MOLECULAR GENETICS OF PATATIN-LIKE PHOSPHOLIPASE DOMAIN-CONTAINING 3 AND CHRONIC LIVER DISEASE

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Cover illustration: The potato eaters (Vincent van Gogh, 1885)

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“Science is organized knowledge. Wisdom is organized life.”

Immanuel Kant

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ABSTRACT

Chronic liver disease is a major health burden worldwide. Major determinants of this condition are viral infections, alcohol abuse and obesity. Genetic background modulates the effect of damaging agents on the liver. The genetic variant rs738409 in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene associates with increased susceptibility to the entire spectrum of chronic liver disease, and in particular with non-alcoholic fatty liver disease. The variant results in an isoleucine to methionine substitution at position 148 (I148M) of the amino acidic sequence and was first associated with increased hepatocyte fat content. Despite the strength of the genetic association, the mechanisms causing liver fat accumulation and hepatocyte damage are not yet understood.

In this thesis, we tested the following hypotheses: 1) PNPLA3 is involved in hepatic very low density lipoprotein secretion 2) the protein acts as a glycerolipid hydrolase and the 148M mutation is a loss of function 3) PNPLA3 has a specific role in retinol metabolism in hepatic stellate cells. We tested the first hypothesis measuring VLDL secretion in a cohort of 55

individuals genotyped for the I148M variant and we found that carriers of the 148M allele secret less VLDL for a given amount of liver fat. We confirmed this result *in vitro* by measuring APOB secretion in cell lines stably overexpressing the 148I or the 148M PNPLA3. We tested the second hypothesis performing enzymatic activity assays using purified 148I and 148M recombinant proteins. The wild type protein had glycerolipid hydrolase activity and the 148M mutation induced a loss of function. Finally, we tested the third hypothesis assessing the effect of PNPLA3 up- and down-regulation on hepatic stellate cell retinyl palmitate content and retinol release. We found that PNPLA3 insulin–mediated up-regulation induces retinol release from hepatic stellate cells and that this effect is abolished by PNPLA3 silencing. We confirmed this finding by looking at human circulating levels of RBP4, a reliable marker of retinol plasma levels, in 146 individuals genotyped for the I148M variant. We found carriers of the M allele to have lower RBP4 plasma levels, confirming the role of PNPLA3 in retinol metabolism.

In conclusion, we identified two possible mechanisms underlying the susceptibility to chronic liver disease in carriers of the PNPLA3 mutation: 1) reduced intracellular triglyceride mobilization leading to hepatocyte damage 2) impaired hepatic stellate cell retinol metabolism causing abnormal response of hepatocytes to damaging agents.

Keywords: NAFLD, PNPLA3, VLDL, retinol, hepatic stellate cells

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SAMMANFATTNING PÅ SVENSKA

Kronisk leversjukdom är ett stort hälsoproblem i hela världen. De viktigaste faktorerna för detta tillstånd är virusinfektioner , alkoholmissbruk och fetma. Den genetiska bakgrunden påverkar effekten av skada på levern. Den genetiska varianten rs738409 i PNPLA3 genen förknippas med ökad mottaglighet för kronisk leversjukdom, särskilt med alkoholfri fettlever. Varianten ger en isoleucin till metionin substitution vid position 148 (I148M) i amino syra sekvensen och sattes först i samband med ökat innehåll av hepatocyte fett. Trots det starka genetiska sambandet, är de mekanismer som orsakar lever ansamling av fett och hepatocyte skador ännu inte klarlagda.

I denna avhandling , testade vi följande hypoteser : 1) PNPLA3 är inblandade i lever VLDL sekretion 2) proteinet fungerar som en glycerolipid hydrolas och 148M mutationen är en förlust av funktion 3) PNPLA3 har en specifik roll i retinol ämnesomsättningen i ”hepatic stellate cells” (HSC). För att testa den första hypotesen mätte vi VLDL sekretion i en kohort av 55 personer genotypade för I148M varianten och vi fann att bärare av 148 M allelen utsöndrar mindre VLDL för en given mängd leverfett. Vi bekräftade detta resultat *in vitro* genom mätning av apoB sekretion i celler som stabilt överuttrycker 148I eller 148M PNPLA3. Vi testade den andra hypotesen genom att göra enzymatiska aktivitetsanalyser med hjälp av renade 148I och 148m rekombinanta proteiner. 148I proteinet hade glycerolipid hydrolasaktivitet och 148M mutationen inducerade en förlust av funktion. Slutligen testade vi den tredje hypotesen genom att bedöma effekten av PNPLA3 upp - och nedreglering på HSC retinylpalmitat innehåll och retinol frisättning. Vi fann att PNPLA3 insulin-medierad uppreglering inducerar retinol frisättning från HSCs och att denna effekt förstörs av PNPLA3

dämpningen. Vi bekräftade detta genom att titta på nivåer av RBP4, en tillförlitlig markör för retinol plasmanivåer, i 146 individer genotypade för I148M varianten. Vi fann att bärare av M allelen har lägre RBP4 plasmanivåer, vilket bekräftar den roll PNPLA3 har i retinol ämnesomsättning.

Sammanfattningsvis identifierade vi två möjliga mekanismer bakom mottaglighet för kronisk leversjukdom hos bärare av PNPLA3 mutationen: 1) minskad intracellulär triglycerid mobilisering leder till hepatocyte skador 2) nedsatt HSC retinol ämnesomsättning orsakar onormalt stark reaktion av hepatocyter på skadliga ämnen.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **C Pirazzi**, M Adiels, MA Burza, RM Mancina, M Levin, M Ståhlman, MR Taskinen, M Orho-Melander, J Perman, A Pujia, L Andersson, C Maglio, T Montalcini, O Wiklund, J Borén, S Romeo

Patatin-like phospholipase domain-containing 3 (PNPLA3) I148M (rs738409) affects hepatic VLDL secretion in humans and in vitro

Journal of Hepatology 57: 1276–1282 2012

II. P Pingitore*, **C Pirazzi***, RM Mancina, BM Motta, C Indiveri, A Pujia, T Montalcini, K Hedfalk, S Romeo
*equal contribution

Recombinant PNPLA3 protein shows triglyceride hydrolase activity and its I148M mutation results in loss of function

Biochim Biophys Acta 1841: 574–580 2014

III. **C Pirazzi**, L Valenti, BM Motta, P Pingitore, K Hedfalk, RM Mancina, MA Burza, C Indiveri, Y Ferro, T Montalcini, C Maglio, P Dongiovanni, S Fargion, R Rametta, A Pujia, L Andersson, S Ghosal, M Levin, O Wiklund, M Iacovino, J Borén, S Romeo

PNPLA3 has retinyl-palmitate lipase activity in human hepatic stellate cells

In manuscript

CONTENT

ABBREVIATIONS

αSMA	alpha-smooth muscle actin
ALT	alanine transaminase
APOB	apolipoprotein B
APOC3	apolipoprotein C3
AGPAT	1-acylglycerol-3-phosphate O-acyltransferase
ATGL	adipose triglyceride lipase
ATP	adenosine triphosphate
ChREBP	carbohydrate-responsive element-binding protein
CoA	coenzyme A
DAG	diacylglycerol
ELISA	enzyme-linked immunosorbent assay
GCKR	carbohydrate-responsive element-binding protein
GPAT	glycerol-3-phosphate acyl transferase
GWAS	genome-wide association study
HSCs	hepatic stellate cells
LPA	lysophosphatidic acid
LPAAT	lysophosphatidic acid acyl transferase
LRAT	lecithin-retinol acyl transferase
LYPLAL1	lysophosphatidic acid
MAG	monoacylglycerol

McA-RH	McArdle rat hepatoma
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTP	microsomal triglyceride transfer protein
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NCAN	neurocan
PA	phosphatidic acid
PDGF(R)	platelet-derived growth factor (receptor)
PNPLA3	patatin-like phospholipase domain-containing 3
PPP1R3B	protein phosphatase 1, regulatory subunit 3B
RAR	retinoic acid receptor
RBP4	retinol binding protein 4
RXR	retinoid X receptor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small interfering ribonucleic acid
SREBP-1c	sterol regulatory element binding protein-1c
TAG	triacylglycerol
TGFβ(R)	transforming growth factor beta (receptor)
TIMP	tissue inhibitor of metalloproteinase
TM6SF2	transmembrane 6 superfamily member 2
VLDL	very low density lipoprotein secretion

1 INTRODUCTION

This thesis examines the function of the patatin-like phospholipase domain-containing 3 (PNPLA3) protein in three different settings: triglyceride secretion from hepatocytes in the form of very low density lipoproteins (VLDL); *in vitro* enzymatic activity of the purified protein; retinol metabolism in hepatics stellate cells (HSCs). In each of this settings, the role of PNPLA3 is investigated together with the effect on the protein function of a common genetic variant in the protein, the isoleucine to methionine substitution at position 148 (I148M), whose association with non-alcoholic fatty liver disease (NAFLD) is well established.

1.1 Hepatic lipid metabolism

Liver is the major metabolic organ in human body[1]. Hepatic lipid metabolism takes place in the hepatocyte[2], the liver cell unit. Lipids are needed to build cell membranes, to create energy in the form of ATP, to synthetize hormones, to transport intracellular signals as second messengers and for many other functions[3,4]. In the hepatocytes, lipids coming from the diet are processed in order to be used, stored, or delivered to the periphery via the blood stream[5]. The hepatocyte is also able to newly synthetize lipids starting from simpler molecules, such as acetyl-Coenzyme A[6].

1.1.1 Intracellular triglyceride metabolsim

Triglycerides are neutral lipids composed of a glycerol backbone esterified by three fatty acids[7]. They have a high energetic content and represent a suitable form of lipid storage[8]. They can also be transported in the blood stream and delivered to the periphery after being packaged in lipoproteins[9].

Triglyceride synthesis begins with the link between a molecule of glycerol phosphate (from glucose catabolism) and an acyl-CoA to obtain lysophosphatidic acid (LPA). This reaction is catalyzed by the enzyme glycerol phosphate acyl transferase (GPAT). In the next step, the LPA is esterified with another acyl-CoA by the lysophosphatidic acid acyl-transferase (LPAAT) to obtain phosphatidic acid (PA). The PA is then dephosphorylated and the resulting diacylglycerol is esterified with a third acyl-CoA to triacylglycerol by the diacylglycerol acyl transferase [10]. Alternatively, a triglyceride can be obtained by consecutive acylation steps of a monoacylglycerol catalyzed by monoacylglycerol-acyl transferase (MAG) and diacylglycerol-acyl transferase (DAG)[11].

On the other hand, triglycerides can be catabolized to diacylglycerol, monoacylglycerol and finally glycerol and free fatty acids by lipase enzymes, which break the ester bounds between the glycerol hydroxyl groups and the carboxylic group on the fatty acid head[12].

1.1.2 Very low density lipoprotein secretion

In order to reach peripheral tissues and organs, lipids and in particular triglycerides need to be transported in the blood stream. Given their hydrophobicity, they cannot be in a water solution such as blood as free molecules. Instead, within the hepatocyte they are packaged in particles called lipoproteins, composed by apolipoproteins and lipids[13]. The protein fraction of these particles allows water solubility and, at the same time, presents hydrophobic domains that can bind lipids[14].

Lipoproteins are classified based on their density[15]. Very low density lipoproteins (VLDL) are characterized by the lowest density and the largest size. VLDLs are responsible for the transport in the blood stream of

triglycerides and in fasting conditions circulating VLDLs correspond to circulating triglycerides[16]. The main apolipoprotein of VLDL particles is the apolipoprotein B100 (APOB100), synthetized by the liver[17]. Each VLDL particle contains one APOB100 protein[17]. In the hepatocyte endoplasmic reticulum, a newly synthetized APOB100 molecule is lapidated with triglycerides by the microsomal triglyceride transfer protein (MTP) and is then secreted in the blood stream[9].

1.1.3 Triglyceride storage

Triglycerides are easy to store. Indeed, their neutral charge makes them not harmful for the intracellular environment. When needed, they can be mobilized by the action of lipases and made available for utilization[18]. In physiological conditions, adipose tissue is the main storage site for triglyceride in human body[19]. However, in conditions of increased caloric intake or reduced energy expenditure, excess triglycerides are stored in ectopic sites such as muscle and liver[20]. In particular, accumulation of triglycerides in the hepatocytes is identified as liver steatosis and consists of formation of lipid droplets within the cytoplasm. Steatosis is not harmful by itself and is reversible upon reduced caloric intake and increased energy expenditure. However, steatosis represents the first step in a process that can progress to various degrees of lever injury. The entire spectrum of steatosis-related liver injury degrees is known as non-alcoholic fatty liver disease.

1.2 Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is characterized by fat accumulation into the liver in individuals without viral, congenital, and autoimmune liver diseases who refer no alcohol consume[21]. It comprehends the entire spectrum of liver injury stages, from simple steatosis to chronic

steatohepatitis, liver cirrhosis and possibly hepatocellular carcinoma (HCC)[22].

1.2.1 Natural history

NAFLD onset is characterized by hepatic steatosis, usually caused by unbalance between caloric intake and energy expenditure[23] **(Figure 1)**. Simple steatosis is asymptomatic and is usually diagnosed by imaging techniques. Increased ALT levels do not discriminate steatosis from normal liver, other liver diseases or more advanced stages of NAFLD[24]. Steatosis prevalence in western countries is 20% in the general population, but increases up to 80% in obese individuals[25]. Uncomplicated steatosis remains stable in most cases and may even regress. However, in 5% of affected individuals a progression to non-alcoholic steatohepatitis (NASH) is observed[26]. NASH is characterized by inflammation, hepatocyte ballooning and, possibly, mild fibrosis[27]. This condition is associated with a reduced life expectancy and progresses to advanced fibrosis in 15-30% of cases[24]. Among individuals with advanced fibrosis, 12-35% develops cirrhosis, the end-stage liver disease[24], for which liver transplantation is the only available therapy. On top of this, one third of individuals with cirrhosis will develop hepatocellular carcinoma[22].

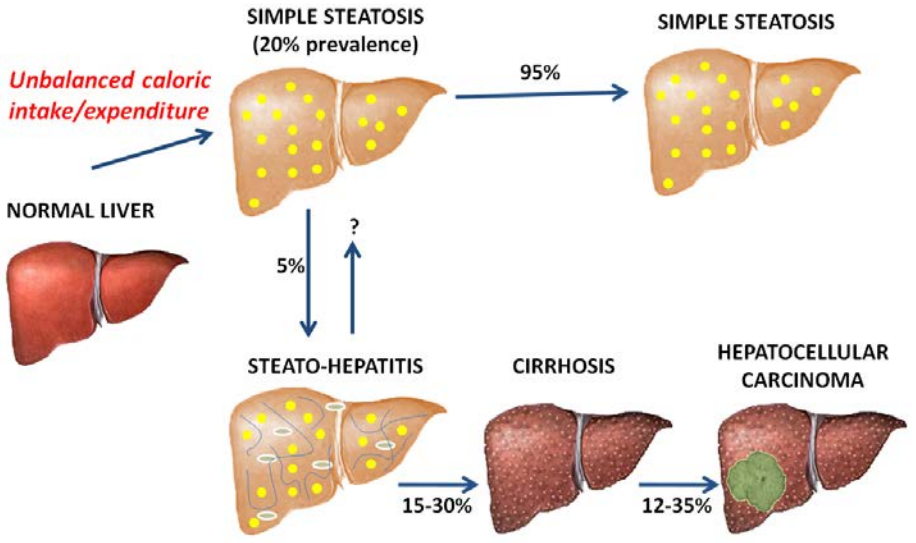

Figure 1. Natural history of non-alcoholic fatty liver disease.

1.2.2 Etiopathogenesis

NAFLD development begins with a net retention of triglycerides within hepatocytes. Alterations in uptake, synthesis, degradation, or secretion of triglycerides can all lead to intracellular accumulation[26]. The major determinant of these alterations is insulin resistance[26]. This condition is characterized by increased levels of circulating insulin and reduced insulin response of the glucose homeostasis mechanisms[28]. However, the insulin anabolic effects on lipid metabolism remain active, determining increased de novo lipogenesis, reduced lipolysis and consequent intracellular lipid accumulation[28]. Unbalanced caloric intake and energy expenditure trigger the mechanisms underlying the onset of insulin resistance and NAFLD, and interact with genetic background in determining the disease phenotype[29].

1.2.3 Human Genetics of steatosis: monogenic and polygenic determinants

Rare mutations in key genes for liver lipid metabolism cause monogenic forms of hepatic steatosis. Among these, abetalipoproteinemia, lipodystrophy and carnitine palmitoyltransferase II deficiency are the best understood. Abetalipoproteinemia is caused by mutations in the microsomal triglyceride transfer protein (MTP) gene, which make impossible for the liver to secrete APOB100-containing lipoproteins[30]. Therefore, fat accumulates in the hepatocytes. Lipodystrophy is characterized by an abnormal body fat distribution and is classified as general or partial. It can be caused by mutation in various genes. As a common feature, mutations causing lipodystrophy affect adipocyte lipid metabolism, thus inducing liver fat accumulation[31]. Carnitine palmitoyltransferase II deficiency impairs fatty acid oxidation within the hepatocyte, causing an increase in liver fat content[32].

The effect of common genetic variants in determining steatosis and NAFLD is well established[33]. Several genes and mutations have been found to associate with increased liver fat content and increased risk of disease progression from simple steatosis towards chronic liver disease. The most recently identified genetic variant is a glutamate to lysine substitution at residue 167 within the *TM6SF2* gene (rs58542926)[34]. Other common (minor allele frequency $\geq$5%) mutations have been found to associate with increased liver fat content in *NCAN* (rs2228603), *PPP1R3B* (rs4240624), *GCKR* (rs780094), *LYPLAL1* (rs12137855)[35] and *APOC3* (rs2854116)[36] genes.

Up to date, the most widely replicated genetic variant to associate with liver fat content[37] and with the entire spectrum of NAFLD[38] is an isoleucine to

methionine substitution at residue 148 (I148M, rs738409) in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene.

1.3 Patatin-like phospholipase domain-containing 3 protein

PNPLA3, also known as adiponutrin, is a phospholipase belonging to the family of patatin-like domain containing phospholipases[39]. Despite the strong evidence that link the I148M mutation in this protein to NAFLD[38], the molecular mechanisms underlying this association are not yet completely understood.

1.3.1 Overview

PNPLA3 belongs to the patatin-like domain containing phospholipase family[40]. The family name originates from the fact that the first family member was discovered in potatoes[41]. These lipases are characterized by an atypical catalytic site constituted of a dyad serine-aspartate[42]. The *PNPLA3* gene, located on chromosome 22, has been identified for the first time as a determinant of adipocyte differentiation in an mRNA screening performed on mouse pre-adipocytes to compare changes in mRNA expression before and after differentiation[43]. While in mice adipose tissue is the main tissue where *pnpla3* is expressed[44], in humans liver and skin show the highest expression[45]. Moreover, the human protein is composed by 481 amino acids[46], whereas the mouse pnpla3 is approximately100 amino acid shorter. The lipase activity is catalyzed by a serine at position 47 and an aspartate at position 166[47].

PNPLA3 expression levels are under the control of insulin signaling, mediated by a sterol regulatory element binding protein 1c (SREBP-1c)

binding site within intron 1 of the gene[45]. *PNPLA3* gene also contains a carbohydrate response element that binds ChREBP[48]. *PNPLA3* mRNA expression is down-regulated by fasting and up-regulated by subsequent re-feeding, as opposed to its closest family member, PNPLA2 (also known as ATGL)[45]. Conflicting data have been published on the specific enzymatic activity of the PNPLA3 protein and on the effect of the I148M mutation.

1.3.2 Genetic association with liver disease: the I148M variant

PNPLA3 rose to the attention of the scientific community after a genome wide association study that found its common genetic variant I148M to associate with liver fat content in a general population of more than 2000 individuals[46]. Virtually at the same time, another GWAS showed the same variant to associate with circulating levels of liver enzymes[49]. Since then, more than 200 papers have been published investigating on the association between PNPLA3 I148M variant and the entire spectrum of liver disease. Results from this massive body of work have provided evidence for association between PNPLA3 and steatosis[35,37,50], liver fibrosis progression[51-53], cirrhosis[54,55], hepatocellular carcinoma[56-58] in individuals with NAFLD, alcoholic liver disease, Hepatitis C virus infection, hereditary hemochromatosis[59] and primary sclerosing cholangitis[60]. Taken as a whole, these data indicate that the I148M variant represents a genetic background that exposes to an increased risk of liver injury induced by various environmental agents.

1.3.3 *In vitro* and *in vivo* functional studies

Numerous studies have been performed on *in vitro* and *in vivo* models to unravel the molecular mechanism underlying the genetic association between

PNPLA3 I148M variant and liver injury. Based on the results of these studies, two main hypotheses have been proposed: PNPLA3 might be a glycerolipid hydrolase, with the 148M mutation resulting in a loss of function; alternatively, PNPLA3 might have lysophosphatidic acid acyl-transferase (LPAAT) activity and the 148M mutation would induce a gain of function. In both cases, the mutation would cause an increase in intracellular triglyceride content.

Supporting the hydrolase hypothesis, in human hepatocyte models overexpression of the wild type protein has been shown to induce lipolysis whereas the 148M mutant protein did not show lipolysis activity[47,48]. These data were confirmed by an in vitro study using wild type or mutant PNPLA3 purified from insect cells, in which the wild type protein showed glycerolipid hydrolysis activity, whereas the mutant protein did not[61]. Furthermore, transgenic mice chronically overexpressing the human mutant PNPLA3 showed decreased glycerol release from triglycerides compared to chronic WT PNPLA3 overexpression[62].

On the other hand, the LPAAT hypothesis was supported by the finding that mice acutely overexpressing the human WT had hepatic histology similar to controls, whereas mice acutely overexpressing the mutant protein showed increased lipid fat content[47]. This data were consistent with mouse knock-out models that did not show any specific phenotype[63,64]. Moreover, PNPLA3 wild type protein purified from *E. coli* was shown to have LPAAT activity in vitro, and the 148M mutation to induce a gain of this function[65].

1.4 Hepatic stellate cells

Hepatic stellate cells (HSCs) are mesenchymal cells that lie within the Disse space around the hepatic sinusoids[66]. Also known as perisinusoidal cells or

Ito cells, they play a major physiological role in retinol metabolism[67] and are key players in liver injury[68].

1.4.1 General characteristics

In physiological conditions, HSCs are in quiescent state and present long protrusions that wrap around the sinusoids[69]. Their cytoplasm is characterized by numerous lipid droplets, containing retinyl esters. The role of HSCs in normal liver is still unclear, although some evidences suggest that they might work as antigen presenting cells specialized in presenting lipid antigens[70].

1.4.2 Role in retinol homeostasis

HSCs represent approximately 10% of the total number of cells in the liver. Despite this, they contain more than 70% of the total retinol stored in the entire body[71]. The retinol is delivered to the HSCs from hepatocytes and is stored in lipid droplets mainly as retinyl palmitate[72]. The enzyme that catalyzes the esterification between palmitic acid and retinol is the lecithin-retinol acyl transferase (LRAT)[73]. The retinol stored in HSCs can be released and made available upon needing. However, the enzyme that catalyzes the hydrolysis of retinyl palmitate to retinol and palmitic acid has not yet been identified. Several candidates from in vitro or animal experiments have been studied, but so far none of them has been shown to function in humans[74,75]. The retinol content of HSCs is completely lost during liver injury and subsequent HCS activation[76].

1.4.3 Role in chronic liver disease

During liver injury, HSCs lose their retinyl palmitate lipid droplets and leave the quiescent state, undergoing a phenotype change towards myofibroblasts[77]. It is not known whether the lipid droplet loss is cause or a consequence of the

activation, nor whether this loss is protective or deleterious for the injured hepatocytes. Nevertheless, activated HSCs become able to contract and secrete collagen, causing fibrosis and subsequent liver cirrhosis[78]. Once activated, they also secrete enzymes that induce extracellular matrix remodeling, such as matrix metalloproteinases (MMPs)[79] and their respective inhibitors (tissue inhibitors of metalloproteinase, TIMPs)[80]. Furthermore, they express the typical myofibroblast marker α-smooth muscle actin (alpha-SMA)[81] and several cytokine receptors, such as transforming growth factor β receptor (TGFR)[82] and platelet-derived growth factor receptor (PDGFR)[83]. In particular, transforming growth factor β (TGFβ) represent the most potent activator of HSCs[84]. Once established, the fibrosis process is virtually not reversible and leads to liver cirrhosis.

1.5 Vitamin A

Vitamin A comprehends various liposoluble unsaturated organic compounds that are divided in two main groups: retinoids (retinol, retinaldheyde and retinoic acid) and carotenoids (mainly β-carotene). Both groups include compounds constituted by a beta-ionone ring attached to an isoprenoid chain. This structure is called retinyl group and retinoids are composed by 1 retinyl group, while carotenoids result from the union of 2 retinyl groups[85]. As a micronutrient, vitamin A plays a role in many physiological functions and it is supplied by diet in two main forms: as retinoids from meat (esters of retinol) and as carotenoids from vegetables (β-carotene).

1.5.1 Metabolism in human body

Once absorbed by the intestine, retinol is packaged in chylomicrons as retinyl ester[86]. B-carotene is first cleaved in two retinol molecules, which are then esterified in chylomicrons. Chylomicrons reach the liver via the venous portal

system and within the hepatocytes retinyl esters are hydrolyzed to retinol and fatty acids by hepatocytes retinyl esterases[75]. At this stage, retinol can undergo various fates: it can be converted to retinoic acid, a potent activator of transcription factors[87]; it can be released in the blood stream bound to the retinol binding protein 4 (RBP4) to reach the periphery[88]; it can be delivered to hepatic stellate cells for storage as retinyl palmitate[89]. The retinol stored in hepatic stellate cells can be in turn released and made available for further uses. The retinyl esterase responsible for release of retinol from retinyl palmitate in hepatic stellate cells has not yet been identified.

1.5.2 Physiological functions

Retinoids represent the active compounds among those included in the group of vitamin A. The specific function of the different retinoids in physiological processes depends on their chemical structure[90].

Retinol is characterized by an alcoholic group at the free end of the isoprenoid chain. Its main functions are storage and transport. For storage, retinol is esterified to a fatty acid, mostly palmitic acid, in order to obtain a neutrally charged molecule that is packaged in lipid droplets[91]. For transport, retinol moves through the blood stream bound to a complex composed by RBP4 and transthyretin[92]. Transthyretin prevents glomerular filtration of the retinol-RBP4 complex.

Retinaldheyde is characterized by an aldehydic group at the free end of the isoprenoid chain. It is formed by oxidation of a retinol molecule, catalyzed by retinol dehydrogenase[93]. This reaction is reversible. In the retinal epithelium, retinaldheyde (also called retinal) is covalently bound to the protein opsin to form rhodopsin. Retinal is present rhodopsin as 11-*cis*-retinal. Upon light activation, 11-*cis*-retinal becomes all-*trans*-retinal, inducing a conformational

change in the opsin structure that is transmitted to coupled G protein. These events represent the bases of the sight function[94].

Retinoic acid is characterized by a carboxylic group at the free end of the isoprenoid chain. It is formed through oxidation of retinaldheyde by the retinaldheyde dehydrogenase[95]. This reaction is not reversible. It mainly exerts its function as a ligand of nuclear receptors. In particular, retinoic acid binds retinoic acid receptor (RAR) and retinoid X receptor (RXR), which are present as a DNA transcription repressor heterodimer. Retinoic acid binding induces a dissociation of the heterodimer and therefore the activation of transcription of more than 500 genes. The retinoic acid-mediated regulation of transcription is fundamental for embryonic development[96].

1.5.3 Deficiency and hypervitaminosis

Vitamin A deficiency is the main cause of blindness in developing countries[97]. The inability to produce enough retinal for the sight function leads to night blindness and, subsequently, to total blindness. Deficiency of vitamin A during pregnancy can cause miscarriage and fetal abnormalities, given the importance of retinoic acid in embryogenesis. Vitamin A deficiency has been also associated with increased infection susceptibility. Other than malnutrition, important causes of vitamin A deficiency are alcohol abuse, iron deficiency and fat malabsorption conditions.

Hypervitaminosis A is a condition determined by excessive intake of pre-formed vitamin A[98]. The vitamin A excess leads to bone metabolism abnormalities, sight function alterations, liver toxicity, seborrhea, skin yellow discoloration and nausea. The symptoms can regress with vitamin A intake suspension. Given the strict regulation of carotenoid conversion to retinal, hypervitaminosis A caused by β-carotene excessive intake is virtually

impossible. On the contrary, animal meet, and in particular the liver of certain species such as seal, moose, walrus and polar bear, is highly enriched in preformed vitamin A and thus high intake can lead to hypervitaminosis A.

2 AIM

The overall aim of this thesis is to understand the molecular mechanisms underlying the genetic association between the PNPLA3 I148M genetic variant and chronic liver disease. The specific aims of the three papers are:

- **Paper I:** to examine the role of PNPLA3 in hepatic VLDL secretion in humans

- **Paper II:** to study the PNPLA3 enzymatic activity and the effect of the 148M mutation *in vitro* using purified proteins

- **Paper III:** to assess the role of PNPLA3 in retinol metabolism in human primary hepatic stellate cells

3 MATHERIALS AND METHODS

In this section, a general description of the methods used in the thesis is provided. A more detailed explanation of the techniques is given in the Materials and Methods section of the papers.

3.1 Göteborg cohort

To study the effect of the PNPLA3 I148M genetic variant on the hepatic VLDL secretion, we used a cohort of 55 overweight individuals genotyped for the PNPLA3 I148M variant. In this cohort, liver fat content measured by nuclear magnetic resonance was available. None of the enrolled individuals was taking lipid lowering medications. Alcohol consumption, diabetes, BMI>40, severe hyperlipidemia, thyroid, kidney, heart, liver and hematological abnormalities were considered exclusion criteria. All individuals gave inform consent to the study and the protocol was approved by local ethical committee.

3.2 VLDL kinetic analysis

Hepatic VLDL secretion was measured as follows. First, a bolus injection of leucine and glycerol containing stable isotopes of the hydrogen was administered. Next, circulating VLDLs were isolated by ultracentrifugation and isotope enrichment in APOB100 (leucine) and triglycerides (glycerol) was assessed by mass spectrometry. Data were finally fit in a mathematical model that allows estimating amount of triglycerides and APOB100 secreted from the liver as VLDL particles.

3.3 McA-RH 7777 stable cell line

McArde rat hepatoma cells (McA-RH 7777) cells were selected to investigate *in vitro* the role of PNPLA3 on VLDL secretion given their ability to secrete VLDL-like sized Apob-containing triglyceride-rich particles. In order to obtain cell lines stably overexpressing human 148I or 148M PNPAL3, cells were first transfected with pcDNA3.1 vectors containing the respective PNPLA3 cDNAs, a V5-tag and G418 resistance. After 48 hours, cells that integrated the wild type or mutant vector in their DNA were selected by adding G418 to the growth medium and 6 single clones were isolated and expanded. Stable overexpression was checked by western blot on cell lysates using an anti-V5 antibody.

3.4 *In vitro* ApoB secretion analysis

ApoB secretion from McA-RH 7777 cells stably overexpressing the 148I and 148M PNPLA3 was measured by pulse-chasing protein secretion with radiolabeled methionine in the growth medium after stopping protein synthesis by incubation with methionine-free medium. Apob was subsequently immune-precipitated using a polyclonal anti-Apob antibody, run on SDS-PAGE and visualized by gel exposure to a phosphor screen.

3.5 PNPLA3 148I and 148M protein purification

PNPLA3 148I and 148M cDNAs were cloned into the expression vectors pPICZB containing a 6his-tag and transformed in the specific host Pichia pastoris. After growth in fermenters, cells were broken and wild type and mutant PNPLA3 proteins were purified from the membrane fraction by Ni-

Ni-affinity chromatography. Protein purity was assessed by western blot using anti PNPLA3 and anti-6his antibodies, Coomassie staining and mass spectrometry analysis.

3.6 Enzymatic activity assays

Purified wild type and mutant PNPLA3 enzymatic activities were tested by incubation with radiolabeled substrates and subsequent reaction product purification by thin layer chromatography and measurement by scintillation counting. The following activities were tested: glycerolipid hydrolase (by incubation with triolein, diolein and monolein), thioesterase (by incubation with oleoyl-CoA) and lysophosphatidic acid acyl-transferase (by incubation with lysophosphatidic acid and oleoyl-CoA.

3.7 *In vitro* and *ex vivo* hepatic stellate cells models

LX-2 cells, an immortalized line of human hepatic stellate cells, were kindly provided by Professor Scott L. Friedman (Mount Sinai School of Medicine, New York, USA). This cell line was obtained by transfecting human HSCs isolated from liver by ultracentrifugation with the SV40 large T antigen (Simian Vacuolating Virus 40 TAg), a proto-oncogene from the polyomavirus SV40. The cell line was next selected for growth in medium containing 2% fetal calf serum.

Primary human hepatic stellate cells were obtained by Sciencell and grown according to the manifacturer's instructions. In particular, cells were plated in flasks or plates after coating with Poly-L-Lysine.

3.8 Retinol metabolism assessment

In all experiments, hepatic stellate cells were incubated with palmitic acid and retinol. Intracellular accumulation of retinyl palmitate was visualized by Oil red O staining of cytoplasmic lipid droplets and measured by radiolabeling [^{3}H]-retinol. Extracellular retinol release was measured in cold medium after incubation with [^{3}H]-retinol and palmitic acid. The effect on retinol metabolism of PNPLA3 up- and down-regulation was assessed by treatment with insulin and PNPLA3-siRNA respectively.

3.9 Milano cohort

PNPLA3 I148M variant was genotyped in a cohort of 146 individuals with biopsy proven non-alcoholic fatty liver disease. Other causes of liver disease were considered as exclusion criteria. All individuals gave inform consent to the study and the protocol was approved by local ethical committee.

Retinol binding protein 4 (RBP4) fasting plasma levels were measured by enzyme-linked immunosorbent assay (ELISA).

3.10 Retinol binding protein 4 analysis

Linear regression model or general linear model were used to assess differences in RBP4 levels across PNPLA3 I148M genotypes. Analysis was adjusted for confounding factors. Differences in RBP4 levels between paired genotypes were tested by post-hoc analysis using a Fisher's Least Significant Difference test.

4 RESULTS

In this section a general description of the main findings from the three papers included in the thesis is presented. Results are fully shown in the Results sections of the full papers.

4.1 Paper I

In paper I, we found PNPLA3 to be involved in hepatic VLDL secretion in humans and *in vitro*. In particular, by analyzing hepatic VLDL secretion in 55 overweight individuals genotyped for the PNPLA3 I148M variant, we found carriers of the 148M mutant allele to secrete less VLDL compared to wild type individuals for a given amount of liver fat.

We confirmed this finding in an *in vitro* model by overexpressing the 148I or the 148M PNPLA3 protein in McA-RH 7777 cells. We found cells overexpressing the mutant protein to secrete less APOB and to hydrolyze less triglycerides.

4.2 Paper II

In paper II, we demonstrated that purified PNPLA3 has a glycerolipid hydrolase activity. We purified the wild type and mutant proteins using the yeast P. pastoris as expression system. Wild type PNPLA3 was active on triolein **(Figure 2)** and diolein, but not on monolein. PNPLA3 showed also thioesterase activity.

The 148M mutant protein did not exhibit any of these activities, suggesting the methionine substitution induces a loss of function of the protein. Only a

mild lysophosphatidic acid acyl transferase activity was observed for the wild type protein, while the mutant protein showed no LPAAT activity.

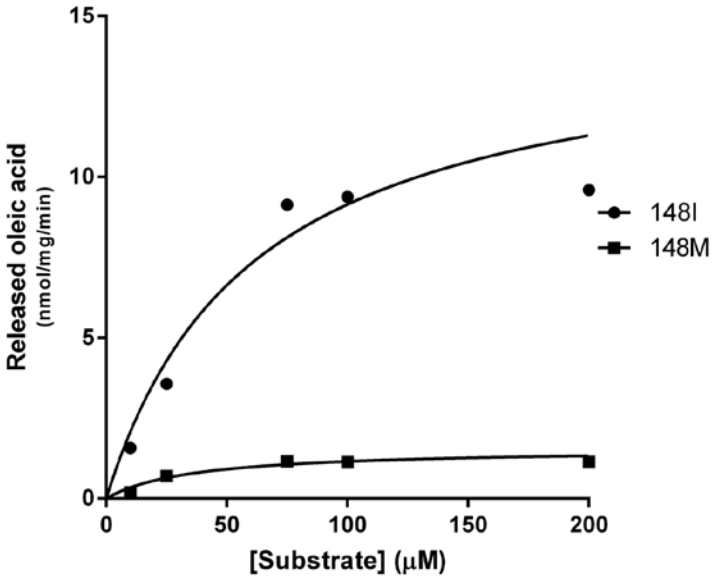

Figure 2. PNPLA3 148I has triglyceride hydrolase activity and the 148M substitution induces a loss of function. Purified wild type and mutant PNPLA3 were incubated with radiolabeled triolein. Released oleic acid was measured by scintillation counting and data were fit into Michaelis-Menten equation.

Presence or not of detergent in protein elution buffer and pH condition changes did not affect PNPLA3 activity.

4.3 Paper III

In paper III, we showed PNPLA3 to be involved in retinol metabolism in human hepatic stellate cells. We demonstrated that PNPLA3 mRNA and protein are highly expressed in HSCs and that this expression is modulated by retinyl palmitate availability.

Using siRNA against PNPLA3, we could demonstrate that insulin-dependent retinol release from HSCs was specifically mediated by PNPLA3. We also showed that HSCs overexpressing the wild type PNPLA3 had less intracellular retinyl palmitate-containing lipid droplets than HSCs overexpressing the mutant protein **(Figure 3)**.

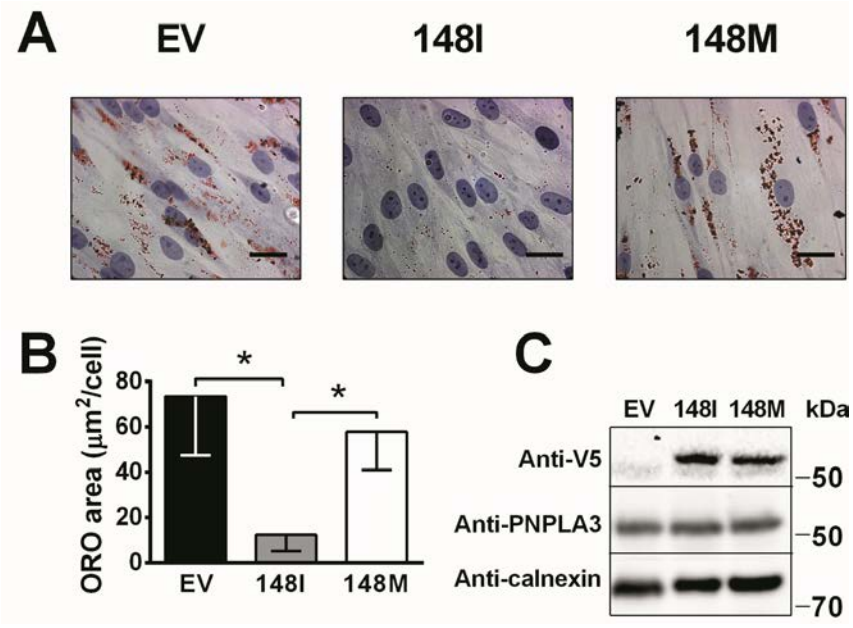

Figure 3. Overexpression of the wild type but not the mutant PNPLA3 induces retinyl palmitate lipid droplet breakdown in human pHSCs. Human primary hepatic stellate cells (pHSCs) were transfected with 148I or 148M PNPLA3 or with an empty vector. Lipid droplet content was visualized by Oil Red O (ORO) staining (A) and quantified by Biopics (B). Transfection efficiency was assessed by western blot (C).

We tested retinyl esterase activity of purified PNPLA3 and found the wild type protein hydrolyzes retinyl palmitate, whereas the mutant protein does not. Finally, we showed that homozygotes for the 148M mutant allele have lower circulating levels of RBP4, a reliable marker of circulating retinol levels, compared to carriers of the wild type allele.

5 DISCUSSION

5.1 PNPLA3 and VLDL secretion

Liver fat accumulation can be driven by several metabolic alterations, including increased de novo lipogenesis and triglyceride synthesis, decreased lipolysis and β-oxidation, increased hepatocyte fat uptake and reduced secretion[99]. Several studies have shown an association between the PNPLA3 148M mutant allele and lower triglyceride levels, especially in obese individuals[100-102]. Individuals with defects in the APOB gene show increased liver fat content due to inability to secrete VLDL[103]. We demonstrated that PNPLA3-dependent liver fat accumulation is driven by a reduced triglyceride secretion by the liver via VLDL particles.

5.1.1 Evidences from human kinetic data

In a cohort of 55 overweight individuals, we first confirmed the increased liver fat content in carriers of the M allele. Given the known positive correlation between liver fat content and VLDL secretion[104], we would have expected carriers of the M allele to secrete more VLDL. However, we could not find any differences in absolute VLDL secretion among genotypes. To explain this unexpected finding, we analyzed the correlation between liver fat content and VLDL secretion across the PNPLA3 genotypes. In the overall cohort, there was a positive correlation between liver fat content. This correlation was preserved within PNPLA3 genotypes, but carriers of the 148M mutant allele showed lower VLDL secretion compared to wild type individuals for the same amount of liver fat.

5.1.2 *In vitro* ApoB secretion

We confirmed our findings on humans using an in vitro model to study ApoB secretion. ApoB is the main VLDL apolipoprotein and each VLDL contains one ApoB molecule[13]. We decided to use McArdle rat hepatoma (McA-RH 7777) cells for our aim given their ability to secrete VLDL-like sized triglyceride-rich lipoprotein particles[105]. We showed that cells stably overexpressing the 148M mutant PNPLA3 secreted less ApoB, had reduced lipolytic activity and increased fat content compared to those overexpressing the wild type protein. These results were consistent with our findings in the human cohort.

5.1.3 Proposed model

Based on our findings and given the intracellular localization of PNPLA3 on lipid droplets and endoplasmic reticulum, we proposed a model **(Figure 4)** in which PNPLA3 mobilize triglycerides stored in lipid droplets, making them available for incorporation in nascent VLDL particles. By inhibiting this function, the 148M mutation causes reduced VLDL secretion and increased liver fat retention.

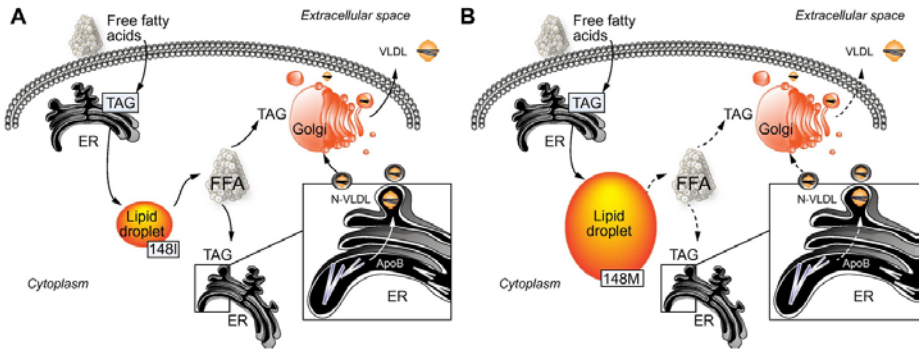

Figure 4. Putative model for PNPLA3 function.

5.2 PNPLA3 enzymatic activity

Conflicting results have been published on the PNPLA3 function and on the effect on it of the 148M mutation. Two main hypotheses have been proposed: PNPLA3 may act as glycerolipid hydrolase, with the mutation resulting in a loss of function[61]; alternatively, PNPLA3 might have lysophosphatidic acid acyl transferase activity and the mutation would be a gain of function[65]. In both cases, the net result of the mutation is increased liver fat content.

5.2.1 Purification system suitability

To clarify these conflicting results, we decided to produce purified wild type and 148M mutant PNPLA3 protein and to test both enzymatic activities. We selected the yeast Pichia pastoris as expression system for the protein, based on the high protein yield that this system allows to obtain[106]. Furthermore, being a eukaryotic host, P. pastoris guarantees correct folding and processing of membrane-attached proteins, such as PNPLA3[107]. Indeed, we were able to purify high amount of wild type and mutant PNPLA3 from the membrane fraction of P. pastoris.

5.2.2 TAG hydrolase vs LPAAT activity

To test the enzymatic activity of PNPLA3, we performed assays using radiolabeled substrates. We found PNPLA3 to have main triglyceride hydrolase activity and a milder LPAAT activity. In both cases, the I148M substitution induced a striking reduction in enzymatic activity, suggesting that the mutation results in a loss of function. The discrepancy of previously published results may depend on the expression systems used to purify the protein. Indeed, while PNPLA3 purified from the eukaryotic insect cells Sf9 showed activities similar to our findings[61], protein purified from prokaryotic

E. coli showed main LPAAT activity and the 148M inducing a gain of function of this activity[65].

5.3 PNPLA3 in hepatic stellate cells

We found PNPLA3 to be highly expressed in liver and retina. These tissues share an important role in vitamin A metabolism and in particular hepatic stellate cells are the main storage site for retinol in the body[72]. Based on this, we hypothesized that PNPLA3 plays a role in retinol metabolism in hepatic stellate cells. We demonstrated that PNPLA3 is highly expressed in hepatic stellate cells at both mRNA and protein levels. Importantly, although several lipases have been proposed as the retinyl esterase of hepatic stellate cells[74,75], no evidences have been produced indicating a clear candidate responsible for this important activity.

5.3.1 Role in retinol metabolism

We found that PNPLA3 protein levels in hepatic stellate cells are regulated by retinol availability, indicating that PNPLA3 might be involved in retinol metabolism. Retinol availability induced PNPLA3 protein level reduction coupled with increased intracellular retinyl palmitate content, whereas retinol depletion increased PNPLA3 protein levels and simultaneous intracellular retinyl palmitate decrease. Consistently, insulin-induced PNPLA3 upregulation was associated with intracellular retinyl palmitate reduction and increase extracellular retinol release, while PNPLA3 down-regulation by siRNA abolished insulin effect, indicating that the phenotype we observed was specifically mediated by PNPLA3.

5.3.2 Effect of the 148M variant

We also investigated the effect of the 148M mutation on retinol metabolism by a triple approach. First, we overexpressed the 148I wild type or the 148M mutant protein or an empty vector in hepatic stellate cells, and we found the wild type protein to induce a reduction in intracellular retinyl palmitate content compared to the mutant protein and the empty vector. Next, we tested the activity of the purified wild type and mutant protein by performing enzymatic assays and we found the wild type protein to have retinyl esterase activity, whereas no activity was observed for the mutant protein. Finally, we tested the effect of the mutation on retinol metabolism in humans by measuring RBP4 plasma levels in 146 individuals genotyped for the PNPLA3 I148M variant. RBP4 is a reliable marker of circulating retinol levels[108]. We found that homozygotes for mutant allele had lower RBP4 levels compared to carriers of the wild type allele. Taken together, these results suggest that PNPLA3 plays a role in retinol release from the hepatic stellate cell storage and that the 148M mutation results in a loss of function. Given the importance of retinol metabolites for sight function and gene expression regulation in embryogenesis and adult liver function[98], these findings offer a possible explanation for the evolutionary selection of the 148M mutation.

5.3.3 Possible implications in liver disease

When activated by liver injury, hepatic stellate cells acquire a myofibroblast-like phenotype and lose their retinol content[66]. Activated HSCs play a major role in chronic liver disease, being responsible for collagen secretion and deposition that leads to fibrosis and cirrhosis[68]. The association between PNPLA3 genotype and chronic liver disease might be mediated by its retinyl esterase activity in HSCs. It is not established whether retinol release upon HSC activation is a cause or a consequence of the activation itself. It is also

not clear whether the retinol release is protective, damaging or neutral to the liver. One might hypothesize that retinol released has a protective function on the hepatocyte during HSCs activation and that carriers of the 148M mutation cannot benefit of this effect, given the retinol retention induced by the mutation.

6 CONCLUSION

The major findings of this thesis are:

1. The PNPLA3 protein contribute to the in hepatic very low density lipoprotein secretion

2. The PNPLA3 protein has a predominant glycerolipid hydrolase activity and the 148M mutation results in a loss of function

3. PNPLA3 has a retinyl esterase activity in hepatic stellate cells with the 148M mutation resulting in intracellular retinyl-palmitate retention

All together, these findings identify two possible independent mechanisms responsible for the increased susceptibility to chronic liver disease in carriers of the PNPLA3 mutation: 1) hepatocyte damage mediated by excessive fat content due to a reduced intracellular triglyceride mobilization 2) hepatocyte damage mediated by changes in hepatic stellate cell retinol metabolism. Targeting PNPLA3 for drug development may result in a new treatment strategy for liver steatosis and the entire spectrum of chronic liver disease.

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REFERENCES

1. Ebrahimkhani MR, Neiman JA, Raredon MS, Hughes DJ, Griffith LG. Bioreactor technologies to support liver function in vitro. Adv Drug Deliv Rev 2014.
2. Shapiro B. Lipid metabolism. Annu Rev Biochem 1967;36:247-70.
3. Subramaniam S, Fahy E, Gupta S, et al. Bioinformatics and systems biology of the lipidome. Chem Rev 2011;111:6452-90.
4. Fahy E, Subramaniam S, Murphy RC, et al. Update of the LIPID MAPS comprehensive classification system for lipids. J Lipid Res 2009;50 Suppl:S9-14.
5. Frayn KN, Arner P, Yki-Järvinen H. Fatty acid metabolism in adipose tissue, muscle and liver in health and disease. Essays Biochem 2006;42:89-103.
6. Back DW, Goldman MJ, Fisch JE, Ochs RS, Goodridge AG. The fatty acid synthase gene in avian liver. Two mRNAs are expressed and regulated in parallel by feeding, primarily at the level of transcription. J Biol Chem 1986;261:4190-7.
7. Vander Wal RJ. Triglyceride structure. Adv Lipid Res 1964;2:1-16.
8. Flatt JP. Use and storage of carbohydrate and fat. Am J Clin Nutr 1995;61:952S-9S.
9. Olofsson SO, Asp L, Borén J. The assembly and secretion of apolipoprotein B-containing lipoproteins. Curr Opin Lipidol 1999;10:341-6.
10. Wendel AA, Lewin TM, Coleman RA. Glycerol-3-phosphate acyltransferases: rate limiting enzymes of triacylglycerol biosynthesis. Biochim Biophys Acta 2009;1791:501-6.
11. Shi Y, Cheng D. Beyond triglyceride synthesis: the dynamic functional roles of MGAT and DGAT enzymes in energy metabolism. Am J Physiol Endocrinol Metab 2009;297:E10-8.
12. Lass A, Zimmermann R, Oberer M, Zechner R. Lipolysis - a highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores. Prog Lipid Res 2011;50:14-27.
13. Havel RJ. Lipoproteins and lipid transport. Adv Exp Med Biol 1975;63:37-59.
14. Li WH, Tanimura M, Luo CC, Datta S, Chan L. The apolipoprotein multigene family: biosynthesis, structure, structure-function relationships, and evolution. J Lipid Res 1988;29:245-71.
15. Krauss RM. Lipoprotein subfractions and cardiovascular disease risk. Curr Opin Lipidol 2010;21:305-11.

16. Yang LY, Kuksis A, Myher JJ, Steiner G. Origin of triacylglycerol moiety of plasma very low density lipoproteins in the rat: structural studies. J Lipid Res 1995;36:125-36.
17. Olofsson SO, Bjursell G, Boström K, et al. Apolipoprotein B: structure, biosynthesis and role in the lipoprotein assembly process. Atherosclerosis 1987;68:1-17.
18. Wiggins D, Gibbons GF. The lipolysis/esterification cycle of hepatic triacylglycerol. Its role in the secretion of very-low-density lipoprotein and its response to hormones and sulphonylureas. Biochem J 1992;284 (Pt 2):457-62.
19. Coppack SW, Jensen MD, Miles JM. In vivo regulation of lipolysis in humans. J Lipid Res 1994;35:177-93.
20. Ravussin E, Smith SR. Increased fat intake, impaired fat oxidation, and failure of fat cell proliferation result in ectopic fat storage, insulin resistance, and type 2 diabetes mellitus. Ann N Y Acad Sci 2002;967:363-78.
21. Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. Science 2011;332:1519-23.
22. Adams LA, Lymp JF, St Sauver J, et al. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. Gastroenterology 2005;129:113-21.
23. Bhala N, Jouness RI, Bugianesi E. Epidemiology and natural history of patients with NAFLD. Curr Pharm Des 2013;19:5169-76.
24. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Aliment Pharmacol Ther 2011;34:274-85.
25. Lazo M, Clark JM. The epidemiology of nonalcoholic fatty liver disease: a global perspective. Semin Liver Dis 2008;28:339-50.
26. Angulo P. Nonalcoholic fatty liver disease. N Engl J Med 2002;346:1221-31.
27. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-21.
28. DeFronzo RA, Ferrannini E. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. Diabetes Care 1991;14:173-94.
29. Zelber-Sagi S, Nitzan-Kaluski D, Goldsmith R, et al. Long term nutritional intake and the risk for non-alcoholic fatty liver disease (NAFLD): a population based study. J Hepatol 2007;47:711-7.
30. Gregg RE, Wetterau JR. The molecular basis of abetalipoproteinemia. Curr Opin Lipidol 1994;5:81-6.
31. Garg A. Clinical review#: Lipodystrophies: genetic and acquired body fat disorders. J Clin Endocrinol Metab 2011;96:3313-25.

32. Sigauke E, Rakheja D, Kitson K, Bennett MJ. Carnitine palmitoyltransferase II deficiency: a clinical, biochemical, and molecular review. Lab Invest 2003;83:1543-54.
33. Anstee QM, Day CP. The genetics of NAFLD. Nat Rev Gastroenterol Hepatol 2013.
34. Kozlitina J, Smagris E, Stender S, et al. Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2014;46:352-6.
35. Speliotes EK, Yerges-Armstrong LM, Wu J, 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.
36. Petersen KF, Dufour S, Hariri A, et al. Apolipoprotein C3 gene variants in nonalcoholic fatty liver disease. N Engl J Med 2010;362:1082-9.
37. Romeo S, Huang-Doran I, Baroni MG, Kotronen A. Unravelling the pathogenesis of fatty liver disease: patatin-like phospholipase domain-containing 3 protein. Curr Opin Lipidol 2010;21:247-52.
38. Daly AK, Ballestri S, Carulli L, Loria P, Day CP. Genetic determinants of susceptibility and severity in nonalcoholic fatty liver disease. Expert Rev Gastroenterol Hepatol 2011;5:253-63.
39. Kienesberger PC, Oberer M, Lass A, Zechner R. Mammalian patatin domain containing proteins: a family with diverse lipolytic activities involved in multiple biological functions. J Lipid Res 2009;50 Suppl:S63-8.
40. Jenkins CM, Mancuso DJ, Yan W, Sims HF, Gibson B, Gross RW. Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. J Biol Chem 2004;279:48968-75.
41. Strickland JA, Orr GL, Walsh TA. Inhibition of Diabrotica Larval Growth by Patatin, the Lipid Acyl Hydrolase from Potato Tubers. Plant Physiol 1995;109:667-74.
42. Rydel TJ, Williams JM, Krieger E, et al. The crystal structure, mutagenesis, and activity studies reveal that patatin is a lipid acyl hydrolase with a Ser-Asp catalytic dyad. Biochemistry 2003;42:6696-708.
43. Baulande S, Lasnier F, Lucas M, Pairault J. Adiponutrin, a transmembrane protein corresponding to a novel dietary- and obesity-linked mRNA specifically expressed in the adipose lineage. J Biol Chem 2001;276:33336-44.
44. Hoekstra M, Li Z, Kruijt JK, Van Eck M, Van Berkel TJ, Kuiper J. The expression level of non-alcoholic fatty liver disease-related gene PNPLA3 in hepatocytes is highly influenced by hepatic lipid status. J Hepatol 2010;52:244-51.

45. Huang Y, He S, Li JZ, et al. A feed-forward loop amplifies nutritional regulation of PNPLA3. Proc Natl Acad Sci U S A 2010;107:7892-7.
46. Romeo SK, J., Xing C, Pertsemlidis A, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2008;40:1461-5.
47. He S, McPhaul C, Li JZ, et al. A sequence variation (I148M) in PNPLA3 associated with nonalcoholic fatty liver disease disrupts triglyceride hydrolysis. J Biol Chem 2010;285:6706-15.
48. Perttilä J, Huaman-Samanez C, Caron S, et al. PNPLA3 IS REGULATED BY GLUCOSE IN HUMAN HEPATOCYTES AND ITS I148M MUTANT SLOWS DOWN TRIGLYCERIDE HYDROLYSIS. Am J Physiol Endocrinol Metab 2012.
49. Yuan X, Waterworth D, Perry JR, 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.
50. Speliotes EK, Butler JL, Palmer CD, et al. PNPLA3 variants specifically confer increased risk for histologic nonalcoholic fatty liver disease but not metabolic disease. Hepatology 2010;52:904-12.
51. Hotta K, Yoneda M, Hyogo H, et al. Association of the rs738409 polymorphism in PNPLA3 with liver damage and the development of nonalcoholic fatty liver disease. BMC Med Genet 2010;11:172.
52. Sookoian S, Pirola CJ. Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease. Hepatology 2011;53:1883-94.
53. Valenti L, Al-Serri A, Daly AK, 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.
54. 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.
55. Valenti L, Rumi M, Galmozzi E, et al. Patatin-like phospholipase domain-containing 3 I148M polymorphism, steatosis, and liver damage in chronic hepatitis C. Hepatology 2011;53:791-9.
56. Burza MA, Pirazzi C, Maglio C, et al. PNPLA3 I148M (rs738409) genetic variant is associated with hepatocellular carcinoma in obese individuals. Dig Liver Dis 2012;44:1037-41.
57. 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 7.
58. Valenti L, Dongiovanni P, Ginanni Corradini S, Burza MA, Romeo S. PNPLA3 I148M variant and hepatocellular carcinoma: A common genetic variant for a rare disease. Dig Liver Dis 2013;45:619-24.

59. Valenti L, Maggioni P, Piperno A, et al. Patatin-like phospholipase domain containing-3 gene I148M polymorphism, steatosis, and liver damage in hereditary hemochromatosis. World J Gastroenterol 2012;18:2813-20.
60. Friedrich K, Rupp C, Hov JR, et al. A frequent PNPLA3 variant is a sex specific disease modifier in PSC patients with bile duct stenosis. PLoS One 2013;8:e58734.
61. 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;286:37085-93.
62. Li JZ, Huang Y, Karaman R, et al. Chronic overexpression of PNPLA3I148M in mouse liver causes hepatic steatosis. J Clin Invest 2012;122:4130-44.
63. Basantani MK, Sitnick MT, Cai L, et al. Pnpla3/Adiponutrin deficiency in mice does not contribute to fatty liver disease or metabolic syndrome. J Lipid Res 2011;52:318-29.
64. 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.
65. Kumari M, Schoiswohl G, Chitraju C, et al. Adiponutrin functions as a nutritionally regulated lysophosphatidic Acid acyltransferase. Cell Metab 2012;15:691-702.
66. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev 2008;88:125-72.
67. Lee YS, Jeong WI. Retinoic acids and hepatic stellate cells in liver disease. J Gastroenterol Hepatol 2012;27 Suppl 2:75-9.
68. Lee UE, Friedman SL. Mechanisms of hepatic fibrogenesis. Best Pract Res Clin Gastroenterol 2011;25:195-206.
69. Melton AC, Yee HF. Hepatic stellate cell protrusions couple platelet-derived growth factor-BB to chemotaxis. Hepatology 2007;45:1446-53.
70. Alisi A, Romania P, Nobili V, Locatelli F, Fruci D. Human hepatic stellate cells are liver-resident antigen-presenting cells. Hepatology 2011;54:1107; author reply 8.
71. Hendriks HF, Blaner WS, Wennekers HM, et al. Distributions of retinoids, retinoid-binding proteins and related parameters in different types of liver cells isolated from young and old rats. Eur J Biochem 1988;171:237-44.
72. Blomhoff R, Rasmussen M, Nilsson A, et al. Hepatic retinol metabolism. Distribution of retinoids, enzymes, and binding proteins in isolated rat liver cells. J Biol Chem 1985;260:13560-5.
73. Shmarakov IO, Jiang H, Yang KJ, Goldberg IJ, Blaner WS. Hepatic retinoid stores are required for normal liver regeneration. J Lipid Res 2013;54:893-908.

74. Mello T, Nakatsuka A, Fears S, et al. Expression of carboxylesterase and lipase genes in rat liver cell-types. Biochem Biophys Res Commun 2008;374:460-4.
75. Schreiber R, Taschler U, Preiss-Landl K, Wongsiriroj N, Zimmermann R, Lass A. Retinyl ester hydrolases and their roles in vitamin A homeostasis. Biochim Biophys Acta 2012;1821:113-23.
76. Natarajan SK, Thomas S, Ramachandran A, Pulimood AB, Balasubramanian KA. Retinoid metabolism during development of liver cirrhosis. Arch Biochem Biophys 2005;443:93-100.
77. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. The myofibroblast: one function, multiple origins. Am J Pathol 2007;170:1807-16.
78. Friedman SL, Roll FJ, Boyles J, Bissell DM. Hepatic lipocytes: the principal collagen-producing cells of normal rat liver. Proc Natl Acad Sci U S A 1985;82:8681-5.
79. Arthur MJ. Collagenases and liver fibrosis. J Hepatol 1995;22:43-8.
80. Arthur MJ. Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. Am J Physiol Gastrointest Liver Physiol 2000;279:G245-9.
81. Geerts A. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. Semin Liver Dis 2001;21:311-35.
82. Inagaki Y, Okazaki I. Emerging insights into Transforming growth factor beta Smad signal in hepatic fibrogenesis. Gut 2007;56:284-92.
83. Wong L, Yamasaki G, Johnson RJ, Friedman SL. Induction of beta-platelet-derived growth factor receptor in rat hepatic lipocytes during cellular activation in vivo and in culture. J Clin Invest 1994;94:1563-9.
84. Castilla A, Prieto J, Fausto N. Transforming growth factors beta 1 and alpha in chronic liver disease. Effects of interferon alfa therapy. N Engl J Med 1991;324:933-40.
85. Pitt GA. Chemical structure and the changing concept of vitamin A activity. Proc Nutr Soc 1983;42:43-51.
86. Goodman DW, Huang HS, Shiratori T. Tissue distribution and metabolism of newly absorbed vitamin a in the rat. J Lipid Res 1965;6:390-6.
87. Freemantle SJ, Spinella MJ, Dmitrovsky E. Retinoids in cancer therapy and chemoprevention: promise meets resistance. Oncogene 2003;22:7305-15.
88. Newcomer ME, Ong DE. Plasma retinol binding protein: structure and function of the prototypic lipocalin. Biochim Biophys Acta 2000;1482:57-64.
89. Blomhoff R, Helgerud P, Rasmussen M, Berg T, Norum KR. In vivo uptake of chylomicron [3H]retinyl ester by rat liver: evidence for retinol transfer from parenchymal to nonparenchymal cells. Proc Natl Acad Sci U S A 1982;79:7326-30.

90. Fields AL, Soprano DR, Soprano KJ. Retinoids in biological control and cancer. J Cell Biochem 2007;102:886-98.
91. Trøen G, Nilsson A, Norum KR, Blomhoff R. Characterization of liver stellate cell retinyl ester storage. Biochem J 1994;300 (Pt 3):793-8.
92. Blomhoff R, Norum KR, Berg T. Hepatic uptake of [3H]retinol bound to the serum retinol binding protein involves both parenchymal and perisinusoidal stellate cells. J Biol Chem 1985;260:13571-5.
93. Weber F. Biochemical mechanisms of vitamin A action. Proc Nutr Soc 1983;42:31-41.
94. Okada T, Palczewski K. Crystal structure of rhodopsin: implications for vision and beyond. Curr Opin Struct Biol 2001;11:420-6.
95. Napoli JL. Retinoic acid biosynthesis and metabolism. FASEB J 1996;10:993-1001.
96. Shiota G, Kanki K. Retinoids and their target genes in liver functions and diseases. J Gastroenterol Hepatol 2013;28 Suppl 1:33-7.
97. von Lintig J. Provitamin A metabolism and functions in mammalian biology. Am J Clin Nutr 2012;96:1234S-44S.
98. McLaren DS, Kraemer K. Retinoids and carotenoids in general medicine. World Rev Nutr Diet 2012;103:137-47.
99. Fabbrini E, Mohammed BS, Magkos F, Korenblat KM, Patterson BW, Klein S. Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. Gastroenterology 2008;134:424-31.
100. Kollerits B, Coassin S, Beckmann ND, et al. Genetic evidence for a role of adiponutrin in the metabolism of apolipoprotein B-containing lipoproteins. Hum Mol Genet 2009;18:4669-76.
101. Krarup NT, Grarup N, Banasik K, et al. The PNPLA3 rs738409 G-Allele Associates with Reduced Fasting Serum Triglyceride and Serum Cholesterol in Danes with Impaired Glucose Regulation. PLoS One 2012;7:e40376.
102. Palmer CN, Maglio C, Pirazzi C, et al. Paradoxical Lower Serum Triglyceride Levels and Higher Type 2 Diabetes Mellitus Susceptibility in Obese Individuals with the PNPLA3 148M Variant. PLoS One 2012;7:e39362.
103. Sen D, Dagdelen S, Erbas T. Hepatosteatosis with hypobetalipoproteinemia. J Natl Med Assoc 2007;99:284-6.
104. Adiels M, Taskinen MR, Packard C, et al. Overproduction of large VLDL particles is driven by increased liver fat content in man. Diabetologia 2006;49:755-65.
105. Hansson PK, Asztély AK, Clapham JC, Schreyer SA. Glucose and fatty acid metabolism in McA-RH7777 hepatoma cells vs. rat primary hepatocytes: responsiveness to nutrient availability. Biochim Biophys Acta 2004;1684:54-62.

106. Hedfalk K. Further advances in the production of membrane proteins in Pichia pastoris. Bioengineered 2013;4.
107. Oberg F, Ekvall M, Nyblom M, Backmark A, Neutze R, Hedfalk K. Insight into factors directing high production of eukaryotic membrane proteins; production of 13 human AQPs in Pichia pastoris. Mol Membr Biol 2009;26:215-27.
108. Erikstrup C, Mortensen OH, Nielsen AR, et al. RBP-to-retinol ratio, but not total RBP, is elevated in patients with type 2 diabetes. Diabetes Obes Metab 2009;11:204-12.