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"THE MOLECULAR BASIS OF HUMAN DISEASES"

"Investigation of the role of the HNF4a transcription factor in the pathogenesis of metabolic diseases using mouse models"

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ΠΕΡΙΛΗΨΗ

Το Μεταβολικό Σύνδρομο είναι ένα σύμπλεγμα από ασθένειες συμπεριλαμβανομένων της παχυσαρκίας, τη υπέρτασης, της δυσλιπιδαιμιας (υπερτιγλυκεριδαιμίας συνδιασμένης από χαμηλά επίπεδα υψηλής πυκνότητας λιποπρωτείνης (HDL) και υψηλά επίπεδα χαμηλής πυκνότητας λιποπρωτείνης (LDL) διαταραγμένη ανοχή στη γλυκόζη ή αντίσταση στην ινσουλίνη που όλα μαζί αυξάνουν τον κίνδυνο για ανάπτυξη καρδιολογικών νοσημάτων. Η αιτιολογία του Μεταβολικού Συνδρόμου παραμένει άγνωστη και πιο αποτελεσματικές θεραπείες για την αντιμετώπιση της νόσου είναι επιτακτικό να ανακαλυφθούν. Το λιπώδες ήπαρ που οφείλεται σε μη αλκοολική κατανάλωση είναι μία ακόμα ασθένεια που προκαλεί την εκτεταμένη συγκέντρωση λιπώδους ιστού στο ήπαρ των ασθενών. Έχει διατυπωθεί από την επιστημονική κοινότητα πώς το λιπώδες ήπαρ είναι μια συνέπεια του Μεταβολικού Συνδρόμου στο ήπαρ. Ο ηπατικός μεταγραφικός παράγοντας 4 άλφα (HNF4α) είναι μέλος των ορμονικών πυρηνικών υποδοχέων της υπεροικογένειας των μεταγραφικών παραγόντων και εκφράζεται σε υψηλά επίπεδα στο ήπαρ, στο έντερο, στους νεφρούς και στο πάγκρεας. Σημειακές μεταλλάξεις στο γονίδιο του HNF4A είναι υπεύθυνες για την ανάπτυξη και εκδήλωση του πρώιμου ώριμου διαβήτη σε νεαρά άτομα. Επίσης, σημειακές μεταλλάξεις στο γονιδιακό τόπο του συγκεκριμένου γονιδίου έχουν συσχετιστεί, με τη χρήση γονιδιακών μελετών συσχέτισης, με μειωμένα επίπεδα υψηλή πυκνότητας χοληστερόλης. Σε ποντίκια, ο μεταγραφικός παράγοντας HNF4α είναι υπεύθυνος για την ανάπτυξη του ήπατος και ελέγχει την έκφραση πολλών γονιδίων που εμπλέκονται στο μεταβολισμό των λιποπρωτεινών.

Κύριος στόχος της παρούσας μελέτης ήταν η διερεύνηση του ρόλου του μεταγραφικού παράγοντα HNF4α στην παθογένεση μεταβολικών ασθενειών και συγκεριμένα του Μεταβολικού Συνδρόμου και του λιπώδους ήπατος. Για την επίτευξη του στόχου, δύο στελέχη ποντικών χρησιμοποιήθηκαν. α) το APOE*3Leiden.CETP διαγονιδιακό στέλεχος ποντικού, το οποίο είναι ένα πολύ καλα χαρακτηρισμένο μοντέλο για την μελέτη του επαγώμενου από τη δίαιτα Μεταβολικού Συνδρόμου και β) το μοντέλο ποντικού όπου ο μεταγραγικός παράγοντας HNF4α έχει αποσιωποιηθεί στοχευμένα στο ήπαρ του (HNF4α Liver KO). Ποντίκια στα οποία ο μεταγραφικός παράγοντας HNF4a δεν εκφράζεται στο ήπαρ τους εμφανίζουν εκτεταμένη συσσώρευση λιπιδίων (τριγλυκεριδίων και χοληστερόλης) στο ήπαρ τους και μπορούν να χρησιμοποιηθούν σαν μοντελο για τη μελέτη του λιπώδους ήπατος που οφείλεται στην μη κατανάλωση αλκοόλης. Το πρώτο βήμα για την επίτευξη του στόχου ήταν ο σχεδιασμός τριών διαφορετικών «μικρών φουρκετών RNA» (shRNA) που στοχεύουν σε διαφορετικά σημεία το ώριμο μετάγραφο του γονιδιου HNF4A. Το

shRNA που εξάλειφε πιο αποδοτικά την παραγωγή του μεταφραγικού παράγοντα HNF4α εισήχθη σε ειδικό πλασμιδιακό φορέα κάτω από τον έλεγχο των εκκινητών H1 και U6. Στη συνέχεια οι πλασμιδιακές κατασκευές πακεταριστηκαν μέσα σε Αδενο-συσχετιζόμενου ιούς (AAVs). Σε επόμενο βήμα οι ιοι AAV-shHNF4α μαζί με τους αντίστοιχους ιούς ελέγχου, θα εισαχθούν στα ποντίκια APOE*3Leiden.CETP όπου θα προκαλέσουν στοχευμένη αποσιώπιση του μεταγραφικού παράγοντα HNF4a στο ήπαρ των ποντικών αυτών. Τα μολυσμένα με τους ιούς ποντίκια θα εισαχθούν σε ειδική δίαιτα για την επαγωγή του Μεταβολικού Συνδρόμου και στη συνέχεια θα πραγματοποιηθεί εκτεταμένη φαινοτυπική και μεταγραφική ανάλυση των ποντικών αυτών. Επίσης, μεταγραφική ανάλυση ήπατος ποντικιών που όπου ο μεταγραγικός παράγοντας HNF4α είχε αποσιωποιηθεί στο ήπαρ HNF4α Liver KO, πραγματοποιήθηκε στο εργαστήριο και αποκάλυψε σημαντικές μεταβολές στα επίπεδα έκφρασης διάφορων γονιδίων που εμπλέκονται στη βιοσύνθεση και το μεταβολισμό λιπιδίων και απολιποπρωτεινών. Στόχος ήταν η πιστοποίηση νέων γονιδίων χωρίς καθορισμένο ρόλο στο μονοπάτι μεταβολισμού της υψηλής πυκνότητας απολιποπρωτείνης που βρέθηκε ότι έχουν διαφορετικά επίπεδα έκφρασης και πιθανόν είναι στόχοι του μεταγραφικού παράγοντα HNF4a. Επιβεβαίωση των αποτελεσμάτων από τις μικροσυστοιχίες πραγματοποιήθηκε με τις τεχνικές «αντίστροφης μεταγραφάσης ποσοτικοποιημένη αλυσιδωτή αντίδραση πολυμεράσης» (RT-qPCR) καθώς και με ανοσοκατακρήμνιση χρωματίνης από δείγματα ήπατος των ποντικών αυτών με απώτερο στόχο την διερεύνηση θέσεων πρόσθεσης του μεταγραφικού παράγοντα HNF4a στους εκκινητές των νέων γονιδίων.

Συμπερασματικά, με τη χρήση ποντικών μοντέλων διερευνήθηκε ο ρόλος του μεταγραφικού παράγοντα HNF4α στην παθογένεση μεταβολικών ασθενειών όπως του Μεταβολικού Συνδρόμου και του λιπώδους ήπατος από τη μη κατανάλωση αλκοόλ, καθώς και ο προσδιορισμός νέων γονιδίων στόχων του μεταγραφικού παράγοντα. Τα αποτελέσματα της έρευνας θα μπορούν να χρησιμοποιηθούν για τον προσδιορισμό καινούριων θεραπευτικών σκευασμάτων που θα μπορούσαν να εξερευνηθούν περαιτέρω στο μέλλον για την αντιμετώπιση και θεραπεία των ασθενειών αυτών.

ABSTRACT

Metabolic Syndrome (MetS) is a cluster of several pathologies including obesity, hypertension, dyslipidemia (hypertriglyceridemia combined with low levels of high density lipoprotein cholesterol (HDL) and high levels of low density lipoprotein cholesterol (LDL)), and impaired glucose tolerance or insulin resistance, all of which increase the risk for developing cardiovascular disease. The etiology of MetS is poorly understood and effective therapies are urgently needed. Nonalcoholic fatty liver disease (NAFLD) is defined as a disorder with excess fat in the liver due to non-alcoholic causes. It has been proposed that NAFLD is the "hepatic manifestation" of MetS. Hepatocyte nuclear factor 4 alpha (HNF4a, NR2A1) is a member of the hormone nuclear receptor superfamily of transcription factors that is expressed mainly in the liver, intestine, kidney and pancreas. Mutations in the HNF4a gene cause Maturity Onset Diabetes of the Young (MODY) in humans whereas SNPs in the HNF4a locus have been associated with reduced HDL levels in GWAS. In mice, HNF4a is required for liver development and for controlling the expression of many genes involved in lipoprotein metabolism.

The main goal of this thesis is to elucidate the role of HNF4a in the pathogenesis of metabolic diseases such as MetS and NAFLD. To achieve this goal, two mouse lines were used: a) the APOE*3Leiden.CETP transgenic mouse which is a well-established model for diet-induced MetS; and b) the HNF4a liver knockout mouse. Mice lacking hepatic HNF4a expression accumulate lipids (triglycerides, cholesterol) in the liver and can be used as a model of NAFLD. As a first step towards the achievement of our goal, three small hairpin RNAs (shRNAs) were designed and screened for efficient targeting of sequences in the mouse HNF4a mRNA. The most efficient shRNA was introduced into an appropriate plasmid vector under the control of the H1 or U6 promoters and the constructs were packaged into Adeno-Associated Viruses (AAVs). In the next step, the AAV-shHNF4a viruses will be used, along with control AAVs, for long term silencing of HNF4a in the livers of the APOE*3Leiden.CETP transgenic mice. The infected mice will be subjected to diet-induced MetS and this will be followed by extensive phenotypic and transcriptomic analysis of these mice. The transcriptomic analysis of the livers of HNF4a Liver KO mice has been completed in our lab and revealed significant changes in the expression of several genes involved in lipid and apolipoprotein biosynthesis and metabolism. The focus of this thesis was on the validation of novel genes with not yet established roles in High Density Lipoprotein metabolism which were found to be differentially expressed and could serve as direct targets of HNF4. Validation of the microarray data was performed

by RT-qPCR and chromatin immunoprecipitation in mouse liver samples was used to verify the direct binding of HNF4a to the promoter regions of these genes.

In summary, using appropriate mouse models we are investigating the contribution of HNF4a in the pathogenesis of metabolic diseases such as MetS and NAFLD and the identification of novel HNF4a target genes. The ultimate goal is to identify novel drug targets which could be explored therapeutically in the future for the treatment of these diseases.

INTRODUCTION

I. METABOLIC SYNDROME

Obesity is a worldwide pandemic and by 2030 it is predicted to affect 10% of the global population (Webber et al., 2014). Obesity is the outcome of an imbalance between caloric intake and energy expenditure, leading to an abundance of energy, which is stored as fat in the white adipose tissue (Chugh and Sharma, 2012; McKenney and Short, 2011). Obesity exacerbates a variety of health problems including type 2 diabetes and cardiovascular disease (Kopelman, 2000). Metabolic alterations associated with obesity have been recognized and clustered to define the metabolic syndrome.

The **Metabolic Syndrome (MetS)** is a cluster of several abnormalities, including hypertension, dyslipidemia (hypertriglyceridemia, low levels of high density lipoprotein cholesterol (HDL), high levels of low density lipoprotein cholesterol (LDL)), abdominal obesity and impaired glucose tolerance or insulin resistance (Reilly and Rader, 2003) all of which create an increased risk of cardiovascular disease (Reaven, 2011). However, this definition is still a matter of debate (Jornayvaz et al., 2010b). Using the Third Adult Treatment Panel (ATPIII) guidlines a new definition for MetS has come out (Alberti et al., 2005). The new definition categorize patients with MetS as those who have central obesity and one of the following factors: raised systolic blood pressure ≥130mmHg or diastolic blood pressure ≥85mmHg, raised triglycerides ≥1.7 mmol/l; reduced HDL-cholesterol <1.03 mmol/l in males and <1.29 mmol/l in females, raised fasting plasma glucose, with fasting plasma glucose ≥5.6 mmol/l or previously diagnosed type II diabetes (Alberti et al., 2005). Although insulin resistance is a significant characteristic of MetS, it remains difficult to measure in clinical practice and it was not included in the new definition. Central obesity is much easier to measure and since it is the major feature of the MetS, it is required to diagnose MetS (Asrih and Jornayvaz, 2015).

The pathogenesis of metabolic syndrome remains debated. However, initiation of the disease is caused by genetic predisposition along with a sedentary lifestyle and a diet with excess calories (Rask-Madsen and Kahn, 2012). In additional to these, abdominal obesity and insulin resistance appear to be the center of the pathophysiology of the MetS (Cornier et al., 2008).

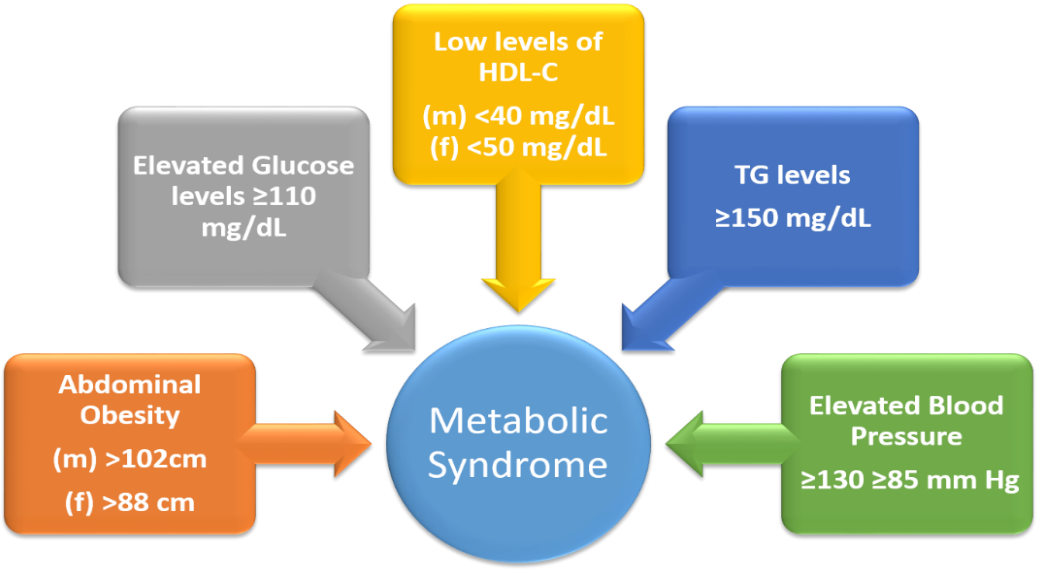


Figure 1. Metabolic Syndrome definition.

MetS is the major cause of morbidity and death in the western countries (Simons et al., 2011). It is the main risk factor for type II diabetes by which 8% of Americans and 11% of Chinese suffer and thus constitutes a major threaten to global public health (Alberti et al., 2005; Cornier et al., 2008; Eckel et al., 2005; Roger et al., 2011). It is estimated that 366 million people worldwide had diabetes in 2011 and that number is predicted to increase to 522 million by 2030 (Whiting et al., 2011). The prevalence of MetS follows the one of obesity and is increasing at a dramatically rate, affecting more than 20% of the worldwide adult population (Onat, 2011). The increasing rate of MetS and its related comorbidities, such as cardiovascular diseases, has been accompanied by an augmentation in liver alterations including nonalcoholic fatty liver disease (NAFLD) (Angulo 2002; Marchesini et al., 2003). Particularly, **it has been proposed that NAFLD may be the cause and the hepatic manifestation of MetS** (Lonardo, 1999; Lonardo et al., 2015; Marchesini et al., 2003; Williams, 2015; Yki-Jarvinen, 2014).

II. NON-ALCOCOLIC FATTY LIVER DISEASES

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome and is defined as the accumulation of lipids in the liver of patients who do not consume

alcohol (Buzzetti et al., 2016). NAFLD is asymptomatic in the majority of affected patients and is associated with obesity and other characteristics of MetS such as hypertension, dyslipidemia and insulin resistance or type II diabetes (Hassan et al., 2014). NAFLD is associated with insulin resistance which is the major risk factor for type II diabetes (Jornayvaz and Shulman, 2012). NAFLD is an inclusive term that encompasses a wide spectrum of conditions, from simple steatosis (accumulation of fat – "fatty liver") to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis (Ratziu et al., 2010).

The NAFLD is the most common chronic liver disease in Western societies, affecting one in four adults in the USA (Jornayvaz and Shulman, 2012). Studies have showed that its prevalence in the adult population is about 20-40% in Western countries (Williamson et al., 2011) and 10-30% in Asian countries (Kim et al., 2014; Wang et al., 2014). The prevalence of NALFD in healthy individuals without the presence of metabolic risk contributing factors is about 16% (Than and Newsome, 2015), increasing to 43-60% among patients with diabetes (Williamson et al., 2011) and up to 90% in patients with hyperlipidemia (Gaggini et al., 2013). Generally, its prevalence is rising over time along with the increase in the prevalence of obesity (Loomba and Sanyal, 2013).

The pathogenesis of NAFLD is not clearly understood. It has been proposed that increased abdominal obesity and insulin resistance with elevated levels of free fatty acids (FFA) may play a significant role in the development of steatosis. There are a diversity of theories that have been formulated, driving initially to the *"two hit hypothesis"* (Day and James, 1998). Based on this, the first hit is the hepatic accumulation of triglycerides, leading to hepatic steatosis, while the second hit is the production of free radicals and activation of inflammatory pathways giving rise to fibrogenesis (Asrih and Jornayvaz, 2015; Tilg and Moschen, 2010). However, more recently, a "*multiple hit hypothesis"* has been raised. According to this, hits come simultaneously from gut and adipose tissue to promote inflammation in the liver, proposing that insulin resistance and inflammation are acting at the same time (Tilg and Moschen, 2010). Dietary habits, environmental and genetic factors lead to insulin resistance, obesity and changes in the gut microbiome (Buzzetti et al., 2016). Insulin resistance plays a major role in the pathogenesis of NAFLD. The adipose tissue, apart from muscle and liver, becomes resistant to the anti-lipolytic effect of insulin resulting in adipose tissue lipolysis which increases flux of fatty acids to the liver and hepatic de novo lipogenesis (DNL) (Bugianesi et al., 2010). De novo lipogenesis is a metabolic pathway that exists only in adipose tissue and liver, where glycolysis, saturated fatty acids biosynthesis and triglyceride formation take place. Along with fat accumulation, increased lipotoxicity happens due to elevated levels of free fatty acids, free cholesterol and other lipid metabolites (Cusi, 2009). The consequences are mitochondria dysfunction

with oxidative stress and endoplasmic reticulum stress (Cusi, 2009). Furthermore, changes in gut flora cause additional production of fatty acids in the bowel and increased small intestine permeability thus leading to increased fatty acid flux and elevated circulating levels of molecules which provide an additional outcome to inflammation (Kirpich et al., 2015).

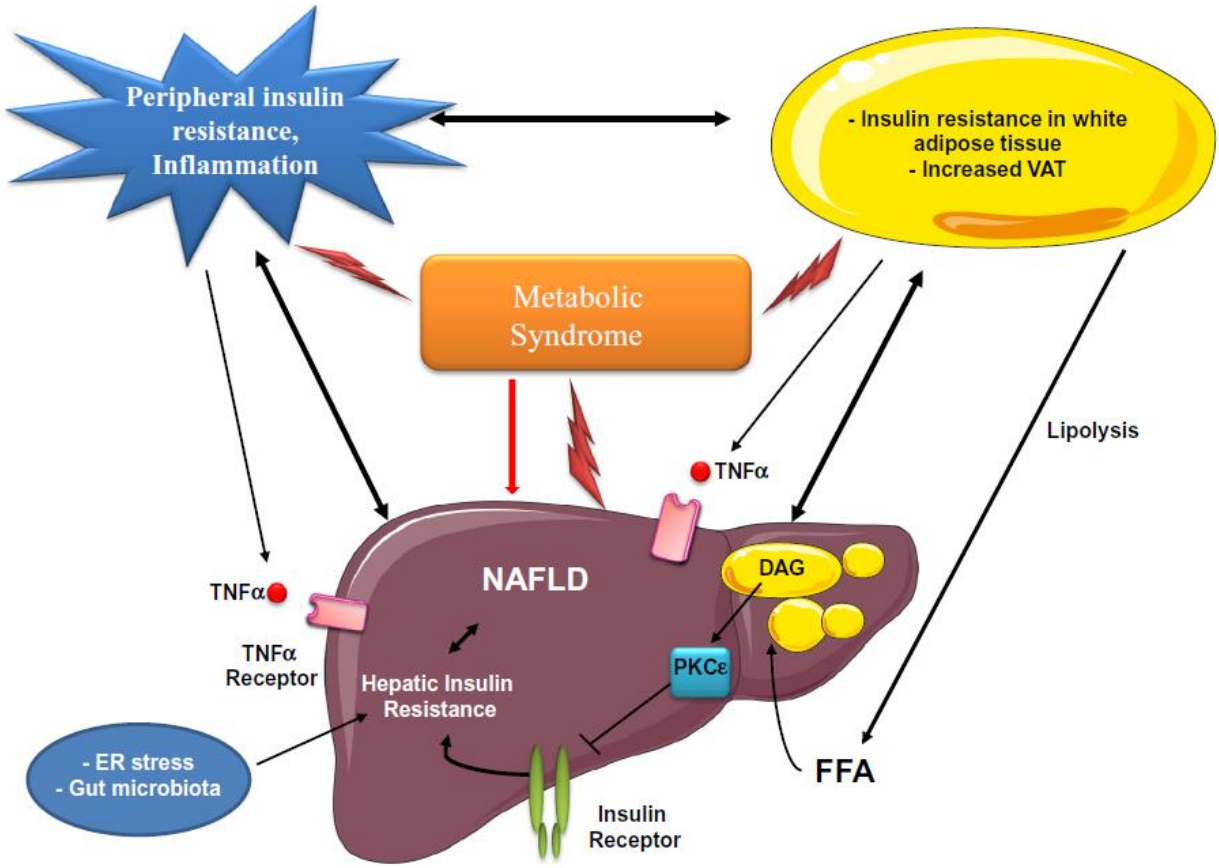


Figure 2. Multiple hit hypothesis in the development of MetS and NAFLD (Asrih and Jornayvaz, 2015).

The diagnosis of NAFLD is based on liver biopsy which is still the gold standard to distinguish fatty liver from NASH and from fibrosis (Buzzetti et al., 2015). NAFLD is usually asymptomatic and the majority of patients have transaminases at normal levels (Browning et al., 2004). Further, alternative methods have been established to assess NAFLD, such as non-invasive imagin, which are presented in the review of *Musso et al.* (Musso et al., 2011). Patients with NAFLD develop nonalcoholic steatohepatitis (NASH) which is characterized by necrosis in the vicinity of steatotic hepatocytes, mild inflammation and fibrosis (Brunt et al., 2011). Cardiovascular disease is the main cause of mortality in patients with NASH (Anstee et al., 2013).

Common pathophysiological mechanisms involved in Metabolic Syndrome and Non-Alcoholic Liver Disease

Over-nutrition and sedentary lifestyle are the main factors leading to an increased prevalence of both MetS and NALFD. The contribution of MetS to NAFLD involves different factors among which insulin resistance, central obesity, inflammation, oxidative stress and genetic predispositions. However, it is unknown which mechanism comes first and how they affect the others promoting MetS and NAFLD.

Insulin resistance has been proposed as the major link between MetS and NAFLD. Alterations in the canonical pathway of insulin lead to insulin resistance, a condition which affects several organs. Hepatic insulin resistance is closely associated with elevated liver fat content, which comes from plasma free fatty acids (Donnelly et al., 2005). Fat accumulates in the liver of patients with NAFLD mainly in the form of triglycerides (Jacome-Sosa and Parks, 2014; Musso et al., 2013). Insulin resistance in the white adipose tissue causes lipolysis and consequently secretion of free fatty acids in the circulation (Eguchi et al., 2006) and increased efflux of FFAs to the liver (Lewis et al., 2002). Free fatty acids in the liver promote hepatic diacylglycerols (DAG) accumulation and activation of inflammatory pathways. DAG is increased from both fatty acid re-esterification and hepatic *de novo* lipogenesis (DNL) and at the same time decreased hepatic lipid clearance (free fatty acid oxidation and very low-density lipoprotein excretion). Furthermore, hepatic de novo lipogenesis is increased by activation of different transcription factors such as sterol regulatory element-binding protein-1 (SREBP-1), carbohydrate response element-binding protein (ChREBP) and peroxisome proliferator-activated receptor (PPAR)-γ (George and Liddle, 2008). SREBP-1c is activated by insulin and regulates the activation of DNL (Schultz et al., 2000); SREBP-2 participates in cellular cholesterol homeostasis (Musso et al., 2013) and ChREBP is activated by glucose and increase DNL. In insulin resistance IR-2 is downregulated and thus SREBP-1c is upregulated leading to DNL upregulation (Stefan et al., 2008). In addition, β-oxidation of FFAs is inhibited, promoting hepatic lipid accumulation (Postic and Girard, 2008). PPARγ is highly expressed in adipocytes and promotes fatty acids uptake into adipose tissue and adipocytes differentiation (Birkenfeld and Shulman, 2014). It has been found that patients with dominant negative mutations in PPARγ develop NAFLD and the metabolic syndrome suggesting

raised triglyceride flux to the liver (Savage et al., 2003). Liver specific PPARγ knockout mice are protected against the development of hepatic steatosis (Gavrilova et al., 2003; Matsusue et al., 2003).

Proximal defects in insulin signaling with decreased insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 by insulin receptor are closely correlated with hepatic steatosis and hepatic DAG accumulation (Shulman 2014). DAG in turn activates the epsilon form of protein kinase C (PKCε), which has been found to be highly expressed in the liver and it is activated in rodent models of nonalcoholic fatty liver disease. Activated PKCε binds to and inhibits the insulin receptor tyrosine kinase, decreasing insulin-stimulated glycogen synthesis and phosphorylation of GSK3. Furthermore, insulin receptor inactivation decreases insulin suppression of hepatic gluconeogenesis through decreased phosphorylation of forkhead box subgroup O (FOXO). This results to increased FOXO translocation to the nucleus, where it promotes increased gene transcription of the gluconeogenic enzymes (Shulman 2014). Studies in multiple transgenic or knockout rodent models of nonalcoholic fatty liver disease have demonstrated the association between DAG- PKCε activation in the liver and hepatic insulin resistance (Choi et al., 2007; Matsuzaka et al., 2007; Savage et al., 2006; Zhang et al., 2007). More significant, elevated hepatic DAG content and PKCε activity are the strongest predictors of hepatic insulin resistance in obese humans with NAFLD (Kumashiro et al., 2011). Supporting study of inhibiting PKCε using antisense oligonucleotide demonstrated that hepatic insulin resistance is prevented despite hepatic lipid accumulation (Samuel et al., 2007). Several studies have been published using animal models of NAFLD which illustrate the straightforward correlation between NAFLD and hepatic insulin resistance (Birkenfeld et al., 2011; Camporez et al., 2013a; Camporez et al., 2013b; Cantley et al., 2013; Jornayvaz et al., 2010a; Jornayvaz and Shulman, 2012). Together these studies showed that NAFLD is caused by hepatic lipid

accumulation and is associated with hepatic insulin resistance and further that insulin resistance could be the missing link between the MetS and NAFLD (Asrih and Jornayvaz, 2015).

Ectopic fat accumulation results from imbalance between energy intake and energy expenditure. This promotes the transfer of energy storage from adipose tissue to skeletal muscle and the liver by forming lipid droplets. Ectopic fat accumulation intracellularly leads to insulin resistance in skeletal muscle and the liver even in the absence of peripheral and visceral adipose tissue. DAGs are lipid-derived metabolites that are responsible for triggering insulin resistance in the liver by activating of PKCε in the liver (Shulman 2014).

Inflammation induces insulin resistance in MetS and NAFLD. Increase levels of free fatty acids along with lipotoxicity, adipose tissue dysfunction and gut endotoxins cause the production and release of pro-inflammatory cytokines, both systematically and locally in the liver. JNK-AP-1 and IKK-NF-κB are the two main inflammatory pathways that get involved in the development of the chronic inflammatory state in NAFLD (Hotamisligil, 2006). Several systemic inflammatory markers such as interleukin-6 (IL-6) and TNFa and C-reactive protein (CRP) are increased in MetS (Haffner, 2003). Moreover, elevated plasma levels of CRP are used as markers for the development of NAFLD (Targher et al., 2008).

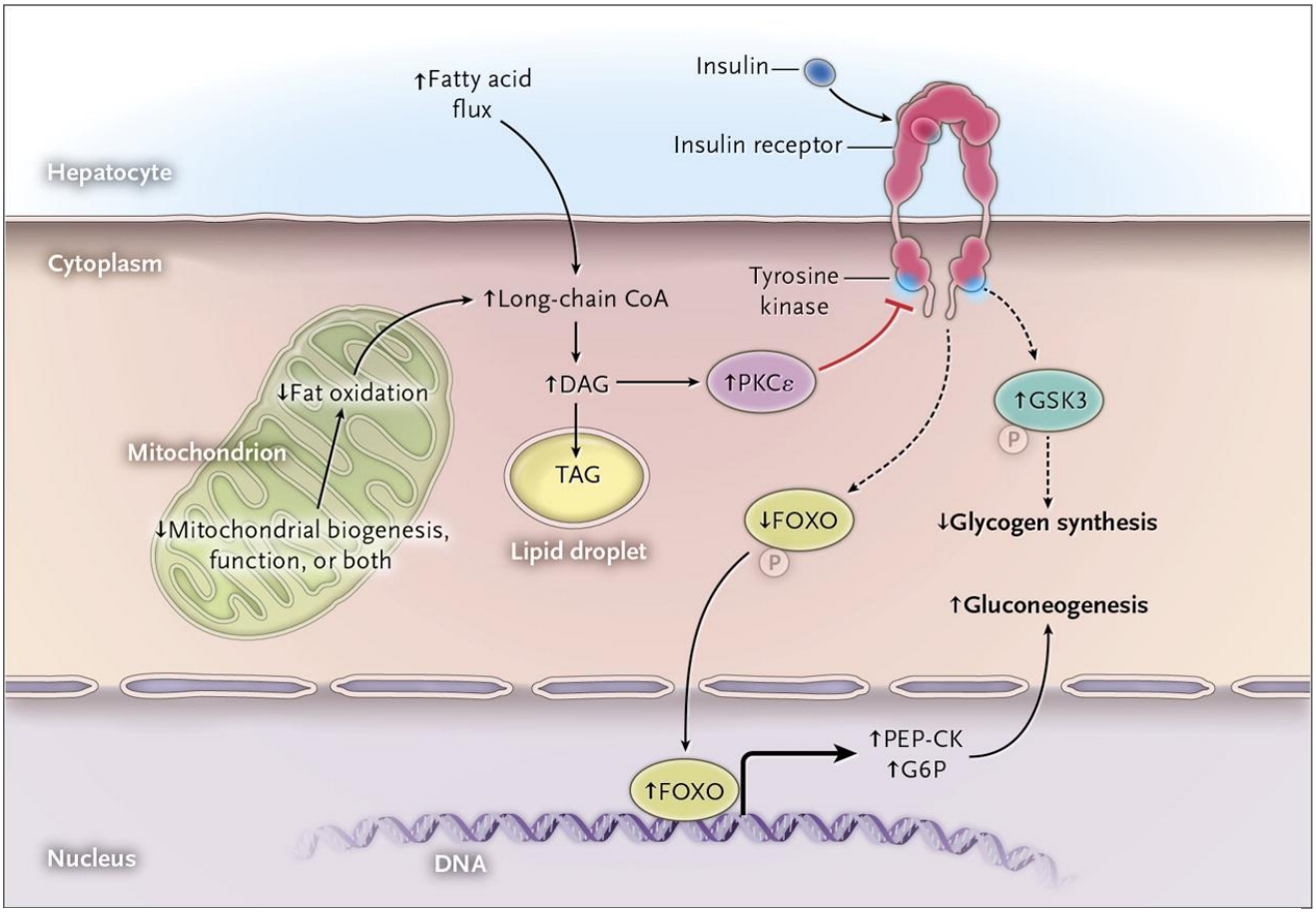


Figure 3.Molecular Mechanisms of Lipid-Induced Hepatic Insulin Resistance (Shulman 2014).

In addition to ectopic fat accumulation and inflammation, gut microbiota has been found to play significant role in the development of obesity and insulin resistance (Asrih and Jornayvaz, 2015; Ley et al., 2005; Palermo et al., 2014). Gut microbiome is involved in the pathogenesis and development of NALFD, through the so-called gut-liver axis. Vrieze and coworkers showed that when insulin resistant male mice with MetS received feces infusion from lean donors developed improved peripheral insulin responsiveness (Vrieze et al., 2012). This was associated with increased butyrate producing bacteria (mainly Roseburia and Eubacterium halii) in feces. Intestinal permeability can be enhanced by certain patterns of microbiome diversities, leading to lipopolysaccharidemia. NAFLD subjects have significantly raised gut permeability and elevated prevalence of small intestine bacterial overgrowth in comparison with healthy subjects (Miele et al., 2009). The significant association between intestine bacterial overgrowth and NASH can be observed even in the absence of increased mucosal permeability (Wigg et al., 2001). Furthermore, intestine bacteria suppress the synthesis of fasting-induced adipocyte factor (Fiaf) leading to elevated activity of lipoprotein lipase and triglyceride accumulation (Tilg and Moschen, 2010). Gut bacteria can also produce enzymes that catalyze the conversion of dietary choline into toxic compounds. These amines can be uptaken by the liver, transformed to trimethylamine-N-oxide and cause inflammation and liver injury (Zeisel et al., 1983). Finally, gut microbiota promotes NAFLD through another mechanism, by altering the bile acid metabolism influencing farnesoid X receptor (FXR) signaling and thus inhibiting hepatic DNL and VLDL export (Tremaroli and Backhed, 2012).

III. METABOLIC SYNDROME, NONALCOHOLIC FATTY LIVER DISEASE AND CARDIOVASCULAR DISEASES

The pathophysiological mechanism that associates liver steatosis with cardiovascular disease is unclear. NALFD is associated with increased risk for CVD and is a predictor for CVD independent of the presence of other metabolic syndrome risk factors such as hypertension, type II diabetes, dyslipidemia and obesity (Than and Newsome, 2015). It is extremely difficult to dissect out the causal relationships responsible for the increased risk of coronary heart disease and other complications observed in patients with NALFD due to the complex interactions including insulin resistance and abdominal obesity (Ballestri et al., 2014). The RISC (Relationship between Insulin Sensitivity and Cardiovascular disease) study illustrated that patients with NAFLD had raised 10 year coronary heart

disease risk score (Edens et al., 2009). Recent phase II trials assessing coronary atherosclerotic plaque in patients with NAFLD have showed that such patients are more likely to have advanced high risk coronary plaque, independently of traditional cardiovascular risk factors as compared with patients without NAFLD (Puchner et al., 2015). In a recent large cohort study of biopsies from proven NAFLD patients, such patients had an increased risk of death from cardiovascular and liver related causes (Ekstedt et al., 2015). NAFLD has been associated with subclinical atherosclerosis assessed by increased arterial stiffness (AS), impaired flow-mediated vasodilation (FMD) and increased carotid intima-media thickness (cIMT) as reported in a recent meta-analysis (Bonci et al., 2015). Coronary and abdominal aortic calcification, impaired left ventricular function and heart failure (HF) are also linked to NAFLD (Katsiki et al., 2016; Mellinger et al., 2015; Oni et al., 2013). A retrospective study showed that NAFLD, assessed by ultrasonography, was significantly associated with increased coronary artery calcification (CAC) score (*i.e*., CAC score > 100), independently of traditional CVD risk factors (Chen et al., 2010).

Dyslipedaemia is the hallmark of MetS and is characterized by elevated TG and low levels of HDL-C (Brunzell and Hokanson, 1999). In patients with NALFD or MetS insulin fails to inhibit lipolysis and production of triglyceride rich VLDL particles occurs from the liver (Adiels et al., 2007). The increase in VLDL leads to decrease in HDL cholesterol and to production of small, dense LDL particles which are highly atherogenic (Tchernof and Despres, 2013). This atherogenic dyslipidemia may be at least partially responsible for the increased CVD risk in NAFLD patients as these lipid abnormalities have been independently associated with CVD morbidity and/or mortality (Graham et al., 2012; Tenenbaum et al., 2014). Lipoproteins are essential parts of the underlying mechanistic action in dyslipedaemia associated with NAFLD. Lipoproteins deliver cholesterol and TG from the liver and intestine to muscle and adipose tissue by forming chylomicron, VLDL and LDL particles. In addition to this fuction, lipoproteins participate in the transport of the excess cholesterol from extra-hepatic tissues to the liver, a function named reverse cholesterol transport-RCT, for the elimination via bile secretion. Both functions are changed in NAFLD. Patients with NAFLD have decreased hepatic insulin sesitivity and glucose uptake despite having high circulating insulin levels which can contribute to dyslipedaemia (Bugianesi et al., 2005; Gaggini et al., 2013)

In addition, a wide range of pro-inflammatory molecules which are secreted by the liver in response to oxidative stress and liver injury may further impact on the associated cardiovascular risk in NAFLD (Williamson et al., 2011). Activation of innate immunity offers a potential link between insulin resistance and dyslipedaemia in MetS. Activation of immunity in animal models causes

changes in lipoproteins, enzymes, transfers proteins and receptors profile with an increase in atherogenic lipoprotein particles (Reilly and Rader, 2003). These changes are similar to those observed in humans with MetS including increased hepatic VLDL production, reduced VLDL clearance, increased small and dense LDL, reduced HDL, and alteration in HDL composition.

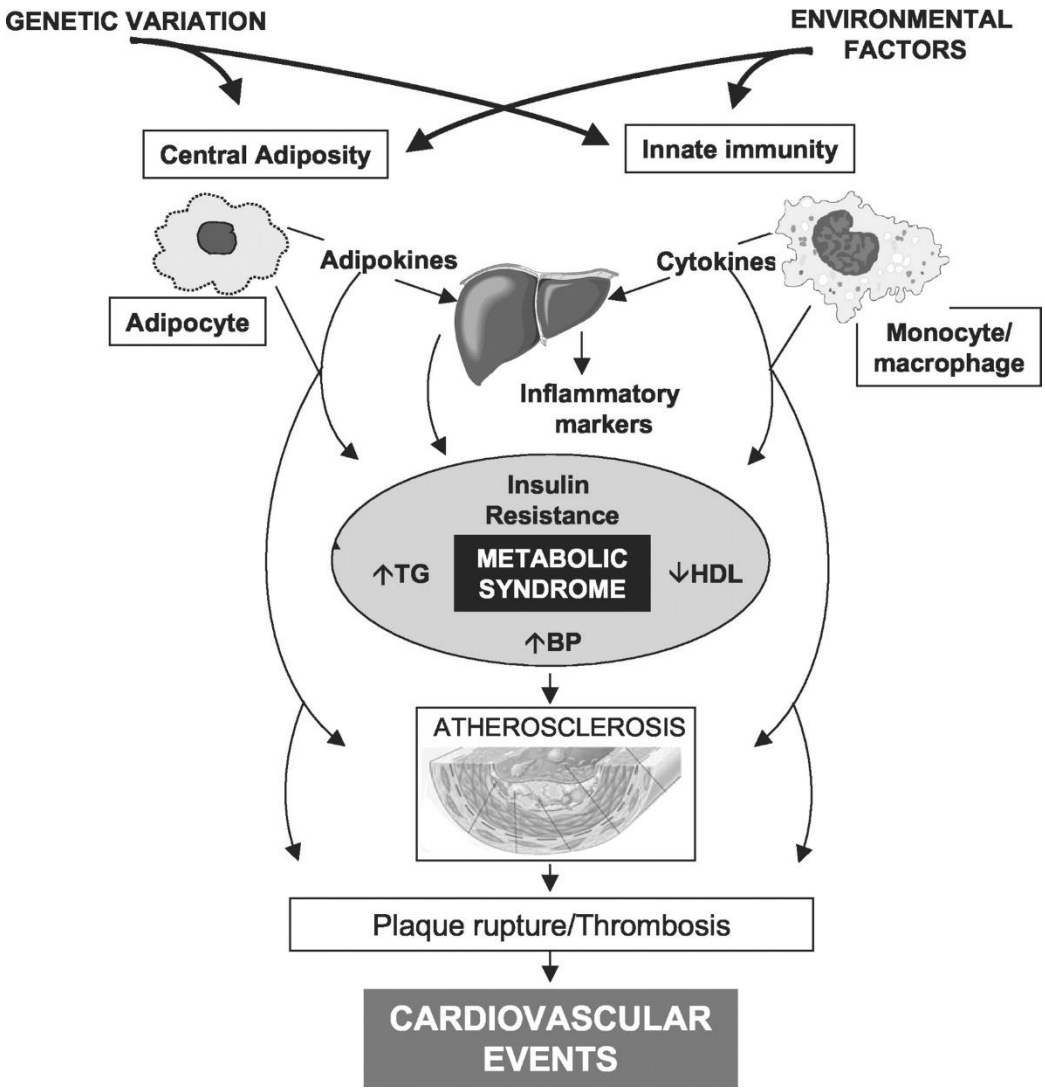


Figure 4. Pathophysiology of atherosclerotic cardiovascular disease in the metabolic syndrome (Reilly and Rader, 2003)

IV. HDL CHOLESTEROL AND CARDIOVASCULAR DISEASE

HDL-C concentration is a reflection of various metabolic and inflammatory processes, and this partly explains its strong inverse association with coronary heart disease. Although the HDL hypothesis has been challenged, it is important to emphasize that its value as a predictor of cardiovascular risk remains largely unchallenged (Rader and Hovingh, 2014). There is straightforward evidence that high-density lipoprotein cholesterol levels in plasma are reciprocally correlated with cardiovascular disease risk. Several studies performed in humans, animals and tissue culture have clear illustrated the beneficial functions of HDL and its significant role in cellular cholesterol efflux and protection against inflammation. It is important to be mentioned that very high HDL cholesterol levels or completely loss of HDL do not indicate protection or acceleration of atherosclerosis, respectively. However, LDL cholesterol levels are directly associated with increased or decreased risk for atherosclerosis. The underlying mechanisms of HDL cardio-protective effect are not well understood, mainly because HDL consists of numerous subpopulations of particles that are highly diverse in terms of shape (discoidal or spherical), density, protein composition, molecular size, and electrophoretic migration (Kuivenhoven and Groen, 2015). More recently, genome-wide association studies (GWAS) have examined the relationship between single-nucleotide polymorphisms (SNPs) associated with changed HDL cholesterol levels and the risk of coronary disease. In addition, several genetically modified mice have been investigated to gain further insights into the role of HDL metabolism and function in cardiovascular and metabolic diseases.

i. HDL Biogenesis, Remodeling and Catabolism

The biosynthesis of HDL is a complex process in which several membrane bound and plasma proteins get involved (Zannis et al., 2004). The first step in the process involves the secretion of the apoA-I by the liver and the intestine (Zannis et al., 1985). The secreted apoA-I interacts functionally with ABCA1 and thus cellular phospholipids and cholesterol are transferred to lipid-poor apoA-I. The lipidated apoA-I is now converted to discoidal particles enriched in unesterified cholesterol. The esterification of free cholesterol is performed by the enzyme lecithin/cholesterol acyltransferase (LCAT), which converts the discoidal (nascent) to spherical (mature) HDL particles (Zannis et al.,

2006). Following the same pathway, apoE and apoA-IV can synthesize HDL particles which contain these proteins (Duka et al., 2013; Kypreos and Zannis, 2007).

After their synthesis, HDL particles are transformed further in a process named “remodeling” by cell receptors and other plasma proteins. Hepatic lipase (HL) and endothelial lipase (EL) are involved in the remodeling of HDL (Maugeais et al., 2003; Santamarina-Fojo et al., 2004). They hydrolyze triglycerides and some phospholipids in the HDL, driving the conversion of HDL2 to HDL3 and preβ-HDL and accelerating the catabolism of HDL. ApoM is required for remodeling of HDL particles (Wolfrum et al., 2005). Part of cholesteryl esters which are formed by LCAT action can be transferred to VLDL/IDL/LDL by cholesteryl ester transfer protein (CETP). Phospholipids from VLDL/IDL can be transferred to the HDL particles during lipolysis by the phospholipid transfer protein (PLTP). Scavenger receptor class B type-I (SR-BI) mediates the selective uptake or efflux of cholesteryl esters by the cells. It is mostly expressed in hepatocytes, macrophages and steroidogenic tissues. In the liver, cholesterol esters are secreted into the bile through the ATP binding cassette half-transporters G5/8 (ABCG5/8) while in the adrenal cells they participate in steroid hormone synthesis.

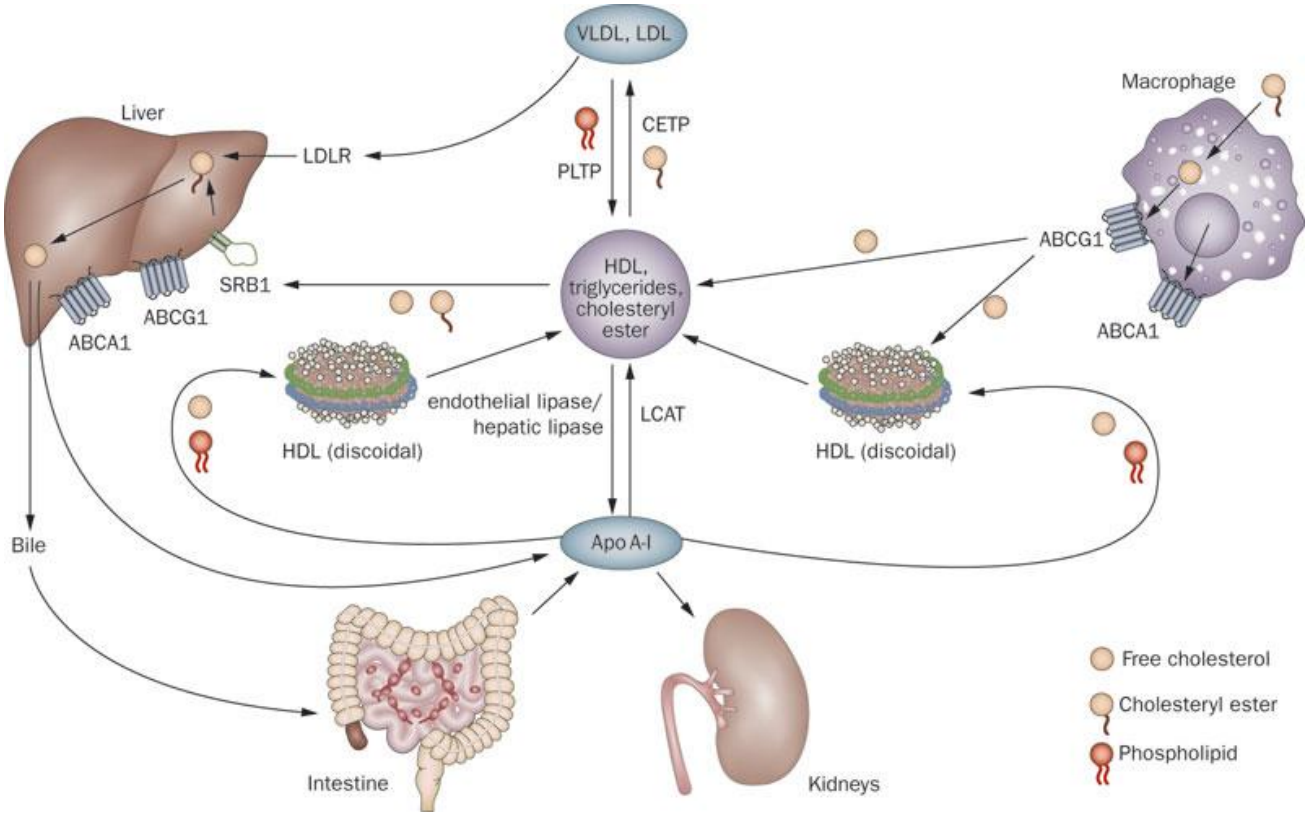


Figure 5. Molecular biosynthesis of HDL (Navab et al., 2011).

Cholesterol efflux from macrophages to α-HDL particles is mediated by the cell membrane transporter ATP binding cassette transporter G1 (ABCG1). Finally, the removal of excess cholesterol from the peripheral tissues, including macrophages and foam cells of the arterial wall, back to the liver for excretion into the bile is traditionally called 'reverse cholesterol transport' (RCT) pathway (Zannis et al., 2015).

ii. Beyond the Genetics of HDL

Twin studies have revealed that both genetic and environmental factors contribute equally to the blood levels of HDL (Goode et al., 2007). GWA studies have yielded more comprehensive knowledge of the mechanisms causing cardio-metabolic diseases in the general population. GWAs discover already established as well as novel regulators of the HDL levels. Recent GWAs have emerged as a new tool to identify common genetic variants that are closely correlated with altered plasma lipids. In these studies SNPs are associated with changes in blood lipids as well as altered risk for cardiovascular disease. Notably, one of the most widely known paper is the one from Teslovich et al. 2010, in which 38 loci are listed with HDL cholesterol association (Teslovich et al., 2010). These studies have demonstrated the most established "HDL genes" which control the HDL synthesis as well as HDL-mediated cholesterol homeostasis, as described in the previous paragraph. A schematic presentation of the established "HDL genes" is depicted in Figure 6.

Furthermore, there are a huge number of other factors that regulate HDL cholesterol concertation modifying the lipolysis of plasma triglycerides. Figure 7 illustrates the most significant of these factors. Most of these genes control the activity of lipoprotein lipase (LPL), the enzyme that hydrolyze the triglycerides from chylomicron and VLDL in the circulation. A comprehensive meta-analysis in the field added 46 more loci which are associated with HDL cholesterol (Willer et al., 2013).

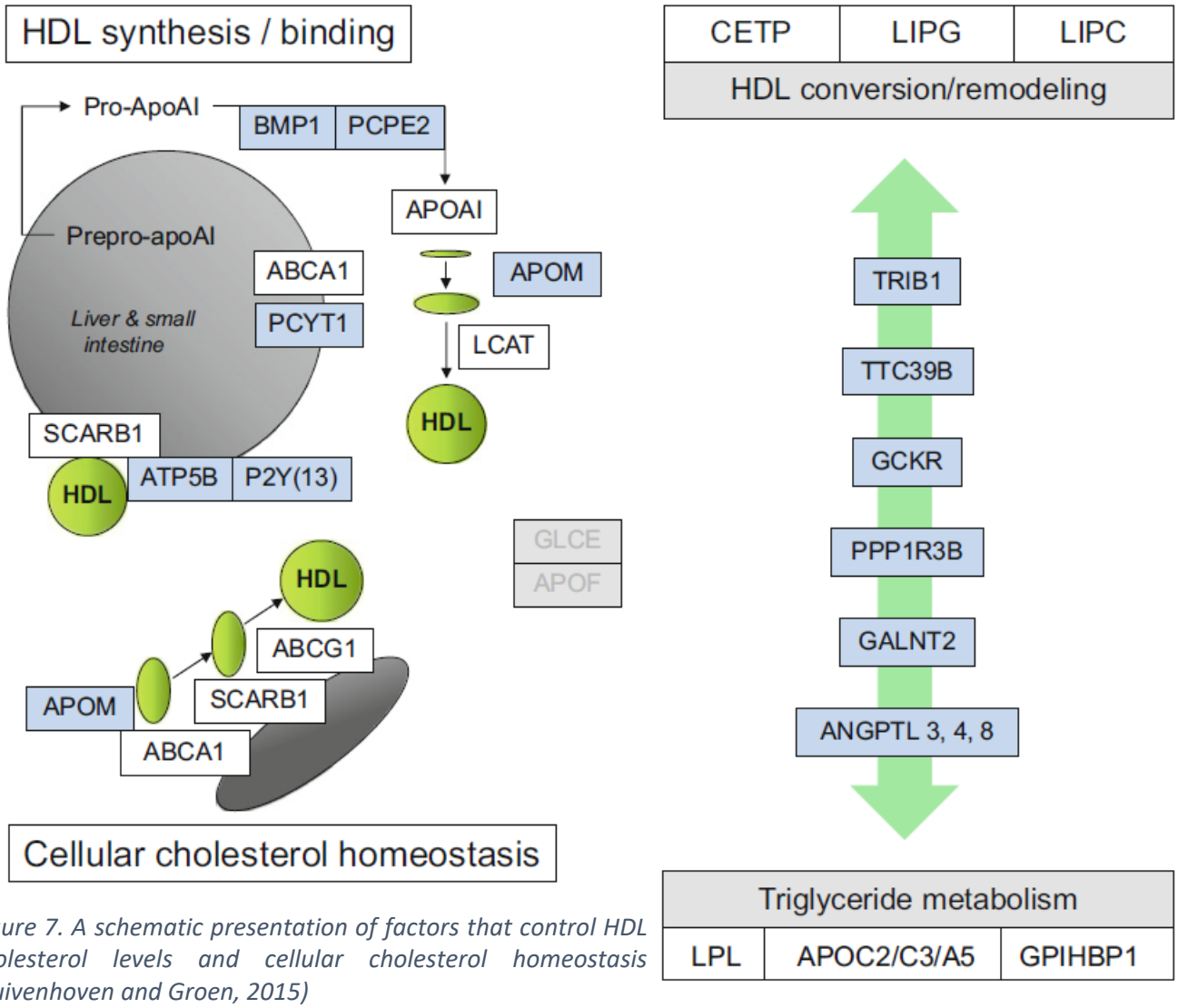


Figure 7. A schematic presentation of factors that control HDL cholesterol levels and cellular cholesterol homeostasis (Kuivenhoven and Groen, 2015)

Figure 6. A schematic presentation of factors that affect HDL through roles in the conversion and remodeling of HDL or through effects on triglyceride/glucose metabolism (Kuivenhoven and Groen, 2015).

CTP: Phosphoethanolamine cytidylyltransferase-PCYT2

Phosphatidylethanolamine (PE) is the primary phospholipid on the inner leaflet of cellular membranes as well as in HDL (Fullerton and Bakovic, 2010). Pcyt2 is the main regulatory enzyme for de novo biosynthesis of phosphatidylethanolamine by the CDP-ethanolamine pathway. Total deletion of Pcyt2 in mice is embryonic lethal (Fullerton et al., 2007), which confirms the necessity of this gene for animal growth and development. Heterozygous mice ($Pcyt2^{+/-}$) (Fullerton and Bakovic, 2010; Fullerton et al., 2009), as well as Pcyt2 liver-specific mice develop liver steatosis. The gene

established a strong physiological connection between PE and triglycerides synthesis through the common intermediate DAG (Leonardi et al., 2009). Pcyt2 knockout mice, in order to reduce the excess DAG, synthesize additional fatty acids from glucose by lipogenesis and thus they inevitably accumulate triglycerides (Leonardi et al., 2009). A recent study demonstrated that Pcyt2 deficient mice exhibit hypertriglyceridemia as a result of multiple metabolic adaptations, including elevated hepatic and intestinal lipoprotein secretion and stimulated expression of genes which get involved in lipid absorption, transport and lipoprotein assembly together with decreased plasma triglycerides clearance (Fullerton et al., 2009; Singh et al., 2012).

Tribbles Homologe 1 – TRIB1

Tribbles encode an evolutionarily conserved protein family that regulates cell proliferation, motility, metabolism and oncogenic transformation. Trib acts as scaffold protein which facilitates the degradation of target proteins (Imajo and Nishida, 2010). Several GWAs have linked the TRIB1 locus with plasma levels of triglycerides (TGs), high-density lipoprotein cholesterol (HDL-C), and coronary artery disease (CAD) (Douvris et al., 2014; Kathiresan et al., 2009; Teslovich et al., 2010; Willer et al., 2013). In previous studies conducted in murine models, hepatic viral-mediated over-expression of Trib1 was shown to increase fatty acid oxidation and decrease triglyceride synthesis, which caused significant reductions in plasma cholesterol and TG (Bauer et al., 2015b; Burkhardt et al., 2010). Whereas Trib1 knockdown mice exhibited hypertriglyceridemia (Burkhardt et al., 2010), liver-specific deletion of Trib1 exhibits increased hepatic triglyceride (TG) content, lipogenic gene transcription, and de novo lipogenesis (Bauer et al., 2015a). The same study revealed that tribbles-1 regulates hepatic lipogenesis through posttranscriptional regulation of C/EBPα, which in turn transcriptionally upregulates Trib1. On the other hand, acute knockdown of TRIB1 in human primary hepatocytes resulted in reduced expression of MTTP and APOB, which are required for VLDL assembly (Soubeyrand et al., 2016). Furthermore, epidemiological studies confirmed that TRIB1 is associated with HDL cholesterol without affecting the concentrations of apoA-I (Varbo et al., 2011). In addition, in a recent study, an unclear correlation between TRIB1 gene and HNF4A gene was demonstrated (Soubeyrand et al., 2016) as well as a function of Trib1 as a negative regulator of the retinoic acid receptor shedding new light on the molecular mechanisms for nuclear receptor-mediated transcriptional repression (Imajo and Nishida, 2010).

24-Dehydrocholesterol reductase - DHCR24

3b-Hydroxysterol D24-reductase (DHCR24) catalyzes the conversion of desmosterol to cholesterol (Zerenturk et al., 2013). Related to its role in cholesterol biosynthesis, DHCR24 modulates lipid raft formation, thus facilitating signal transduction and trafficking, regulates oxidative stress, is neuroprotective, anti-apoptotic and anti-inflammatory (Luu et al., 2014). Dhcr24 has been linked with cardiovascular disease (Spann et al., 2012; Wu et al., 2013) and patients with mutations in DHCR24 have elevated desmosterol and lowered cholesterol levels (Horvat et al., 2011). Furthermore, it has been found that DHCR24 is transcriptionally regulated by sterols via the sterol-regulatory element-binding protein-2 transcription factor (Horton et al., 2003). Finally, the enzyme activity is inhibited at a post-translational level by certain oxysterols (Zerenturk et al., 2012) and steroid hormones (Jansen et al., 2013).

Protein Phosphatase 1 Regulatory Subunit 3B - PPP1R3B

PPP1R3B gene encodes for glycogen targeting PP1 (protein phosphatase 1) subunit G(L), which has been associated with type II diabetes and MODY-1 (Dunn et al., 2006). GWAs linked the gene with HDL cholesterol levels and viral-mediated overexpression of the gene was shown to reduce HDL levels in mice as well as the triglyceride levels (Teslovich et al., 2010).

Carboxylesterase 3 –CES3B

Carboxylesterase 3/triacylglycerol hydrolase (Ces3/TGH) participates in hepatic VLDL assembly and in adipose tissue basal lipolysis (Dolinsky et al., 2004a; Dolinsky et al., 2004b). Hepatic inhibition of TGH decreased VLDL secretion both in vitro and in vivo (Gilham et al., 2003; Lehner and Vance, 1999; Wei et al., 2010). Blocking hepatic VLDL assembly and secretion can lead to severe hepatic lipid accumulation (Raabe et al., 1999), a condition which is prevented in TGH deficient ($Tgh^{-/-}$) mice. This is a compensatory mechanism by decreasing non-esterified fatty acid (NEFA) flux from the adipose tissue to liver and increasing hepatic fatty acid oxidation (Lian et al., 2012b; Wei et al., 2010). In humans, elevated TGH expression was observed in patients with steatosis and NASH (Ashla et al., 2010), but the role in liver steatosis has not been determined. There are a significant number of studies which clearly demonstrate that liver deficiency of Ces3/TGH decreases blood lipids, improves glucose tolerance and attenuates steatohepatitis (Lian et al., 2012a; Lian et al., 2016;

Lian et al., 2012b; Wei et al., 2010), suggesting the potential therapeutic target that may hold for lowering blood lipid levels.

V. NUCLEAR RECEPTORS

Nuclear receptors (NR) discovered more than 3 decades ago are members of a large superfamily protein that function as principal sensor, signal transducers and transcription regulators of metabolic pathways. Small molecule compounds (endocrine hormones, lipid metabolites, xenobiotics, pharmaceutics) can directly bind to the NR as ligands and regulate NR's activity. These NRs respond to ligands and promote or inhibit the signaling pathways, which they are involved by controlling the transcription of target genes via recruitment of coactivators or corepressors respectively. NRs consist of 48 distinct receptors which are differentially expressed in metabolic tissues and cell types and regulate a wide range of biological processes including development, homeostasis, metabolism, circadian rhythms, endocrine function, reproduction, inflammation, and immunity (Giudici et al., 2016).

The NR superfamily is sorted into seven families, NR1 (thyroid hormone like), NR2 (HNF4-like), NR3 (estrogen like), NR4 (nerve growth factor IB-like), NR5 (fushi tarazu-F1 like), NR6 (germ cell nuclear factor like), and NR0 (knirps or DAX like) (Xiao et al., 2013). Based on their ligand binding, NRs are classified into three superfamilies (Figure 4): a) the *steroid hormone receptors* that particiapate in the majority of biological actions when the ligand is steroid hormone (e.g. estrogen receptor (ER) and glucocorticoid receptor (GR)), b) the *adopted orphan receptors* that were identified prior to the discovery of their regulatory ligands and now have been deorphanized with the aid of chemical, structural and genomic technologies (e.g. Liver-X-receptors (LXRs) and the peroxisome proliferator-activated receptor gamma (PPARγ)) and c) the *orphan receptors* whose endogenous ligands are still not identified and it remains unclear if these NRs require ligand binding to activate transcription (e.g. nerve growth factor IB (NGFIB) and the neuronderived orphan receptor 1 (NOR1)) (Glass and Ogawa, 2006).

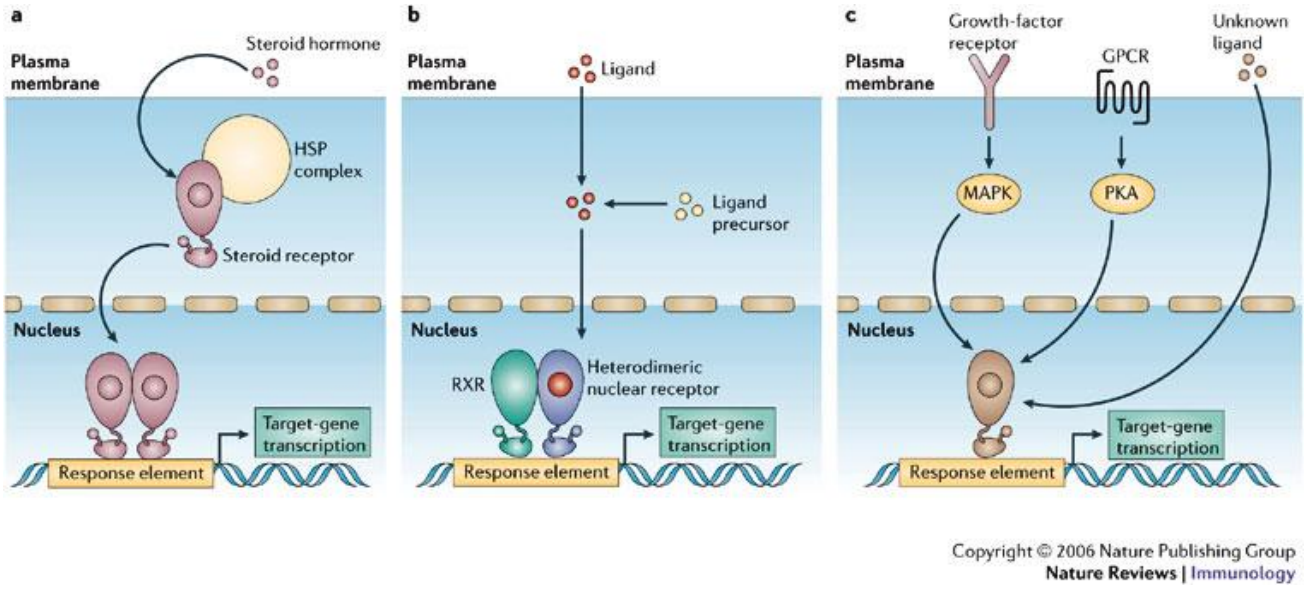


Figure 8. The nuclear-receptor superfamily can be subdivided into three classes: the classical steroid-hormone receptors, the so-called 'adopted' orphan receptors that bind DNA as heterodimers with retinoid X receptors (RXRs), and the orphan receptors that have not been linked to naturally occurring ligands (Glass and Ogawa, 2006).

All NRs are evolutionarily conserved and have a common structure consisting of four domains (Germain et al., 2006). The DNA binding domain (region C) and the ligand binding domain (region E) are the most conserved domains. The other two domains, the N-terminal A/B domain and the D region, are comparatively less well conserved. The *A/B domain* contains an activation domain that stimulates transcription in a ligand-independent manner. The DNA binding domain consists of two cysteine-rich zinc finger motifs, two α-helices and a C-terminal extension, and plays important roles in both nuclear localization and in the interaction with other transcription factors (Germain et al., 2006). The *D region* serves as a linker between the DNA-binding domain and the ligand-binding domain and contains a nuclear localization signal. The ligand binding domain is contained in the C-terminal *E region* and has four functionally interconnected regions including the ligand binding pocket, a dimerization surface and a transcriptional co-regulator binding surface which participates in protein-protein interactions with other transcription factors, and an activation helix known as AF-2, which mediates ligand dependent transactivation (Warnmark et al., 2003).

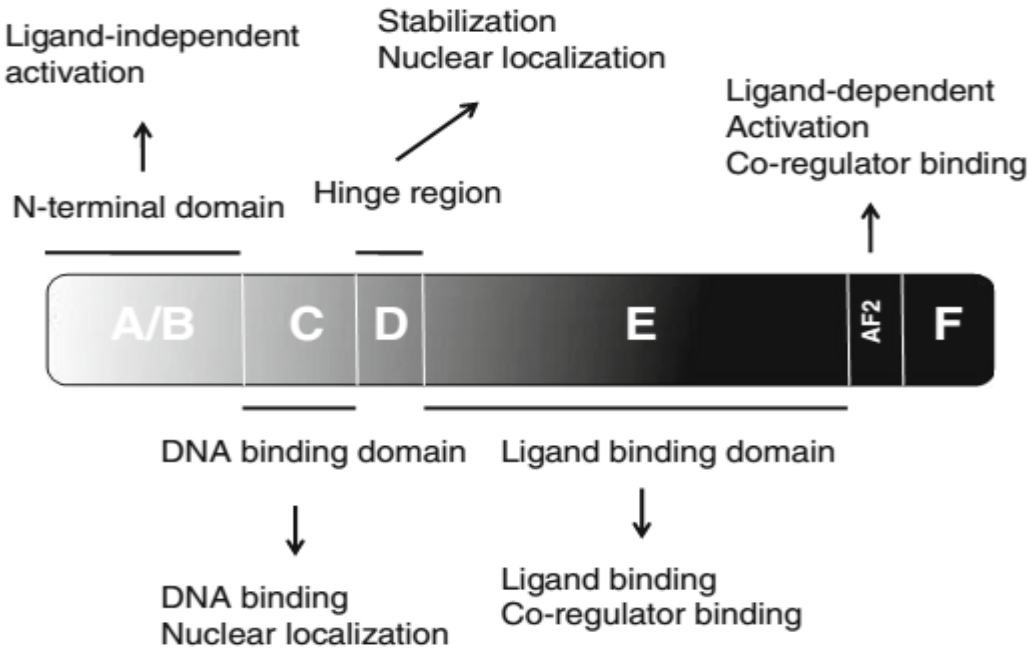


Figure 9. Schematic representation of the domain structure of a typical nuclear (Khurana et al., 2012).

NRs bind DNA at highly specific nucleotide sequence motifs of 5-10 base pairs known as hormone response elements (HREs) (Schwabe and Teichmann, 2004). NRs bind to HREs in their target promoters through their DBD. HREs contain direct repeats (DRs), inverted repeats (IRs) or palindromic repeats (PRs) of the sequence 5'-AG(G/T)TCA-3'. These repeats are separated by one, two, three, four, or five nucleotides and are designated DR1, DR2, etc. (for direct repeats), IR1, IR2, etc. (for the inverted repeats), and PR1, PR2, etc. (for the palindromic repeats) (Cotnoir-White et al., 2011). The exact binding sequence of different nuclear receptors defines their specificity. Furthermore, NRs are able to regulate the expression of genes lacking canonical HREs through physical and functional interactions with other promoter-bound transcription factors acting as super-activators or trans-repressors (Glass, 2006).

NRs that are activated by steroids form homodimers and bind to HREs on the promoters of their target genes in a ligand-dependent manner while NRs that are regulated by other ligands form heterodimers with Retinoid X Receptor (RXR) and bind to HREs in the presence or absence of ligands, activating or repressing transcription, respectively. Upon ligand binding heterodimeric nuclear receptors undergo conformational changes that lead to the dissociation of co-repressor complexes and the subsequent recruitment of co-activators resulting in a switch from repression to gene activation (Bourguet et al., 2000).

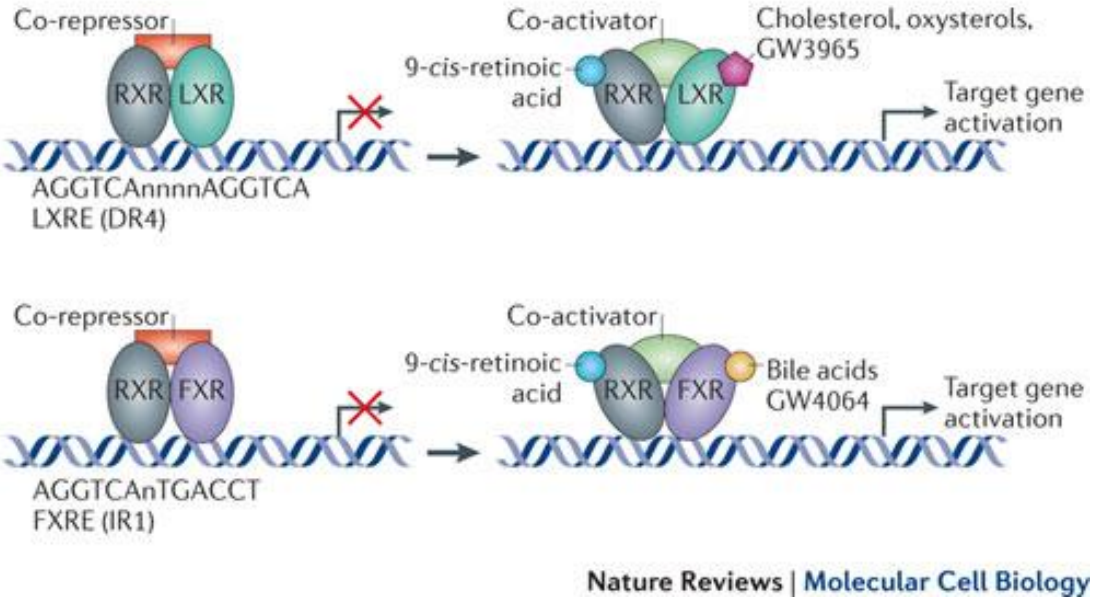


Figure 10. NRs form heterodimer that binds to a DR4 (direct repeat spaced by four nucleotides) FXRE (FXR response element) in the regulatory regions of target genes, thereby repressing gene expression. Following ligand binding, the heterodimer changes conformation, which leads to the release of co-repressors and the recruitment of co-activators. This results in the transcription of target genes (Calkin and Tontonoz, 2012).

Hepatic Nuclear Factor 4 alpha

Hepatic nuclear factor 4α (HNF4α, NR2A1) is a member of the nuclear receptor superfamily but exhibits properties that are rather distinct from other proteins of the superfamily (Schrem et al., 2002; Sladek et al., 1990). HNF4α has been widely associated with the transcriptional regulation of hepatocyte genes specifically implicated in lipid metabolism, glucose metabolism, differentiation and morphogenesis (Babeu and Boudreau, 2014). HNF4α was isolated as a protein from rat liver nuclear extracts bound on DNA element required for transcription of the gene encoding transthyretin and apolipoprotein CIII (Sladek et al., 1990). HNF4α is expressed at high levels in liver and to a lesser degree in kidney, small intestine, colon and pancreatic β cells (Drewes et al., 1996; Jiang et al., 2003). Human HNF4 gene is located to 20q12-q13.1 as has been found by genetic linkage analysis (Argyrokastritis et al., 1997). *HNF4A* locus is transcriptionally regulated through the use of two distinct promoters that are physically separated by more than 45 kb (Thomas et al., 2001). Isoforms produced by the activity of the closer promoter are classified P1 whereas isoforms produced by the second and more distant promoter are designated P2. This difference causes P2 isoforms to be

shorter than P1 isoforms and, more importantly, to lack the cofactor interacting domain designed as activating function (AF)-1. These structural differences between P1 and P2 isoforms may display distinct roles by interacting differently with specific cofactors and being differently regulated in specific contexts of physiological importance. Isoforms from P1 promoter are produced mainly in adult liver, kidney, intestine and colon whereas those from P2 promoter are expressed mainly in fetal liver and in adult intestine, pancreas and colon.

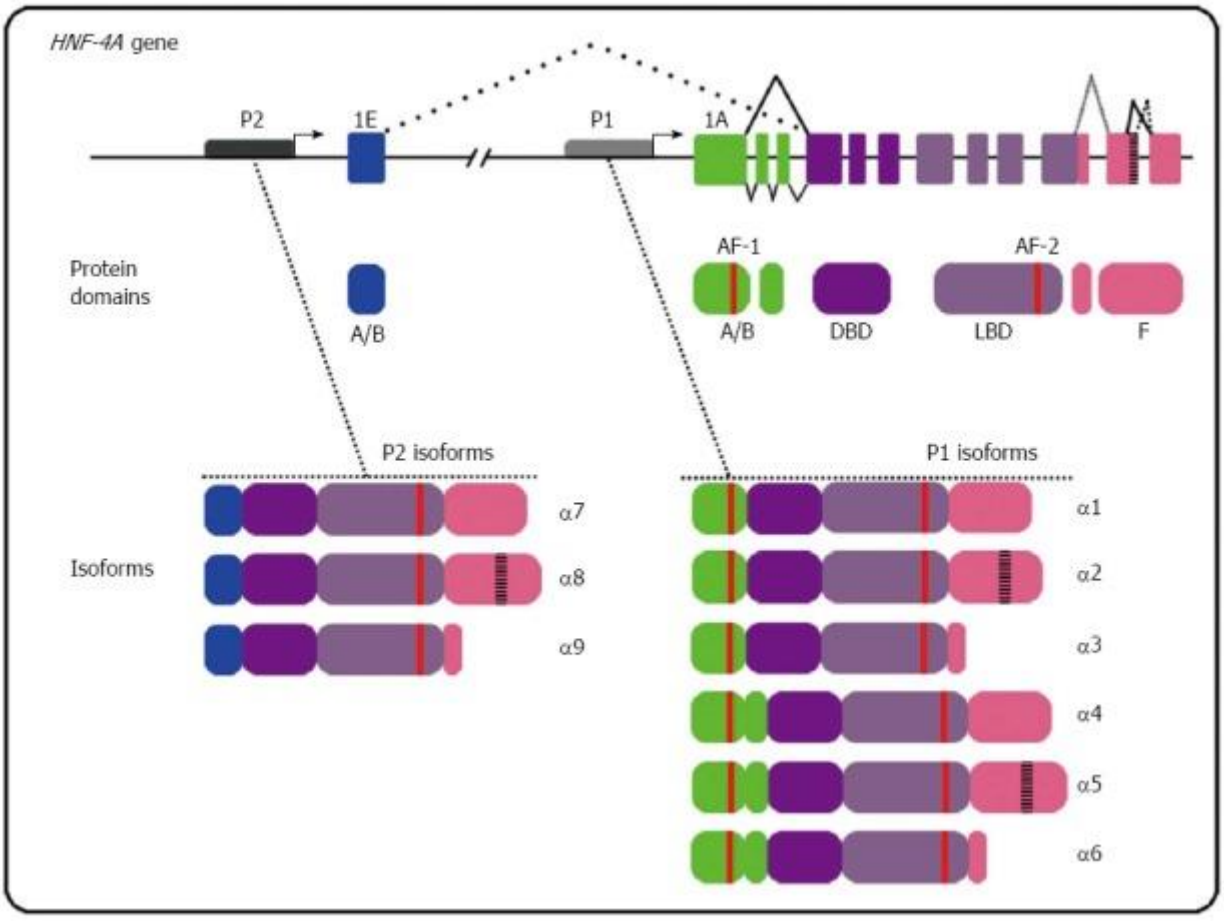


Figure 11. Hepatocyte nuclear factor 4-alpha P1 and P2 isoforms classes originate from alternative promoters and splicing (Babeu and Boudreau, 2014).

HNF-4α belongs to *orphan nuclear receptors* superfamily and is characterized by the presence of two well-conserved functional domains, the DNA-binding domain (DBD) and the ligand-binding domain (LBD). HNF4α binds as a homodimer to its DNA recognition site, a direct consensus repeat element 5' xxxxCAAAGTCCA 3' with either one or two nucleotide spacer (DR1 or DR2), as well as a previously unrecognized polarity in the classical DR1 motif (AGGTCAxAGGTCA) (Fang et al., 2012).

Upon binding to DNA, it recruits transcriptional co-activators and other additional components and regulates positively the expression of the target genes (Gonzalez, 2008; Kardassis et al., 2014). HNF4α in the liver is localized only in the nucleus where is required for development of the liver and interacts with regulatory elements on promoters of genes which encode for enzymes, transporters and other nuclear receptors and whose products are associated with a number of critical metabolic pathways.

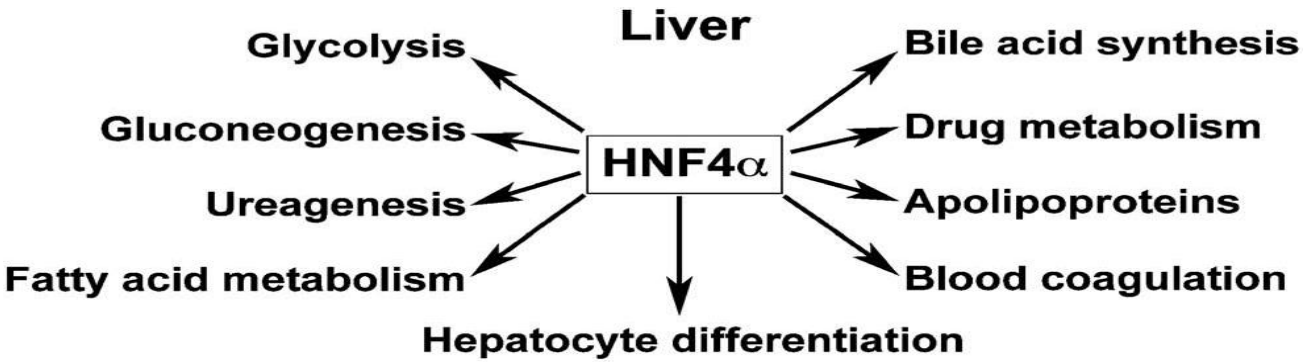


Figure 12. Role of HNF4α in control of gene expression in the liver (Gonzalez, 2008).

HNF4α adopts spontaneously a transcriptionally active conformation after it captures a suitable endogenous fatty acid in the binding pocket within the cell (Wisely et al., 2002). In addition to that, there is some evidence that HNF4α can bind exogenous fatty acyl-CoA ester (Hertz et al., 1998; Schroeder et al., 2005), however, it is still not clear whether it transcriptional activity is changed by binding to these or other ligands.

In humans, heterozygous mutations of HNF4α causes maturity onset diabetes of the young 1 (MODY1) which is characterized by autosomal dominant inheritance (Yamagata et al., 1996). These patients display normal insulin sensitivity and liver and kidney function, however, they have impaired glucose-stimulated insulin secretion from the pancreatic β cells (Fajans et al., 2001; Gupta et al., 2005). These symptoms appear to be due to deficiency in HNF4α signal transduction in the pancreas. In addition, MODY1 disorders have reduced levels of plasma triglycerides and cholesterol (Lehto et al., 1999; Shih et al., 2000).

Very interestingly, no homozygous mutations of HNF4α gene have been found in humans. This is in agreement with the neonatal lethality which was observed in embryonic targeted gene disruption (Chen et al., 1994), these embryos fail to undergo normal gastrulation (Duncan et al., 1997). The embryonic lethality of HNF4α KO mice is maybe due to the necessity of HNF4α during the liver development (Duncan, 2003; Kyrmizi et al., 2006).

Conditional liver-specific deletion of the HNF4α gene in mice results in marked metabolic dysregulation and increased mortality (Hayhurst et al., 2001). HNF4-LivKO mice exhibit altered lipid levels and are characterized by massive lipid accumulation in hepatocytes (steatosis), reduced serum triglyceride, total cholesterol and HDL-C and elevated serum bile acids due to impaired expression of genes involved in lipid metabolism, including apolipoproteins, cholesterol synthesis enzymes and bile acid transporters (Hayhurst et al., 2001; Inoue et al., 2006; Martinez-Jimenez et al., 2010; Yin et al., 2011).

Table 1. Genes that alter in HNF4α liver-specific knockout mice

Function	Genes
VLDL secretion	MTP, APOB
De novo cholesterol biosynthesis	HMGCR, HMGCS, SREBP-2, FAS, DGAT1, DGAT2
Cholesterol catabolism	CYP7A1, CYP8B1
Cholesterol esterification	ACAT2, LCAT
Cholesterol uptake	LDLR, SR-BI
Transporters	ABCA1, ABCG5, ABCG8, MDR2, L-FABP
Apolipoproteins	APOA1*, APOA2, APOA4, APOC2, APOC3, APOE*
Nuclear receptors	PPARa, PPARg

Furthermore, HNF4α LivKO mice lost weight after 5 weeks and die at 8 week and morphologically they exhibit hepatomegaly and hepatocyte hypertrophy (Hayhurst et al., 2001).

The bile acid imbalance that is exhibited in liver-specific *Hnf4α*$^{-/-}$ (L*Hnf4α*$^{-/-}$) mice is a result of reduced hepatic expression of genes involved in bile acid biosynthesis and bile acid uptake from blood. Hypotriglyceridemia may be due to reduced hepatic expression of microsomal transport protein (MTP) and apolipoprotein B (ApoB), which both play a significant role in very-low-density lipoprotein (VLDL) secretion. Hayhurst *et al.* suggested that the elevated expression of scavenger receptor class B type I (SR-BI), which selectively uptake cholesteryl esters from HDL into the hepatocyte from plasma HDL, is responsible for hypocholesterolemia in liver-specific *Hnf4α*$^{-/-}$ mice. In the liver cholesterol nay be converted to bile acids by cholesterol 7α- hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8B1), both enzymes controlling the biosynthesis of bile acids. Hepatic cholesterol is also secreted directly to the bile via ABC transporter G5 (ABCG5) and G8 (ABCG8)

(Wittenburg and Carey, 2002; Yu et al., 2002) or SR-BI (Kozarsky et al., 1997). Yin et al. demonstrated that via adenovirus liver delivery of a small hairpin of Hnf4α which causes acute loss of hepatic HNF4α causes hypotriglyceridemia and development of fatty liver via reducing VLDL secretion (Yin et al., 2011). Furthermore, the finding that VLDL receptor (Vldr) and Abcg1 were induced in Hnf4α-deficient mice suggests that Hnf4α deficiency does not cause a global repression of genes involved in lipid metabolism. A recently study revealed that chronic loss of hepatic HNF4a in $Ldlr^{-/-}$ mice increases energy expenditure and protects against the development of atherosclerosis whereas, acute loss of hepatic HNF4a reduces the development of atherosclerosis in $Apoe^{-/-}$mice (Xu et al., 2015). In addition the same study proposed that suppression of hepatic HNF4a expression together with approaches that alleviate hepatic steatosis may be useful for preventing the development of atherosclerosis.

VI. MetS AND NALFD TREATMENT

i. RNAi technology

RNA interference (RNAi) is a strong gene-specific silencing mechanism that is initiated by double-stranded (ds) RNA (Cullen, 2005). The discovery of RNAi, for which A.Z. Fire and C.C. Mello were awarded a Nobel Prize in 2006 revealed that RNAi degrades mRNAs complementary to the antisense strands of double-stranded RNA (Czech et al., 2011). RNAi resulting from the endogenous miRNA pathway or artificial exogenous siRNA or shRNA regulate gene expression by controlling the synthesis of protein through posttranscriptional gene silencing (Borel et al., 2014). RNAi is a widespread and natural phenomenon found in fungi, plants and animals and utilizes cellular mechanisms catalyzed by molecular machines that direct the antisense sequences of dsRNA to bind to mRNA and exert nuclease activity to the mRNA (Rana, 2007). Fundamental insights into mammalian RNAi came from biochemical studies that revealed that vital components of the RNAi machinery were conserved across the species. The mRNA degradation is guided by 21-23 nucleotide (nt) RNA fragments from long ds-RNA.

The potential uses and therapeutic benefits of RNAi are tremendous. Recent insight into RNAi technologies have revealed their roles by analyzing the functions and regulation of gene expression in eukaryotes and moreover applying this information for identification and amelioration of several

diseases. RNAi mediated post-transcriptional gene silencing (PTGS) mechanism in mammals includes gene regulation systems such as small interfering RNAs (siRNA), micro RNA (miRNA) and small hairpin RNAs (shRNA).

A recent review from Michael Czech *et al.* demonstrates the new technological approaches and functions of short sequences of double-stranded RNAs in order to address novel potential targets for the treatment of metabolic disease (Czech et al., 2011). They recently engineered a complex 5 component glucan-based encapsulation system for siRNA (small interfering RNA) delivery to phagocytes. The resulting glucan particles containing fluorescently labelled siRNA were readily internalized by macrophages, but not other cell types, and released the labelled siRNA into the macrophage cytoplasm (Cohen et al., 2016; Tesz et al., 2011).

New technologies that combine small interfering RNA molecules with antibodies or other targeting molecules also appear encouraging. Although still at an early stage, the emergence of RNAi-based therapeutics has the potential to markedly influence our clinical future.

Small hairpin RNA

The ability of RNAi to target any gene makes it an intriguing platform for developing of next generation therapeutics. It was discovered that smaller synthetic sequences of 21 to 23 nucleotides such as shRNAs were more efficient than dsRNA to trigger RNAi (Bofill-De Ros and Gu, 2016). ShRNAs are synthesized in the nucleus of the cells and then transported to the cytoplasm, incorporated into the RNA induced silencing complex (RISC) for activity, to target mRNA (Cullen, 2005). ShRNA is transcribed by the RNA polymerase III. The product mimics pri-microRNA (pri-miRNA) and is processed by Drosha protein. The resulting product which is named pre-shRNA is exported from the nucleus by Exportin 5. Then it is processed by Dicer and loaded into the RNA-induced silencing complex (RISC). The sense (passenger) strand is degraded. The antisense which is the guide strand directs RISC to mRNA that has a complementary sequence. If the complementarity is absolute, RISC cleaves the mRNA. In the case of imperfect complementarity, RISC represses translation of the mRNA (Borel et al., 2014; Czech et al., 2011).

ShRNAs are encoded by DNA vectors, they can be delivered to cells in any of the numerous ways that have been devised for delivery of DNA constructs that allow ectopic mRNA expression. These include standard transient transfection, stable transfection and delivery using viruses ranging

from retroviruses to adenoviruses (Hannon and Rossi, 2004). Expression can also be driven by either constitutive or inducible promoter systems (Paddison et al., 2004).

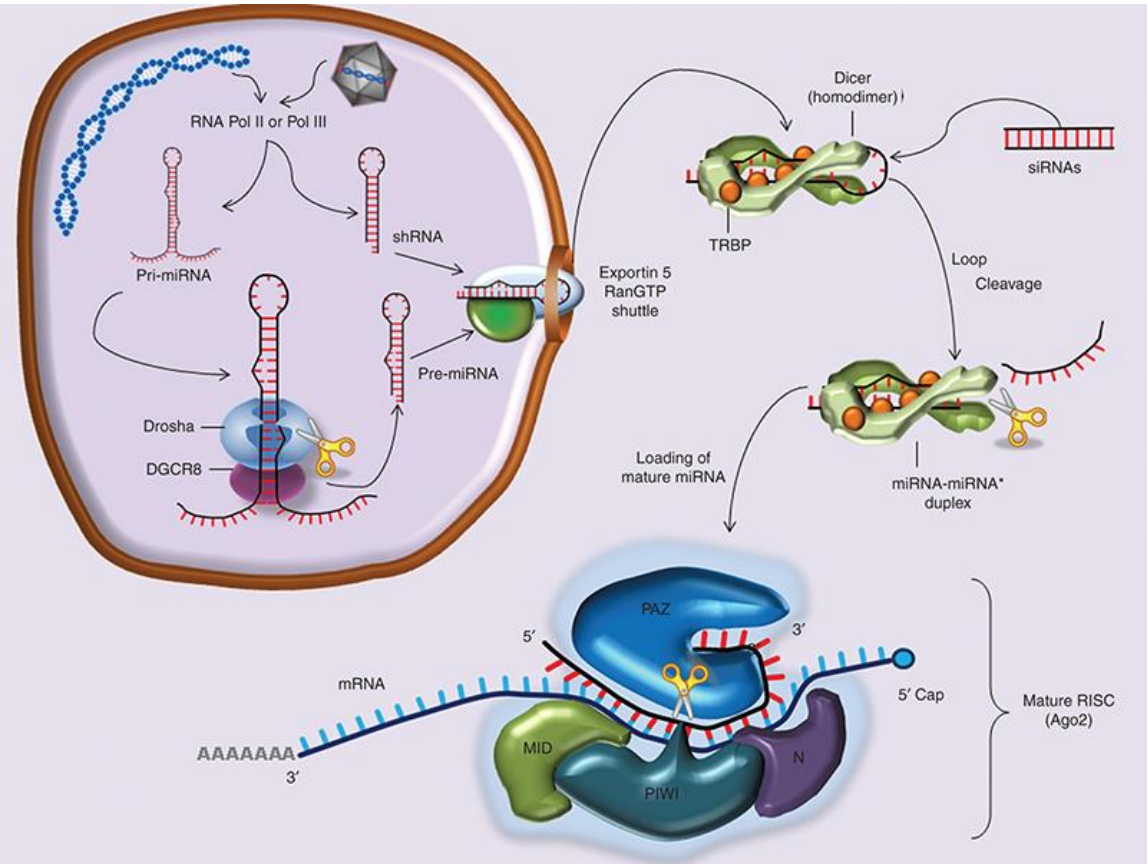


Figure 13. RNAi and shRNA Biogenesis (Borel et al., 2014).

ii. Adeno-associated virus vector technology

Adeno-associated virus (AAV) is a single-stranded DNA parvovirus and is one of the leading gene therapy vectors due to its absence of pathogenicity and low immunogenicity, stability and the potential to integrate site-specifically without known side-effects (Buning et al., 2008; Selot et al., 2014). A large number of recombinant AAV vector types has been created with the purpose of optimizing efficiency, specificity and the safety of *in vitro* and *in vivo* gene transfer. The AAVs are classified according to the coinfection of an unrelated helper virus for productive infection (e.g. adenovirus, herpesvirus, human cytomegalovirus, or papillomavirus).

AAV is a very simple virus with two genes one encoding for the viruses replication (REP) and the other is a protein capsid composed of 60 capsid subunits, and a ~4.7-kb single-stranded linear DNA genome that is framed by inverted terminal repeat sequences (ITRs), which form T-shaped

hairpin ends (Carter and Samulski, 2000). The genome contains three open reading frames (ORFs) under the control of three promoters –p5, p19, p40. The Rep gene gives rise to four different viral replicate proteins—Rep78, Rep68, Rep52, andRep40. The Rep proteins are required not only for viral replication but also for transcription, encapsidation, integration, and rescue of the virus from the latent state. The Cap gene encodes three different capsid proteins known as VP1, VP2, and VP3 (Buning et al., 2008; Lagor et al., 2013).

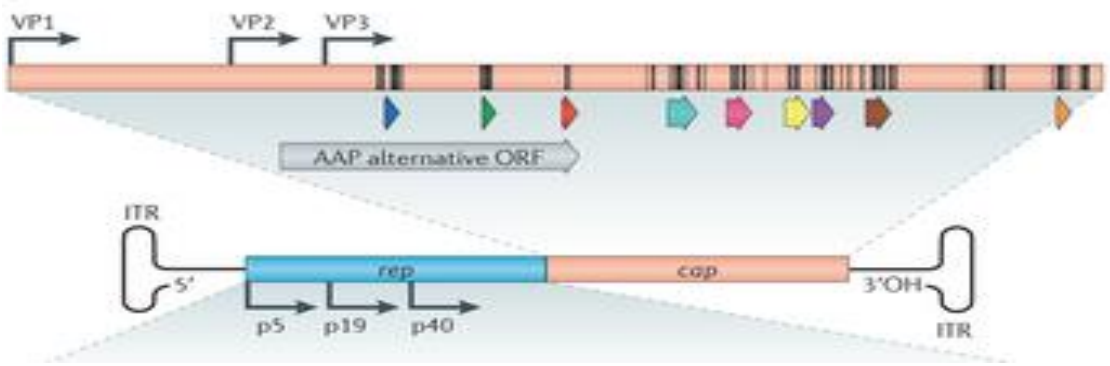


Figure 14. Organization of the AAVs genome (Kotterman and Schaffer, 2014).

To generate a recombinant version of AAV for laboratory use, the vector plasmid contains the transgene expression cassette of interest is inserted between the ITRs, and replaces both *rep* and *cap.* The two AAV-specific ORFs, rep and cap, required *in trans* are encoded on the AAV helper plasmid, which does not contain ITR sequences. These two plasmids are co-transfected with a third plasmid which contains the necessary information for AAV replication. Otherwise, rep and cap and the adenoviral helper genes can be combined into a single plasmid (Buning et al., 2008; Kotterman and Schaffer, 2014). After transcription and translation of Rep and Cap proteins the ITR flanked gene of interest cassette of the vector plasmid is replicated and the single stranded DNA molecules are encapsidated into AAV capsids (Dubielzig et al., 1999; King et al., 2001).

To this day more than 100 human and nonhuman primate AAVs have been identified, including 12 serotypes all of them and variants of them could transduce human cell lines in culture (Lisowski et al., 2015; Lochrie et al., 2006; Wu et al., 2006). The several serotypes differ in the receptors that the virus use to enter into the cell and in the epitopes that are recognized by the immune system. The ability to cross-package the prototypic AAV2 vector genome into different capsids is a powerful tool of novel tropism and biology, with evolving capsid engineering technologies

and directed evolution approaches which further enhance the utility and flexibility of these vectors (Kotterman and Schaffer, 2014; Lisowski et al., 2015).

Clinical trials of AAV-mediated gene delivery to accessible tissues have enabled successful treatment of several recessive monogenic disorders, which has provided strong momentum to the research field. Up to 20 clinical trials have been set up to prove therapeutic effect of AAV vectors on neurodegenerative diseases, retinopathies, neuromuscular diseases (Ortolano et al., 2012), cardiovascular diseases (Zacchigna et al., 2014), atherosclerosis (Lehrke and Lebherz, 2014) and lipoprotein metabolism (Lagor et al., 2013) even more for specific knockdown of apolipoprotein B in mice (Koornneef et al., 2011; Maczuga et al., 2014).

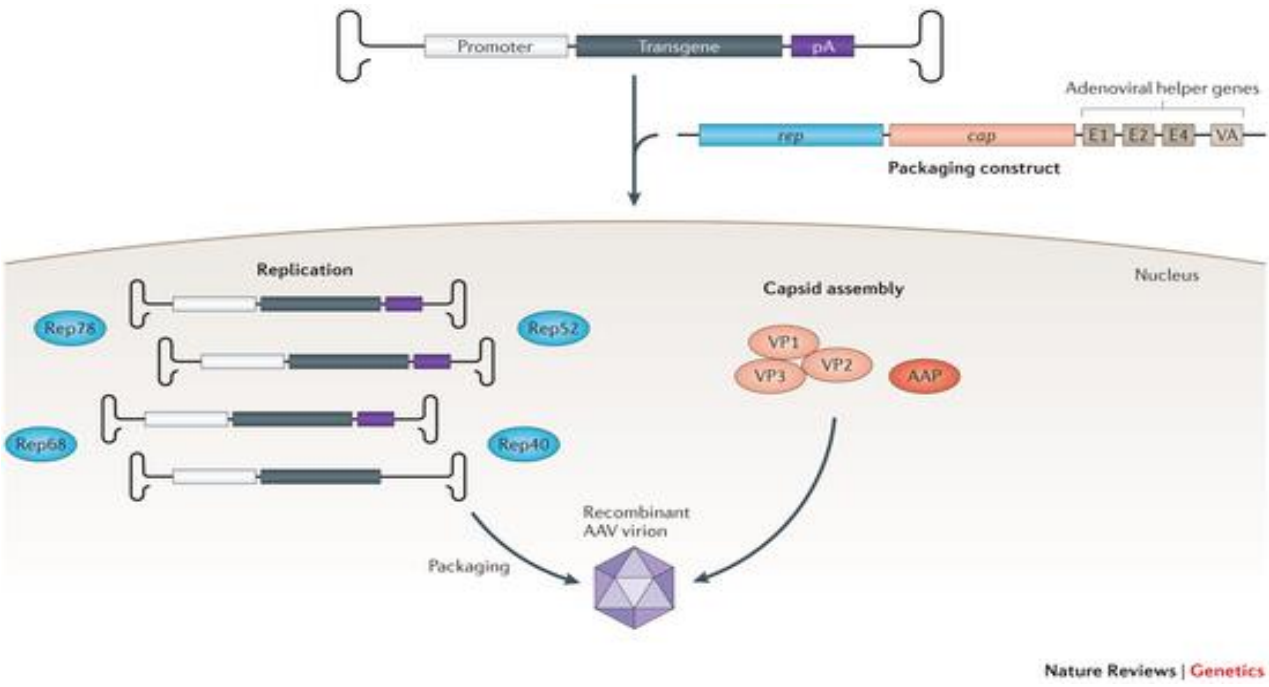


Figure 15. Adeno-associated virus variant generation (Kotterman and Schaffer, 2014).

Nowadays, most of the AAV-RNAi approaches takes either the form of a shRNA or pri-miRNA as the effector molecule. ShRNAs have been widely used for gene knockdown applications because of their simple design, when it expressed from an AAV vector for their long-term expression and stability. The shRNA is located in the plasmid vector under the human U6 small nuclear RNA promoter and the human H1-RNA gene promoter(Baer et al., 1990), with U6 being stronger than H1 (Borel et al., 2014; Grimm et al., 2005).

PURPOSE OF THE STUDY AND SIGNIFICANCE

The rising prevalence worldwide for metabolic disorders such as Metabolic Syndrome and Nonalcoholic fatty liver disease has led to the prediction that patients of these diseases have increased risk for cardiovascular disease and a shorter life expectancy. MetS, on one side, includes central obesity, impaired glucose tolerance or diabetes, hypertension, and dyslipidemia with high plasma concentrations of triglyceride and low concentrations of high density lipoprotein cholesterol, all which can contribute to cardiovascular risk. NAFLD, on the other side, has become a major cause of chronic liver disease in Western societies and has been characterized as the hepatic manifestation of MetS. MetS is the major health problem throughout the word and according to clinical studies it is estimated that around 90 million people suffer from MetS and its comorbidities and die from them. Thus, it is necessary to treat the MetS and NAFLD. To achieve this goal, the pathophysiological mechanisms behind these diseases should be explored. In the first part, the project targets to unravel the molecular mechanisms through which the silencing of transcription factor HNF4a in the liver of APOE*3Leiden.CETP transgenic mice may mediate in the development of MetS. For this purpose, small hairpin RNA, which target the HNF4α mRNA, were cloned into appropriate plasmid vectors and then packaged into Adeno-associated viruses. The injected mice will be subjected to diet-induced MetS and this will be followed by extensive phenotypic and transcriptomic analysis. The second part the project aims to discover novel genes with not yet established roles in High Density Lipoprotein metabolism which were found through transcriptomic analysis of HNF4α liver knockout mice to be differentially expressed and may serve as direct targets of HNF4α.

In summary, using two different well established mouse models the projects aim to shed light into the role of HNF4α transcription factor in the pathogenesis of metabolic diseases and further to identify novel target genes which could be used as therapeutic drug targets in the future for the treatment of this cluster of diseases.

MATERIAL AND METHODS

Project I: Silencing of HNF4a in the livers of the APOE*3Leiden.CETP transgenic mice

For the purpose of this study, plasmid vectors containing the ideal DNA sequence, able to delete the complementary in the hepatocytes of APOE*3Leiden.CETP mice, were amplified and purified from bacteria. Molecular cloning techniques were used.

I. Cloning producing pcDNA3-6myc-mHNF4a.cDNA vector

DNA constructs

The mouse HNF4A gene was amplified from mouse cDNA library with PCR using 5' and 3' primers containing restriction sites for EcoRI and EcoRV, respectively. The PCR template is mouse cDNA library from C57BL/6 mice. The construct produced after annealing of complementary oligonucleotides and ligation with T4 DNA ligase (Biolabs) into the EcoRI and EcoRV site of pcDNA3-6myc plasmid vector (available in the lab).

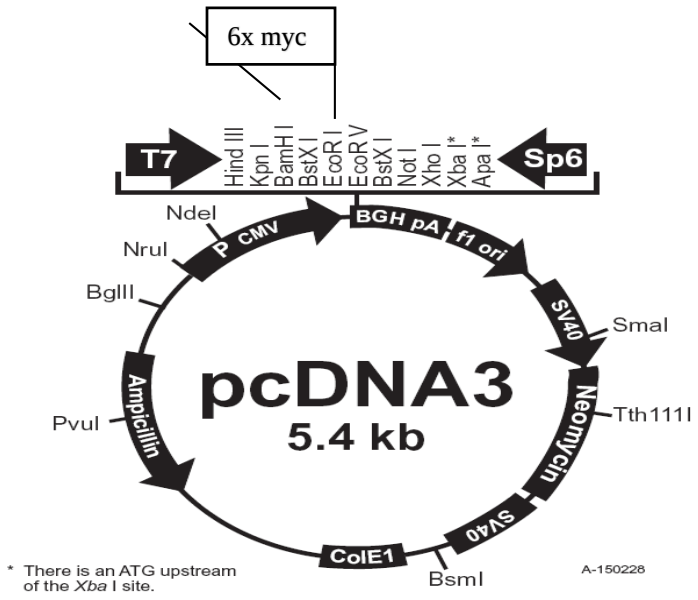


Figure 16. Modified map of pcDNA3 6-myc vector (Invitrogen, Life technologies).

Polymerase chain reaction

The sequences of the primers are the following:

Table 2. Sequences of primers for amplification of HNF4a cDNA from C57BL/6 mice

Name	Sequence
Hnf4a.cDNA_FW	5'...GCGAGAATTCCGACTCTCTAAAACCCTT...3'
Hnf4a.cDNA_REV	5'...GGAGTGATATCCTAGATGGCTTCTTGCTT...3'

Table 3, 4. PCR reaction for amplification of mHNF4A cDNA fragment and thermocycler conditions

PCR protocol			
mHNF4a-cDNA (1ng)			2 µl
Buffer 10X (Invitrogen, Life technologies, #M0273)			2.5 µl
$MgCl_2$ (25mM) (Invitrogen, Life technologies)			1.5 µl
dNTPs (2mM) (Invitrogen, Life technologies)			3 µl
Forward primer (10µM) (Eurofins)			1 µl
Reverse primer (10µM) (Eurofins)			1 µl
Taq DNA polymerase (Invitrogen, Life technologies, #M0273)			0.25 µl
Nuclease-free water			13.75 µl
Total volume			25 µl
Thermocycling conditions for PCR			
Initial Denaturation		94°C	3 min
30 cycles	**Denaturation**	94 °C	30 sec
	Primer Annealing	64 °C	45 sec
	Elongation	72 °C	2 min
Final Elongation		72 °C	5 min
Hold		4 °C	∞

PCR clean up

The PCR product (HNF4α cDNA) was cleaned up using NucleoSpin® Gel and PCR Clean-up kit from MACHEREY-NAGEL according to the respective protocol.

Digestion and purification of insert and vector

After clean up the mHNF4a-cDNA amplicon is ready for restriction digestion. The insert was digested with EcoRI HF (NEB) and EcoRV (Minotch) enzymes and cloned into corresponding sites of the backbone pcDNA3 6-myc vector (Figure 3), which has been cut with the same enzymes in order to obtain complementary ends for subsequent ligation. The restriction reactions were performed at 37°C for 1 hour, both followed by heat inactivation of the enzyme at 65°C for 20 minutes.

Furthermore, the free phosphorylated ends of the restricted vector were dephosphorylated to avoid self-ligation of the vector using Antarctic phosphatase (£B0289S, NEB) in a reaction at 37 °C for 15 minutes, followed again by heat inactivation of the enzyme at 65°C for 20 minutes.

The digested products were electophorated in 1% agarose gel, extracted and cleaned up from agarose gel protocol according to Macherey-Nagel protocols. The eluted DNAs were loaded into a 1% agarose gel.

Table 5. Digestion reactions of pGL3 promoter vector with EcoRI HF and EcorV enzymes

Restriction Digestion Protocol	
DNA	1 µl (1µg DNA)
Cut Smart buffer 10x (#B7204S, NEB)	2 µl (1:10 of total volume)
EcoRI HF (20.000 U/mL, NEB)	0.5 µl
EcoRV (12.000 U/mL, Minotech)	0.5 µl
Nuclease-free water	16 µl
Total volume	20 µl

Ligation of mHNF4a cDNA with pcDNA3 6-myc

Set up a 10 µl ligation reaction using a molar ratio of 1:2 vector to insert. The T4 DNA ligase (NEB) was used. The reaction incubated at 4°C overnight.

Table 6. Ligation reaction between pcDNA3-6myc vector and mHNF4a.cDNA

Component	Self ligation	Ligation
Insert DNA (332 ng/µl)	----	2 µl
Vector DNA (33.2 ng/µl)	1 µl	1 µl
10X T4 DNA Ligase Buffer (NEB)	1 µl	1 µl
T4 DNA Ligase (NEB)	1 µl	1 µl
Nuclease-free water	7 µl	5 µl
Total volume	10 µl	10 µl

Transformation

DH10β competent bacteria were transformed either with the pcDNA3-6myc_mHNF4a.cDNA vector or with the self-ligated vector. 100μL of bacteria cells were transferred to each tube containing the above vectors, and after 30 minutes on ice they were heat-shocked at 42oC for 45 seconds. 900mL of LB medium were added in each tube and bacteria were incubated at 37oC for 1 hour. Afterwards, the cells were centrifuged, redissolved at 100μL final volume of LB, poured in petri dishes and let to develop colonies at 37oC overnight. The next day, 15 distinct bacteria colonies were inoculated in 2mL LB medium and let to proliferate at 37oC overnight under shaking.

Minipreps

Plasmid isolation and purification from the 5 bacteria colonies with pcDNA3-6myc_mHNF4a.cDNA vector were performed by using the NucleoSpin® Plasmid kit (MACHEREY-NAGEL) according to the manufacturer's protocol. Plasmid DNAs were measured at TECAN Infinite 200® PRO instrument and afterwards they were subjected to restriction digestion with EcoRI HF and EcoRV. The colonies were checked with restriction which of them contained the insert.

Cell culture and transfection

HEK293T cell, mouse hepatocytes (HEPA) cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin, at 37°C and 5% CO_2.

For mHNF4a expression analysis assay, HEK293T cells were seeded in 6-well plate at a density 3 x 10^5 cells per well, in Dulbecco's modified Eagle's medium 1 day prior transfection. Transfections were performed with Calcium Phosphate [$Ca_3(PO_4)_2$] co-precipitation method. Briefly, one day before transfection, cells were plated in the appropriate amount of growth medium such that they will be 60% confluent at the time of transfection. For each transfection sample, prepare complexes as follows:

1. Dilute $CaCl_2$ (2M) and DNA in dH_2O
2. Add equal volume of 2x Hepes Buffered Saline (HBS) while vortexing
3. Incubate for 20min at RT

4. Add transfection complexes to cells. Mix gently by rocking the plate back and forth
5. Incubate the cells at 37°C in a CO_2 incubator for 8-18h
6. Replace medium with fresh medium
7. Incubate the cells at 37°C in a CO_2 incubator for 24h
8. Harvest cells

Isolation of cytoplasmic and nuclear protein extracts

In order to isolate the proteins from cells to perform Western blot analysis assay and detect the expression of mhnf4a protein, the following protocol was used:

1. Remove the medium from the wells
2. Wash with 1 ml/well PBS 1X
3. Add 0.5 ml/well PBS 1X and then scrape cells. Break cells accumulation by pipeting up and down.
4. Transfer in a clean 1.5mL eppendorf tube
5. Centrifuge at 5000rpm for 5min at 4oC
6. Remove PBS 1X
7. Add 50-100 µl/ sample lysis buffer 1X (Promega Lysis Buffer 5X). Dissolve the pellet.
8. Store in aliquots at -80oC for 10 minutes and then allow in ice
9. Centrifuge at 13000rpm for 5 minutes at 4oC
10. Transfer the supernatant into new 1,5 ml eppendorf tubes

Calculation of protein concentration (Lawry)

Protein concentration in cell extracts is calculated from the absorbance at 750 nm using the Biorad DC protein Assay kit

1. Transfer 5 µl of sample into a well of a 96-well plate
2. Prepare the dilution of A' : 500 µl of reagent A + 10 µl reagent S
3. Transfer 25 µl of dilution A' to each well
4. Start the reaction by adding 200µl of reagent B and incubate for 15min at room temperature at least
5. Measure OD at 750nm

Western blot analysis for detection of mouse *Hnf4a* and *6-myc* protein

Cell proteins were separated by electrophoresis on a 10-12% Tris-Acetate gel and blotted onto a 0.45 µm nitrocellulose membrane (Invitrogen). The blot incubated with a 1:2000 dilution of a goat polyclonal anti-mHnf4a antibody (Santa Cruz Biotechnology, #sc-6556) and a mouse polyclonal anti-c-myc antibody (Sigma-Aldrich) and mouse a-actin (Chemicon International Inc.), followed by incubation with a 1:10.000 dilution of anti-goat, anti-mouse and anti-rabbit antibody conjugated to horseradish peroxidase(HRP) (Sigma-Aldrich). Antibody binding was detected by chemiluminescent detection kit.

II. Cloning producing pSUPER vector with shRNAs

DNA constructs

Three shRNA constructs simultaneously targeting mouse Hnf4a were made by annealing of complementary oligonucleotides and ligation into BglII and HindIII sites of the pSUPER gfp.neo vector (OligoEngine, Seattle, WA). The sequences of the oligonucleotides which were used in this study are listed in Table 6. The shRNA_1 is located close to 5' end of CDS (437-457bp), the shRNA_2 close to middle of CDS (671-691bp) and the shRNA_3 close to 3' end of CDS (1196-1216bp).

Table 7. Sequences of oligonucleotides used for shRNAs

Name	Sequence
1) HNF4a shRNA sense	5' - GATCCCC**GCAGGTTTAGCCGACAATGTG**TTCAAGAGA**CACATTGTCGGCTAAACCTGC**TTTTTA - 3'
1) HNF4a shRNA antisense	5' - AGCTTAAAAA**GCAGGTTTAGCCGACAATGTG**TCTCTTGAA**CACATTGTCGGCTAAACCTGC**GGG - 3'
2) HNF4a shRNA sense	5' - GATCCCC**GCGACATTCGGGCAAAGAAGA**TTCAAGAGA**TCTTCTTTGCCCGAATGTCGC**TTTTTA - 3'
2) HNF4a shRNA antisense	5' - AGCTTAAAAA**GCGACATTCGGGCAAAGAAGA**TCTCTTGAA**TCTTCTTTGCCCGAATGTCGC**GGG - 3'
3) HNF4a shRNA sense	5' - GATCCCC**GGCAGATGATCGAACAGATCC**TTCAAGAGA**GGATCTGTTCGATCATCTGCC**TTTTTA - 3'

3) HNF4a shRNA antisense	5' - AGCTTAAAAA**GGCAGATGATCGAACAGATCC**TCTCTTGAA**GGATCTGTTCGATCATCTGCC**GGG - 3'
4) scrambled shRNA sense	5'-GATCCCC**TTCTCCGAACGTGTCACGT**TTCAAGAGA**ACGTGACACGTTCGGAGAA**TTTTTA- 3'
4) scrambled shRNA antisense	5'-AGCTTAAAAA**TTCTCCGAACGTGTCACGT**TCTCTTGAA**ACGTGACACGTTCGGAGAA**GGG- 3'

The single stranded oligonucleotides were annealed in order to produce double stranded DNAs according to manufacturer's instructions. The concentration of each oligonucleotide was measured at TECAN Infinite 200® PRO instrument.

1. Anneal the forward and reverse strands of the oligos that contain the siRNA-expressing sequence targeting HNF4A gene. A reaction of total volume 20μl was performed with 5μg of sense and antisense oligonucleotide, respectively and NEB2 10X Buffer (NEB). The reaction boiled at 92°C for 2 minutes and then it is cooled until it reach approximately 10°C. After annealing the double stranded DNA contains single stranded sticky ends as presented below.

2. Linearize the pSUPER vector with BglII (10.000 U/ml, Minotech) and HindIII (10.000 U/ml, Minotech)
3. Clone the annealed oligonucleotides into the pSUPER vector
4. Transform the vector in DH10β bacteria

After the transformation of competent cells with the pSUPER.neo+gfp_shRNAs, minipreps were performed and the plasmid DNA isolated by using the NucleoSpin® Plasmid kit (MACHEREY-NAGEL) according to the manufacturer's protocol. Plasmid DNAs were measured at TECAN Infinite 200® PRO instrument and afterwards they were subjected to restriction digestion with EcoRI and HindIII enzymes.

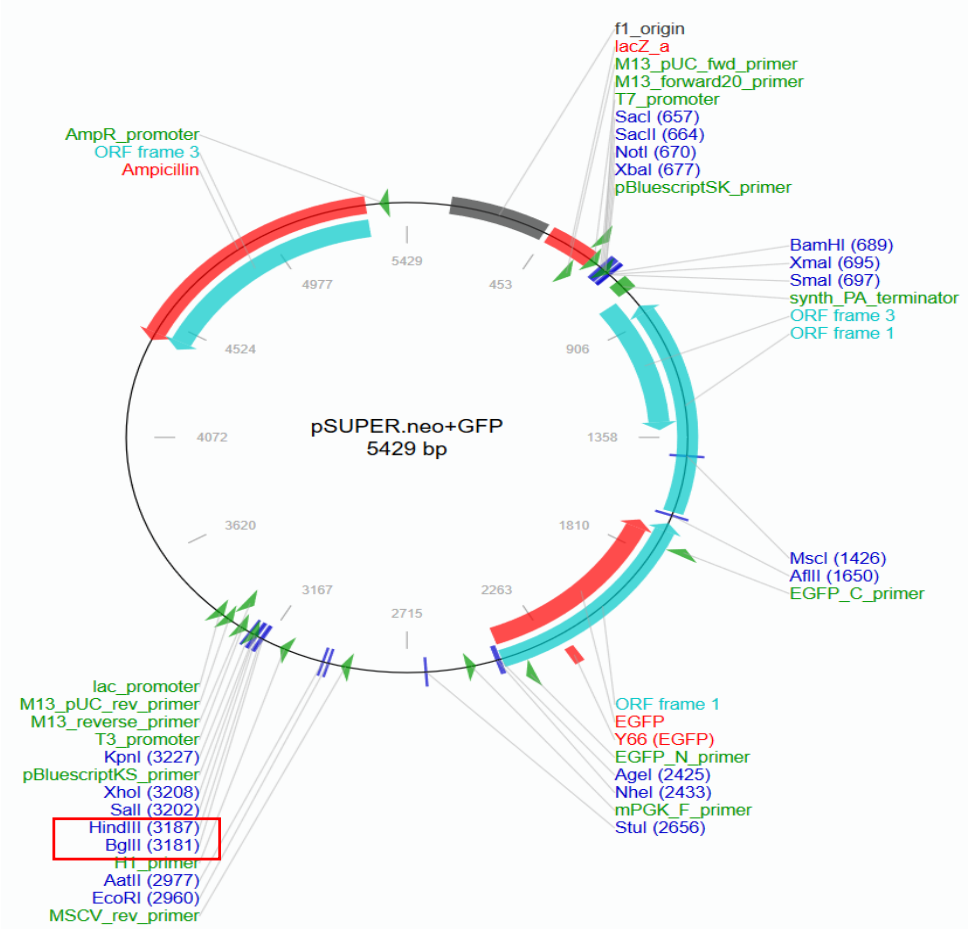


Figure 17. Map of pSUPER vector indicating the sites for shRNAs cloning.

The shRNAs generated in a large scale and the plasmid DNAs purified with midipreps using the NucleoSpin® Plasmid kit (MACHEREY-NAGEL) according to the manufacturer's protocol.

The plasmid DNAs transfected into HEK293Tcells using calcium phosphate method in order to evaluate the effectiveness of shRNAs to delete the mouse hnf4a protein. After protein isolation and SDS-PAGE with western blot analysis

III. Cloning producing dsAAV-H1/U6-RSV-eGFP-FF vector with shRNA_3

DNA constructs

ShRNA_3 reduced more efficiently the mouse hnf4a protein and the following experiments were performed with this shRNA. In order to generate the Adeno-associated viruses' backbone, shRNA expression cassettes were subcloned in 931-dsAAV-U6-RSV-eGFP-FF and 933-dsAAV-H1-RSV-

eGFP-FF vectors (kindly provided by Dr. Kuivenhoven (University of Groningen)), which are under the control of U6 and H1 promoter, respectively. The shRNA_3 oligonucleotide designed with the BbsI site in the 5' end.

The sense and antisense oligonucleotides annealed as previous and the double stranded oligonucleotide cloned into 931-dsAAV-U6-RSV-eGFP-FF and 933-dsAAV-H1-RSV-eGFP-FF vectors, which first digested with the BbsI (10.000 U/ml, NEB #R0539S) restriction enzyme.

5' CACC G/A (sense) – TCAAGAG – (anti--) 3'

3' C/T (anti--) – AGTTCTC – (sense) AAAA 5'

Table 8. Sequences of oligonucleotides used for AAVs generation

Name	Sequence
HNF4a shRNA sense (BbsI site)	5' - cacc***GGCAGATGATCGAACAGATCC***TCAAGAG***GGATCTGTTCGATCATCTGCC*** - 3'
HNF4a shRNA antisense (BbsI site)	5' - aaaa***GGCAGATGATCGAACAGATCC***CTCTTGA***GGATCTGTTCGATCATCTGCC*** - 3'
Scrambled shRNA sense (BbsI site)	5 '-cacc***TTCTCCGAACGTGTCACGT***TCAAGAG***ACGTGACACGTTCGGAGAA*** - 3'
Scrambled shRNA antisense (BbsI site)	5'- aaaa***TTCTCCGAACGTGTCACGT***CTCTTGA***ACGTGACACGTTCGGAGAA*** - 3'

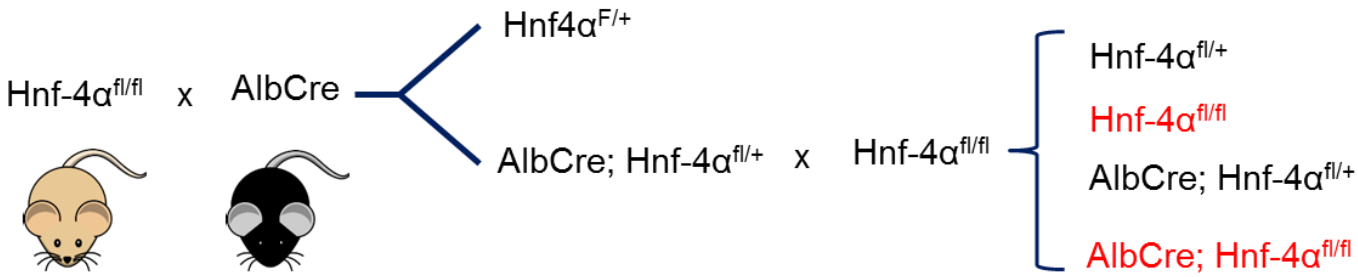
After the transformation of competent cells with the dsAAV-H1/U6-RSV-eGFP-FF_shRNAs, minipreps were performed and the plasmid DNA isolated by using the NucleoSpin® Plasmid kit (MACHEREY-NAGEL) according to the manufacturer's protocol. Plasmid DNAs were measured at TECAN Infinite 200® PRO instrument and afterwards they were subjected to restriction digestion with BbsI enzyme.

The plasmid DNAs transfected into HEK293Tcells using calcium phosphate method in order to evaluate the effectiveness of shRNAs to delete the mouse hnf4a protein. After protein isolation and SDS-PAGE with western blot analysis.

Project II: Transcriptomic analysis of the livers of HNF4a liver specific knockout mice

Mouse model

A conditional knockout mouse model (HNF4α Liver Knockout (L-HNF4α$^{-/-}$)) which is wide known that develop liver steatosis (Hayhurst et al., 2001) was developed at lab with Cre-loxP system. Mice lacking hepatic HNF4a expression accumulated lipid in the liver and exhibited greatly reduced serum cholesterol and triglyceride levels and increased serum bile acid concentrations (Hayhurst et al., 2001).



Homozygous animals HNF4αfl/fl carrying two floxed (fl; flanked by loxP) allele were crossed with animals hemizygous for albumin-Cre transgene (AlbCre). Heterozygous (fl/+) animals carrying one copy of the AlbCre transgene were then interbred with fl/fl littermates lacking Cre to generate HNF4a liver knockout mice (H4LivKO - HNF4α$^{fl/fl}$;Alb-Cre) and littermate control mice (HNF4α$^{fl/fl}$).

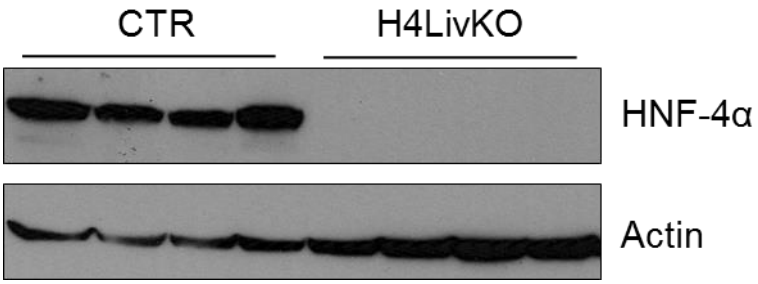


Figure 18. Western blot analysis of hnf4α protein from liver of CTR and HNF4α mice.

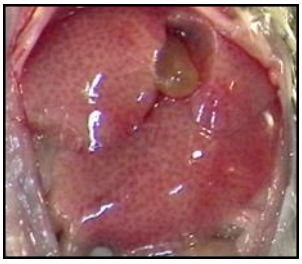


Figure 19. Macroscopic image showing the development of lipid deposition in the liver of these mice.

Tissue collection

Mice were subjected to anatomy and collection of specific tissues such as liver, adipose tissue, small intestine, large intestine and other organs. In this project, we analyzed livers from the two HNF4α LivKO and control mice.

Genomic DNA extraction

DNA extraction was performed by following the Phenol-Chloroform protocol and several genes were detected by performing PCR.

1. Add one volume of phenol:chloroform:isoamyl alcohol (25:24:1) to your sample, and vortex or shake by hand thoroughly for approximately 20 seconds
2. Centrifuge at 13000rpm for 20mins at room temperature. Prepare tubes with 450µl Chloroform
3. Carefully remove the upper aqueous phase, and transfer the layer to a fresh tube with 450µl Chloroform and do vortex
4. Centrifuge at 13000rpm for 20mins at room temperature
5. Carefully remove 400µl from the upper phase and transfer the layer to a fresh sterile tube
6. Add 280µl Isopropanol (70% of the initial Volume 400 µl)
7. Centrifuge at 13000rpm for 15mins at room temperature
8. Carefully remove the supernatant without disturbing the DNA pellet
9. Resuspend the DNA pellet with 500µl Ethanol 75%
10. Centrifuge at 13000rpm for 10mins at room temperature
11. Carefully remove the supernatant without disturbing the DNA pellet. Remove as much of the remaining ethanol as possible
12. Dry the DNA pellet in the hood for 2 minutes or at room temperature for 5–10 minutes
13. Resuspend the DNA pellet in 100µl of dH2O DNAse/RNAse free or Water for Injection by pipetting up and down 30–40 times
14. Centrifuge briefly to collect the sample, and place the tube on ice (4oC overnight or store at -20oC)

RNA isolation protocol

The RNA isolation of cells either from cell cultures or tissues performed with the TRIZOL RNA isolation protocol as following:

1. Add 1ml Trizol per sample (up to 100mg tissue) into a 2ml eppedorf tube
2. Homogenize on ice and then incubate for 5mins at room temperature
3. Add 200µl Chloroform and vortex for 30s. Incubate for 3mins at room temperature
4. Centrifuge at 12.000xg for 15mins at 4°C
5. Carefully remove the upper aqueous phase, and transfer the layer of 400-500µl to a fresh tube with 500µl Isopropanol (250µl Isopropanol + 250µl High salt Buffer)
6. Incubate for 30mins on ice (-20°C)
7. Centrifuge at 12.000xg for 10mins at 4°C
8. Carefully remove the supernatant without disturbing the RNA pellet
9. Resuspend the RNA pellet in 1ml Ethanol 75%
10. Centrifuge at 12.000xg for 10mins at 4°C
11. Carefully remove the supernatant without disturbing the RNA pellet
12. Repeat the proceed from step 9
13. Resuspend the RNA pellet in 50µl WFI

cDNA synthesis

In order to use the total isolated RNA for analysis of expression levels of several genes we should to synthesize cDNA using M-MLV Reverse Transcriptase (Invitrogen, #28025). The protocol that performed is the following:

1. Add the following components to a nuclease-free microcentrifuge tube:
   - 10 µl **RNA** (100ng/µl)_ 1µg in total
   - 3 µl **Random Primers** (100ng/µl)
   - 5 µl **dNTPs** (2mM)
   - 15 µl **H_2O**
2. Heat mixture to 65°C for 5 min and **quick chill** on ice

3. Collect the contents of the tube by brief centrifugation
4. Add
    - 10 µl **5x first strand buffer**
    - 5 µl **DTT 0,1M**
    - 1 µl **RNase OUT** Recombinant Ribonuclease Inhibitor (Cat. No. 10777-019; 40 units/µl)
5. Mix contents of the tube gently and incubate at 37°C for 2 min
6. Add 1 µl (200 units) of **M-MLV RT** (Cat. No. 28025; 200 U/µl) and mix by pipetting gently up and down
7. Incubate tube at 25°C for 10 min
8. Incubate 50 min at 37°C
9. Inactivate the reaction by heating at 70°C for 15 min

All incubations take place in a PCR machine and the final reaction volume is 50 µl.

Real Time quantitative PCR (RT-qPCR)

QPCR experiments were performed in order to validate the findings of liver transcriptomic analysis done in HNF4α LivKO mice. Specific primers for mRNA, genomic DNA and promoter region for selected genes were designed by using the DNASTAR and Fast PCR programs.

Chromatin immunoprecipitation assay

In order to identify the binding of HNF4α transcription factor on the promoter regions of gene which get involved in lipid and glucose metabolism chromatin immunoprecipitation assay (ChIP) was performed according to the given protocol.

Day 1: Preparation of chromatin

1. Grow or harvest cells in a 100mm dish.
2. Remove medium and wash with 7ml DMEM or PBS.
3. Add 9ml DMEM or PBS (prewarmed at 37ºC).
4. Add 1ml **formaldehyde** (from 10% stock solution) drop wise and mix immediately.
5. Incubate for 10min at RT (or 37ºC).

6. Add 1ml **glycine** (from 1.375M stock solution) drop wise, mix immediately with gentle agitation.
7. Place the plate on ice and wash 3 times with 10 ml **1x PBS/0.5mM PMSF** (ice cold).
8. Add 7 ml **1x PBS / 0.5% NP-40 / 0.5 mM PMSF**.
9. Scape cells and transfer to a 15ml falcon tube.
10. Centrifuge at 1000rpm, 5min, 4°C.
11. Resuspend the cell pellet in 2-5ml **Swelling buffer**.
12. Incubate on ice for 10 min, flickering the tube occasionally.
13. Homogenize cells using the Dounce machine (speed 4-5), 20-30 times for each sample.

Check the efficiency of the process:

- mix on a slide 5µl Trypan Blue (0,4% in PBS) and 5µl from the sample
- check under the microscope – the more blue cells the more efficient the process. Repeat the homogenization if not enough cells are stained.

14. Centrifuge at 2000 rpm, 5 min, 4°C.
15. Remove supernatant.
16. Resuspend pellet (nuclei) in 1ml **Sonication buffer**.

* *If there are too many cells, dilute them in Sonication buffer and perform the sonication to aliquots of 1ml.*

17. Fragment chromatin by sonication (the number of sonications depends on the cell type):

12 times x 30 sec at 50% amplitude (cool the sample between sonications by leaving it on ice for 4-5 min)

18. Centrifuge at 13000rpm, 15min, 4°C.
19. Transfer supernatant to 1.5 ml eppendorf.
20. Centrifuge at 13000rpm, 15min, 4°C.
21. Transfer supernatant to a 2ml eppendorf and add 1ml **Sonication Buffer** (optional).
22. 25-50µl are kept to check the quality of the chromatin and the rest is stored at -80°C.

* *25-50 µl samples can be kept from intermediate steps of sonication to be used for comparison and evaluation of the sonication efficiency.*

23. Add **150µl H_2O** and **10.5µl NaCl 4M** to the 50µl of chromatin.
24. Incubated O/N at 65°C.

Day 2: Clean-up of the test sample

1. Add **150µl H_2O** and **10.5µl NaCl 4M** to the 50µl of chromatin.
2. Incubated O/N at 65ºC.
3. Add **2µl RNAse A** (10mg/ml, DNase-free).
4. Incubate for 1h at 37ºC.
5. Add **2µl EDTA** (0.5M).
6. Add equal volume of **Phenol:Chloroform:Isoamyl 25:24:1**.
7. Mix by vortexing until the sample is homogeneous.
8. Centrifuge at 13000rpm, 5min, RT.
9. Transfer upper phase to 1.5ml eppendorf.
10. Add equal volume of **Chloroform** and **4µl glycogen** (5µg/µl).
11. Centrifuge at 13000rpm, 5min, RT.
12. Transfer upper phase to 1.5ml eppendorf.
13. Add **1/10 V CH_3COONa 3M** and **$2^{1/2}$ V EtOH absolute**.
14. Mix well and place at -80ºC for 30min.
15. Centrifuge at 13000rpm, 10min, RT.
16. Remove supernatant.
17. Add **500µl 75% EtOH**.
18. Centrifuge at 13000rpm, 10min, RT.
19. Remove supernatant.
20. Air-dry the pellet and resuspend DNA in **20µl H_2O**.
21. Check the quality of the fragmented chromatin (10-20µl) on a 2% agarose gel.

If chromatin fragments are less than 1000bp proceed to the next step:

Day 3: Equilibration/Blocking/Preclearing/IP

1. Take the chromatin out of -80ºC and thaw on ice (it takes a long time).

Equilibration and blocking of beads

2. For each sample use 160 µl protein G sepharose beads (50% slurry):

   - 40µl protein G sepharose beads for control reaction (IgG or w/o ab)
   - 40µl protein G sepharose beads for IP
   - 80µl protein G sepharose beads for preclearing.

3. Centrifuge the protein G sepharose beads at 3000rpm, 3min, 4ºC and remove supernatant.
4. Add **1.5ml Sonication Buffer**.
5. Rotate 10min at 4ºC.
6. Centrifuge at 3000rpm, 3min, 4ºC.

22. Remove supernatant.

7. Repeat the wash 2 more times.
8. Add **494µl Sonication Buffer + 5µl BSA (100mg/ml) + 1µl sonicated λDNA (0.5µg/µl)**.
9. Rotate for 2h at 4ºC.
10. Transfer 130µl beads in 2 x 1.5ml eppendorf tubes and store at 4ºC (they are going to be used later for the IP and control reactions).

Preclearing of chromatin

11. Centrifuge the rest of the beads at 3000rpm, 3min, 4ºC.
12. Remove supernatant.
13. Measure concentration and ratio at 260nm/280nm.
14. Add **1500µl of chromatin** to the beads.

When there are more than one sample, divide each concentration with the smallest one to produce a factor.

Add to the beads 1500 µl of the sample with the smallest concentration and (1500 / factor) µl of the rest of the samples (add sonication buffer up to 1500µl).

15. Add **15µl BSA (100mg/ml)** and **3µl λ DNA (0.5µg/µl)**.
16. Rotation for 2h at 4ºC.
17. Centrifuge at 3000rpm, 3min, 4ºC.
18. Transfer supernatant to a new tube and estimate the volume.
19. Keep 1/10 V of the amount that will be used in each IP and store it at -20ºC (**input**).

Immunoprecipitation

✓ Chromatin is split in **2** 1.5ml tubes:

(a) Control – add IgG or nothing

(b) IP – add **5µg** of antibody

20. Rotation for O/N at 4ºC.

Day 4: Beads/Washes/Elution/De-crosslinking

---

1. Centrifuge the beads stored at 4ºC (after equilibration/blocking), at 3000rpm, 3min, 4ºC.
2. Remove supernatant.
3. Add the IP samples (-ab, +ab) to the beads.
4. Rotation 2h at 4ºC.
5. Centrifuge the samples at 3000rpm, 3min, 4ºC and remove supernatant.
6. Wash 2 times with **Wash Buffer A**:

   Add 1 ml Wash Buffer A.

   Rotate for 10min at 4ºC.

   Centrifuge at 3000rpm, 3min, 4ºC.
7. Wash 2 times with 1 ml **Wash Buffer B**.
8. Wash 2 times with 1 ml **Wash Buffer C**.
9. Wash 2 times with 1 ml **TE buffer**.
10. Add 150µl **Elution Buffer** (freshly prepared and prewarmed at 65ºC).
11. Vortex.
12. Incubate at 65ºC for 10min. Flick the tube frequently.
13. Vortex.
14. Centrifuge at 13000rpm, 1min, RT.
15. Transfer supernatant (150µl) to a new 1.5 ml tube.
16. Add 150µl **Elution Buffer** to the beads.
17. Vortex.
18. Incubate at 65ºC for 10min. Flick the tube frequently.
19. Vortex.
20. Centrifuge at 13000 rpm, 1min, RT.
21. Transfer supernatant (150µl) to the tube containing the first eluate (final volume 300µl).
22. Add 100µl H_2O and 21µl NaCl 4M.

23. To the **input** add Elution Buffer up to 300µl, 100µl H_2O and 21µl NaCl 4M.
24. Mix and incubate O/N at 65ºC.

Day 5: DNA clean-up

1. Add **2µl RNAse A** (10mg/ml, DNase-free).
2. Incubate for 1h at 37ºC.
3. Add **2µl EDTA** (0.5M) and **2µl Proteinase K** (10mg/ml).
4. Incubate for 2 h at 42ºC.
5. Add 200µl H_2O.
6. Add 1/10 V **CH_3COONa 3M**.
7. Add equal volume of **Phenol:Chloroform:Isoamylalchohol 25:24:1**.
8. Vortex (the mix should be homogeneous).
9. Centrifuge at 13000rpm, 5min, RT.
10. Transfer upper phase to a new 1.5ml tube.
11. Add equal volume of **Chloroform**.
12. Vortex.
13. Centrifuge at 13000rpm, 5min, RT.
14. Transfer upper phase to a 2ml tube.
15. Add **4µl glycogen** (5µg/µl) and **$2^{1/2}$ V EtOH absolute**.
16. Mix and incubate O/N at -20ºC.

Day 6: DNA clean-up

1. Centrifuge at 13000rpm, 30min, 4ºC.
2. Remove supernatant.
3. Add 1ml **75% EtOH**.
4. Centrifuge at 13000rpm, 10min, RT.
5. Remove supernatant.
6. Airdry the pellet.
7. Resuspend input in 100µl **10mM Tris (pH 7.5)** and the control/IP samples in 50µl **10mM Tris (pH 7.5)**.

Buffers

Swelling Buffer							
	final [C]	stock [C]	pH	5 ml	10 ml	20 ml	30 ml
Hepes	25 mM	0.5 M	7.9	250,0	500	1000	1500
$MgCl_2$	1.5 mM	1 M		7,5	15	30	45
KCl	10 mM	3 M		16,5	33	66	99
NP-40	0.5%	10%		250,0	500	1000	1500
DTT	1 mM	1 M		5,0	10	20	30
Aprotinin	2 µg/ml	2mg/ml		5,0	10	20	30
PMSF	0.5 mM	0.1 M		25,0	50	100	150
Protease Inhibitor	1 x	25 x		200,0	400	800	1200
H_2O				4241	8482	16964	25446

Sonication Buffer							
	final [C]	stock [C]	pH	2.5 ml	5 ml	10 ml	25 ml
Hepes	50 mM	0.5 M	7.9	250	500	1000	2500
NaCl	140 mM	5 M		70	140	280	700
EDTA	1 mM	0.5 M	8.0	5	10	20	50
Triton X-100	1%	20%		125	250	500	1250
Na-deoxycholic acid	0.1%	5%		50	100	200	500
SDS	0.1%	20%		12,5	25	50	125
Aprotinin	2 µg/ml	2mg/ml		2,5	5	10	25
PMSF	0.5 mM	0.1 M		12,5	25	50	125
Protease Inhibitor	1 x	25 x		100	200	400	1000
H_2O				1872,5	3745	7490	18725

PBS / NP-40 / PMSF							
	final [C]	stock [C]	pH	10 ml	20 ml	30 ml	40 ml
PBS	1x	10x		250	500	750	1000
NP-40	0.5%	10%		500	1000	1500	2000
PMSF	0.5 mM	0.1 M		50	100	150	200
H_2O				9200	18400	27600	36800

Wash Buffer A						
	final [C]	stock [C]	pH	5 ml	10 ml	25 ml
Hepes	50 mM	0.5 M	7.9	500	1000	2500
NaCl	140 mM	5 M		140	280	700
EDTA	1 mM	0.5 M	8.0	10	20	50
Triton X-100	1%	20%		250	500	1250
Na-deoxycholic acid	0.1%	5%		100	200	500
SDS	0.1%	20%		25	50	125
Aprotinin	2 µg/ml	2 mg/ml		5	10	25
PMSF	0.5 mM	0.1 M		25	50	125
H_2O				3945	7890	19725

Wash Buffer B						
	final [C]	stock [C]	pH	5 ml	10 ml	25 ml
Hepes	50 mM	0.5 M	7.9	500	1000	2500
NaCl	500 mM	5 M		500	1000	2500
EDTA	1 mM	0.5 M	8.0	10	20	50
Triton X-100	1%	20%		250	500	1250
Na-deoxycholic acid	0.1%	5%		100	200	500
SDS	0.1%	20%		25	50	125
Aprotinin	2 µg/ml	2 mg/ml		5	10	25
PMSF	0.5 mM	0.1 M		25	50	125
H_2O				3585	7170	17925

Wash Buffer C						
	final [C]	stock [C]	pH	5 ml	10 ml	25 ml
Tris-Cl	20 mM	1 M	8.0	100	200	500
EDTA	1 mM	0.5 M	8.0	10	20	50
LiCl	250 mM	2.5 M		500	1000	2500
NP-40	0.5%	10%		250	500	1250
Na-deoxycholic acid	0.5%	5%		500	1000	2500
Aprotinin	2 µg/ml	2 mg/ml		5	10	25
PMSF	0.5 mM	0.1 M		25	50	125
H_2O				3610	7220	18050

TE						
	final [C]	stock [C]	pH	5 ml	10 ml	25 ml
Tris-Cl	10 mM	1 M	8.0	50	100	250
EDTA	1 mM	0.5 M	8.0	10	20	50
Aprotinin	2 µg/ml	2 mg/ml		5	10	25
PMSF	0.5 mM	0.1 M		25	50	125
H_2O				4910	9820	24550

Elution Buffer						
	final [C]	stock [C]	pH	1 ml	5 ml	10 ml
Tris-Cl	50 mM	1 M	8.0	50	250	500
EDTA	1 mM	0.5 M	8.0	2,5	12,5	25
SDS	1%	20%		50	250	500
$NaHC0_3$	50 mM	1M		50	250	500
H_2O				847,5	4237,5	8475

Luciferase and β-gal (lacZ) reporter assays

Before beginning the assays, prepare the Luciferase Assay Reagent, the Lysis Buffer and the β-gal mix.

A. Preparation of Lysis Buffers

1. Make aliquots of Lysis Buffer 5X to 1X by adding sterile dH_2O and mix gently

B. Preparation of Mammalian Cell Lysate

2. Remove growth medium from cultured cells
3. Rinse cells in 1ml PBS 1X/well. Do not dislodge cells. Remove as much of the final wash as possible
4. Dispense a minimal volume of 150µl Lysis Buffer 1X/well. mix gently for 10mins at 260rpm
5. Scrape attached cells from well and use a micropipettor to transfer the cells to a 1,5ml microcentrifuge tube. Pipetting gently
6. Transfer the tubes at -80°C for at least 10min [END POINT]
7. Transfer the tubes immediately at 37°C for ~1-2mins

8. Centrifuge at 13ooo rpm for 5mins
9. Transfer the supernatant to a new tube
10. Vortex and short spin the samples

} **ON ICE**

For 5µl cell lysate, change only the volume of the Buffer P to 149µl

C. <u>Preparation of Luciferase assay for single-tube Luminometer</u>

11. Program the luminometer to perform a 2-second measurement delay followed by a 5-second measurement read for luciferase activity
12. Add 60µl of cell lysate to a luminometer tube
13. Add 60µl of Luciferin (Luciferase Assay Reagen) into 60µl of the cell lysate tube. Mix by pipetting 2–3 times or vortex briefly
14. Place the tube in the luminometer and initiate reading

<u>Note:</u> If the measurements are not be detected by the luminometer, then it is appropriate to dilute our samples (dilution 1:10) with Lysis Buffer.

D. <u>Preparation for β-galactosidase (lacZ) assay</u>

1. Warm up the ONPG Buffer
2. Prepare the mix for 10µl cell lysate:
    - 144µl Buffer P
    - 44µl ONPG
    - 2µl Salt Buffer 100X

= 190µl solution

3. Mix gently the solution
4. Add 10µl cell lysate in each well and then put the plate in the TECAN machine

RESULTS AND DISCUSSION

Project I: Silencing of HNF4a in the livers of the APOE*3Leiden.CETP transgenic mice

Previous studies have demonstrated the significant role of HNF4a transcription factor in the lipid and apolipoprotein biosynthesis and metabolism. Furthermore, GWA studies have revealed the correlation of single nucleotide mutations in the HNF4A locus with low HDL cholesterol levels. The above observations indicate that finding new ways of inducing the expression of key enzymes of HDL metabolism could have important consequences for the therapy of patients with CVDs.

Since the expression of lipoprotein-related genes is regulated mainly at the transcriptional level, our aim was to identify the role of HNF4a in the expression of HDL genes in hepatic cells. Moreover, a recent publication from Xu and colleagues laboratory proposed that suppression of hepatic HNF4a expression together with approaches that alleviate hepatic steatosis may be useful for preventing the development of atherosclerosis (Xu et al., 2015). Lipid deposition in the liver and atherosclerosis are two of the main clinical disorders of metabolic syndrome.

For this purpose, the apoE*3Leiden.CETP transgenic mouse model was used. This mouse model is a useful translational model for MetS, in which obesity and IR can be combined with dyslipidemia. In addition to that, E3L.CETP mouse is a well-established model for hyperlipidemia and atherosclerosis.

To functionally establish the role of HNF4a in regulating HDL genes, the goal was to generate shRNA expressing vectors for specific silencing of HNF4A gene. At a first step, the mouse hnf4a cDNA was inserted in pcDNA3-6myc plasmid vector. After production of this expression vector it was used in transient transfections in human embryonic kidney 293 (HEK 293) cell line in order to test the protein expression. Western blot analysis as well as the map of the expressing plasmid vector are shown below.

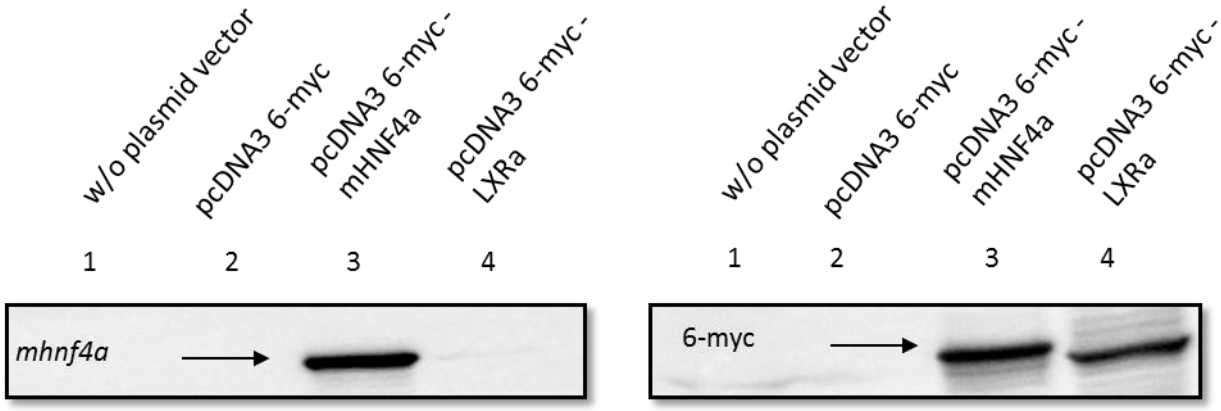


Figure 20. Transfection expression analysis of mouse Hnf4a and 6-myc into HEK293T cells.

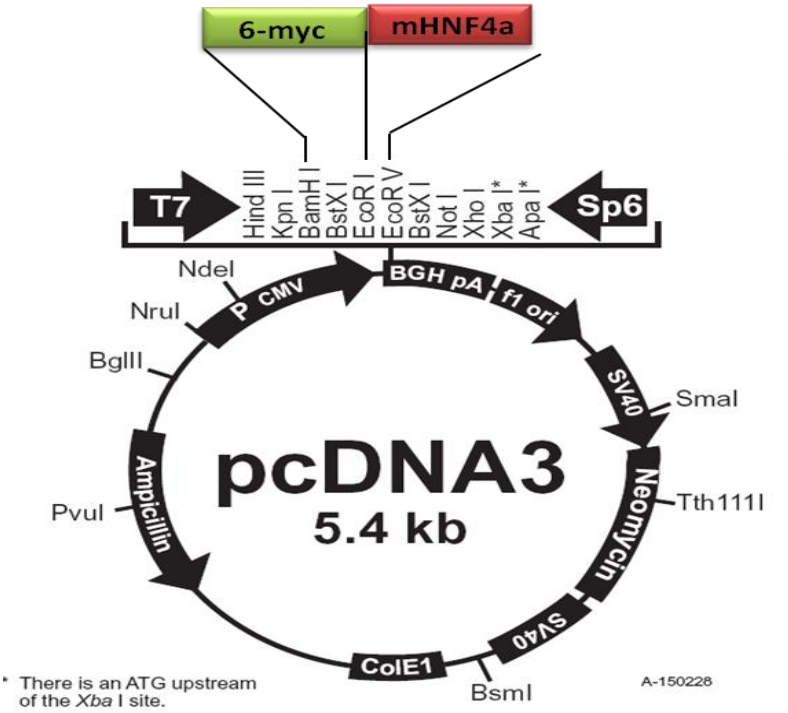


Figure 21. Map of pcDNA3-6myc_mHNF4a.cDNA vector (modified).

At a second step, three different shRNAs which target the messenger RNA of HNF4A gene at different sites were designed. The oligonucleotides were inserted in the pSUPER.neo/gfp plasmid vector. Transient transfections with both plasmids - pcDNA3-6myc_mHNF4a.cDNA and pSUPER.neo/gfp - were performed in HEK 293 T cells in order to check which of these cause the most

effective ablation of HNF4A transcripts. As it is shown in the graphs below, the shmHNF4a_3 is the most effective small hairpin RNA.

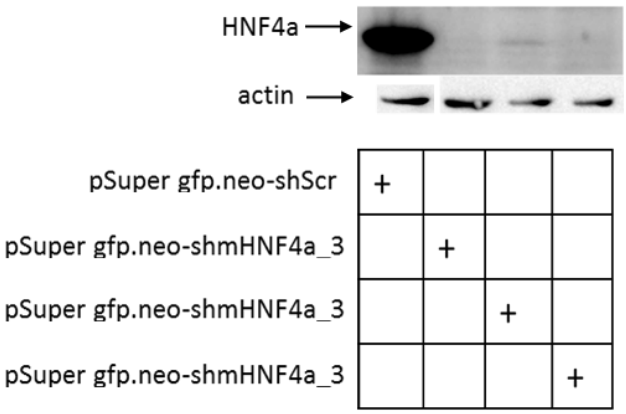


Figure 23. Western blot analysis of produced mhnf4a protein upon administration of shRNAs in HEK293T cells.

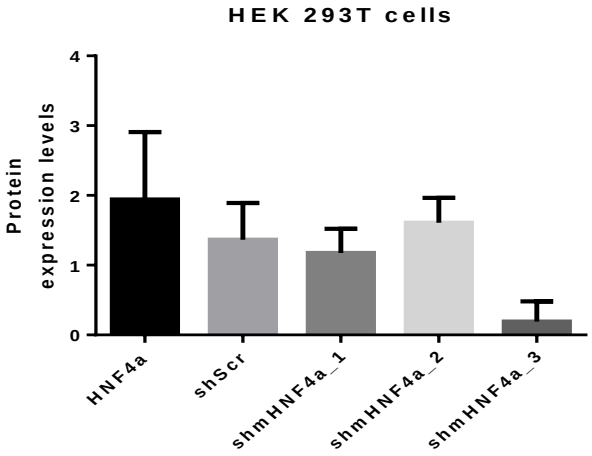


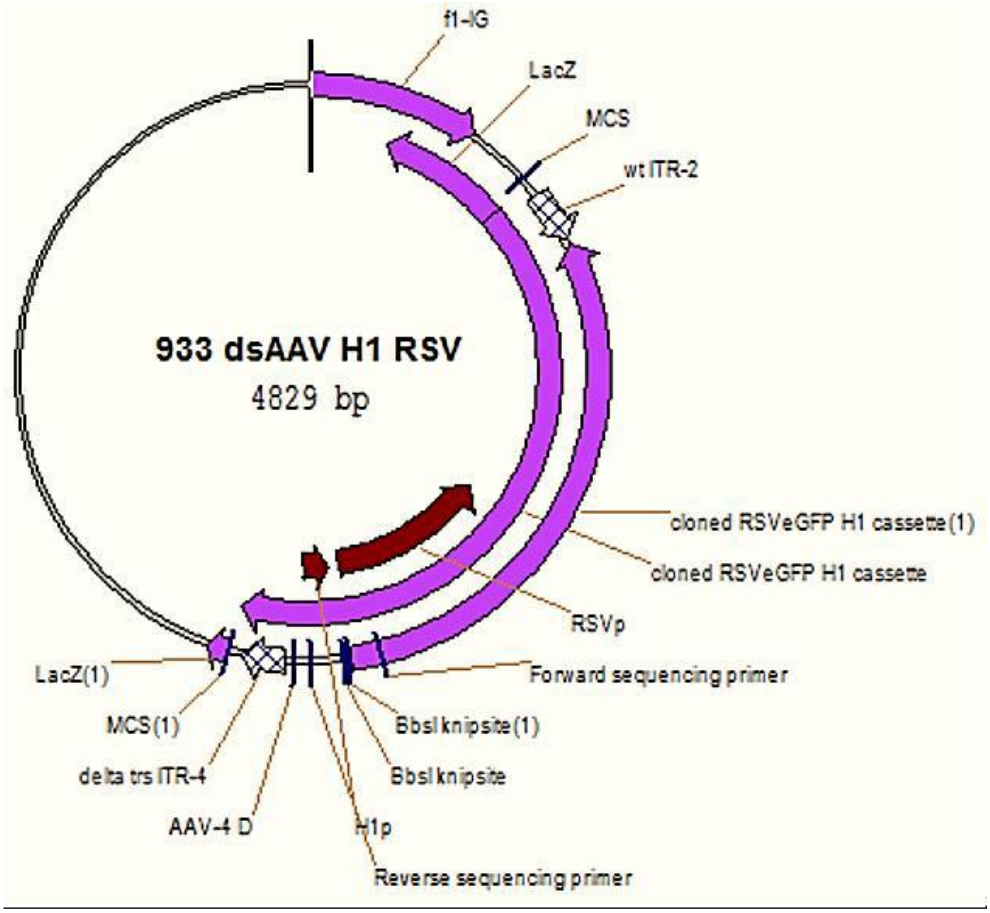
Figure 22. Quantification of protein expression levels of mhnf4a after co- transfection of HEK293T cells with pcDNA3-6myc_mHNF4a.cDNA and pSUPER.neo+gfp_shRNAs.

Various groups in the past have established the great potential of RNAi as a revolutionary method for studying gene functions in whole animals. The advantage of RNAi technology over conventional knock-out models is that more target genes can be assessed in less time, thus saving efforts and resources. Although the currently available technologies for expression of shRNAs from plasmid DNAs or from any other form of non-viral vector are easy and useful for studies in isolated cultured cells, there is no methodology on hand to efficiently deliver non-viral vectors to the entire cell mass within an intact organ in vivo. For this reason, more researchers have recently developed virus-derived shRNA expression vectors, which will provide a significantly higher efficiency for shRNA delivery in whole organisms and will permit better control over tissue specificity of shRNA expression (Nicklin and Baker, 2002).

Several publications in the past have documented the successful development and use of gene transfer vectors based on adeno-associated virus (AAV) for expressing shRNA. AAVs represent an optimal system for delivery of shRNA expression cassettes, as they fulfill all the theoretical requirements of an ideal shRNA transfer system: a) they are generally very effective as a vehicle for transgene delivery in vitro and in vivo; b) they are regarded as the safest of all known viral vector

systems, with wild-type and recombinant AAV believed to be non-pathogenic; c) they have a very broad host range, including quiescent and dividing cells; d) they typically result in strong and persistent transgene expression in the infected cell, with a much lower risk of insertional mutagenesis than retroviral vectors; and e) they can be manufactured easily and efficiently. Importantly, in cultured mammalian cells and in whole animals, infection with these vectors was shown to result in specific, efficient, and stable knockdown of various targeted endo- or exogenous genes (Grimm et al., 2005).

In order to use the produced shRNA for HNF4a silencing in whole organism experiments, we generated shRNA expressing AAV plasmid vectors (H1-shmHNF4a_3 and U6-shmHNF4a_3). The aim is to use the above vectors along with helper vectors in order to produce AAVs. The ultimate goal is to generate Adeno-Associated Virus (AAV) vectors for expressing shRNAs. In cultured mammalian cells and in whole animals, infection with these vectors was shown to result in specific, efficient, and stable knockdown of various targeted endo- or exogenous genes. The generation of AAV particles expressing shRNAs follows the basic rules and protocols that have been established for conventional transgenes, using typical vector and helper plasmids. For the construction of the shRNA expressing AAV vectors we used the dsAAVU6-RSV-eGFP and dsAAV-H1-RSV-eGFP vectors kindly provided to us by Dr Jean Albert Kuivenhoven (University Medical Center Groningen, European Research Institute for the Biology of Ageing, Groningen, the Netherlands). The double-stranded oligonucleotides of shmHNF4a_3 with the BbsI protruding ends were cloned in the corresponding sites of the dsAAV-U6-RSV-eGFP and dsAAV-H1-RSV-eGFP vectors. As control in our AAV experiments, we are going to use short-hairpin vectors targeting LacZ (H1-shLacZ and U6-shLacZ).



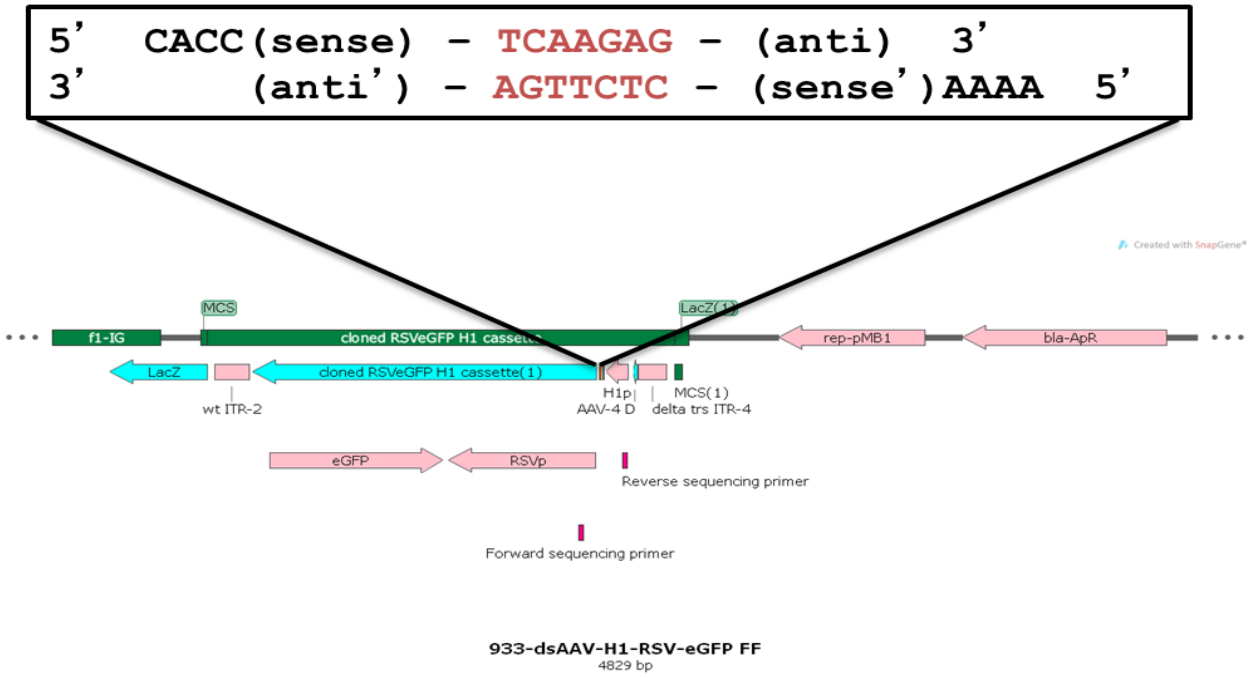


Figure 24. Map of 933-dsAAV-H1-RSV-eGFP FF vector with the cloning site of shRNA_3.

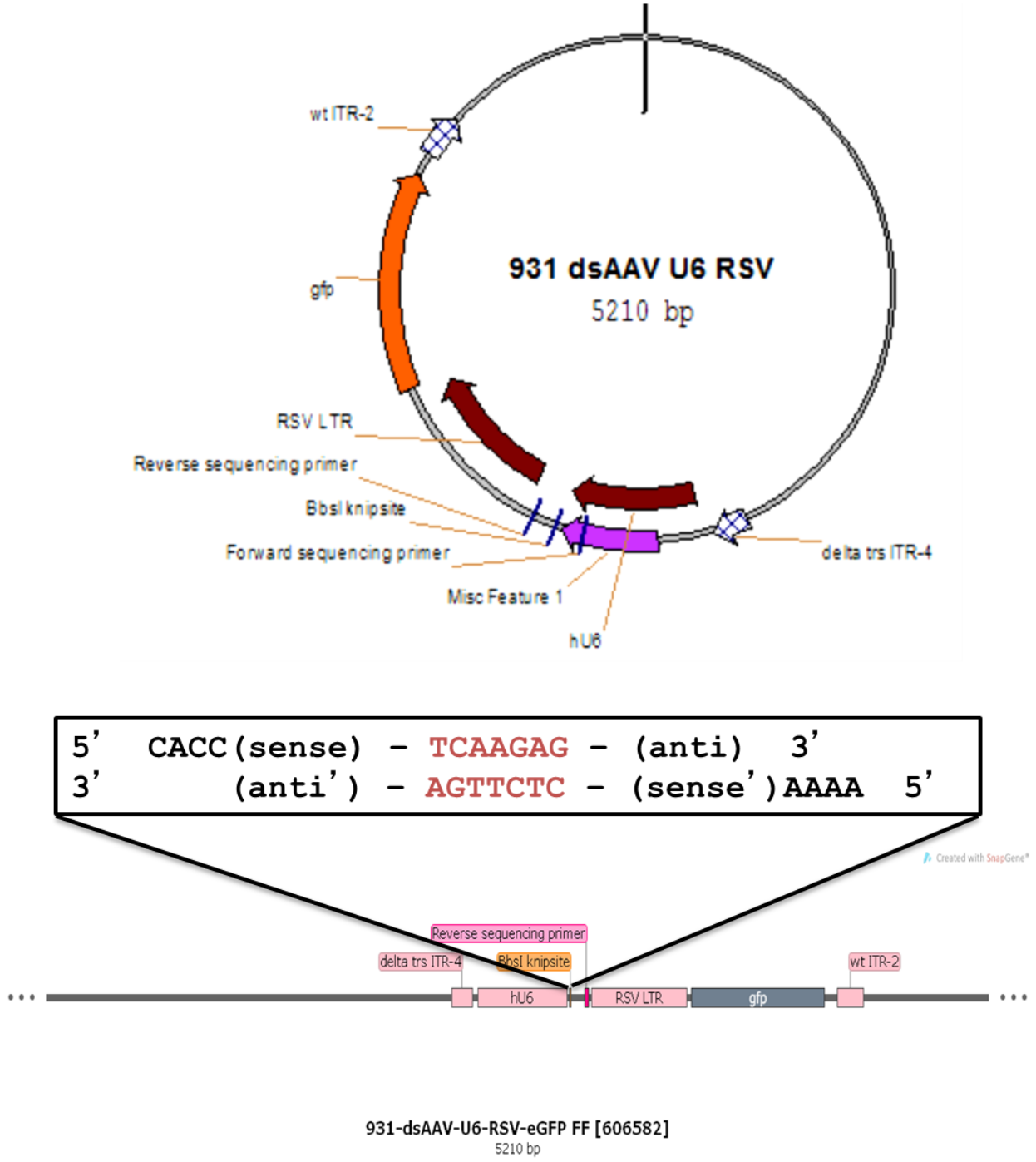


Figure 25. Map of 931-dsAAV-U6-RSV-eGFP FF vector with the cloning site of shRNA_3.

Transient transfections were performed with the corresponding expressing vectors of shHNF4a_3 with the pcDNA3-6myc_mHNF4a.cDNA in order to elucidate their efficacy. As it is shown below from the graphs, shHN4a_3 is able to reduce the hnf4a protein levels with both H1 and U6 plasmid vectors.

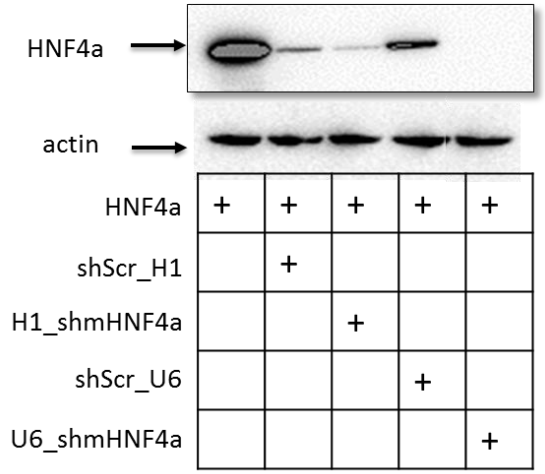


Figure 27. Western blot analysis of produced mhnf4a protein upon administration of shRNAs in HEK293T cells.

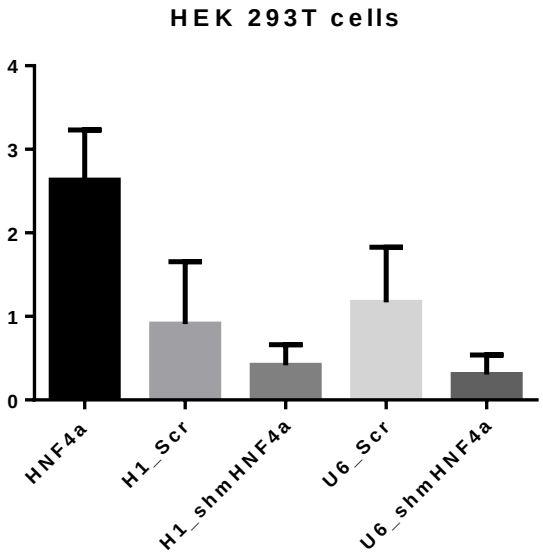


Figure 26. Quantification of protein expression levels of mhnf4a after co- transfection of HEK293T cells with pcDNA3-6myc_mHNF4a.cDNA and dsAAV-H1/U6-RSV-eGFP-FF_shRNAs

These AAVs are going to be used for mice infection in order to achieve liver specific suppression of the HNF4A gene. The infected apoE*3Leiden.CETP transgenic mice will be subjected to diet-induced MetS and this will be followed by extensive phenotypic and transcriptomic analysis of these mice. Afterwards, we will examine the changes in the expression levels of HDL and lipid related genes in order to identify new HNF4a target genes and study the mechanisms of their regulation by HNF4a.

Project II: Transcriptomic analysis of the livers of HNF4a liver specific knockout mice

Previous studies reported that HNF4a liver specific knock out mice exhibit lipid deposition in the liver. Transcriptomic analysis of the livers of these mice has been completed in our lab and revealed significant changes in the expression levels of several genes involved in lipid and apolipoprotein biosynthesis and metabolism. Transcriptomic analysis was performed in Genomic Facility in IMBB-FORTH and revealed also changes in expression levels of several other genes that get involved in several diseases such as Cardiovascular Disease, Metabolic Disease and many other as it is shown the table below. The analysis was performed with Affymetrix Mouse Gene 2.0 ST arrays. Four mice were used in each group and the threshold was 2.0.

Table 9. List of diseases according to the different pathways that genes organized

Top Diseases and Bio Functions		
Diseases and Disorders		
Name	p-value	#Molecules
Gastrointestinal Disease	1,17E-03 - 7,87E-13	172
Hepatic System Disease	2,46E-04 - 7,87E-13	98
Metabolic Disease	1,19E-03 - 7,87E-13	159
Endocrine System Disorders	1,12E-03 - 5,47E-11	157
Cardiovascular Disease	1,45E-03 - 5,73E-11	115
Lipid Metabolism	1,46E-03 - 3,46E-20	200
Molecular Transport	1,32E-03 - 3,46E-20	216
Small Molecule Biochemistry	1,46E-03 - 3,46E-20	244
Vitamin and Mineral Metabolism	1,46E-03 - 1,36E-19	81
Energy Production	9,07E-04 - 3,82E-13	51

In general, 591 and 492 genes were downregulated and upregulated, respectively. Among these genes there were several that get involved in lipoprotein metabolism and especially in HDL and LDL biosynthesis and metabolism. Some of the genes that found in microarray data analysis shown in Figure 22.

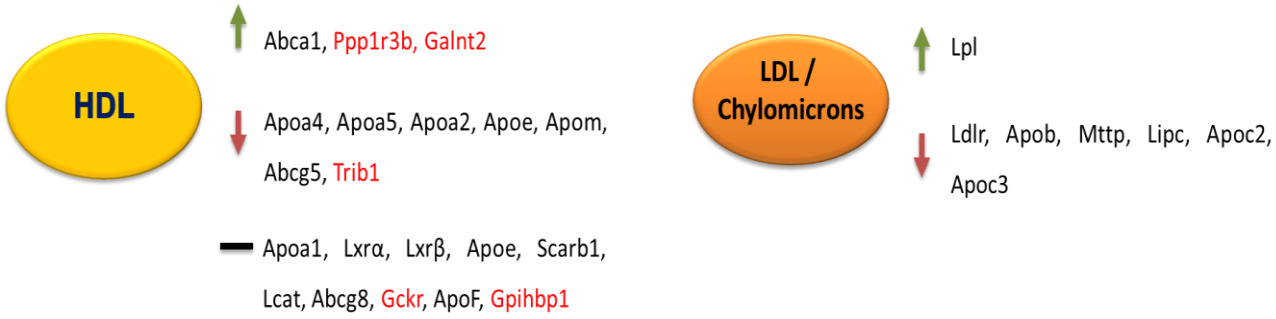


Figure 28. Several genes as discovered from microarray data analysis.

Validation of the microarray data was performed with RT-qPCR and Chromatin Immunoprecipitation (ChIP) assays only for a set of genes which participate in HDL biosynthesis pathway. Furthermore, the genes were selected according to the fold change expression levels between the two groups of mice and the predicted Hnf4a binding site in the promoter regions of these genes.

The total transcriptome from the livers of the hepatic Hnf4a deficient and control mice were used to synthesize cDNA. The cDNA was used to verify the expression levels of several genes. As far as the ChIP experiments are concerned, liver tissues from these mice homogenized and from the total chromatin we precipitate only the one which was bound with the Hnf4a antibody. This was used in order to verify already established genes and find novel genes with binding site for Hnf4a transcription factor.

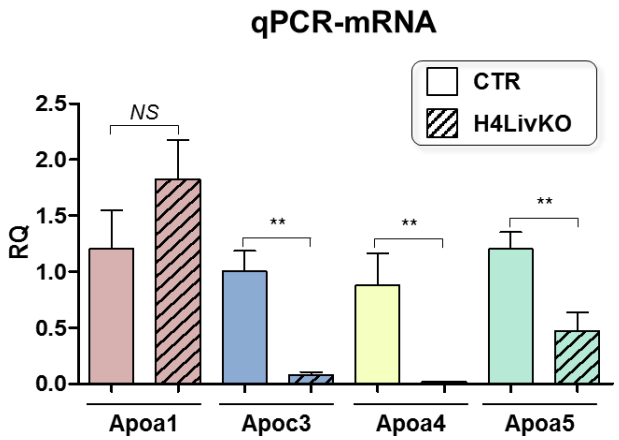


Figure 29. Hepatic HNF4α deficiency affects the expression of genes in apoa1-a4-a5-c3 cluster.

The data show that the mRNA levels of apolipoproteins Apoc3, Apoa4 and Apoa5 gene were all significantly reduced in Hnf4α-deficient mice compared those from control mice. The mRNA levels of Apoa1 were unaffected by disruption of Hnf4a gene. Our date are consistent with the previous study (Hayhurst et al., 2001), but another study in which generated hepatic Hnf4a deficiency mice through adenovirus expressing small hairpin RNA of HNF4a, the Apoa1 mRNA levels were significantly reduced (Yin et al., 2011).

The above results confirmed with the ChIP experiments. As we can see, chromatin for the specific set of genes precipitated with Hnf4a antibody. This clear demonstrate a specific hnf4a binding site in the promoter region of these genes.

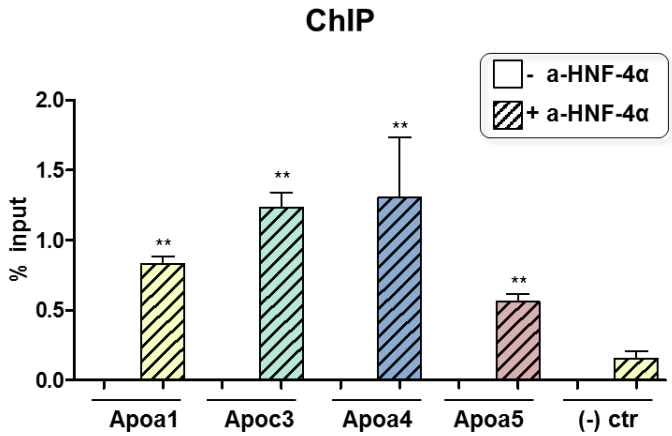


Figure 30. Hepatic HNF4α deficiency affects the expression of genes in apoa1-a4-a5-c3 cluster.

The results of the above are presented in the following cartoon.

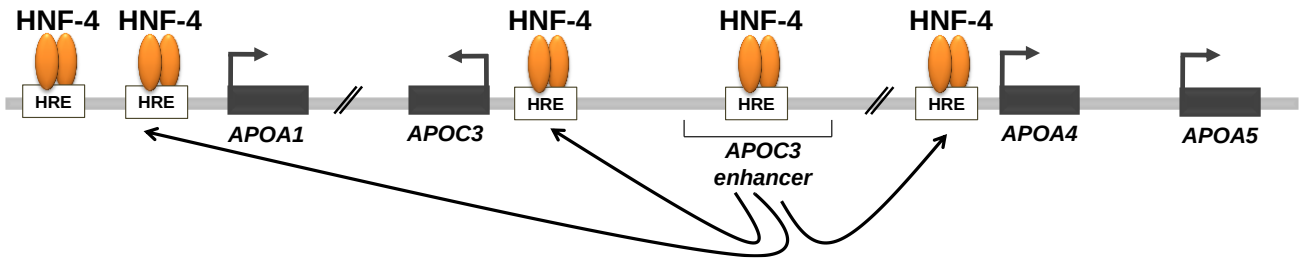


Figure 31. Schematic presentation of the Hnf4a binding sites on the APOA1-A4-A5-C3 cluster.

In addition, another set of genes are assessed with RT-qPCR to identify the differentially expressed mRNA levels. The majority of the genes participates in lipid metabolism. The mRNA levels of Ces3b, Dhcr24, Pcyt2 and Trib1 were significantly reduced in Hnf4a liver knock out mice compared with the control mice, indicating a link between those genes and the Hnf4a transcription factor. The mRNA levels of Ppp1r3b were dramatically increased three folds in hepatic Hnf4a deficient mice compared to control mice.

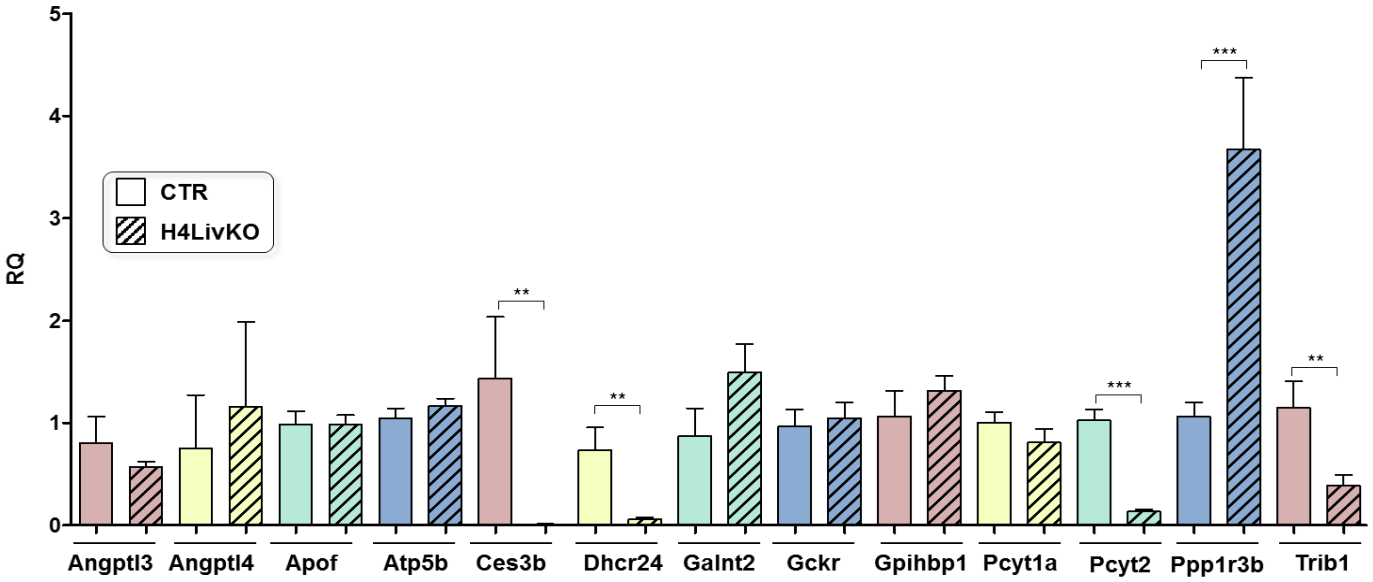


Figure 32. Hepatic mRNA levels of several genes determined by quantitative real-time PCR.

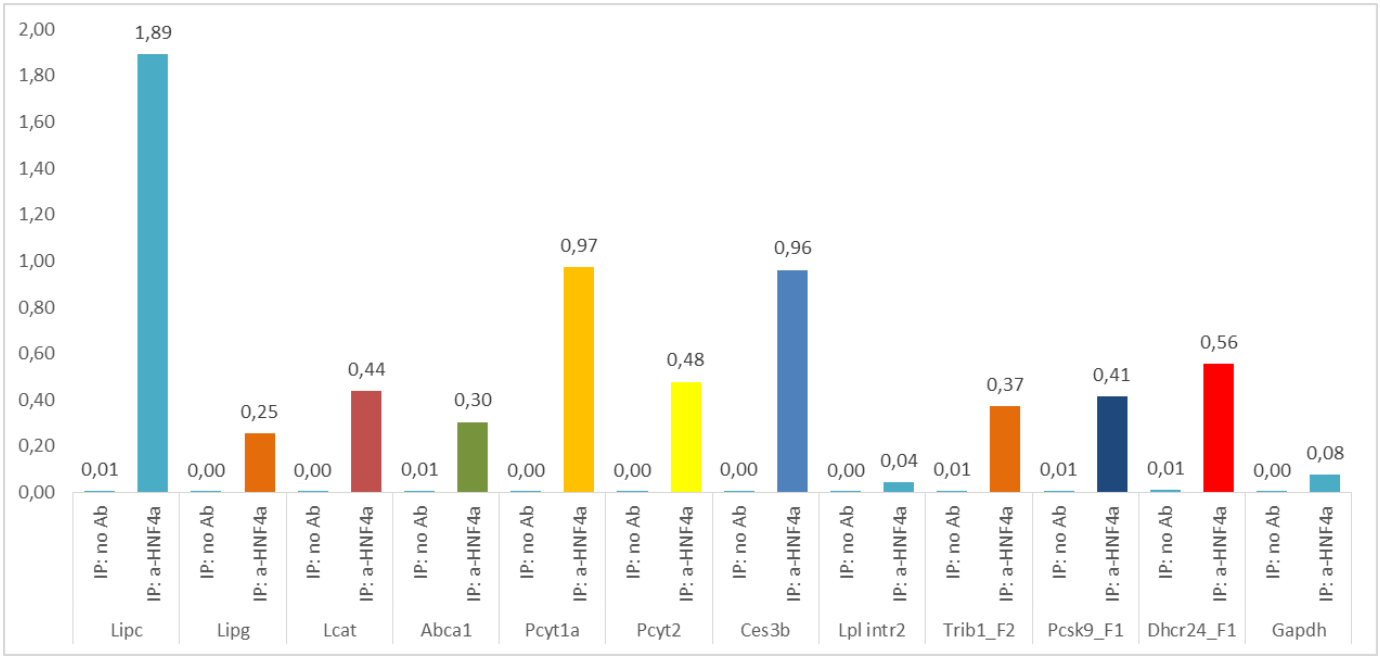


Figure 33. ChIP analysis using HNF4a antibody.

Together, these data indicate that HNF4α is important for normal expression of multiple genes such as Lipc, Pcyt2, Ces3b, Trib1, Dhcr24 that are involved in apolipoprotein and lipid metabolism.

The next experiments should be the luciferase reporter assay in order to strongly validate that the HNF4a bind in the promoter of these genes. So, the promoter regions of these genes should be cloned upstream of luciferase gene. The construct should be co-transfected in HEK 293 T cells, which do not express endogenous the HNF4a, with the construct, pcDNA3-6myc_mHNF4a.cDNA, which express the mouse Hnf4a. The luciferase activity will measured and it will compared with the control experiment.

In summary, in this project we use a conditional knock out mice to decipher the role of hepatic HNF4a in lipid homeostasis. We show that a significant number of genes that are involved in apolipoprotein and lipid metabolism altered. Furthermore, we clear demonstrate a link between these genes, which expression changed in hepatic Hnf4a knock out mice, with the HNF4a itself and verify the direct binding of HNF4a to the promoter regions of these genes.

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