Non-alcoholic fatty liver disease

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Chapter 1

General Introduction

Obesity and the metabolic syndrome: a problem of excess energy

Obesity is not a new problem; humans have been dealing with this for a long time. Yet nowadays it has increased to become a worldwide epidemic health problem (Ng *et al.*, 2014). The Venus of Willendorf, stemming back to the Paleolithic era, already exhibits one of the classic forms of obesity (Figure 1) (Cheng, 2006). Whether the Venus sculptures were only a symbol of fertility or a real representative of people of the era is not clear (Haslam and Rigby, 2010). Nevertheless, it is a testimony that obesity was already present from early times.

Figure 1) Venus of Willendorf (25,000 BCE); Upper Paleolithic

Obesity is a growing source of concern, well-known as a risk factor in the development of various metabolic disorders such as type 2 diabetes (T2D), nonalcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD) (figure 2). Obesity associated diseases are the leading cause of mortality worldwide (Barness *et al.*, 2007; Flegal *et al.*, 2005), estimated as causing 3.4 million deaths in 2010 (Ng *et al.*, 2014). Between 1980 and 2013 the body-mass index (BMI) increased worldwide, in men from 28.8% to 36.9%, and in women from 29.8% to 38.0% (Ng *et al.*, 2014). This increase is not just limited to adults, but also includes children and adolescents in developed countries, where in 2013 about 23% were overweight or obese (Ng *et al.*, 2014)

Obesity is a problem of positive energy balance, favoring an increase in energy storage over energy utilization. Unfortunately, overeating, the consumption of energy-dense foods high in sugar and fat, particularly saturated fat and trans fat, and overconsumption of sugar sweetened beverages, considered to be the result of a gastronomic revolution (Unger and Scherer, 2010), can cause or contribute to obesity and other dietary related health problems. Dense population, more passive transportation methods, the globalization of food markets and fast-food chains encouraging consumption of processed foods and large portions of high-calorie snacks are features of urban environments that contribute to the obesity epidemic (Figure 3). It is estimated that during the last 50 years the daily caloric intake per capita has increased as much as 500 kcal/day (Unger and Scherer, 2010)

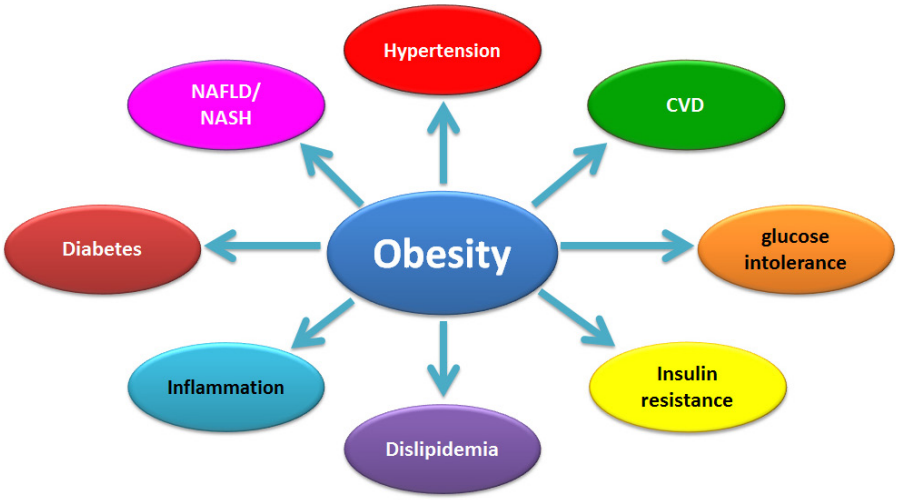

Figure 2) Obesity leads to the development of several metabolical complications

Besides these life-style related and environmental factors, genetics and interaction between multiple genes, and epigenetic related factors also cause or contribute to obesity (Brownson *et al.*, 2005; Kokkoris and Pi-Sunyer, 2003; Walley *et al.*, 2006) (Figure 3).

Although obesity and many of its metabolic co-morbidities including insulin resistance, type 2 diabetes (T2D), dyslipidemia, hypertension, inflammation, cardiovascular disease (CVD) and nonalcoholic fatty liver disease (NAFLD) (Hui *et al.*, 2013; Parekh and Anania, 2007; Paschos and Paletas,

Figure 3) Factors that predict risk of obesity

2009; Rahimi and Landaverde, 2013; Unger and Scherer, 2010) (Figure 2) are largely preventable with correct diet and regular exercise, it does not come as a surprise that in times of plenty these seemingly easy tasks are extremely difficult to perform. Moreover, obesity rates do not yet seem likely to decline, a fact which contributes to growing health care costs.

Mechanistic view of obesity

To protect the lipid spillover from adipose tissue to other organs, adipocytes store extra lipids as triglycerides (TG), and then they secrete leptin to control food intake and to trigger fatty acid (FA) oxidation. Therefore, adipocytes control both hypothalamus-regulated food intake (Pelleymounter *et al.*, 1995) and peripherally-regulated fatty acid oxidation in non-adipose tissues (Shimabukuro *et al.*, 1997; Unger *et al.*, 2010; Zhou *et al.*, 1997). Once the adipose tissue reaches its maximum expandability, excessive TG spills over to non-adipose tissues such as skeletal muscle and the liver, leading to ectopic accumulation of lipids. These tissues are able to store TG, but only to a limited extent (Slawik and Vidal-Puig, 2007).

In addition, excessive expansion of adipose tissue is accompanied by a chronic low grade systemic inflammation (Wellen and Hotamisligil, 2003), characterized by the abnormal cytokine production, increased acute-phase reactants, and activation of inflammatory signaling pathways (Wellen and Hotamisligil, 2003). Inflammation is believed to be an important factor, linking obesity to the development of diseases, including T2D, CVD, NAFLD and cancer.

However, while adipose tissue is not able to store excess lipids in a "classical" manner, this loss of capacity leads to increased lipid stores in the form of ectopic fat in other tissues such as liver and muscle. This phenomenon whereby ectopic lipid overload damages these muscle and liver tissues is also common in lipodystrophy, a metabolic condition with markedly reduced body fat (Garg, 2006). Lipodystrophy, in association with an altered/incomplete FA oxidation, results in a severe malfunction of both glucose and lipid homeostasis (Garg, 2006).

Insulin resistance and Type 2 diabetes

Insulin resistance occurs when insulin targeted tissues, liver, skeletal muscle and adipose tissue, become less responsive to the physiological effects of circulating insulin. This obesity associated insulin resistance is recognized as the major cause and most powerful predictor for the development of type 2 diabetes (T2D). In type 2 diabetes a high amount of glucose remains in the blood because an increased level of insulin is no longer capable of reducing it (Bratanova-

Tochkova *et al.*, 2002; Wilcox, 2005). Over time, this phenomenon will burn out the beta cells in the pancreatic islets of Langherhans, which are responsible for insulin production (Himsworth, 2011). As a result, lack of beta cell function will bring about a hyperglycemic status in the body (Wilcox, 2005), and the initial stage of T2D may subsequently progress towards the more severe insulin dependent T2D. T2D is one of the most costly and burdensome chronic diseases of our time. According to the International Diabetes Federation, the worldwide T2D patient population is currently reported to be over 258 million and is expected to increase to about 438 million by 2030 (Hu, 2011). Thus the epidemic of obesity is expected to lead to a further rise in the T2D population.

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is one of the worst problems of the obese population, and is known as the hepatic component of the metabolic syndrome (Bedogni *et al.*, 2005). NAFLD was first recognized in 1980 (Hui *et al.*, 2013; Ludwig *et al.*, 1980) and soon known to be the most common chronic liver disease (Hui *et al.*, 2013). NAFLD is associated with visceral obesity, insulin resistance and dyslipidemia, and can have severe clinical consequences. NAFLD has been detected in between 25 and 30% of the general population and 90% of morbidly obese patients (Bhala *et al.*, 2013; Torres and Harrison, 2008). NAFLD is a term used to describe a broad spectrum of liver related disorders (de Alwis and Day, 2008), of which the mildest form is hepatic steatosis. While in a normal liver hepatic lipids are not more than 5% of the total liver weight (Brumbaugh and Friedman, 2014), accumulation of excessive lipids in the liver is the main characteristic of steatosis (Figure 4). This can be caused by an imbalance in the processes of fatty acid uptake, synthesis and oxidation of fatty acids, or it can be caused by a combination of these circumstances (Figure 5) (Cohen *et al.*, 2011). The condition of steatosis is benign and reversible, but it can also progress to the next stage of NAFLD, which is non-alcoholic steatohepatitis (NASH). NASH can be distinguished from steatosis by the presence of inflammation and fibrosis (Figure 4) (Mirza, 2011). Hepatic steatosis progresses to NASH in only 10-20% of the NAFLD patients. Chronic hepatic inflammation can then lead to fibrosis, cirrhosis, and in some individuals to end-stage liver disease with hepatocellular carcinoma (liver cancer) (Zhang *et al.*, 2014). Between 10 and 29% of individuals with NASH develop cirrhosis within 10 years (Argo and Caldwell, 2009) and between 4% and 27% of individuals with cirrhosis develop hepatocellular carcinoma (Starley *et al.*, 2010). NAFLD includes several other symptoms like portal fibrosis and lymphocytic inflammation (Brunt *et al.*, 1999; Tilg and Moschen, 2010). With the rise of

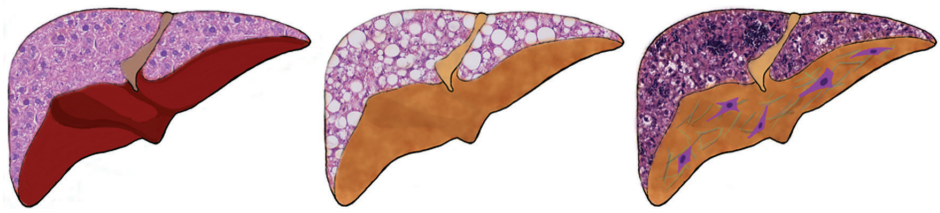

Figure 4) Representation of normal (left), steatotic (middle) and NASH (right) livers.

obesity, it is predicted that NAFLD will be the most common etiology for liver transplantation in the 21st century (Agopian *et al.*, 2012).

Origin of hepatic fat and steatosis

There are three ways to supply FFA used for hepatic TG formation: [1] diet, [2] *de novo* synthesis, and [3] adipose tissue. The free fatty acids available in the liver have three typical destinations: re-esterified to TG and able to be stored in lipid droplets; oxidized in the mitochondria to produce energy and ketone bodies; or combined with apolipoproteins and secreted as an essential compound for very-low-density lipoproteins (VLDL) (Cohen *et al.*, 2011). When hepatic fatty acids exceed the normal amount, due to an imbalance between FA influx and

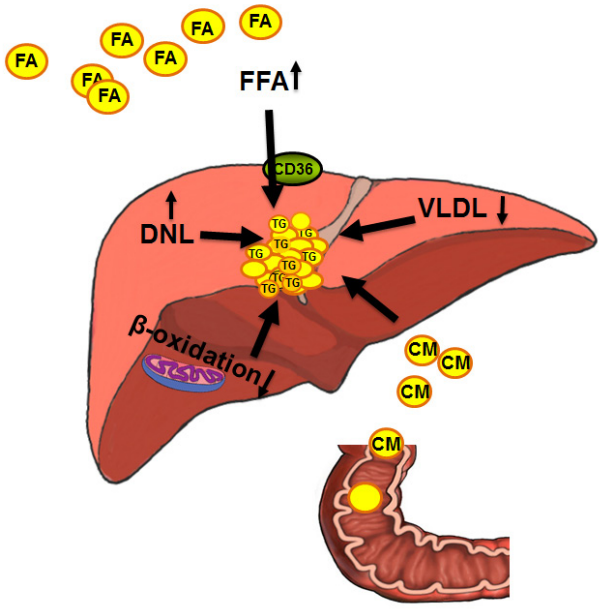

Figure 5) Causes of increased lipid accumulation in the liver

FA efflux, steatosis occurs. Steatosis development can be due to an increase in hepatic TG and *de novo* lipogenesis and/or reduced beta oxidation of fatty acids (Cohen *et al.*, 2011). Steatosis can also occur with an increase in TG-rich chylomicron (CM) particles containing dietary fat, which are transported from intestine to liver (Figure 5). Steatosis also may occur during fasting, when DNL is stimulated.

The first regulatory step in hepatic fat homeostasis is fatty acid uptake at the plasma membrane. Short- and medium-chain fatty acids can diffuse passively from the plasma membrane, but long-chain fatty acid uptake is via both passive diffusion and by integral or membrane-associated proteins, one of which is CD36 (Ehehalt *et al.*, 2006) (Figure 6).

The lipid transporter CD36

The class B scavenger receptor, CD36, was first discovered in platelets over 30 years ago. CD36 is a membrane glycoprotein ubiquitously expressed on a variety of cell types including adipocytes, myocytes, macrophages, platelets, hepatocytes, vascular endothelial cells, smooth muscle cells, and intestinal enterocytes (Silverstein and Febbraio, 2009). It is now known that CD36 is a multifunctional protein at various locations in the body. CD36 is known as fatty acid translocase, and is involved in the uptake of long-chain fatty acids (Aitman *et al.*, 1999; Glazier *et al.*, 2002). It has been demonstrated that the role of CD36 as an orosensory receptor of dietary long-chain fatty acids (LCFA) in both rodents and humans is associated with the detection of dietary fatty acids (Simons *et al.*, 2011). CD36 expression

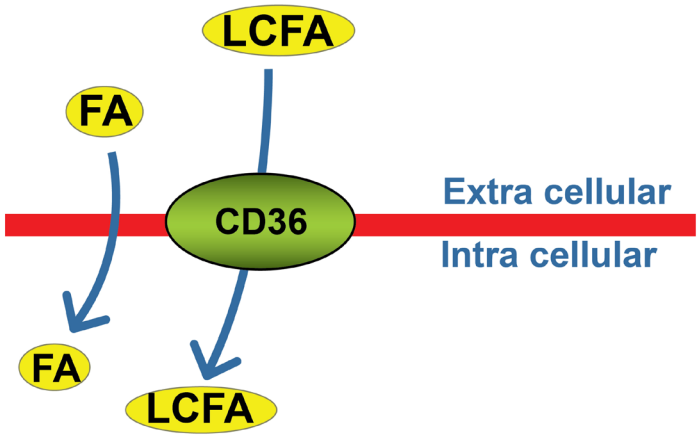

Figure 6) Fatty acid transportation is mediated by both passive diffusion and a transporter protein like CD36

in endothelial cells is associated with angiogenesis (Clugston *et al.*, 2014).

CD36 is a receptor for a wide range of ligands in monocytes and macrophages. CD36 mediates the phagocytosis of apoptotic cells and the uptake of oxidatively damaged low-density lipoproteins (ox-LDL). CD36 is involved in the recognition of ligands of gram positive bacteria cell walls (Hoebe *et al.*, 2005a; Stuart *et al.*, 2005). In addition, CD36 acts as a co-receptor for recognition of di-acylglyceride via the TLR2/6 complex (Hoebe *et al.*, 2005b). Therefore, CD36 is reported to be involved in the development of inflammation either via direct CD36-mediated pro-inflammatory signaling (Baranova *et al.*, 2008; Moore *et al.*, 2002) or via presenting ligands to TLRs (Hoebe *et al.*, 2005a).

The role of CD36 in NAFLD

Importantly, it was discovered that CD36 is one of the crucial downstream targets of PPARγ (Yu *et al.*, 2003; Zhou *et al.*, 2008). Thereby, CD36 supports the role of PPARγ in adipocyte differentiation by binding and uptaking long-chain fatty acids in the heart, adipose tissue and muscle. The oxidation of FA in these tissues provides much of the energy needed for proper function. However, the detailed pathways regulating CD36 expression and function are still unclear. The CD36 knock out mouse exhibits a huge decrease in FA uptake and utilization by the heart, skeletal muscle, and adipose tissues; it is therefore believed that FA delivery to the liver increases hepatic steatosis (Febbraio *et al.*, 1999). In the liver CD36 may contribute to hepatic steatosis by facilitating high affinity uptake of fatty acids from the circulation (Febbraio and Silverstein, 2007). CD36 expression is normally weak in hepatocytes (Luiken *et al.*, 2002), but its expression is increased upon a high fat diet (HFD) (Koonen *et al.*, 2007a). Hepatic CD36 gene and protein expression levels are correlated with hepatic triglyceride storage and secretion, suggesting that CD36 may play a causative role in the pathogenesis of NAFLD.

There is a growing body of evidence suggesting that in addition to LCFA uptake, CD36 has a role in inflammatory signal transduction (Sheedy *et al.*, 2013). Ox-LDL has been shown to activate CD36-dependent signaling, resulting in JNK activation, which can consequently lead to inflammation (Rahaman *et al.*, 2006). Targeted deletion of CD36 in mice has been shown to reduce macrophage content and levels of pro-inflammatory cytokines in the plasma (Kuchibhotla *et al.*, 2008; Moore *et al.*, 2005; Rahaman *et al.*, 2006; Stewart *et al.*, 2010; Stuart *et al.*, 2005). In addition, CD36 has been shown to facilitate Toll-like receptor (TLR) signaling, in the presence of ox-LDL via TLR4/TLR6 and in response to diacylglyceride via TLR2/6, and thus activate the innate host response (Hoebe *et al.*, 2005b; Rahaman *et al.*, 2006; Stewart *et al.*, 2010; Stuart *et*

al., 2005; Triantafilou *et al.*, 2006). As a result, CD36 has been implicated in the systemic inflammation associated with chronic diseases.

NASH progression

Inflammation is not only a driver of NASH pathology, but also a hallmark of NASH. NASH was originally defined in the pathological samples of alcoholic steatohepatitis by Ludwig and co-workers in 1980 (Ludwig *et al.*, 1980). Liver biopsy evaluation is currently the method used to distinguish the presence of NASH. The histopathological symptoms of NASH include: infiltration of inflammatory cells; ballooned hepatocytes with Mallory-Denk hyaline bodies; lobular inflammation typically localized in the acinar zone 3; portal chronic mononuclear cell inflammation; apoptotic (acidophil) bodies; megamitochondria (giant mitochondria) and/or the presence of collagen deposition (fibrosis) (Brunt and Tiniakos, 2010). If there is no inflammation, the NASH score is low. Since inflammation is the local protective response to tissue injury, knowledge of whether inflammation occurs from the steatotic liver or independent of the steatotic liver is largely based on association studies; the actual mechanisms leading to liver inflammation and the development of NASH are largely unclear. Some examples of contributors are mentioned below:

The inflammatory response: NF-κB

An increasing body of evidence supports the fundamental role of the nuclear factor-kappaB (NF-κB) family of transcription factors in the inflammatory responses. The family consists of five members of the heterodimeric or homodimeric complex, of which p65 and p50 are highly expressed in the liver and involved in the regulation of expression of several pro-inflammatory cytokines (Oeckinghaus and Ghosh, 2009). Of the pro-inflammatory cytokines, particularly the tumor necrosis factor (TNF), Interleukin 6 (IL-6) (Feldstein, 2010) and Interleukin-1 beta (IL-1β) play a central role in the development of NASH. Hepatic inflammation has been suggested to directly induce insulin resistance by interfering with insulin signaling pathways (Lagathu *et al.*, 2006). Therefore, for the treatment of both insulin resistance and NASH there is increasing interest in finding a therapeutic agent that targets NF-κB family members.

The defensive response : Kupffer cells

Kupffer cells, or the hepatic resident macrophages, are responsible for clearance by phagocytosis of apoptotic cells and other exogenous cells, particularly in the liver. Upon recognition of potential danger Kupffer cells can become activated; they can then release a variety of biologically active mediators, including pro-inflammatory cytokines

and chemokines, thereby initiating inflammation (Baffy, 2009). The released pro-inflammatory cytokines can interact with hepatic stellate cells and contribute to collagen production (Tacke, 2012). Furthermore, pro-inflammatory cytokines and chemokines can also stimulate the recruitment of neutrophils, monocytes, and natural killer T cells to the liver. Because Kupffer cells are involved in the pathogenesis of NASH, specific targeting of Kupffer cells may lead to more effective strategies in NASH treatment.

Epigenetic mechanisms and NAFLD: macroH2A

The past few years have seen a growing interest in epigenetic mechanisms involved in the onset of liver diseases like NAFLD (Podrini *et al.*, 2013; Rappa *et al.*, 2013). In particular, it has been noted that severe metabolic perturbations, such as hunger, lead to long lasting changes that render individuals susceptible to metabolic syndrome. Strikingly, this condition appeared to be transmittable to subsequent generations. Also in rodents, metabolic programming of fatty liver mechanisms responsible for these long lasting effects may be either developmental or epigenetic changes. However, only epigenetic changes can explain the multigenerational effects. Therefore, much attention is being focused on epigenetics.

The term 'epigenetic' has been coined to indicate information that does not rely on DNA sequence, but can still be transmitted from mother to daughter cells (Wolffe and Matzke, 1999). This information includes DNA methylation at CpG sites and epigenetic marks determined by chromatin. DNA is decorated by chromatin, which consists mostly of nucleosomes composed of two molecules of each of the core histone proteins: H2A, H2B, H3, and H4. In addition to their structural role, histones are integration sites for various signals and are highly modified on their N-terminal tails. These modifications serve as binding platforms for other proteins and are crucially involved in the regulation of all chromatin functions, such as DNA repair and transcriptional regulation (Buschbeck *et al.*, 2009; Kouzarides, 2007). Moreover, the incorporation of histone variants into chromatin can provide additional regulation. In higher organisms, each histone subtype is represented by a family of genes encoding multiple variants that, in most cases, are highly similar and likely redundant. However, some histone variants show unique features and play very precise roles in critical aspects of chromatin structure regulation (Barrero *et al.*, 2013). The macroH2A variants contain an amino-terminal histone-like region that is similar to histone H2A and a carboxy-terminal globular domain called macrodomain (Pehrson and Fried, 1992). These variants are coded by two different

genes, H2AFY and H2AFY2, which produce macroH2A1 and macroH2A2, respectively (Chadwick and Willard, 2001). MacroH2A1, reported to regulate hepatic fat accumulation and its absence (Changolkar *et al.*, 2007), worsens hepatic steatosis and systemic imbalances upon a high fat regimen in mice (Boulard *et al.*, 2010). Moreover, macroH2A1 knock out (KO) mice revealed an increased hepatic expression of genes in the area of lipid metabolism, including CD36 (Changolkar *et al.*, 2010). Thus, macroH2A1 KO are an interesting model to study the role of epigenetic alterations during the development of obesity and NAFLD.

Aging pathophysiology and NAFLD

Although aging is not a disease, it is considered the leading risk factor for all chronic diseases, including obesity, T2D and NAFLD (Frith *et al.*, 2009a, 2009b). According to the hyperfunction theory proposed by Blagosklonny (Blagosklonny, 2007, 2012), several processes responsible for growth and development in early life become excessive (hyperfunctions) in later life, leading to aging pathology and eventually death.

Since T2D, obesity and NAFLD are all considered as systemic manifestations of cellular hyperfunction and loss of homeostasis, a diversity of forms of hyperfunction can be said to cause various pathologies in aging (Gems and Partridge, 2013). Aging is shown to increase lipid accumulation in the liver and the development of hepatic steatosis (Gan *et al.*, 2011; Ghosh *et al.*, 2012; Honma *et al.*, 2011; Petersen *et al.*, 2003). β-oxidation has been reported to decline in livers with old age, with the involvement of hepatic expression of nuclear receptor peroxisome proliferator-activated receptor α (PPARα) reduction (Sanguino *et al.*, 2004). Recently, Fontana *et al.* demonstrated that aging induces liver damage and increases inflammatory response and the development of NASH in mice (Fontana *et al.*, 2013). However, the role of aging in the progression of NAFLD is not yet clear, one of the aims of this thesis is to determine that role.

Thesis outline

The pathogenesis of NAFLD and the mechanisms responsible for disease progression remain inadequately understood. Although risk factors such as excessive fat accumulation, oxidative stress, mitochondrial injury, pro-inflammatory cytokines and aging have been implicated in the development of hepatic inflammation, the actual trigger for tissue inflammation and hepatocyte degeneration remains uncertain. Better understanding of NAFLD pathogenesis will aid the development of new strategies to treat NAFLD patients in the early stages of disease progression. The research described in this thesis was performed to gain more insights into the etiology, progression and effect of risk factors in the development of NAFLD, obesity and its complications. An introduction for that is provided in **Chapter 1.**

The first part of the thesis focuses on the relationship between old age and inflammation, both of which are risk factors for the development of NAFLD. Understanding how aging could affect the molecular pathology of liver diseases is particularly important, and only a few studies to date have tackled this complex process. **Chapter 2** describes the current knowledge on the interaction between aging processes and NAFLD, and discusses the molecular pathology of liver aging that may aggravate the development of NAFLD. The extent to which obesity-induced metabolic inflammation in the adipose tissue and the liver contribute to metabolic complications remains unknown. We therefore wanted to understand the origin of metabolic inflammation in obesity and define whether obesity triggers adipose tissue and hepatic inflammation simultaneously or in sequence. In **Chapter 3** we evaluate the progression of inflammation in visceral adipose tissue and in the liver, using young to middle-aged (over 1 year old) mice, to roughly represent 60 year old humans. To understand more about the underlying mechanisms related to NAFLD and aging, we studied the livers of aged mice with and without a HFD. In addition, an increase in CD36 (a transmembrane protein mediating long-chain fatty acid uptake) expression is common in models of obesity, insulin resistance and type 2 diabetes (T2D), and is observed in the physiological aging process (Koonen *et al.*, 2007b). The effect of CD36 in the development of age-associated NAFLD is evaluated in **Chapter 4**, where it is suggested that an increase in CD36 may play an important role.

The second part of this thesis describes studies on the role of target gene ablations in the development of NAFLD. CD36 is involved in lipid metabolism and the development of age-associated NAFLD. To further unravel the role of CD36 in NAFLD, we investigated the effect of dietary fat in CD36 knock out (KO) mice in **Chapter 5**. Recent studies highlight

the importance of an epigenetic basis for the development of steatosis based on macroH2A1. MacroH2A1, a variant of histone H2A, emerged as a key chromatin regulator, sensing small nutrients during cell proliferation and differentiation. When incorporated into the chromatin of hepatocytes, macroH2A1 regulates gene expression. In **Chapter 6** we determine the *in vivo* role of macroH2A1 in response to nutritional excess and we also evaluate macroH2A1 KO mice in the development of NAFLD. **Chapter 7** provides a general discussion, major challenge and perspectives for future research on NAFLD progression. The results of the studies outlined above are summarized in **Chapter 8**. To be able to share my work with those less proficient in English, in **Chapter 9** I have summarized my work in the Dutch, in **Chpater 10**, in Turkish and in **Chapter 11**, in Persian languages.

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