

What is the influence of complement factor C₃ and lipid metabolism of adipose tissue on hepatic steatosis in mice?

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Abstract

NAFLD (non-alcoholic fatty liver disease) represents a fatty liver whereby lipid accumulation (steatosis) occurs. In recent decades, the prevalence of NAFLD has dramatically increased. Activation of the complement system, which is an important subset of the immune system, and especially complement factor C₃, may have a significant role in the progression of NAFLD. In this study is examined if there are, next to alterations in hepatic lipid metabolism, also alterations present in the lipid metabolism of adipose tissue. It appears that acylation stimulating protein (ASP) has an important role in linking the activation of complement and lipid metabolism of adipose tissue in NAFLD. In this study the following research question is stated: What is the influence of complement factor C₃ and the lipid metabolism of adipose tissue on hepatic steatosis in mice? It is expected that wild type mice on a HFD diet will show the highest hepatic lipid accumulation. Furthermore the expression of some enzymes involved in lipid metabolism will be altered. Liver and adipose tissue from C₃^{+/+} (n=17) and C₃^{-/-} (n=17) mice is used. The control group (n=12) received a chow diet and the experimental group (n=22) a high fat diet (HFD). The hepatic lipid accumulation and the expression of some enzymes, involved in the lipid metabolism of adipose tissue, are determined in these mice. The results, showed no significant differences in lipid accumulation between both mouse groups. The expression levels of the enzymes FASN, SCD1, PPAR γ and DGAT1 were significantly different between the diets in one or both mouse groups. The expression of the enzymes HSL and DGAT1 were significantly higher in the C₃^{+/+} mice compared to the C₃^{-/-} mice. It can be concluded from histological stainings and preliminary scoring results that hepatic lipid accumulation occurs the most in C₃^{+/+} mice on a HFD. However it seems that lipids do not only accumulate by C₃ activation,

but also other mechanisms seems to play a role. It is assumed that in $C3^{-/-}$ mice the ASP pathway is disturbed, which will lead to an increased flux of fatty acids to the liver. In $C3^{+/+}$ mice there is a higher adipose tissue lipolysis and lipogenesis present since the expression of HSL and DGAT were increased.

Keywords

NAFLD, hepatic steatosis, complement system, complement factor C3, adipose tissue and hepatic lipid metabolism, ASP.

Introduction

NAFLD is characterized by a fatty liver or hepatic steatosis, whereby the hepatic fat percentage exceeds 5% of the wet weight of the liver [1]. This hepatic steatosis results from an imbalance between lipid availability and lipid release. Simple steatosis is a relatively early, non-risky stage, since no inflammation is present in the liver [2]. NAFLD can however progress in nonalcoholic steatohepatitis (NASH), a more detrimental and advanced stage of fatty liver disease, characterized by hepatic inflammation. Patients with NASH can develop hepatic fibrosis, cirrhosis and finally hepatocellular carcinoma, which will lead to increased mortality [3].

The hepatic lipid metabolism is altered in patients with NAFLD. It seems that high hepatic lipid content and insulin resistance are also associated with alterations of proteins and enzymes involved in lipid metabolism of adipose tissue [4]. A well-known fact is the increase in the rate of adipose tissue lipolysis in subjects with NAFLD [5]. From previous data is known that mice fed a HFD exhibit adipose tissue dysfunction, characterized by down- or upregulation of several genes involved in lipid metabolism of adipose tissue [6]. In the previous section, it is explained that NAFLD is often associated with alterations in the lipid metabolism of liver and adipose tissue. There seems to be a crosstalk between the immune system and adipose tissue. Obesity is related to adipose dysfunction and represents a state of chronic low-grade inflammation. It is believed that infiltration of macrophages in liver or adipose tissue contributes to the development of NAFLD and induces the production of reactive oxidative species (ROS). ROS will result in higher oxidative stress, which contributes to the progression of NAFLD [7]. The inflammatory response present in NASH is activated by the innate immune system [8]. The complement system is a subset of the innate immune system [9]. Preliminary data suggest that complement activation may have a significant role in the pathogenesis and progression of NAFLD and NASH [3,8]. Complement activation occurs through three pathways, the classical, alternative and mannose binding lectin pathway. Activation of these pathways

depends on different molecules for their initiation and amplification [10]. These three pathways will, however, converge at the central component C₃, which is the most important and abundant component in the complement system. The cleavage product of C₃ is C_{3a}. C_{3a} can influence, via activation of nuclear factors and cytokines, the process of liver regeneration. Deficiency of C_{3a} diminishes liver regeneration [10]. C_{3a}, however, also stimulates the production of prostanoids and pro-inflammatory cytokines by Kupffer cells thereby contributing to a pro-inflammatory environment [11].

The complement system has, next to its part in innate immunity, as described above, also a role in the lipid metabolism of adipose tissue. The protein ASP exerts an important function herein. C₃ is rapidly converted, by plasma carboxypeptidase N or R, to acylation-stimulating protein (ASP). ASP, also referred to as C₃adesArg, is the biologically inactive fragment of C_{3a} [12]. Both in human and mice, levels of ASP are elevated in obese subjects, diabetics, and associated metabolic disorders. ASP levels are decreased upon weight loss or exercise [13]. Thus, it is believed that both ASP and its precursor C_{3a} have an important role in the regulation of fatty acid uptake in adipose tissue and body lipid homeostasis [11], and these processes show a connection between the alternative complement pathway and metabolism of the adipose tissue [12].

It seems that both complement factor C₃ and ASP may have a great influence on both the development of NAFLD and lipid metabolism in liver and adipose tissue. In this study, it will be investigated whether C₃ affects hepatic steatosis in mice. Also, the potential inhibitory and stimulating effects of ASP on genes involved in lipid metabolism in adipose will be studied.

The aims of this study were firstly, to determine if preliminary data, which suggested that complement factor C₃ deficiency diminishes steatosis in NASH, corresponds with own found results in this study. Secondly, to examine if the expression of candidate genes involved in the lipid metabolism of adipose tissue are altered in C₃ wild type and knock out mice. Evaluation of histological stainings by an experienced liver pathologist revealed that wild type mice had higher NASH grades compared to C₃ knockout mice. Mice fed the HFD appeared to have more hepatic lipid accumulation compared to mice that received the chow diet. Thus, it is expected that C₃ wild type mice receiving the HFD diet will have the highest hepatic lipid accumulation.

Material and methods

Twelve-weeks-old specific pathogen-free male C₅₇BL/6 (C₃^{+/+}) and C₃ knockout (C₃^{-/-}) mice were used for all experiments. All mice were housed individually and maintained in a controlled environment under standard conditions.

In this study, liver and adipose tissue from wild-type mice ($C3^{+/+}$) and $C3$ knockout ($C3^{-/-}$) is used to examine the research question. The control group of this study consisted of $C3^{+/+}$ mice ($n=6$) and $C3^{-/-}$ mice ($n=6$), which were fed a standard chow diet for 12 weeks. The experimental group consisted of $C3^{+/+}$ mice ($n=11$) and $C3^{-/-}$ mice ($n=11$), which were fed a high fat diet (HFD) (D12492, Research Diets) for 12 weeks, to examine the effect of a mild NAFLD inducing diet on complement activation. Mice from the control and experimental group were littermates. All mice had ad libitum access to food and water. The HFD contained 60% energy as triglycerides. Mice were sacrificed by CO₂ asphyxiation. Snap frozen liver and adipose tissue was used for histological staining and RNA isolation, respectively.

Hepatic lipid accumulation of each mouse was determined by Oil Red O staining. Deep-frozen liver pieces were cut in 4 μ m thick liver sections using a cryostat and subsequently stained. The liver morphology was evaluated using a light microscope. Lipid accumulation was scored on a 0-3 point scale resulting in 4 groups. The sections were scored taking into account the size and intensity of the red colour, which represents lipid droplets. It was also checked if the droplets were evenly distributed over the tissue.

RNA from adipose tissue was isolated and used to synthesize cDNA according to the protocols. The produced cDNA was used as a template for the quantitative polymerase chain reactions (qPCR). Primers of several enzymes, involved in the lipid metabolism of adipose tissue, were used. Eventually the relative gene expression of these enzymes was calculated according to the delta-Ct relative quantification method.

Results

Body weight gain and liver weight in $C3^{+/+}$ and $C3^{-/-}$ mice fed a HFD or chow diet

To study the effect of $C3$ activation on hepatic steatosis, two mice groups, a wild-type ($C3^{+/+}$) and knockout mice group ($C3^{-/-}$), received a HFD or chow diet for 12 weeks. It is expected that the HFD will induce mild hepatic steatosis. Preliminary data show that mice from all four groups gained weight during the 12-week diet treatment. Although only $C3^{-/-}$ mice fed a HFD gained significantly more body weight (Fig. 1, Table 2, $7,21 \pm 4,12$; $8,39 \pm 3,86$, respectively) compared to $C3^{-/-}$ mice fed a chow diet ($3,74 \pm 1,59$; $3,49 \pm 0,91$, respectively) ($p=0,0005$). The body weight gain is also significantly different between the two mouse groups ($p=0,0096$).

Additional preliminary data also showed the hepatic effect of the diets. Liver weight as percentage of body weight was significantly higher in $C3^{+/+}$ and $C3^{-/-}$ fed a chow diet (Fig. 2, Table 2, $4,53 \pm 0,27$; $4,12 \pm 0,13$, respectively) compared to mice fed a HFD ($3,79 \pm 0,67$; $3,40 \pm$

0,36, respectively) ($p=0,0001$). The liver weight also differed significantly between the two mice groups ($p=0,0216$).

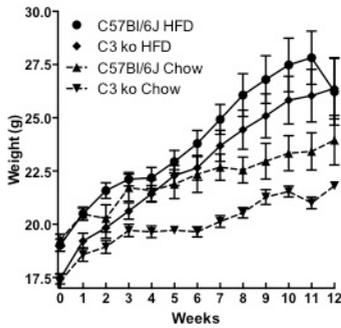


Figure 1. Body weight change over 12 weeks in $C3^{+/+}$ and $C3^{-/-}$ mice fed a HFD or chow diet.

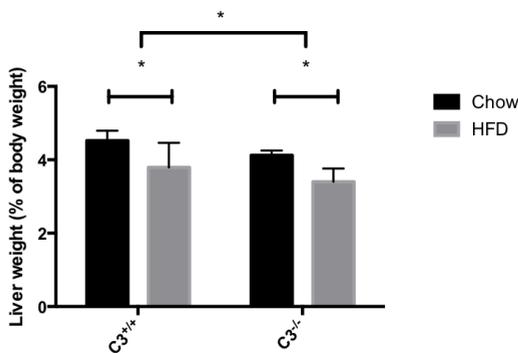


Figure 2. Liver weight expressed as percentage of total body weight, in $C3^{+/+}$ and $C3^{-/-}$ mice fed a HFD or chow diet

Development of hepatic steatosis in $C3^{+/+}$ and $C3^{-/-}$ mice fed a HFD or chow diet

Liver sections of the four mouse subgroups were stained with an Oil-Red-O staining to detect lipid droplets (Fig. 3). The histology of the liver sections was evaluated within each subgroup. The red colour represents lipid droplets, which is an indication for hepatic steatosis. In the figure below, representative images of liver histology of each subgroup are demonstrated. It can be seen that the $C3^{+/+}$ mice on HFD have the highest intensity of red colour and the largest lipid droplets. $C3^{-/-}$ mice on a HFD also display lipid droplets, however, these are smaller and more evenly distributed. Both mice groups on the chow diet demonstrate less red colour.

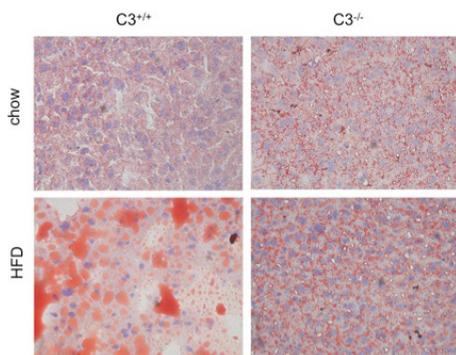


Figure 3. Representative images of Oil red O (ORO) staining of liver sections, showing hepatic steatosis in $C3^{+/+}$ and $C3^{-/-}$ mice fed a chow diet (upper panel). Lower panel: representative images of ORO staining showing hepatic steatosis in $C3^{+/+}$ and $C3^{-/-}$ mice fed a HFD. Magnification at 40x.

Scoring of the ORO stainings resulted in a subdivision of four scoring groups, each with a different degree of triglyceride accumulation. The resulting scoring of the stainings is displayed in table 1. Group 0 showed a relatively normal liver morphology, with a slightly red colour, indicating little triglyceride accumulation (n=7). Group 1 demonstrated the first signs of hepatic steatosis (n=6). Group 2 displayed little macrovesicular lipid droplets (n=9). Group 3 showed larger macrovesicular lipid droplets (n=11).

Table 1. Results scoring ORO stainings in $C3^{+/+}$ and $C3^{-/-}$ mice fed a HFD or chow diet.

	$C3^{+/+}$		$C3^{-/-}$	
	Chow (n=6)	HFD (n=11)	Chow (n=6)	HFD (n=10*)
Score 0 (n=7)	3	1	2	1
Score 1 (n=6)	0	3	2	1
Score 2 (n=9)	3	0	2	4
Score 3 (n=11)	0	7	0	4

* Mouse 16 of $C3^{-/-}$ HFD group was not present.

In table 2 an overall summary of body parameters of $C3^{+/+}$ and $C3^{-/-}$ mice is given.

Table 2. Effect of HFD and chow diet for 12 weeks on body parameters of C3^{+/+} and C3^{-/-} mice.

	C3 ^{+/+}		C3 ^{-/-}	
	Chow (n=6)	HFD (n=11)	Chow (n=6)	HFD (n=11*)
Body weight gain (g)	3,74 ± 1,59	7,21 ± 4,12	3,49 ± 0,91	8,39 ± 3,86
Liver/body weight %	4,53 ± 0,27	3,79 ± 0,67	4,12 ± 0,13	3,40 ± 0,36
Steatosis score (0–3)	1,5 ± 0,866	2,750 ± 1,548	1,5 ± 0,500	2,5 ± 0,866

* n=10 for Steatosis score, mouse 16 of C3^{-/-} HFD group was not present.

mRNA expression of genes involved in lipid metabolism of adipose tissue in C3^{+/+} and C3^{-/-} mice fed a HFD or chow diet

To understand the involvement of the lipid metabolism of adipose tissue in the development of NASH, the expression of several genes involved in fatty acid synthesis, degradation and development, was examined in relation to hepatic steatosis in mice. Messenger RNA (mRNA) expression of some adipose tissue enzymes involved in lipid metabolism was significantly different between the genotype of mice and between the diets the mice received.

The expression of the enzymes FASN and SCD1, important for lipid synthesis, was significantly higher in the chow diet group compared to the HFD in both mice genotypes for FASN and in the C3^{-/-} mice group for SCD1 (Fig. 4, $p < 0,0001$ and $p = 0,0024$, respectively). The expression of the adipogenic gene PPAR γ was significantly higher in the HFD compared to the chow diet in the C3^{+/+} mice group (Fig. 4, $p = 0,0420$). The statistical analysis of the PPAR γ expression revealed an interaction between the diets and genotypes, which means that the effects of diets were significantly different between C3^{+/+} mice, where a HFD results in higher expression of PPAR γ compared to C3^{-/-} mice, where the chow diet leads to a higher expression level ($p = 0,0138$). Gene expression of FASN, SCD1 and PPAR γ was not significantly different between the two mouse groups.

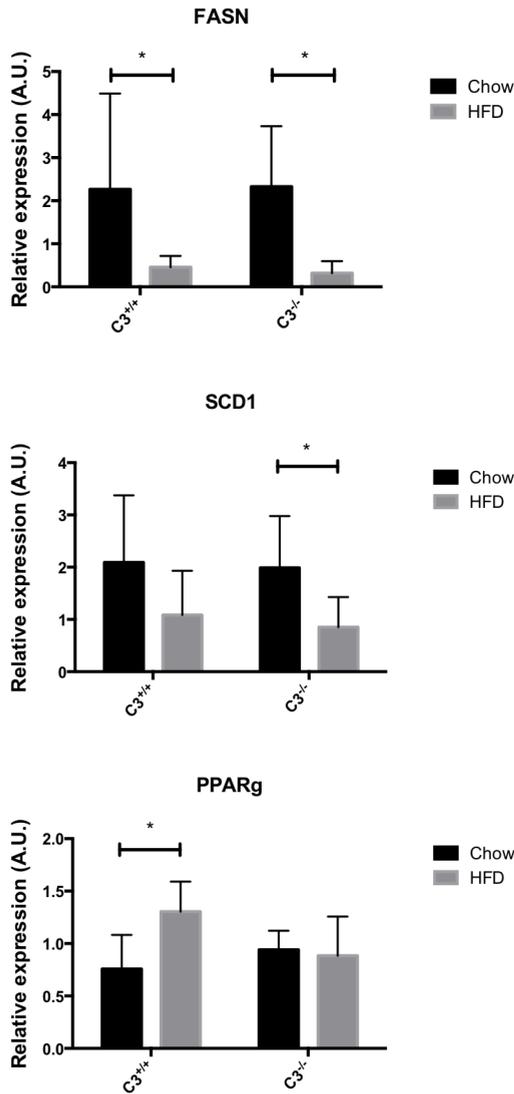
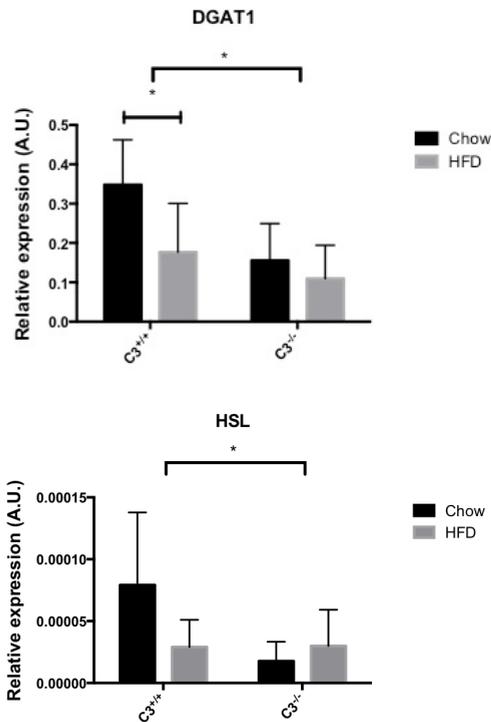


Figure 4. Adipose tissue gene expression of FASN, SCD1 and PPAR γ . The expression of FASN was significantly higher in both mice groups, which received the chow diet. The expression of SCD1 was significantly higher for the chow diet in the $C3^{-/-}$ mice group. For PPAR γ the expression was higher in the $C3^{+/+}$ mice group, which received the HFD. The statistical analysis of PPAR γ showed a significant interaction between the diets and mouse genotypes.

The expression level of the enzyme DGAT1, also involved in lipid synthesis differed significantly between both mouse groups and was, in the $C3^{+/+}$ mice group, significantly higher in the chow diet group compared to the HFD (Fig. 5, $p=0,0024$ and $p=0,0094$, respectively). For the enzyme HSL, involved in adipose lipolysis, the expression was significantly different between the two mouse groups (Fig. 5, $p=0,0138$). Both the statistical analysis of HSL and LPL revealed a significant interaction between the diets and genotypes. The effects of the diets were significantly different between $C3^{+/+}$ mice, where a HFD results in lower expression of HSL compared to $C3^{-/-}$ mice, where the chow diet leads to a lower expression level ($p=0,0116$). For the enzyme LPL, the HFD resulted in a higher expression in $C3^{+/+}$ mice compared to $C3^{-/-}$ mice, whereby the chow diet resulted in a higher expression level ($p=0,0286$).



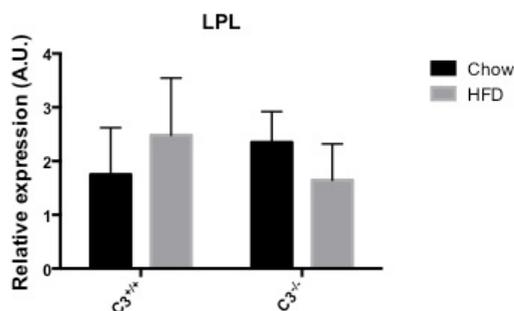


Figure 5. Adipose tissue gene expression of DGAT1, HSL and LPL. Expression levels of both DGAT1 and HSL differed significantly between the two mouse genotypes and the expression of DGAT was significantly higher in the C3^{+/+} mice group, which received the chow diet. Statistical analysis of both HSL and LPL showed a significant interaction between the diets and mouse genotypes.

Discussion/Conclusion

In the present study, C57Bl/6 mice fed a HFD, were used as representative animal model for human hepatic steatosis. To examine the influence of complement factor C3 on hepatic lipid accumulation and genes involved in lipid metabolism of adipose tissue, C3 knock out and C3 wild type mice were used. Thus the data obtained during this research demonstrate the different effects of a HFD and chow diet on the presence of steatosis and alterations in genes involved in lipid metabolism of adipose tissue, in C3^{+/+} and C3^{-/-} mice. As expected high fat feeding resulted in higher weight gain in mice compared to chow fed mice (Fig 1).

Histological stainings and steatosis scoring

Taking into account the scores of hepatic lipid accumulation after ORO staining, C3^{-/-} mice did not have significantly lower mean scores for both diets compared to C3^{+/+} mice. This finding is in contrast with the hypothesis and earlier found results, which stated that mice deficient in C3 have significantly lower steatosis scores in NASH [11]. From the results can be derived that there is no significant difference in steatosis scores between the HFD and chow diet. There is, however, a trend visible towards higher steatosis scores in the HFD groups (Table 2). From the histological staining images can be seen that both mice groups on the HFD have the highest accumulation of lipids in their liver, which corresponds with preliminary steatosis scores in NASH. From the images can be concluded that the HFD indeed triggers mild steatosis in the liver.

Because there is also lipid accumulation present in C3^{-/-} mice on a HFD, it appears that lipids can also accumulate in the liver without C3 activation. The complement system has both

detrimental and beneficial effects in the liver. On the one hand activation of complement results in inflammation and tissue damage, on the other hand it is involved in the clean-up process of dead cells. In $C3^{-/-}$ mice, the protective function of the complement system may be absent, which could contribute to hepatic lipid accumulation in $C3^{-/-}$ mice fed a HFD [11]. In addition, $C3$ activation normally results in the formation of $C3a$, which is converted to ASP. This protein clears postprandial triglycerides and promotes the uptake of FFA to peripheral fat tissue. Research states that disturbances in the ASP pathway will result in hypertriglyceridemia and an increased flux of FFAs to the liver, leading to hepatic steatosis [2].

mRNA expression levels of lipid genes in adipose tissue

There exists a relation between $C3$ activation and lipid metabolism. Two pathways play a role herein. Firstly, the expression of the $C3a$ receptor ($C3aR$) is very high in white adipose tissue and up regulated after a HFD. In mice deficient in this $C3aR$, no insulin resistance occurs [14]. Secondly, also in $C5a$ -like receptor 2 ($C5L2$) knockout mice, multiple metabolic effects are present, such as reduced triacylglycerol synthesis in adipose tissue and delayed clearance of triacylglycerol and glucose. ASP resistance, due to impaired signalling of ASP via $C5L2$, plays an important role in the altered lipid metabolism in adipose tissue [14]. ASP deficient mice are resistant to diet-induced obesity and insulin resistance, and have decreased adipose tissue depots [2,15]. This is also observed in figure 1, where the $C3^{-/-}$ mice exhibit lower weight gain in both the HFD and chow diet group compared to the $C3^{+/+}$ mice.

In this study, mRNA expression of several enzymes involved in lipid metabolism was examined to investigate a possible link between altered lipid metabolism in adipose tissue and $C3$ /ASP deficiency. Expression levels of FASN were significantly higher in both mice groups, and expression of SCD1 was significantly higher in $C3^{-/-}$ mice, fed a chow diet compared to mice fed a HFD. These results do not correspond to the stated hypothesis, which expected that expression of FASN and SCD1 would be higher in high fat feeding mice, due to the stimulating effect on lipogenesis by ASP. The expression of PPAR γ was significantly higher in the $C3^{+/+}$ mice that received a HFD in contrast to the chow diet. This is in accordance with the hypothesis, which stated that mice having NAFLD should have higher expression of PPAR γ . Mice deficient in $C3$ have abnormal lipid metabolism and delayed triglyceride clearance [11,15]. Since mice deficient in $C3$ cannot generate ASP, the effects of ASP on lipid metabolism are diminished. Normally ASP inhibits HSL and stimulates triglyceride synthesis via DGAT. Both effects are lacking in $C3^{-/-}$ mice, which will result in higher expression of HSL and down regulation of DGAT. Our results show that expression of DGAT is indeed significantly lower in $C3^{-/-}$ mice but the expression of HSL is also significantly lower, contradictory to this literature.

However, other literature describes that subjects with NAFLD have higher adipose tissue lipolysis rate [5]. This effect is contradictory to the inhibitory effect of ASP on HSL. Our data show that adipose tissue expression of HSL is significantly higher in the $C3^{+/+}$ mice group compared to the $C3^{-/-}$ mice group. This corresponds with the earlier stated hypothesis, which expected an increased expression of enzymes involved in lipolysis such as HSL. The expression of the enzyme DGAT1 is higher in mice that received the chow diet compared to mice that received the HFD, this is not in agreement with the hypothesis, which expected higher expression of lipogenic genes in $C3^{+/+}$ mice fed a HFD.

Since the expression of HSL is higher in $C3^{+/+}$ mice instead of $C3^{-/-}$ mice, the inhibitory effect of ASP on HSL is exceeded by the higher lipolysis rate present in $C3^{+/+}$ mice with NAFLD.

Because the expression of HSL and DGAT1 is higher in adipose tissue of $C3^{+/+}$ mice, there will be an elevated flux of fatty acids to the bloodstream and eventually the liver, as depicted in the figure below (Fig. 6).

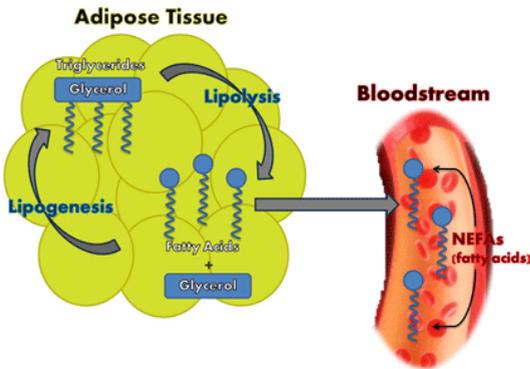


Figure 6. Enzymes involved in lipogenesis and lipolysis of adipose tissue. Both HSL and DGAT1 show higher expression levels in $C3^{+/+}$ mice, resulting in higher flux of fatty acids to the bloodstream.

In conclusion, analysis of histological stainings and preliminary scoring results indicated that $C3^{+/+}$ mice on a HFD display the highest hepatic lipid accumulation. Analysis of mRNA expression levels of lipid genes showed that adipose tissue expression levels of DGAT1 and HSL were higher in the $C3^{+/+}$ mice group, which corresponds with literature. The expression of the transcription factor PPAR γ was higher in adipose tissue of mice fed a HFD, in agreement with the literature.

These data support a link between adipose tissue dysfunction and progression of hepatic steatosis, and the role of the enzymes HSL and DGAT herein.

Role of the student

Esmee Moreau was an undergraduate student in Biomedical Sciences working under the supervision of Dr Sander Rensen when the research in this report was performed. The supervisor proposed the topic and practical experiments. The practical work, statistical processing of the data as well formulation of the conclusions and the writing were done by the student.

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