in Hispanics, the ethnic group that is also most susceptible to NAFLD and remained highly significant after adjusting for BMI, diabetes mellitus, alcohol abuses as well as global and local ancestry [18, 19]. These findings were quickly confirmed by several population based-studies around the world [20].

In addition, Sookoian et al. showed that this variant also predisposes towards all stages of liver damage starting from simple steatosis to steatohepatitis and progressive fibrosis elevated by histological assessment of liver biopsies [21]. Singal et al. even demonstrated in a meta-analysis that PNPLA3 is associated with an increased risk of advanced fibrosis among patients with different underlying liver disease and is an independent risk factor for hepatocellular carcinoma (HCC) in patients with nonalcoholic steatohepatitis or alcohol-related cirrhosis [22].

Because of the similarities between NAFLD and ALD, these findings stimulated similar genetic analysis in patients with ALD. Tian and coworkers confirmed an association between PNPLA3 and steatohepatitis to cirrhosis in a large Mestizo population (mixed European and Native American ancestry, n = 1221) [23–25]. The study of the genetic variant in the well-characterized Heidelberg ALD cohort of 521 patients (148 females/ 369 males, age range 22–87 years) revealed a significant correlation between the GG genotype with histological steatohepatitis (r = 0.404, P < 0.005), ballooning (r = 0.319, P < 0.005) but less with steatosis (r = 0.264, P < 0.05) (Fig. 12.1) [26]. Furthermore, we found that GG genotype carriers had a shorter duration of alcohol consumption (17.2 vs 18.3 years, Fig. 12.2a) and generally consumed less alcohol than CC type carriers without reaching significance (181 vs. 194 g/day)(Fig. 12.2b and Table 12.1). To our surprise, GG carriers showed also a different drinking behavior; interestingly GG Carriers demonstrated significantly reduced consumption of high percentage beverages such as liquor (23% vs 55%, P < 0.001, Fig. 12.2c). This was not due to advanced liver disease or age. No significant differences were recorded for beer and wine intake (data not shown).
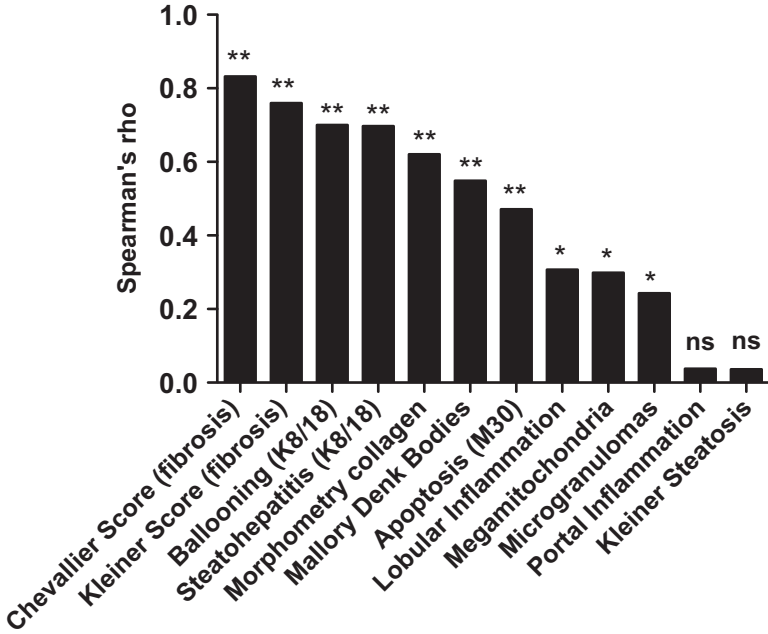
Fig. 12.1 Spearman rank correlation analysis of PNPLA3 GG genotype carriers with histological parameters. * $p < 0.05$; ** $p < 0.01$; ns = not significant
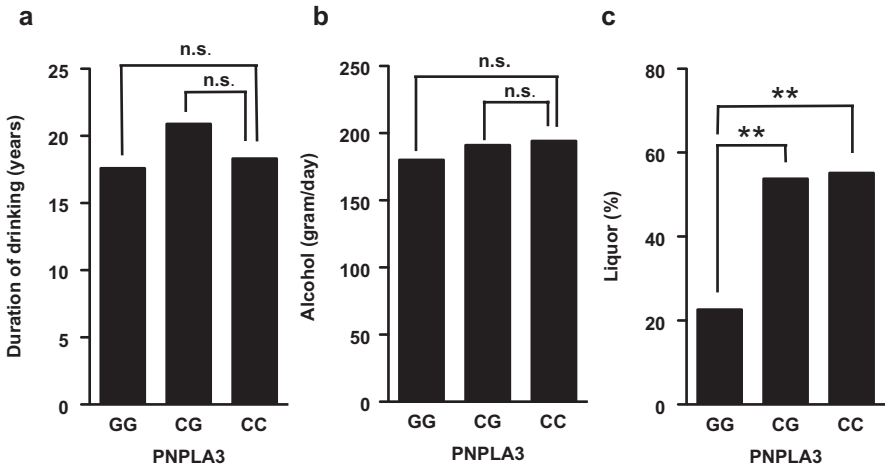
Fig. 12.2 Alcohol drinking characteristics such as (a) duration, (b) daily consumption, (c) liquor for ALD patients depending on the PNPLA3 genotype. GG carrier drink significantly less high percentage alcoholic beverages such as liquor. ** $p < 0.001$

Table 12.1 Characteristics of ALD sub-cohorts based on genotype distribution of rs738409 polymorphism

Parameters	PNPLA3 CC (n = 204)		PNPLA3 CG (n = 274)		PNPLA3 GG (n = 43)		PNPLA3 G (CG + GG) (n = 317)	
Demographic characteristics								
Patients (%)	39.2		52.6		8.2		60.8	
Age (years)	49.5	± 11.0	50.7	± 11.8	50.1	± 9.7	50.7	± 11.5
Risk factors								
BMI (kg/m2)	25.4	± 4.9	25.1	± 4.5	25.6	± 3.9	25.2	± 4.4
H/W ratio	1.0	± 0.1	1.0	± 0.1	1.0	± 0.1	1.0	± 0.1
Alcohol consumption (g/day)	194	± 136.1	190.8	± 146.2	181.2	± 116.1	189.4	± 142
Duration (years)	18.3	± 13.3	20.9	± 13.1	17.2	± 14.2	20.4	± 13.3
Noninvasive parameters								
Hepatic steatosis (0–3, US)	1.8	± 0.9	2.0	± 0.8	1.9	± 0.8	2.0	± 0.8
Liver stiffness (kPA)	13.1	± 17.7	17.6	± 23.0*	17.2	± 22.2	17.5	± 22.9+
CAP (dB/m)	288	± 52	290	± 56	298	± 49	291	± 55
Laboratory parameter								
AST (U/l)	95.2	± 100.8	102.8	± 111.4	111.2	± 116.2	104.0	± 111.9
ALT (U/l)	66.0	± 59.4	71.9	± 93.0	75.1	± 60.3	72.4	± 89.1
GGT (U/l)	406.4	± 572.2	365.9	± 516.1	525.9	± 863.0	388.4	± 578.4
AP (U/l)	105.5	± 76.2	111.6	± 75.8	111.5	± 72.1	111.6	± 75.2
Bilirubin (mg/dl)	1.2	± 2.8	1.4	± 3.0	0.9	± 1.1	1.3	± 2.8
Albumin (g/dL)	4.7	± 4.7	5.3	± 7.2	4.5	± 0.5	5.2	± 6.7
INR	1.4	± 5.4	1.0	± 0.4	0.9	± 0.2	1.0	± 0.4
Urea	20.6	± 10.8	24.6	± 20.2*	20.1	± 9.9	24.0	± 19.2+
Creatinine	0.7	± 0.2	0.7	± 0.3	0.7	± 0.2	0.7	± 0.3
Hemoglobin (g/dl)	14.2	± 1.8	14.2	± 2.5	14.6	± 2.0	14.2	± 2.4
Platelets (/nl)	216.7	± 92.7	201.1	± 80.0*	224.2	± 91.4	204.5	± 82.0
Glucose (mg/dL)	112.0	± 46.2	107.7	± 28.5	110.7	± 34.6	108.1	± 29.3
HbA1C (%)	5.6	± 1.1	5.6	± 0.8	5.8	± 1.3	5.6	± 0.9
Triglycerides (mg/dL)	190.6	± 202.2	192.0	± 205.8	240.9	± 230.4	198.7	± 209.6
Cholesterol (mg/dL)	219.9	± 55.0	213.1	± 61.1	222.9	± 53.4	214.4	± 60.1
HDL cholesterol (mg/dL)	73.2	± 35.9	71.4	± 37.6	75.6	± 37.3	71.9	± 37.5
LDL cholesterol (mg/dL)	113.5	± 46.3	112.4	± 45.5	118.0	± 44.7	113.0	± 45.3
Lipase (U/L)	48.5	± 45.9	75.9	± 216.5	45.3	± 26.0	72.0	± 202.7
Ferritin (ng/ml)	546.1	± 611.6	599.6	± 668.3	685.2	± 708.2	610.8	± 673.1
CRP (mg/dl)	4.7	± 11.1	7.1	± 18.9	6.0	± 12.0	7.0	± 18.1

(n = 521). Data are presented as mean +/− SD or in %

*$p < 0.05$ (CC vs CG); + $p < 0.05$ (CC vs G)

12.5 Role of PNPLA3 rs738409 in Alcoholic Cirrhosis/Advanced Fibrosis

Tian et al. was the first who reported that PNPLA3 rs738409 GG is strongly associated with alcoholic liver disease, especially with clinically evident alcoholic cirrhosis in Mestizo subjects (unadjusted OR = 2.25, P = 1.7 x10^{-10}; ancestry-adjusted OR = 1.79, P = 1.9x10^{-5}) [23]. A small study in 266 patients with alcoholic cirrhosis and 182 heavy drinkers from the UK and Australia confirmed the results in Caucasians and demonstrated that carrying of the PNPLA3 rs738409 G allele and GG genotype were also significantly associated with alcoholic cirrhosis (OR = 2.2, P = 2x10^{-5} and OR = 5.57, P = 1.2x10^{-3}) [27, 28]. A similar study with a Belgian ALD cohort and corresponding controls (n = 328) showed that the PNPLA3 rs738409 G allele was more frequent in ALD patients and identified as risk factor for cirrhosis (OR = 2.1, P = 0.001) [29]. Furthermore, a large European study performed by Stickel et al. in alcoholics from different centers in Germany also identified an association between PNPLA3 rs738409 GG genotype and liver cirrhosis (OR = 2.79, P$_{genotype}$ = 1.2x10^{-5} and P$_{allele}$ = 1.6x10^{-6}) [24]. Falleti as well as Rosendahl et al. confirmed that PNPLA3 rs738409 G allele is more frequent in Caucasians in a study with 483 cirrhotic patients from Italy [30] or 135 patients with alcoholic cirrhosis from Germany and the Netherlands [31]. In addition, Falleti was also the first who identified this SNP as risk factor for HCC development in alcoholic patients with cirrhosis. Only one Asian study in Indians with 120 alcoholics (60 with cirrhosis and 60 without) and 100 controls, confirmed the association between PNPLA3 rs738409 and cirrhosis [32]. The most recent study (n = 387 alcoholics including 206 cirrhotics) performed by Way and Morgan found also an association between GG genotype and cirrhosis with an OR = 2.71 [33]. We also confirmed a weak association in our ALD study cohort and calculated an OR to develop cirrhosis corrected for age, gender and BMI of 1.295 (95% CI 0.787–2.131) for the G genotype [26]. Taken together, all studies including a recent meta-analysis showed an association between PNPLA3 rs738409 G carrier and cirrhosis in patients with alcoholic liver disease (Table 12.2) [34].

12.6 Role of PNPLA3 in Cancer Progression (HCC)

Since PNPLA3 rs738409 was shown to be associated with advanced fibrosis and cirrhosis and this is accompanied in 8–20% of alcoholic cirrhotics with the development of HCC, it was tempting to speculate that this sequence variation might play also a role in liver carcinogenesis. Therefore, case control studies were conducted in ALD patients complicated by HCC or not. Most studies were performed in Europe including Caucasians, except one study performed in 2013 in Japan including

Table 12.2 ORs and 95% CIs of studies analyzing the association between PNPLA3 rs738409 and advanced fibrosis/cirrhosis (adapted from Stickel et al. 2016)

Study (reference)	Type of study	Cases-controls (cirrhotics)	OR or HR (95% CI)	P
Tian et al. 2010 [23]	Case-control	1221 (482)	2.25 (1.74–2.90)	1.7×10^{-10}
Seth et al. 2010 [27]	Case-control	448 (266)	5.57 (1.68–18.43)	1.2×10^{-3}
Trepo et al. 2011 [29]	Case-control	658 (256)	2.08 (1.15–3.77)	0.02
Stickel et al. 2011 [35]	Case-control	1043 (210)	2.79 (1.55–5.04)	1.2×10^{-5}
Falleti et al. 2011 [30]	Case-control	911 (483)	1.76 (1.06–2.92	0.02
Nguyen-Khac et al. 2011 [36]	Case-control	210 (40)	2.5 (1.4–4.4)	0.002
Rosendahl et al. 2012 [31]	Case-control	1510–2781 (135)	2.3 (1.6–3.3)	<0.0001
Dutta et al. 2013 [32]	Case-control	120–100 (60)	2.12 (1.29–3.4)	0.037
Way and Morgan et al. 2013 [33]	Case-control	1106–1058 (212)	2.13 (1.66–2.73)	1.46×10^{-9}

OR, odds ratio; HR, Hazardous ratio; CI, confidence interval

patients diagnosed with HCC without confounding virus infections (hepatitis B or C)(n = 104) [37]. Falleti et al. was the first who identified this SNP as risk factor for HCC development in 483 alcoholic patients from Italy where 166 had cirrhosis and calculated an overall OR of 1.76 (95% CI 1.06–2.92, P < 0.05) [30]. Nischalke and Hamza et al. who calculated a 2.8 and 2.6 fold higher HCC risk in patients with ALD confirmed these findings [38, 39]. Trepo et al. even reported a stronger predisposing effect of PNPLA3 genotype and HCC risk in ALD patients [40]. Another study by Guyot et al. also provides data that confirm the influence of PNPLA3 rs738409 G genotype on the occurrence of HCC in patients with alcoholic cirrhosis with a slightly lower OR of 1.72 (95% CI 1.21–2.45, P = 0.002) [41]. A recent meta-analysis of individual patient data from candidate gene association studies (GWAS) found that PNPLA3 rs738409 is strongly associated with overall HCC and found that this association was more pronounced in ALD (OR = 2.20, 95% CI 1.80–2.67) than in patients with HCV-related HCC [40]. Two subsequent meta-analyses confirmed this association [22, 42]. To conclude, several case-control studies and recent meta-analyses have confirmed that PNPLA3 rs738409 G allele is associated with an around two fold HCC risk in alcoholic cirrhotics, but also other modifiers are likely to play a role in liver carcinogenesis (Table 12.3).

Table 12.3 ORs or HR and 95% CIs of studies analyzing the association between PNPLA3 rs738409 and HCC development (adapted from Trepo et al. 2016)

Study (reference)	Type of study (n = number of studies in meta-analysis)	Cases (cirrhotics)	OR or HR (95% CI)	P
Falleti et al. 2011 [30]	Case-control	483 (166)	1.76 (1.06–2.92)	<0.05
Nischalke et al. 2011 [38]	Case-control	350 (160)	2.83 (1.24–6.42)	0.013
Hamza et al. 2012 [39]	Case-control	304	2,59 (1.29–5.20)	0.007
Trepo et al. 2012 [40]	Case-control	571	4.7 (2.63–8.42)	1.83×10^{-7}
Guyot et al. 2013 [41]	Prospective	279	1.72 (1.21–2.45)	0.02
Trepo et al. 2014 [43]	Meta-analysis (n = 5)	1374	2.20 (1.80–2.67)	4.71×10^{-15}
Nischalke et al. 2014 [44]	Case-control (with replication)	864 (+229)	2.32 (1.36–4.68)	0.00002
Singal et al. 2014 [22]	Meta-analysis (n = 9)	2937	1.4 (1.12–1.75	n.i.
Salameh et al. 2015 [42]	Meta-analysis (n = 4)	1207	2.81 (1.57–5.01)	n.i.
Friedrich et al. 2015 [45]	Retrospective	421	2.4 (1.29–4.46)	0.008
Falleti et al. 2016 [46]	Case-control	226	2.20 (1.03–4.66)	0.039

OR, odds ratio; HR, Hazardous ratio; CI, confidence interval; n.a. not indicated

12.7 Potential Physiological Functions of PNPLA3

So far, the physiological function of PNPLA3 and the effect of the amino acid substitution remain controversial and the structure of PNPLA3 has never been resolved by crystallization or nuclear magnetic resonance spectroscopy [28]. A clear association of the genetic variant between the GG genotype was found with various liver disease stages including steatosis, steatohepatitis (inflammation and ballooning), fibrosis and cancer [26, 28]. PNPLA3 (adiponutrin) is located on chromosome 22 and encodes a 53 kDa protein with 481-aa length that is closely related to PNPLA2 (also called ATGL), the major hormone-sensitive TAG hydrolase of adipose tissue, sharing 56% amino acid identity in the patatin-like domain [47, 48]. The progenitor of this family, patatin, is a major storage protein of potato tubers but also involved in lipolysis of fatty acids. Structural analysis demonstrated, that the mutation has no influence on the catalytic center but the substrate-binding groove, thereby possibly blocking the access of substrates to the catalytic center [49]. In humans, PNPLA3 is expressed in adipocytes, hepatocytes and hepatic stellate cells [50–53]. PNPLA3 is localized on membranes suggesting an involvement in receptor-like interactions with extracellular signals [54], but also associated with the endoplasmatic reticulum

and lipid droplets (LD) most likely due to the Brummer box [55]. Lipid droplets are the main organelles responsible for neutral lipid storage (primarily TAG and cholesteryl ester) and hydrolysis. Studies demonstrated an upregulation during adipocyte differentiation, and a response to fasting and feeding cycles pointing towards a role in regulation of energy mobilization and lipid storage in adipose tissue and the liver [56]. Furthermore, PNPLA3 mRNA expression was increased in subcutaneous and visceral adipose tissue of obese subjects [57].

12.7.1 Enzyme Function

Three independent *ex vivo* studies demonstrated that the isolated PNPLA3 protein has an enzymatic activity on triglycerides (TG) by using radiolabeled triolein and measuring the release of oleic acid. In two studies they used SF-9 insect cells and one study examined the enzyme function in a yeast system (*Pichia pastoris*) [49, 58, 59]. Although the enzyme showed a predominant lipase activity with oleic acid as major substrate which was also confirmed in Huh7 cells [60], Pingitore et al. found a mild lysophosphatidic acid acyltransferase activity (LPAAT). In addition, Jenkins et al. also described an acylglycerol transacylase activity for PNPLA3. In sum, all three papers demonstrated membrane localization and that the I148M mutation results in a loss of function mutation.

Most *in vitro* studies are performed in immortalized hepatoma cell lines, since this protein is highly expressed in the liver. In these studies PNPLA3 seems to possess triacyl-hydrolase activity on TG embedded in lipid droplets in Huh7 cells and the overexpression of the I148M isoform led to a marked reduction of this enzyme activity when compared with wild type PNPLA3 pointing also towards a loss-of-function mutation and resulting in an accumulation of TG, but not those newly synthesized and thereby trapping lipids in the liver [52]. Furthermore, it has been proposed that PNPLA3 148 M may promote intracellular lipid accumulation by reducing hepatic very low density lipoprotein (VLDL) synthesis [61]. VLDLs are particles highly enriched in TG and secreted by the liver to provide different lipids for peripheral tissues [62]. Retention of VLDLs in the liver causes increased hepatic fat content [63]. These findings were also confirmed in humans by measuring hepatic VLDL secretion by injection of stable isotypes in different PNPLA3 carriers [28]. Furthermore, studies showed that silencing of the carbohydrate response element-binding protein (ChREBP) abolished the induction of PNPLA3 mRNA by glucose in immortalized human hepatocytes suggesting a glucose dependent regulation of PNPLA3 [64]. Finally, a recent study by Pirazzi et al. suggested a retinyl-palmitate hydrolase activity for PNPLA3 in hepatic stellate cells in humans [53] and demonstrated that I148M mutation is associated with reduced levels of retinol binding protein 4 (RBP4), the major transport protein for vitamin A in the blood [65]. It is known that HSCs lose their

retinol-storing ability while activation and differentiation into myofibroblasts-like cells that secrete collagen thereby contributing to liver fibrosis. However, this indicates a potential novel link between HSCs, retinoid metabolism and PNPLA3 in determining the susceptibility to chronic liver disease whereas the specific role of PNPLA3 in this process remains still unclear.

12.7.2   In Vivo Studies

However, *in vivo* studies using genetically modified mice overexpressing or silencing PNPLA3 (knockout mice models) proved to be difficult, since deletion of PNPLA3 lead to no altered phenotype, suggesting a distinct enzyme function in mice or that other enzymes may compensate for the lack of PNPLA3 activity. The first approach studying PNPLA3 function *in vivo* was performed via adenoviral PNPLA3 overexpression. He et al. observed that overexpression of wild type PNPLA3 in mouse liver showed no obvious phenotype and did not lead to reduced hepatic TG content, while the I148M mutant protein increases liver fat content [52]. This could suggest a gain of function and a lipogenic role for PNPLA3 that is enhanced in the presence of the mutant variant. In addition, two other studies performed in PNPLA3 knockout mice indicated no major role for PNPLA3 in steatosis development [66, 67]. However, studies in transgenic mice overexpressing the sterol regulatory element binding protein-1c (SREBP-1c) showed an upregulation of PNPLA3 mRNA implicating an important link between PNPLA3 and the insulin signaling pathway, since SREBP-1c is a downstream target [68]. Another study performed in high-fat diet fed rats, in which endogenous PNPLA3 was silenced using specific antisense oligonucleotides also pointed towards a possible role of PNPLA3 in insulin signaling [69]. Furthermore, in obese humans PNPLA3 I148M mutation was shown to be associated with an increased risk of type 2 diabetes [70]. A more recent knock-in mouse model used to either overexpress the mouse I148M mutant protein or the S47A variant resulted in hepatic lipid droplets accumulation and steatosis development when mice were fed with a high sucrose diet, indicating a loss of function mutation [71]. Today, this mouse model is the one most closely resembling the human physiologic condition.

12.8   Recent Findings from the Heidelberg Mono–Center Cohort of Heavy Drinkers – The Role of Alcohol Withdrawal

We recently analyzed the role of the PNPLA3 genotype in over 500 Caucasian heavy drinkers admitted for alcohol detoxification. Expression levels of various mRNA transcripts, histology, serum markers and many clinical parameters including

fibrosis (transient elastography, Fibroscan) and steatosis (CAP, controlled attenuation parameter) were performed. The PNPLA3 rs738409 genotype distribution for CC, CG and GG was 39.2%, 52.6% and 8.2%. Mean LS was lowest in CC carriers (13.1 kPa) as compared to CG and GG carriers (17.6 and 17.2 kPa) (Table 12.1). Interestingly, almost no change was observed in CC carriers after alcohol withdrawal (12.0 kPa, LS2) while LS significantly decreased in CG carriers to comparable 12.7 kPa. Despite a longer observation interval of 6.6 days, LS decreased slower in GG most likely due to sustained inflammation/liver injury as reflected by enhanced AST levels (26). Moreover, hepatic fat content (CAP) was only non-significantly increased in GG carriers and it decreased equally in all groups by 30 dB/m (Table 12.1). Like LS, no significant decrease of CAP was seen in GG carriers (26). In summary, LS was highest in G (GG and CG) carriers resolving to baseline levels comparable to the CC genotype after alcohol withdrawal. In contrast, fat content as measured by CAP was almost identical and decreased equally in all groups after alcohol detoxification. In conclusion, in heavy drinkers, PNPLA3 GG primarily correlates with liver damage but not steatosis resulting in a delayed inflammation-associated resolution of LS as reflected by increased AST levels. Consequently, sustained LS elevation could be a major risk factor in PNPLA3 GG carriers.

12.9   Evidence of Suppressed Fat Mobilization in the Heidelberg Cohort

Interestingly, we could identify several lines of evidence that the PNPLA3 GG associated liver damage is associated with reduced fat mobilization to prevent further hepatic fat loading at various levels. First, we detected that the recently discovered transcriptional cofactor transducin beta-like-related 1 (TBLR1), identified as protective factor against hepatic steatosis in the metabolic syndrome was drastically reduced (Fig. 12.3a) and correlated negatively with the GG genotype ($r = -0.537$; $p < 0.01$) (Table 12.4). Its ligand TBL1 was also negatively correlated although not reaching levels of significance. Surprisingly, the C genotype was significantly associated with liver-synthesized ApoA1 ($r = 0.38$; $p < 0.01$, data not shown), which is known to promote fat efflux from adipose tissues. Significantly decreased ApoA1 levels were seen in G carriers (Table 12.5). In addition, analysis of the expression of lipid droplet-associated proteins of the perilipin family that regulate lipid droplet biogenesis, maintenance and degradation in hepatocytes revealed that Plin5 mRNA was markedly reduced in CG carriers and in the G genotype while no significant differences were observed for Plin3, though both perilipins represent exchangeable proteins shuttling between cytoplasm and lipid droplets dependent on functional state of cells (Straub et al. in press). Similar results were found for plin5 on protein level, where Plin5 was less recruited to lipid droplets in CG and in GG carriers showing a rather diffuse weak staining (Fig. 12.3c and Straub et al. in press). Likewise, Spearman correlation analysis demonstrated a significant negative
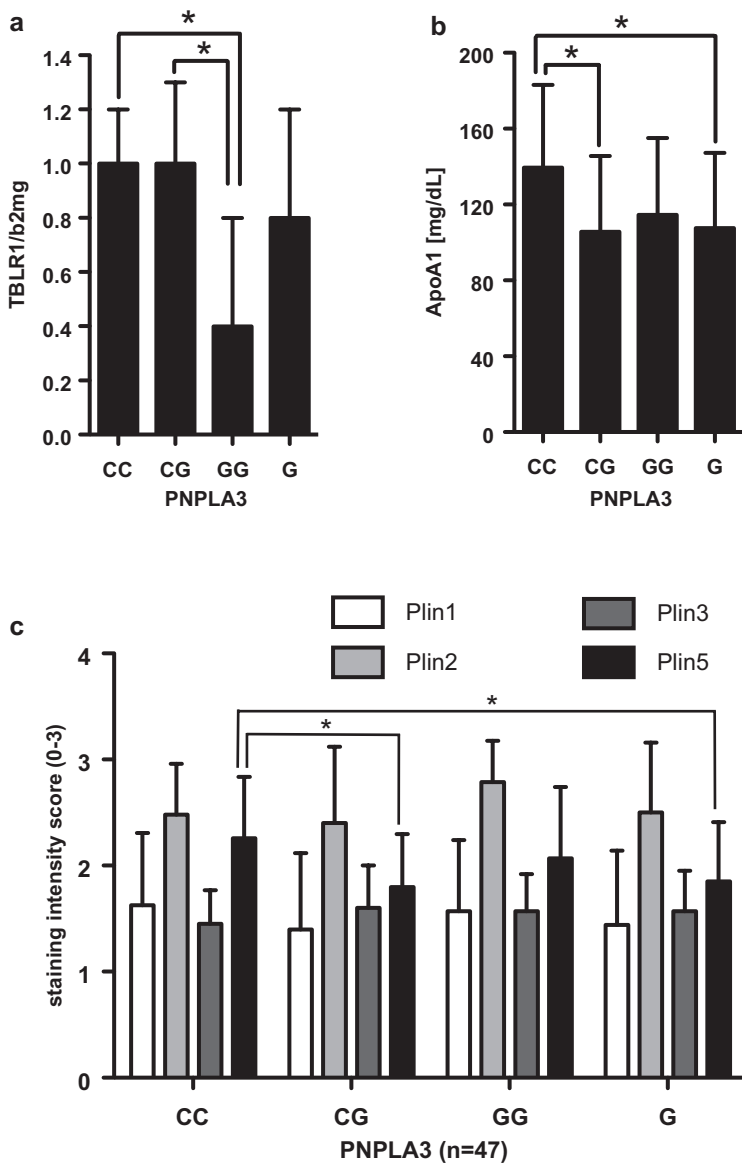
Fig. 12.3 PNPLA3 genotype-dependent expression of (a) TBLR1 mRNA, (b) serum ApoA1 levels and (c) perilipin (plin1, 2, 3 and 5) mRNAs. TBLR1, ApoA1 and plin5 are suppressed in GG carriers. Data are presented as mean+/− SD. * p < 0.05. mRNA expression analysis of n = 24 liver biopsy samples

Table 12.4 Spearman rank correlation analysis of PNPLA3 GG genotype carriers with clinical, morphological, histological and molecular parameters

PNPLA3 GG		
Parameter	Method	rho
Ballooning	Histology	0.332**
Microgranulomas	Histology	0.315**
Steatosis (Kleiner score)	Histology	0.235*
ALT (U/L) 2	Serum	0.106*
Triglycerides (mg/dL)	Serum	0.096*
Liver size	Ultrasound	0.089
AST (U/L) 2	Serum	0.087
TBLR1	Liver mRNA	−0.537**
Plin5	Liver mRNA	−0.442*
Portal inflammation	Histology	−0.186
Liquor	Medical history	−0.167**

* p < 0.05; ** p < 0.01; 1 = before alcohol withdrawal, 2 = after alcohol withdrawal

Table 12.5 Lipid stores and parameters in PNPLA3 genotypes

| Compartment	Parameter	PNPLA3 CC	PNPLA3 CG	PNPLA3 GG
Peripheral fat	BMI (kg/m2)	25.4 ± 4.9	25.1 ± 4.5	25.6 ± 3.9
Serum lipids	Triglycerides (mg/dL)	190.6 ± 202.2	192.0 ± 205.8	240.9 ± 230.4
	Cholesterol (mg/dL)	219.9 ± 55	213.1 ± 61.1	222.9 ± 53.4
Lipid trafficking	ApoA1 (mg/dL)	139.4 ± 43	**105.6 ± 39.2***	**114.5 ± 37.6***
	MLDP/Plin5	4.4 ± 1.3	4.0 ± 2.0	**2.0 ± 0.2***
	TBLR1	1.0 ± 0.2	1.0 ± 0.3	**0.4 ± 0.4***
Liver fat	Steatosis (US)	1.8 ± 0.9	2.0 ± 0.8	1.9 ± 0.8
	Steatosis (histology)	1.9 ± 1.0	1.9 ± 0.1	**2.6 ± 0.5***
	Steatosis (CAP)	288 ± 52	290 ± 56	298 ± 49
Liver damage	AST (U/L) (bw)	95.2 ± 100.8	102.8 ± 111.4	111.2 ± 116.2
	AST (U/L) (aw)	47.8 ± 32.8	52.6 ± 45.9	**82.8 ± 87.8***
	Ballooning (histology)	0.7 ± 0.7	0.8 ± 0.8	**1.4 ± 0.5***
	Steatohepatitis (histology)	1.1 ± 0.8	1.1 ± 0.7	**2.0 ± 0.0***

Data are presented as mean +/− SD
*p < 0.05 (CC vs CG or CC vs GG)

correlation between plin5 and GG type (r = −0.443; p < 0.05) (Table 12.4). In line with our findings, a recent study showed that PNPLA3 I148M is associated with lower *de novo* lipogenesis and a reduction of liver SREBP-1c mRNA levels, despite increased hepatic fat content [72]. This may point towards a compensatory mechanism of hepatic fat increase in subjects with the I148M allele. Taken together, our findings on liver-expressed ApoA1, TBLR1 and plin5 point towards reduced hepatocellular lipolysis and fatty acid mobilization in the liver in PNPLA3 GG carriers. Furthermore, the data on decreased plin5 levels in PNPLA3 G genotype could provide a novel link between PNPLA3 pathophysiology on lipid droplet-formation.

12.10   Reduced Fat Mobilization Due to PNPLA3-Associated Liver Damage: The Liver Damage Feedback Hypothesis

Based on the observations above we propose the following hypothesis named liver damage feedback hypothesis: In heavy drinkers PNPLA3 G seems to predispose to primary liver damage leading to reduced backflow of fatty acids to the liver via ApoA1 (serum lipid trafficking) and inhibited fatty acid utilization via TBLR1 (hepatocellular lipolysis signaling) and plin5 (regulation of fat flux to mitochondria) (Fig. 12.4), which may be a compensatory negative feedback loop in G carriers to prevent further hepatic lipid loading. To sum up, the naturally occurring mutation in PNPLA3 leads to inflammation and fibrosis development combined with disturbed intrahepatic lipid remodeling via suppressed lipolysis and fatty acid mobilization. Due to the similarities between NAFLD and ALD pathophysiology, and the fact that alcohol directly converts into fat, it is reasonable to assume, that the latter is the real problem. Since the variant of PNPLA3 is not directly linked with insulin sensitivity or BMI and does not affect related metabolic disorders such as dyslipidemia or type 2 diabetes [73], and we cannot detect a significant difference in liver fat content in heavy drinking G carriers (CAP), we hypothesize that they might have a facilitated/accelerated fatty acid oxidation which in addition increases liver disease progression. This facilitated FA/TG hydrolysis might be under the control of Plin5 acting as a lipolytic barrier to prevent uncontrolled TG mobilization/shuttling and FA oxidation in the mitochondria [74, 75]. It remains unclear whether the I148M substitution independently of fibrosis directly causes steatosis, lipotoxicity, or both and how it influences hepatocarcinogenesis.
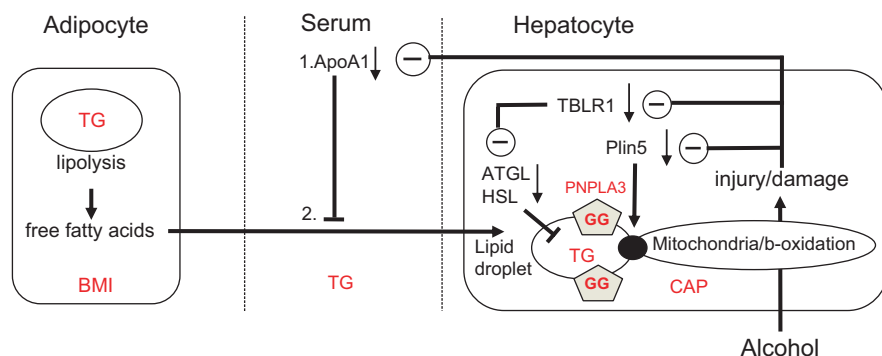
Fig. 12.4 The liver damage feedback hypothesis: PNPLA3 GG seems to cause primary liver damage leading to reduced backflow of fatty acids to the liver via ApoA1 (serum lipid trafficking) and inhibited FA utilization via TBLR1 (hepatocellular lipolysis signaling) and plin5 (regulation of fat flux to mitochondria)

12.11   Other Genetic Variations that Promote ALD

In 2015, a GWAS performed by Buch et al. in patients with ALD comparing 1426 heavy drinkers without indicated liver injury to 712 patients with alcohol-induced cirrhosis in Europeans with a subsequent validation in two independent European cohorts (922 controls and 1148 cases) reported that TM6SF2 (P = 7.89×10^{-10}) and MBOAT7 (P = 1.03×10^{-9}) are important risk loci for alcohol-related cirrhosis and confirmed the role of rs738409 in PNPLA3 (P = 1.54×10^{-48}) at a genome-wide level of significance. These three independent loci are all involved in lipid metabolism, suggesting that lipid turnover is important in the pathogenesis of alcohol-related cirrhosis [76]. Furthermore, Falleti et al. identified TM6SF2 in conjunction with PNPLA3 as potential genetic risk factors for developing HCC in alcohol-related cirrhosis (P = 0.0007) [46]. Overall, it is likely that in future more GWAS studies are performed leading to the identification of additional variants robustly associated with alcohol-induced liver damage.

12.12   Conclusion and Future Perspectives

In the search for genetic risk factors rendering man more susceptible for-alcohol-induced liver disease, PNPLA3 was the first locus to be reproducibly and strongly associated with steatosis, fibrosis/cirrhosis in various liver diseases with different etiologies including NAFLD, ALD, and CHC and even HCC. The various studies on PNPLA3 allow the conclusion that PNPLA3 rs738409 GG carrier represent a subpopulation of high-risk subjects susceptible to develop ALD and cirrhosis. Genetic association studies highlighted a role of PNPLA3 in fat metabolism and a major impact on the development of liver disease but lipodomic analyses have not been performed, yet. If lipid turnover by the mitochondria is disturbed or another mechanism/pathway is influenced by the I148M variant still remains an open question and definitely needs further investigations.

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