

## Perinatal programming of metabolic dysfunction and obesity-induced inflammation

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# Perinatal programming of metabolic dysfunction and obesity-induced inflammation

PhD Thesis

Camilla Ingvorsen

2013

## Preface

This thesis is the outcome of a 3 year PhD study of perinatal programming of metabolic dysfunction and obesity-induced inflammation and written in order to fulfill one of the requirements for obtaining a PhD degree at the Technical University of Denmark (DTU).

The project has primarily been conducted at Centre for Biological Sequence Analysis, Department of Systems Biology at DTU, Lyngby, Denmark. However, the project is a part of the animal research pillar in Centre for Fetal Programming (Strategic Research Council Grant no 09-067124). I also spend 9 months in Dr. Sue Ozannes lab at Institute of Metabolic Science, University of Cambridge, UK. The research was funded by Centre for Fetal programming, Centre for Biological Sequence Analysis DTU, Master of Engineering Frants Allings foundation, Otto Mønsted foundation, Aase and Ejnar Danielsen's foundation and Reinholdt W. Jorck and wife's foundation.

August 2013, Copenhagen, Denmark

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Camilla Ingvorsen



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Many people have contributed to this project and I will express my sincere gratitude to all who put hours, expertise and hard work into this project.

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Then I need to thank Jannie F. Agersten for running innumerable qPCR plates and lipid analyses for me; Anne-Marie Nepper also for running innumerable lipids analyses and for helping out with the animal experiments; Pernille Wehler Gülich for taking care of all the animals; Jeppe Madura Larsen and Anna Hammerich Thysen for their help in optimizing and running the immunological experiments; Khoa Nguyen Do for letting me borrow various equipments at DMAC and for keeping my badminton skills up to date; Lisbeth Buus Rosholm for her excellent technical assistance in lab, for making sure that the weekly cake intake was balanced out with badminton and runs and for ensuring fun and games, so everything doesn't become too scientific; and thanks to all my colleagues in the System Biology of Immune Regulation- group, at Centre for Biological Sequence analysis and at Centre for Fetal Programming for good company and sparring.

Then I want to thank Sue Ozanne for inviting me to her lab and for sparring on the project. From Institute of Metabolic Science is also would like to thank: Denise Fernandez-Twinn for helping with the animal experiment; Keli Phillips for training me in histology and immunohistochemistry; Adrian P. Wayman for taking care of my animal; Sam Virtue, Maarten Soeters and Chong Yew Tan for many fruitful scientific discussions and off cause colleagues in the Ozanne lab and the lunch team for taking good care of me during my time in Cambridge

I also would like to thank my collaborators on the project: Pernille Nordby and Kristian Fog Nielsen (DTU-Systems Biology) for running lipid analyses on the mice study; Karsten Kristiansen (Copenhagen University) and Beijing Genomic Institute for running epigenome sequencing on adipose tissues from the rat studies; Anders Bergström and Bodil Madsen at DTU-FOOD and my two bachelor students Steffen Pedersen and Natasha Yde Larsen for analysing microbiota composition on faecal samples from the rat studies; Knud Josefsen and Morten Schou (Copenhagen University) for letting me borrow their histology lab.

Then I want to thank Sarah Barnes, Marie Kragh and Rikke Mette Guldhammer Bennike for reading and giving me feedback on the introduction to the thesis

Finally, and most importantly, I would like to thank my family and friends for their endless support. They are the reason I still have my head above water.



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## Abstract

The number of obese women in the childbearing age is drastically increasing globally. As a consequence, more children are born by obese mothers. Unfortunately, maternal obesity and/ or high fat intake during pregnancy increase the risk of developing obesity, type-2 diabetes, cardiovascular disease and non-alcoholic fatty liver disease in the children, which passes obesity and metabolic dysfunction on from generation to generation. Several studies try to elucidate causative effects of maternal metabolic markers on the metabolic imprinting in the children; however diet-induced obesity is also associated with chronic low grade inflammation. Nobody have yet investigated the role of this inflammatory phenotype, but here we demonstrate that obesity induced inflammation is reversed during pregnancy in mice, and is therefore less likely to affect the fetal programming of metabolic dysfunction. Instead, we suggest that an early elevated lipid exposure caused by a maternal high fat feeding might be more important for long term metabolic imprinting in the offspring. Therefore, we study the effect of maternal high fat/high sucrose diet during gestation, lactation or both to elucidate if perinatal adaptations to a high fat/high sucrose diet makes the offspring more capable of dealing with a high fat diet later in life. We demonstrate that a dietary mismatch between pre- and post-natal life alters the phenotype in an obese prone rat model at weaning. Thus, exposure to a control diet *in utero* and a high fat/high sucrose diet during lactation cause more severe phenotypic alteration in the offspring at weaning than pups exposed to the high fat/high sucrose diet both *in utero* and during lactation. The same pattern is seen in the adult offspring after being challenged with a high fat diet for 6 weeks. However HFHS exposure during fetal life protected against hyperleptinemia in the adult offspring during the challenge. Additionally, offspring expose to high fat/high sucrose diet during lactation displayed a decrease level of inflammatory genes in the blood, which could indicated that perinatal HFHS exposure protect against the detrimental effects of high fat feeding leading to metabolic disease.



## Resumé

Antallet af overvægtige kvinder i den fødedygtige alder er drastisk stigende på globalt plan. Som følge heraf fødes flere børn af overvægtige mødre. Desværre, vil moderens forøgede indtag af fedt og sukker under graviditeten øge barnets risiko for at udvikle fedme, type 2-diabetes, hjerte-kar sygdomme og ikke-alkoholisk fedtlever, hvilket fører fedme og metabolisk dysfunktion videre fra generation til generation. Adskillige studier forsøger at belyse årsagen til at moderens metaboliske markører fører til metabolisk prægning i børnene; men, kost-induceret fedme er også forbundet med en kronisk lav inflammation. Ingen har endnu undersøgt rollen af denne inflammatoriske fænotype, men her viser vi, at fedme-induceret inflammation mindskes under graviditet i mus og derfor er mindre tilbøjelig til at påvirke den føtale programmering af metabolisk dysfunktion. I stedet antyder vi, at en tidlig forhøjet fedt-eksponering, forårsaget af moderens fedt-indtag, kan være vigtigere for langsigtet metabolisk prægning hos afkommet. Derfor undersøger vi effekten af et højt fedt og sukker indtag hos moderen under graviditet, diegivning eller begge, for at undersøge om perinatal tilpasninger til et højt fedt og sukker indtag gør afkommet bedre i stand til at håndtere en fedt-rig kost senere i livet. Vi påviser, at et misforhold mellem præ- og postnatal miljø ændrer fænotypen i en rottemodel, der er tilbøjelig til at udvikle fedme, ved fravæning. Således vil en eksponering for et kontrol kost i fosterlivet, men en kost rig på fedt og sukker under diegivning, forårsage mere alvorlige fænotypisk ændringer i afkommet ved fravæning, end unger udsat for forhøjet fedt og sukker i både fosterlivet og under diegivning. Det samme mønster ses i de voksne unger efter at være eksponeret for en fedt-rig kost i 6 uger. Dog beskytter eksponeringen af fedt og sukker i fosterlivet mod forhøjet leptin niveauer i de voksne unger, når de bliver fodret med en fedtholdig kost. Yderligere, unger der har diet ved en mor med højt fedt og sukker indtag viser et fald i udtryk af inflammatoriske gener i blodet, hvilket kan indikere, at perinatal fedt og sukker eksponering beskytter mod de skadelige virkninger af en fedt-rig kost, der kan føre til metaboliske sygdomme.



## List of papers included in the thesis

- Ingvorsen C, Brix S, Ozanne SE, Hellgren LI (2013) The effect of maternal inflammation on fetal development and the risk of metabolic disease later in life. *In preparation*
- Ingvorsen C, Thyssen AH, Fernandez-Twinn D, Nordby P, Nielsen KF, Ozanne SE, Brix S, Hellgren LI (2013) Effects of pregnancy on obesity-induced inflammation in a mouse model of fetal programming. *Submitted to International Journal of Obesity*
- Ingvorsen C, Hellgren LI (2013) Effect of maternal high fat/high sucrose intake during gestation and/or lactation on metabolic markers of hepatic lipid metabolism at weaning. *In preparation*
- Ingvorsen C, Hellgren LI (2013) The impact of maternal pre- and post-natal intake of high fat/high sucrose diet on metabolic adaptability to a high fat diet in adult rat offspring. *In preparation*
- Ingvorsen C, Larsen JM, Thyssen AH, Hellgren LI, Brix S (2013) Maternal high fat/high sucrose feeding during lactation alters systemic and adipose tissue inflammation in young and adult rat offspring. *In preparation*





## Presentation of work at scientific meetings

The results from the thesis have also been presented at the following:

- The role of endotoxemia in adipose tissue inflammation. Oral presentation at *DanORC-Young Investigators Network*. Institute of Preventive Medicine, November 2010
- Leptin levels in the young offspring from dams fed hypercaloric diets during gestation are decoupled from body-weight, regardless of maternal post-natal diets. Poster presentation at a scientific meeting in *Centre for Fetal Programming*, Statens Serum Institut, May 2011
- Effects of maternal high energy intake during gestation and lactation on leptin levels in the young and adult pups. Oral presentation at the annual meeting in *Dansk Selskab for Adipositasforskning* (In English: The Danish society for the Study of Obesity), Odense, November 2011
- Maternal energy intake during lactation and gestation has an effect on leptin levels in the offspring. Poster presentation at *Symposium for Biotech Research*, DTU- Systems Biology, November 2011
- Fetal programming of metabolic syndrome in rat offspring exposed to maternal overnutrition. Oral presentation at *Academic Open Mic*. DTU-Systems Biology, November 2011
- Maternal high-fat/high-sucrose diet during lactation results in increased adipose tissue mass, and altered hepatic fatty acid metabolism at weaning. Poster presentation at *Benzon symposium no. 58 Adipose tissue in health and disease*. Copenhagen, August 2012.
- Maternal high-fat/high-sucrose diet during lactation results in increased adipose tissue mass, and altered hepatic fatty acid metabolism at weaning. Poster presentation at *Symposium for Biotech Research*, DTU- Systems Biology, November 2012.
- Gestation modulates the inflammatory response to an obesogenic diet in mice. Oral presentation at the weekly seminar at *DTU-Systems Biology*, November 2012

- Gestation reverses obesity-induced hepatic inflammation in mice. Poster presentation at the *Joint symposium of Centre for Fetal programming and Early Nutrition Consortium: Fetal and childhood programming, Preventing type 2 generations in the next generation*, Hellerup, March 2013. Poster award winner.
- Effect of gestation on obesity-induced hepatic and placental inflammation in mice. Poster presentation at *Abcam symposium: Programming obesity: Central and peripheral contributors*. Cambridge, UK, April 2013
- Obesity-induced hepatic and placental inflammation are absent in obese gestating mice compared to control fed. Poster presentation at *European Congress of Endocrinology*, Copenhagen, May 2013.

## Popular science communication

- Pitch your PhD in 3 minutes. Oral presentation in the science communication competition for PhD students at part of the official opening of *Forskningens Døgn* (In English: Festival of Research), Copenhagen City Hall, May 2011. Awarded a 3<sup>rd</sup> price as communication talent of the year.
- Bliver vores krop programmeret til overvægt? (In English: Are our bodies programmed to obesity?) Oral presentations at Naturvidenskabsfestival (In English: Festival of Natural Science) at 2 lower and 2 upper secondary schools in the Copenhagen area, September 2012



## Abbreviations

ACACA	Acetyl-CoA carboxylase
ACOX	Acyl-CoA oxidase
Arg-1	Arginase-1
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
BMI	Body mass index
BSA	Bovine serum albumin
CCL2	C-C motif ligand 2
CCR2	C-C chemokine receptor type 2
CD	Cluster of differentiation
Cera	Ceramide
ChREBP	Carbohydrate-responsive element-binding protein
CLS	Crown like structure
CPT1	Carnitine palmitoyltransferase 1
CRP	C reactive protein
CVD	Cardio-vascular disease
DAG	Diacylglyceride
DC	Dendritic cell
DHA	Docosahexaenoic acid
DNL	De novo lipogenesis
DPA	Docosapentaenoic acid
ELOVL	Fatty acid elongase
EPA	Eicosapentanoic acid
ER	Endoplasmatic reticulum
FA	Fatty acid
FABP	Fatty acid binding protein
FADS	Fatty acid desaturase
Fasn	Fatty acid synthase
FAT	Fatty acid translocase
FATP	Fatty acid transport protein
FFA	Free fatty acid
GD	Gestation day
Gluc	Glucose
GLUT4	Glucose transporter type 4

GOSR1	Golgi SNAP receptor complex member 1
hCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HFHS	High fat/high sucrose
HSL	Hormone sensitive lipase
IDL	Intermediate lipoprotein
IFN	Inferon
IKK	IκB kinase
IL	Interleukin
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinases
KC	Chemokine ligand 1
LAL	Limulus Amebocyte Lysate
LC-PUFA	Long chain polyunsaturated fatty acids
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LPS	Lipopolysaccharid
MAG	Monoacylglyceride
MCP	Monocyte chemotactic protein
MGL	Monoacylglycerol lipase
Mono	Monocyte
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
Neu	Neutrophil
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH cell	Natural helper cell
NK cell	Natural Killer cells
NLRP3	NOD-like receptor 3
Nos	Nitric oxide synthase
OPN	Ostepontin
PBMC	Peripheral blood mononuclear cell
PC	Principal component
PCA	Principal component analysis
Pck1	Phosphoenolpyruvate carboxykinase 1
PFA	Paraformaldehyde

PHA	Phytohaemagglutinin
PIBF	Progesterone-induced binding factor
PL	Phospholipid
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
QUICKI	Quantitative insulin sensitivity check index
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
ROS	Reactive oxygen species
SCD1	Stearoyl-CoA desaturase 1
SCFA	Short chain fatty acid
srebf1	Sterol Regulatory Element-Binding factor
TAG	Triacylglyceride
TCA	Tricarboxylic acid
TLR	Toll like receptor
TNF	Tumor necrosis factors
TRAIL	TNF-related apoptosis inducing ligand
Treg	Regulatory T cell
ucp1	Uncoupling protein 1
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
$\beta$ -ox	$\beta$ -oxidation
$\Delta$ 5	$\Delta$ 5-desaturase
$\Delta$ 6	$\Delta$ 6-desaturase





*“Nothing in biology makes sense except in the light of evolution”*

-Theodosius Dobzhansky, 1973

## 1. Introduction

In nature, obesity occurs in Svalbard reindeers, seals and polar bears, which makes them capable of dealing with their harsh cold habitat<sup>1</sup>. Thus, for these animals, obesity is a survival strategy. In the human populations, however, obesity leads to metabolic dysfunction and diseases, which eventually shortens lifespan. Today, 65% of the world's population lives in countries where overnutrition kills more people than undernutrition<sup>2</sup> making obesity the biggest threat to human health. Many initiatives have been made to elucidate the underlying mechanisms behind this global obesity epidemic. In the late 80's, the first studies emerged that suggested that obesity and impaired metabolic function had origin in fetal development<sup>3</sup>. Since then, growing evidence has demonstrated that maternal overnutrition increases the risk of obesity and the metabolic syndrome in the offspring in adulthood<sup>4</sup>. Metabolic dysfunction is associated with a chronic low-grade inflammatory phenotype<sup>5</sup>, but to our knowledge nobody has yet explored the role of obesity-induced inflammation in fetal programming of metabolic syndrome.

The aims of this thesis were:

1. To investigate how maternal obesity-induced inflammation is affected by pregnancy in mice.
2. To investigate if maternal high fat/high sucrose (HFHS) diet during perinatal life affects the offspring's metabolic and inflammatory phenotype in a rat model at weaning
3. To investigate if maternal high fat/high sucrose (HFHS) diet during perinatal life affects the offspring's metabolic flexibility in a rat model when challenged with a high fat diet in adulthood.

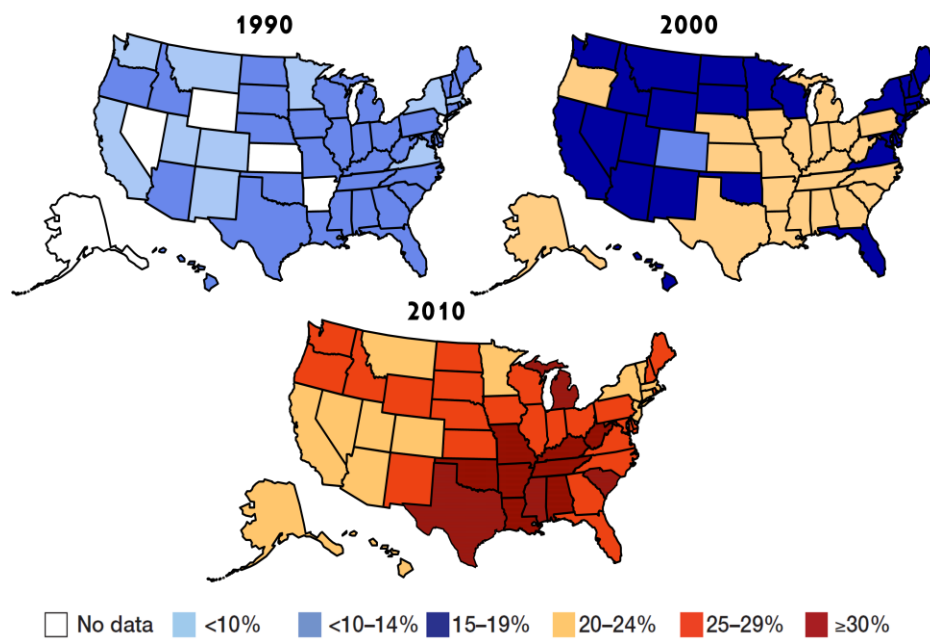
The first part of this thesis focuses on the maternal phenotype and consists of a review (Chapter 2) and mouse study (Chapter 3). In the review, we surveyed the literature to set up a hypothesis regarding the role of maternal obesity-induced inflammation in fetal programming of the metabolic syndrome. In the mouse study, we investigated the effect of pregnancy on maternal obesity-induced inflammation in an established mouse model fetal programming.

The second part of this thesis focuses on the offspring's phenotype and consists of two studies performed in a rat model of maternal high fat/high sucrose feeding (Chapter 4-6). In the first

study, we explored the effect of maternal HFHS intake during gestation and/or lactation on the metabolic (Chapter 4) and inflammatory phenotype (first part of Chapter 6) in the young offspring terminated at weaning. In the second study, we investigated how the offspring's metabolism (Chapter 5) and immune system (second part of chapter 6) coped with a high fat dietary challenge in adulthood after exposure to a maternal HFHS diet during gestation and/or lactation.

## **Obesity and the metabolic syndrome**

Obesity has become a pandemic of the 21<sup>st</sup> century. Obesity and the development of the metabolic syndrome has been a health concern in the western world for decades (Figure 1). Today the world is also experiencing a rise in the prevalence of obesity in developing countries. On a global scale, 1.4 billion people were overweight (BMI>25) in 2008, of those 500 million were considered obese (BMI>30)<sup>2</sup>. It is self-evident, that these numbers are alarming, but of more concern is the rise in obesity among children under the age of 5, especially in the developing world with 30 million overweight children<sup>2</sup>. Childhood obesity is a risk factor for development of the metabolic syndrome already before the onset of puberty<sup>6,7</sup>. The metabolic syndrome is defined as a cluster of symptoms; central obesity, hypertension, dyslipidemia (elevated triacylglyceride, TAG, and reduced high density lipoprotein (HDL)-cholesterol) and hyperglycemia, (Figure 2) which increase the risk of developing type 2 diabetes and cardiovascular disease (CVD)<sup>8</sup>. In clinic, metabolic syndrome is diagnosed when 3 out of the 5 criteria are present. Interestingly, waist circumference ratio is population- and country-specific, and it is speculated that genetic variations among ethnic groups might explain why Asian people tend to have an increased risk of diabetes at a lower BMI compared to Caucasians<sup>7,9</sup>. Currently, theories are being investigated, to elucidate the etiology of the obesity pandemic. In 1988, Barker and Osmond published a study that showed an inverse relationship between birth weight and hypertension later in life<sup>3</sup>. In 1992, Hales and Barker proposed a hypothesis, which suggested that early malnutrition could have long-term consequences and be linked to obesity and metabolic dysfunction in adulthood<sup>10,11</sup>.



**Figure 1. Obesity tendency among adults in the US.** Obesity has become a pandemic in the 21<sup>st</sup> century, no longer only affecting the western world but also to a large extent the developing countries. The colour code indicates the percentage of the population that have a BMI > 30. From Centers for Disease Control and Prevention<sup>12</sup>

### Metabolic syndrome

The presence of 3 out of the following 5 symptoms would diagnose a patient with metabolic syndrome

1. **Elevated waist circumference** (population- and country-specific definition) \*
2. **Elevated triglycerides** ( $\geq 150$  mg/dl or  $\geq 1.7$  mM)
3. **Reduced HDL-cholesterol** ( $< 40$  mg/dL or  $< 1.0$  mM for men,  $< 50$  mg/dL or  $1.3$  mM for women)
4. **Elevated blood pressure** (Systolic  $\geq 130$  mm Hg and/or diastolic  $\geq 85$  mm Hg)
5. **Elevated fasting glucose** ( $\geq 100$  mg/dL or  $5.6$  mM)

\* The values for Europeans are  $\geq 94$ cm for men,  $\geq 80$ cm for women

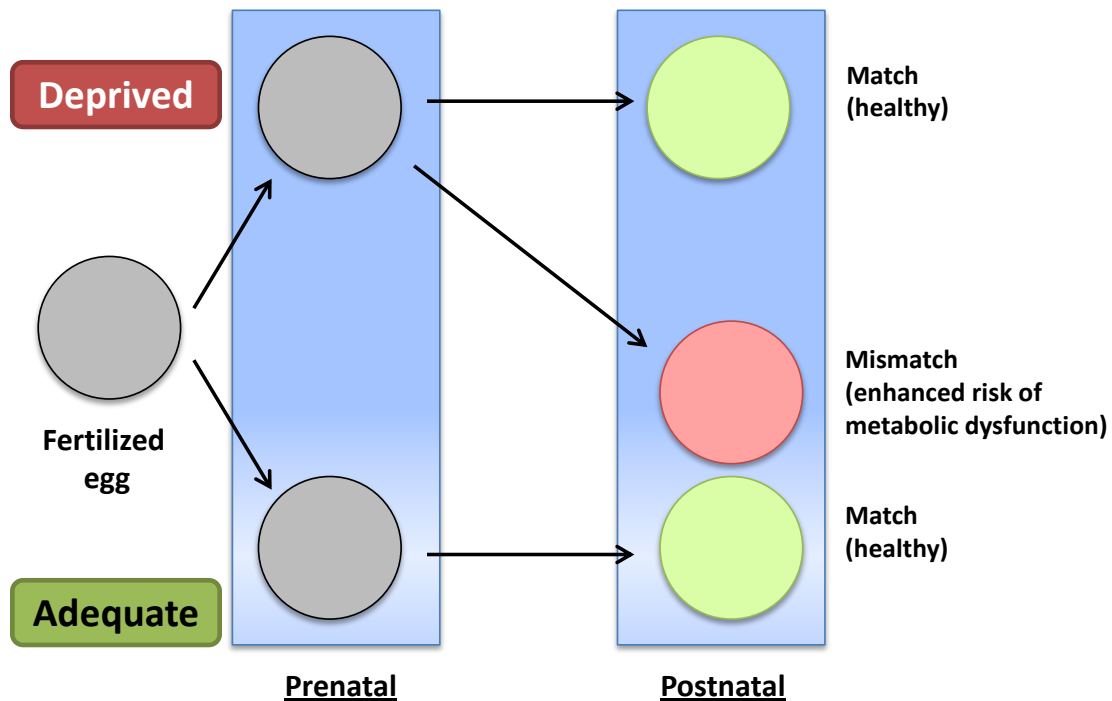
**Figure 2. Cut-off values for the five symptoms which constitutes the metabolic syndrome<sup>8</sup>.**

## **Fetal programming of metabolic syndrome**

Fetal programming is defined as a maternal stimulus or insult during a critical time of development (gestation or early life), which permanently affects the offspring's physiology, structure or metabolism<sup>13</sup>. The initial research within fetal programming evolved around maternal undernutrition and protein restriction during pregnancy and many of the hypotheses regarding programming of metabolic dysfunction focus on maternal undernutrition. Recently, malnutrition due to overnutrition has been investigated as a maternal insult. To understand the framework of fetal programming research, an introduction to one of the first epidemiological studies involving maternal undernutrition and the hypotheses that arose is relevant.

### **The historical background**

Famine in the Netherlands was a tragic consequence of World War 2, but it led to a unique cohort of individuals that were exposed to undernutrition during pregnancy<sup>14</sup>. Children born during and after the Dutch famine demonstrated that uterine undernutrition led to an increased prevalence of obesity, hypertension, CVD, impaired glucose tolerance and dyslipidemia<sup>14</sup>. Additionally, it was noticed that the different symptoms were linked to specific developmental stages, so that famine exposure in either the first or second trimester resulted in different metabolic phenotypes in the children<sup>15</sup>. In 1992, Hales and Barker explained these results by "the thrifty phenotype hypothesis" which suggested that poor nutrition during perinatal life has a detrimental effect on the offspring, leading to a higher risk of type 2 diabetes<sup>16</sup> and the metabolic syndrome<sup>17</sup>. In the wake of the thrifty phenotype hypothesis, Gluckman and Hanson hypothesised that a foetus is capable of "sensing" the environment surrounding the mother, and based on the maternal stimuli, predicts the post-natal environment. The foetus can then adapt its metabolism and physiology to ensure survival after birth ("predictive adaptive response")<sup>18</sup>. E.g. if the offspring is exposed to undernutrition during fetal life, it can adapt a metabolic set point that is more suitable for a post-natal life with limited food availability to ensure survival<sup>19</sup>. Developmental plasticity has probably been an excellent strategy for survival in mammals through changing environments, but it has been suggested that metabolic disease might have arisen due to a mismatch between the predicted environment and the actual post-natal life<sup>18,19</sup>.



**Figure 3: The predictive adaptive response hypothesis.** The hypothesis states that a foetus is capable of adapting its metabolism, physiology or structure to ensure survival, by predicting the post-natal environment based on maternal stimuli during pregnancy. From Gluckman et al. 2007<sup>19</sup>

### Maternal obesity

There is a clear inverse association between birth weight and the risk of developing obesity and the metabolic syndrome. However, the curve is not linear, it is U shaped, demonstrating that children born either small or large for gestational age have increased risk of metabolic dysfunction<sup>20</sup>. Elevated birth weight and exposure to maternal obesity during fetal life are equally strong predictors of the metabolic syndrome<sup>6</sup> and several epidemiological studies have linked maternal obesity to an increased risk of obesity in the children<sup>21-23</sup>. In light of the increasing prevalence of obesity among women of childbearing age<sup>24</sup>, it is important to elucidate the mechanisms behind metabolic dysfunction in children exposed to maternal overnutrition to avoid further acceleration of the obesity pandemic. The effect of maternal obesity and high fat feeding on the metabolic imprinting of the offspring has been studied in-depth in various animal models. A brief overview of the various metabolic outcomes in the offspring is listed in table 1. Studies with relevance to results reported in this thesis are described in greater detail in the following chapters.

**Table 1. The effect of maternal obesity or high fat diets on the offspring's risk of developing obesity and metabolic dysfunction**

<b>Maternal phenotype</b>	<b>Model</b>	<b>Outcomes in the offspring</b>
<b><u>Obesity</u></b>	<i>Mouse</i>	hyperphagia <sup>25</sup> , ↑body weight <sup>25-27</sup> , ↑adiposity <sup>25,27</sup> , ↑plasma leptin <sup>25,26</sup> , ↑plasma insulin <sup>25,26</sup> , ↑blood glucose <sup>25</sup> , ↑plasma TAG <sup>25</sup> , impaired glucose homeostasis <sup>25</sup> , hypertension <sup>25</sup> , hepatic lipid accumulation <sup>26,27</sup> , hepatic inflammation and fibrosis <sup>26,27</sup> , reduced pancreatic beta-cell function <sup>28</sup>
	<i>Rat</i>	hyperphagia <sup>29</sup> , ↑body weight <sup>29-31</sup> , ↑adiposity <sup>29,30,32</sup> , ↑plasma leptin <sup>29,33</sup> , ↑plasma insulin <sup>31,33</sup> , ↑blood glucose <sup>30,31</sup> , ↑plasma TAG <sup>30,31</sup> , ↑plasma FFA <sup>29-31</sup> , Impaired glucose homeostasis <sup>29,30</sup> , hypertension <sup>32</sup>
	<i>Sheep</i>	↑adiposity <sup>34</sup> , Impaired glucose homeostasis <sup>34</sup> , reduced pancreatic beta-cell number and function <sup>35</sup>
<b><u>High fat diets</u></b>	<i>Mouse</i>	↑body weight <sup>36</sup> , dyslipidemia <sup>36</sup> , hepatic lipid accumulation <sup>36</sup> , impaired glucose homeostasis <sup>36</sup>
	<i>Rat</i>	↑body weight <sup>37,38</sup> , ↑adiposity <sup>37,39,40</sup> , ↑plasma leptin <sup>37,38</sup> , ↑plasma insulin <sup>37,40</sup> , ↑blood glucose <sup>37</sup> , ↑plasma TAG, Impaired glucose homeostasis <sup>37</sup> , hepatic lipid accumulation <sup>39,40</sup> , hepatic inflammation <sup>38</sup>
	<i>Non-human primate</i>	↑adiposity <sup>41,42</sup> , hepatic lipid accumulation <sup>41</sup>

Since fetal imprinting persists into adulthood, it is speculated that “malprogramming” is linked to the genome. However, the fast development of the obesity epidemic suggests that the answer is not found within normal evolutionary adaptation. Instead it has been hypothesized that programming of metabolic dysfunction is linked to the epigenome, which fine tunes gene expression to a continuously changing environment without altering the underlying DNA sequence<sup>43</sup>.

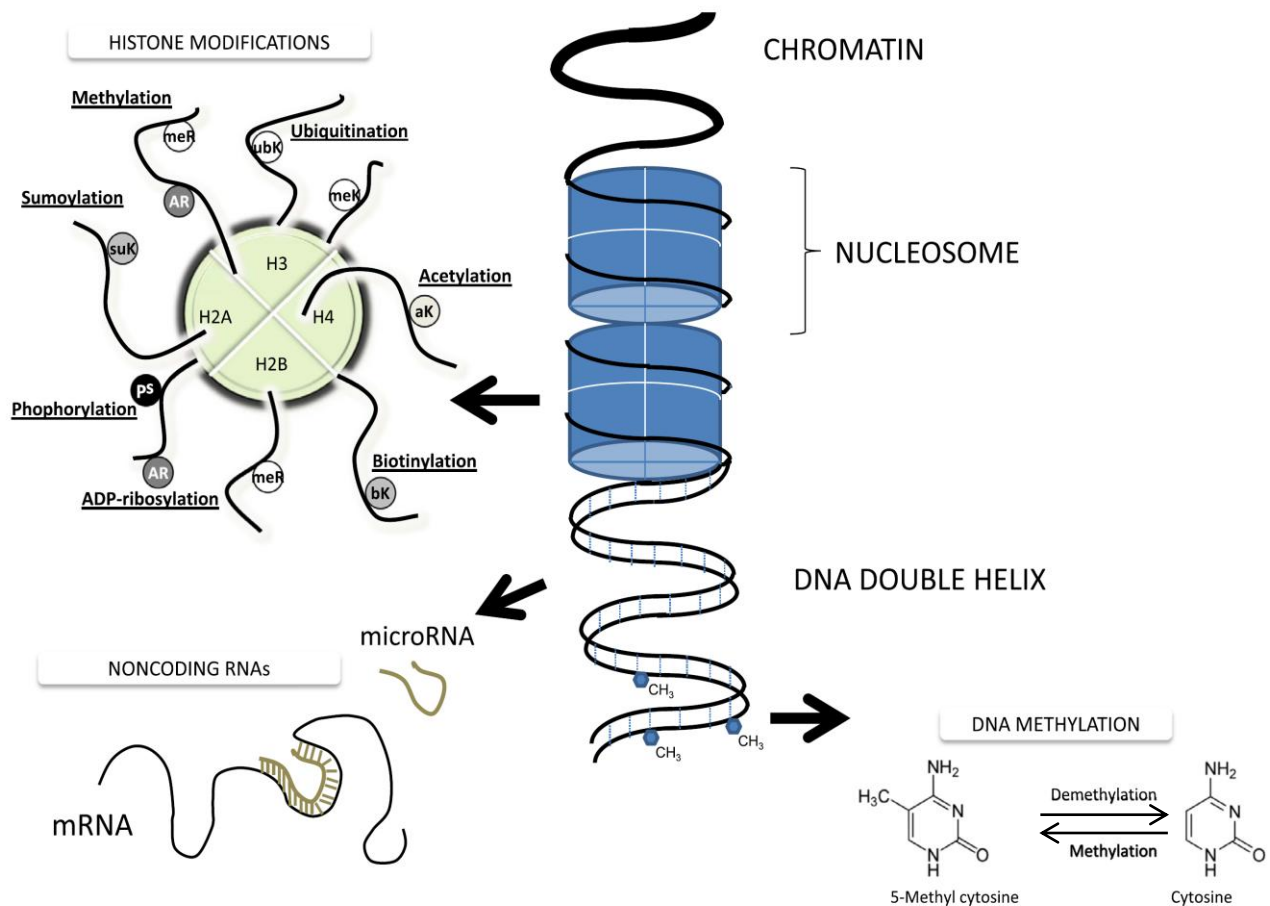
## Epigenetics

Epigenetic means “outside conventional genetics” and therefore covers the alterations in gene expression, which cannot be explained by the DNA sequence<sup>44</sup>. Monozygotic twins are two individuals that share the same genotype but have different phenotypes, as a result of different epigenomes<sup>45</sup>. The phenotypes in monozygotic twins are similar when they are young but differences start to accumulate as the twins grow older, since the epigenome is altered over the years<sup>46</sup>. The epigenome can adapt to environmental changes by modifying gene expression to optimize survival rate<sup>43,47</sup>, but the epigenetic pattern that is established during early development might be more stable than the alterations we accumulate after birth<sup>43</sup>.

Mammalian epigenetics cover a range of modifications that can alter the structure of the DNA molecule without altering the sequence. DNA methylation and histone modifications have been the primary focus of research, but now studies on non-coding RNAs, like microRNAs, are emerging<sup>48</sup> (Figure 4). Normally, DNA methylation occurs on a cytosine within a CpG dinucleotide (cytosine followed by a guanine in the DNA sequence), but a few studies have reported methylation outside CpG’s in embryonic stem cells<sup>49</sup>. The primary function of DNA methylation within promoter regions is gene silencing, and the degree of methylation often correlates with gene expression<sup>45</sup>. Hypermethylation blocks the DNA strand, which prevents the anchoring of DNA binding protein that initiates gene transcription<sup>44,45</sup>. Methylation patterns are also found in the introns and exons of genes, but the reasons for this are unclear, it might enhance rather than silence gene expression<sup>43</sup>. DNA is organized into nucleosomes and chromatins by wrapping around a histone complex. Modifications of the histones happen at the histone tails, and until now acetylation of the lysine residue have been the primary focus. Modification of the histone tails alters the DNA availability; e.g. when the lysine residue is acetylated, the DNA is unwrapped from the histone complex and is therefore available for protein binding and transcription of the gene.



DNA methylation and histone modifications enhance or prevent gene expression by altering the transcription of the mRNA in the cell (transcriptional level). MicroRNA regulates gene expression differently than DNA methylation and histone modifications, via hybridizing to the complementary mRNA from a target gene which prevents translation into protein (translational level).



**Figure 4. Epigenetic regulation of gene expression.** Epigenetic regulation alters the accessibility of the genes by modulating the DNA structure without changing the sequence. DNA methylation and modification of the histone complex are currently the most studied, but studies of noncoding RNA's are emerging. From Milagro et al. 2013<sup>48</sup>

Epigenetics, and in particular DNA methylation, play a central role in cell and tissue differentiation during fetal development and explains why all the different cells and tissues in an organism contain only one genotype, but innumerable phenotypes<sup>45</sup>. Within hours of fertilization DNA is stripped for methylations to make the fertilized egg pluripotent. Shortly after the implantation and throughout fetal development, the DNA is re-methylated giving rise to a variety of tissues and cell types. A major part of the tissue-specific epigenome, which is established throughout fetal life, is

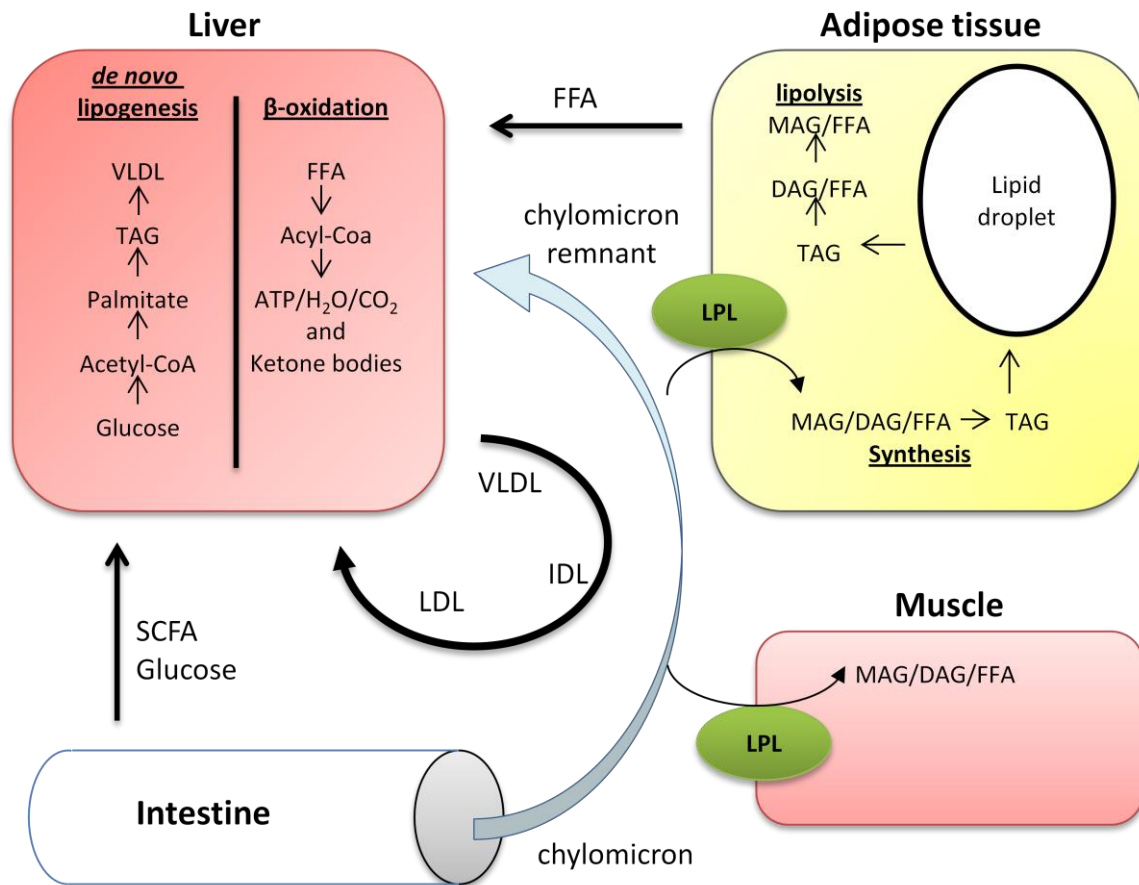
stable and heritable through mitosis, for example when a hepatocyte divides the daughter cell also develops into a hepatocyte. Epigenetic variations, as observed in monozygotic twins, arises as a consequence of the aging process and environmental exposures (nutrition, behaviour, pharmacological treatments)<sup>43</sup>. For instance, it has been shown that the estrogen receptor becomes hypermethylated with age<sup>50</sup>; the methylation pattern of the leptin promoter is altered by a high fat diet<sup>51</sup>; and physical inactivity reduces GLUT4 expression in skeletal muscle due to deacetylation of the histone complex<sup>43,52</sup>

### **Epigenetics in the light of the predictive adaptive response**

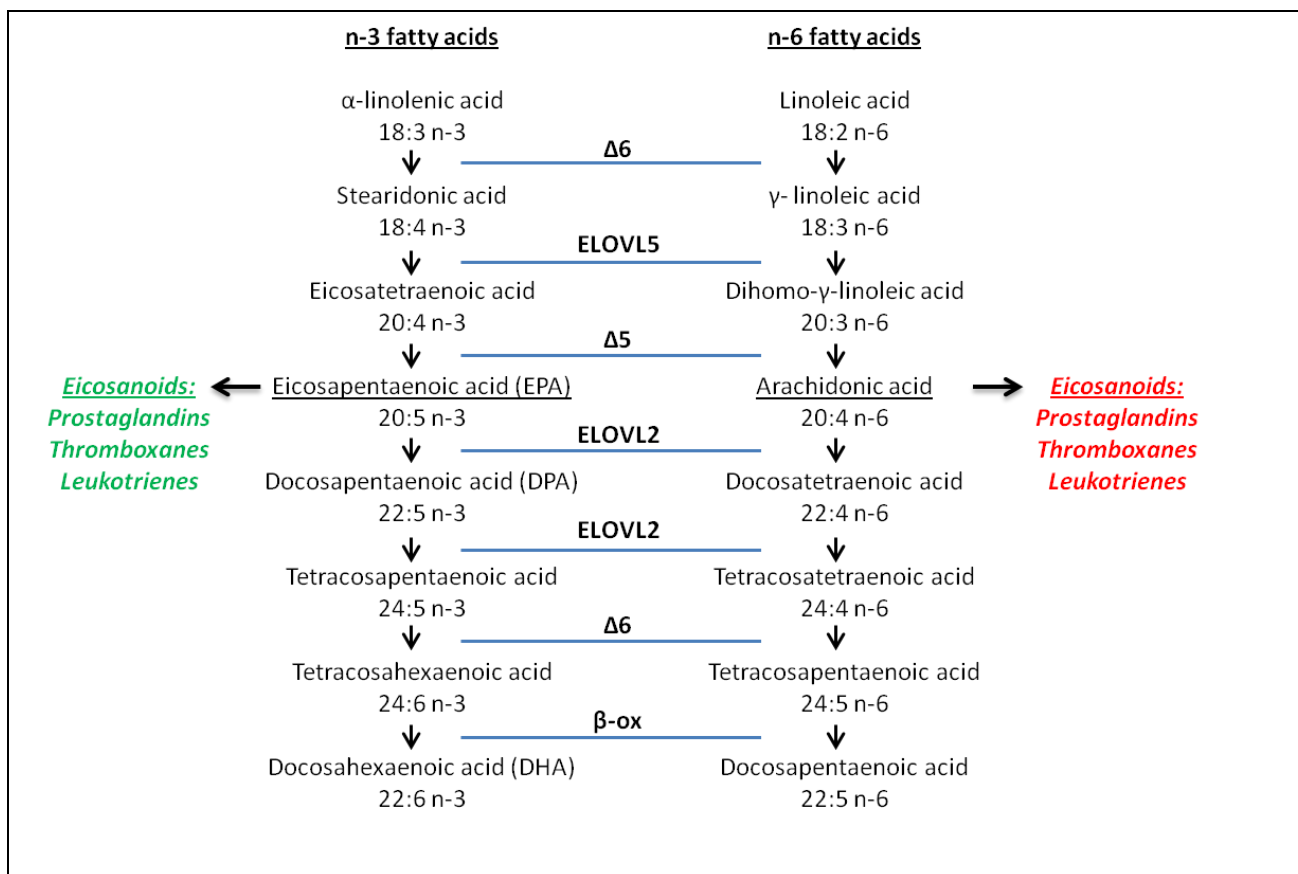
As previously described, the predictive adaptive response is an evolutionary strategy that ensures optimal fitness in a range of environments via maternal stimuli. Epigenetic modifications are believed to play a key role in fetal adaptation and therefore also a central element when mismatch arises between prenatal and postnatal life<sup>47</sup>. Maternal undernutrition has been reported to affect hepatic metabolic function and pancreatic islet formation<sup>53</sup>. While maternal high fat feeding can hyperacetylate hepatic histones<sup>54</sup>, increase gluconeogenesis via epigenetic modifications of *Pck1*<sup>55</sup> and alter leptin and adiponectin expression from adipose tissue via histone modifications<sup>56</sup>. Finally it has also been reported that children born with a low birth weight are less flexible in their metabolic adaptation via DNA methylation when challenged with overfeeding in adulthood<sup>57</sup>. Sookoian and colleagues<sup>58</sup> have used a systems biological approach to elucidate if maternal undernutrition and overnutrition share the same pathways when programming the offspring. Their model predicts that metabolic programming during maternal undernutrition targets genes involved with gene transcription, chromatin structure and DNA methylation, while maternal overnutrition is based on genes implicated in cellular control of glucose, lipid and lipoprotein metabolism and hormone activity. Additionally they suggest that exposure to overnutrition during fetal life is associated with metabolic programming of the liver, insulin resistance and ectopic lipid accumulation. Finally, their model demonstrated that an impaired liver phenotype (abnormal liver weight or hepatic steatosis) was a common denominator for both maternal under- and overnutrition<sup>58</sup>. This emphasizes the critical role of hepatic lipid and glucose metabolism in the development of metabolic syndrome in the adult offspring.

## Lipid metabolism

Dietary lipids are absorbed in the intestine. Short chain fatty acids are transferred directly to the liver via the portal vein<sup>59</sup> (Figure 5) and the larger lipids (TAG, PL and sterols) are packaged into chylomicrons, transferred to the lymphatic system and eventually the blood circulation. The chylomicrons deliver lipids to adipose tissue and muscle via lipoprotein lipase (LPL) situated on the endothelial cell surface of the organs capillaries. TAG from the chylomicrons is hydrolyzed by LPL resulting in the release of free fatty acids (FFA) and diacylglycerides (DAG), which are transported into the cells via fatty acid transport proteins (CD36/FAT, FATP, FABP). When hydrolysis of TAG from the chylomicrons exceeds the fatty acid uptake, there is a spillover of lipids contributing to the pool of FFA in the blood. After delivering TAG to the muscle and adipose tissue the chylomicron remnant are transported to the liver, where they are cleared from the circulation. Therefore, the liver receives exogenous lipids from chylomicron remnants and from LPL spillover, but the majority of lipids, which are trafficked through the liver, are endogenous lipids from de novo lipogenesis (DNL) (fed state) and FFA released by the adipose tissue (fasting state). DNL is only elevated during the postprandial phase and accounts for 26% of the lipids found in the liver. Glucose is absorbed directly from the intestine to the liver via the portal vein and converted to acetyl-CoA via glycolysis and the oxidation of pyruvate<sup>60</sup>. Acetyl-CoA is further converted to Malonyl-CoA by acetyl-CoA carboxylase (ACACA). Acetyl-CoA and Malonyl-CoA constitutes the building blocks used in the formation of the fatty acid palmitate (C16:0), a reaction catalysed by fatty acid synthase (Fasn). Palmitate can be elongated by long chain fatty acid elongase 6 (ELOVL6) to stearic acid (C18:0), when double bonds are introduced by stearyl-CoA desaturase 1 (SCD1) it then forms oleic acid (C18:1). Oleic acid is an important substrate in the formation of TAG, which are packed into very-low density lipoproteins (VLDL) and secreted from the liver. VLDL also delivers lipids to muscle and adipose tissue via LPL on the endothelial cells, similar to the chylomicrons. The release of TAG from VLDL increases the density of the lipoprotein creating smaller denser lipoproteins: intermediate density lipoproteins (IDL) and low density lipoproteins (LDL), which can be reabsorbed by the liver. Not all fatty acids can be synthesised from acetyl-CoA, since mammals are not capable of introducing double bonds beyond the 9<sup>th</sup> carbon. Linoleic acid (n-6) and  $\alpha$ -linolenic acid (n-3) are therefore essential exogenous fatty acids for synthesis of a large range of lipids (Figure 6)



**Figure 5. Lipid metabolism.** Short chain fatty acids (SCFA) are absorbed straight to the portal vein and later the liver. Other dietary lipids are absorbed in the intestine and packaged in chylomicron, before they are released into circulation. LPL in adipose tissue and muscles releases FFA from the chylomicrons, but while the FFAs are absorbed by the muscle and adipose tissue, the chylomicron remnants are cleared by the liver. During feeding, the liver synthesises TAG from glucose by de novo lipogenesis. TAG is packaged in VLDL particles and secreted into circulation, which delivers FFA to the adipose tissue and muscles via LPL, like the chylomicrons. VLDL is converted to IDL and LDL as the TAG core decreases and LDL return to the liver for clearance. During fasting, DNL is inhibited and FFAs are mobilised from the lipolysis of TAG in the adipose tissue. The FFA are transferred to the liver, where it can be used for ATP production via β-oxidation or the formation of ketone bodies. DAG, diacylglyceride; FFA, free fatty acid; IDL, intermediate lipoprotein; LDL, low density lipoprotein; LPL, Lipoprotein lipase; MAG, monoacylglyceride; SCFA, short chain fatty acid; TAG, triacylglyceride; VLDL, very low density lipoprotein



**Figure 6. Synthesis of long chain polyunsaturated fatty acids.** Mammals are not capable of introducing double bonds beyond the 9<sup>th</sup> carbon, which makes n-3 and n-6 fatty acids essential. Linoleic acid is the primary PUFA found in the western diet followed by  $\alpha$ -linolenic acid<sup>61</sup>. Both of them can be metabolised to long chain PUFA and eicosanoid by the same enzymes. Eicosanoids from EPA are in general believed to be anti-inflammatory, while eicosanoids from arachidonic acid are believed to be inflammatory<sup>61</sup>.  $\beta$ -ox:  $\beta$ -oxidation, ELOVL2: fatty acid elongase 2, ELOVL5: fatty acid elongase 5,  $\Delta 5$ :  $\Delta 5$ -desaturase,  $\Delta 6$ :  $\Delta 6$ -desaturase. From Schmitz and Ecker 2008<sup>61</sup>.

During the fasting state, lipids are returned to the liver from the adipose tissue. This process occurs when TAG is hydrolysed to DAG by adipose triglyceride lipase (ATGL). Then, DAG is hydrolysed to monoacylglycerides (MAG) by hormone sensitive lipase (HSL). Finally, MAGs are hydrolysed to FFA and glycerol by monoacylglycerol lipase (MGL)<sup>59,62</sup>. Both FFA and glycerol are released into the blood and reabsorbed by the liver, where the fatty acids are converted to acyl-CoA. Acyl-CoA is catabolised into acetyl-CoA by  $\beta$ -oxidation in the mitochondria, which then can be used in the tricarboxylic acid (TCA) cycle and eventually ATP production, or for the generation of ketone bodies. The mitochondrial membrane is impermeable for acyl-CoA, so it is dependent on the carnitine transport system to enter the mitochondrial matrix. This is regulated by carnitine palmitoyltransferase 1 (CPT1) and the availability of carnitine<sup>63</sup>. Very long chain acyl-CoAs (C>20) are toxic to the mitochondria, so they need shortening by  $\beta$ -oxidation in the peroxisomes

beforehand. This process is mediated by acyl-CoA oxidase (ACOX) and a multi-enzyme complex in the peroxisomes.

### **Regulation of lipid metabolism**

Lipid metabolism is regulated in various ways to ensure that fatty acid oxidation is silenced and DNL is elevated during feeding, and vice versa during fasting. Secretion of insulin from the pancreatic beta cells in response to increased blood glucose during feeding stimulates DNL, so that excess glucose is stored as lipids. Additionally, insulin activates the transcription factor SREBP1c, which up-regulates the expression of Fasn, ACACA and SCD1 in DNL. Fasn and ACACA is also up-regulated by the transcription factor ChREBP. When glucose levels rise, ChREBP is dephosphorylated and translocates from the cytosol to the nucleus. The increased DNL elevates levels of malonyl-CoA, which inhibit CPT1 and the delivery of acyl-CoA to the mitochondria, hereby shutting down oxidation in periods of excess nutrients. VLDL secretion from the liver is also decreased upon insulin stimulation to ensure that lipids are delivered to the muscle and adipose tissue, primarily from the chylomicrons in the postprandial phase. Insulin also inhibits hydrolysis of TAG in the adipose tissue, which decreases the availability of FFA for fatty acid oxidation in the liver. During fasting, insulin levels are low and the adipose tissue starts to shuttle FFA to the liver. Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that are essential for optimal regulation of lipid metabolism. PPAR $\alpha$  is found in hepatocytes, muscle, pancreas and kidney. It is activated by fatty acids, eicosanoids and fibrates, which results in increased uptake and oxidation of fatty acids<sup>63</sup>. PPAR $\gamma$  is primarily found in adipose tissue, where it increases insulin sensitivity and promotes fatty acid uptake and adipocyte differentiation. However, PPAR $\gamma$  is also found to a lesser extent in macrophages<sup>63</sup>, where it can induce an anti-inflammatory phenotype, which is important to maintain homeostasis in the adipose tissue (see below)<sup>64,65</sup>.

### **Non-alcoholic fatty liver disease and dyslipidemia**

Non-alcoholic fatty liver disease (NAFLD) is believed to be the hepatic manifestation of the metabolic syndrome. When the balance between lipid uptake and utilization becomes impaired lipid accumulation in the liver occurs. Patients with NAFLD have increased DNL and VLDL secretion from the liver that results in dyslipidemia, characterized by elevated levels of circulating TAG and/or cholesterol. The trafficking of hepatic lipids in NAFLD is further elevated due to increased amounts of FFA being released from the adipose tissue. This is caused by a decreased insulin

action on the suppression of TAG hydrolysis. Simple steatosis is considered benign and accumulation of TAG might protect the liver against insulin resistance and the concomitant inflammation<sup>66</sup>. However, metabolic overload might not only cause TAG accumulation in the form of lipid droplets, it can also accumulate lipid intermediate products, since the various metabolic pathways often have a limited capacity; Peroxisomal  $\beta$ -oxidation increases when the capacity of mitochondrial  $\beta$ -oxidation is reached. This generates reactive oxygen species (ROS), non-complete oxidation of lipids in the mitochondrial  $\beta$ -oxidation that results in toxic lipids<sup>66,67</sup> and palmitate that is shuttled into alternative pathways such as ceramide synthesis. This accumulation of by-products can induce inflammation, liver injury and fibrosis, also known as non-alcoholic steatohepatitis (NASH). Kupffer cells (hepatic resident macrophages)<sup>68</sup>, regulatory T cells (Treg) and dendritic cells (DCs)<sup>69</sup> normally maintain the non-inflammatory phenotype of the liver. With NASH development, immune cells (macrophages and neutrophils<sup>70</sup>) are recruited to the liver<sup>68</sup>, due to a Th1-skewed (pro-inflammatory) cytokine profile<sup>68</sup>. Various mechanisms have been shown to play a role in development of the inflammatory phenotype. Ibrahim and colleagues<sup>69</sup> demonstrated that the liver contains two distinct populations of DCs with either high (high-DC) or low (low-DC) lipid content. DC-low stimulates the proliferation of Treg and IL-10 production, which maintains a tolerogenic response in the liver. DC-high shows increased lipogenesis and ER stress, which lead to the production of pro-inflammatory cytokines (IL-12, IFN- $\gamma$ , IL-6, TNF $\alpha$ , KC, MCP-1, RANTES, IL-1, IL-8). Additionally DC-high was capable of inducing proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Osteopontin is a matrix glycoprotein expressed by hepatocytes, macrophages and T cells, which plays a role in the production of pro-inflammatory cytokines in PBMCs, migration of monocytes/macrophages/DCs and in matrix degradation<sup>65,71,72</sup>. Osteopontin has been shown to be elevated in steatosis<sup>73</sup> and osteopontin deficiency protects the liver against diet-induced steatosis<sup>72,74</sup>.

## Obesity-induced inflammation

### The healthy link between metabolism and the immune system

The immune system needs to mobilize large amount of energy during infections, and is therefore naturally linked to the metabolism. Bacterial and viral infections induce a Th1 immune response, which is characterized by activation of neutrophils, macrophages, DCs, B cells and T cells. The immune cells need nutrients when activated for; generation of reactive oxygen species from glucose via the pentose phosphate pathway, altering lipid composition in the cell membrane for phagocytosis of pathogens and antigen presentation, cytokine production and migration to the lymph nodes or site of infection<sup>64,65,75</sup>. All these mechanisms are generally fuelled by glucose and lipids and it is therefore necessary to direct nutrients towards the immune cells and away from storage. This is achieved by inducing insulin resistance in the insulin sensing organs, which in return increases gluconeogenesis in the liver and lipolysis in the adipose tissue<sup>65</sup>. The opposite is seen during infections with parasitic worms (helminth). Parasite infections are characterised by a Th2 response via an increase in eosinophils, which gives rise to increased insulin sensitivity instead of the decrease sensitivity observed in the Th1 response. This evolutionary combat strategy is beneficial since the parasite is dependent on the host nutrients to grow. By increasing insulin sensitivity, the nutrient availability is decreased and the parasite is therefore starved<sup>76</sup>. The immune system manipulates the metabolism during an immunological response, but the metabolism also utilizes immunological components to function. Muscles secrete IL-6, which enhance lipolysis and release FFA from the adipose tissue, to accommodate the increased energy consumption during exercise<sup>1,77</sup>. Finally, the immune system is important in tissue remodelling, playing a crucial role in adipose tissue expansion, due to excess nutrient intake (see below).

### Obesity-induced tissue inflammation

Obesity is associated with hepatic lipid accumulation and steatosis, as previously described, but obesity-induced inflammation is not limited to the liver and can affect adipose tissue, pancreas<sup>1,5</sup> muscle<sup>78,79</sup> and hypothalamus<sup>80</sup>. Inflammation in association with obesity was first described in adipose tissue<sup>81</sup>, and therefore the most studied (reviewed in the next section). The pancreas secretes insulin in response to elevated blood glucose. If basal insulin is increased due to a sustained hyperglycaemic diet, the pancreatic islets starts to produce the pro-inflammatory cytokine IL-1 $\beta$ , recruit macrophages and undergoes apoptosis (due to ER stress) to decrease



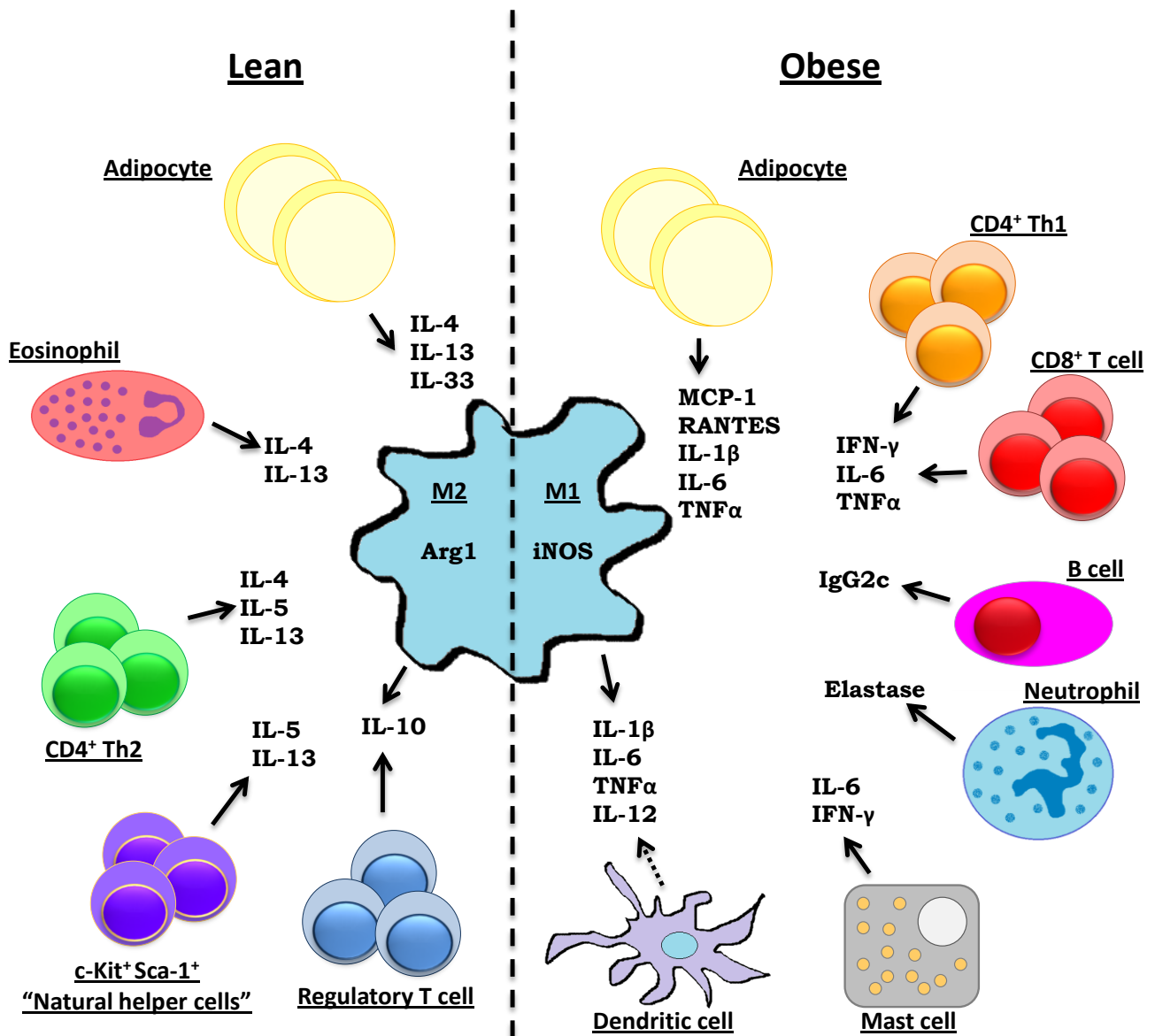
insulin secretion. It is hypothesized that the ER stress activates the NLRP3 inflammasome<sup>82</sup> which generates IL-1 $\beta$  by caspase-1<sup>1,5,83</sup>. The hypothalamus also produces pro-inflammatory cytokines (IL-6, TNF $\alpha$  and IL-1 $\beta$ )<sup>80</sup> and undergoes apoptosis in rats fed a high fat diet<sup>84</sup>. It is speculated that hypothalamic inflammation leads to the insulin and leptin resistance observed in obese subjects, which impairs appetite regulation and exacerbate glucose intolerance<sup>1</sup>. Muscles are central insulin sensing organs, which secretes IL-6 during exercise and requires a proinflammatory response via NF- $\kappa$ B and JNK pathways for tissue repair and muscle regeneration after exercise<sup>1,85</sup>. However, inflammation and macrophage infiltration in the muscles is also associated with insulin resistance and obesity<sup>85</sup>. It is suggested that obesity-associated muscle inflammation is mediated by elevated levels of FFA, which promotes expression of proinflammatory cytokine (TNF $\alpha$ , IL-6 and MCP-1) in myocytes<sup>78,79,85</sup>.

### *Adipose tissue inflammation*

Adipocytes collaborate with immune cells to maintain insulin sensitivity and tissue homeostasis of the adipose tissue in the lean state (Figure 7). Healthy adipose tissue is characterized by the presence of alternatively activated macrophages (M2), which are characterized by the expression of IL-10 and arginase-1(Arg-1)<sup>65,86</sup>. The M2 phenotype is orchestrated by cytokines secreted from eosinophils<sup>76</sup>, CD4<sup>+</sup> Th2 cells (Th2)<sup>87</sup>, Treg<sup>88</sup> and the newly identified “Natural helper cell”<sup>89</sup> (NH cells), which also are present in adipose tissue. Adipocytes<sup>90</sup>, eosinophils<sup>76</sup> and Th2 cells produce IL-4 and IL-13 during homeostasis, the majority of which is secreted from the eosinophils<sup>76,89,90</sup>. Eosinophils are therefore important for suppression of the pro- and cellular inflammatory responses within the adipose tissue. Recently it was proposed that eosinophils are maintained in the tissue by the NH cells<sup>91</sup>. NH cells were identified by Moro and colleagues in 2010<sup>92</sup>, and they are also known as nuocytes<sup>93</sup>, Innate helper type 2 cells<sup>94</sup>, and Innate Lymphoid type 2 cells<sup>95</sup>. They are widely distributed in mammalian tissues<sup>91,95</sup> and have a function similar to the Th2 cells<sup>94</sup>. They secrete large amount of IL-5 and IL-13 upon stimulation with IL-33 in combination with IL-2 and IL-25<sup>92</sup>. IL-33 is secreted from the adipocytes<sup>96</sup> while IL-25 is produced by activated eosinophils and basophils<sup>97</sup> and IL-2 is produced by CD4<sup>+</sup> T cells and DCs<sup>98,99</sup>.

As previously mentioned, inflammation is induced during tissue remodelling, which is necessary to store excess nutrients in the adipose tissue<sup>64</sup>. Adipocytes need to expand to deposit lipids during

sustained high fat feeding, but the adipocytes are surrounded by an extracellular matrix that needs to be broken down and re-built again after the expansion<sup>64,100</sup>. Infiltrating mast cells might be important for this remodelling, since they are associated with degradation and deposition of collagen by the release of proteases, IL-6 and IFN- $\gamma$ <sup>64,101</sup>. If the tissue expands faster than the vasculature, the nutrient and oxygen supply will decrease (hypoxia) and angiogenesis is stimulated by the secretion of pro-angiogenic factors (VEGF) and pro-inflammatory cytokines from macrophages, which accumulate around the hypoxic site<sup>5,102</sup>. IL-33 produced by endothelial cells during obesity development has also been shown to stimulate angiogenesis<sup>103,104</sup>. IL-33 might be increased due to elevated leptin secretion from the adipocytes<sup>104</sup>, but is also hypothesised to be an "alarmin" secreted from necrotic cells before they undergo apoptosis<sup>105</sup>. Since IL-33 secretion normally results in accumulation of eosinophils<sup>91</sup>, it is speculated that endothelial cells release high amounts of IL-33 during angiogenesis to control the inflammatory response needed to enhance vascularisation. Apoptotic cells and cell death is common during hypertrophy and hyperplasia<sup>106</sup>. Dead adipocytes are surrounded by macrophages that engulf cell debris and the lipids that leak from the cells. These phenomena are called Crown Like Structures (CLS) and have been identified in obese adipose tissue<sup>107</sup>. The presence of CLS has been an important hallmark for identification of obesity associated adipose tissue inflammation throughout the literature.



**Figure 7. Immunological phenotypes in adipose tissue in lean and obese states.** Lean adipose tissue is characterized by M2 macrophages, eosinophils, Treg, Th2 and natural helper cells who maintain tissue homeostasis. During development of obesity and insulin resistance, the macrophages differentiate into pro-inflammatory M1 macrophages, which are mediated by infiltration of Th1, CD8<sup>+</sup> T cells, B cells, neutrophils, mast cells and dendritic cells. Immune cell recruitment and inflammation are induced by the increased lipid turnover in the adipose tissue, which generates toxic lipids, ER stress, hypoxia, formation of reactive oxygen species and activation of the inflammasome. Inspiration to the figures comes from Winer and Winer 2012<sup>89</sup>

Chronically elevated lipid deposition in the adipose tissue alters the adipocytes secretion profile (Figure 7). The adipocytes produce pro-inflammatory cytokines in obese adipose tissue (MCP-1, OPN, RANTES, IL-6 IL-1 $\beta$  and TNF $\alpha$ <sup>65,108-110</sup>), which in return recruits a number of different inflammatory immune cells. Neutrophils are recruited in an early transient phase and secrete neutrophil elastase, which degrades the insulin receptor substrate, hereby decreasing insulin signalling in the adipose tissue<sup>64,70</sup>. B cells are also recruited in the early phase, but they stimulate IFN- $\gamma$  secretion from Th1 and CD8<sup>+</sup> T cells, which might be recruited by secretion of the cytokine RANTES<sup>89,111,112</sup>. Additionally, Th1 and CD8<sup>+</sup> T cells also secrete IL-6 and TNF $\alpha$  upon activation<sup>89,106</sup>. The B cells also produce IgG2c whose primary target is GOSR1, which shuttles proteins from the ER to Golgi<sup>113</sup>. Secretion of MCP-1 from the adipocytes recruits monocytes/macrophages that adapt a pro-inflammatory M1 phenotype (characterized by iNOS, IL-1 $\beta$ , IL-6, TNF $\alpha$  and IL-12, MCP-1 and RANTES expression<sup>5,89,106,112</sup>), due to the presence of pro-inflammatory cytokines (e.g. TNF $\alpha$ ) in the tissue<sup>110</sup>. Because M1 macrophages play an essential role in adipose tissue inflammation, several studies have unsuccessfully tried to prevent the induction of macrophage infiltration by inhibiting MCP-1<sup>114,115</sup>. Osteopontin have been suggested as an alternative route, since neutralisation prevents both obesity-induced inflammation and insulin resistance<sup>74,116,117</sup>. Previously, the M1 macrophages were identified by their expression of CD11c on the surface, but CD11c is also expressed on DCs and they have similar cytokine secretion patterns<sup>112</sup>. Stefanovic-Racic and co-workers have demonstrated that DCs account for a large proportion of the CD11c<sup>+</sup> infiltrating immune cells<sup>118</sup>. Only recently, researchers have started to investigate the role of DC in the initiation of obesity-induced adipose tissue inflammation<sup>118,119</sup> but DC are known to recruit and activate macrophages to the site of inflammation<sup>120</sup>. The presence of DCs are also positively correlated with insulin resistance<sup>119</sup> and inhibition of the inflammatory pathway NF- $\kappa$ B in the DCs increase insulin sensitivity<sup>121,122</sup>. These studies provide evidence that DC play an important role in the development of obesity-induced inflammation and insulin resistance, but further research is needed to elucidate the underlying mechanisms.

Many avenues have been explored to elucidate the initial mechanism responsible for obesity-induced inflammation in adipose tissue. Obesity is associated with elevated lipolysis in the adipose tissue, which is known to recruit macrophages to engulf excess fatty acid release, hereby increasing lipid turnover in the tissue<sup>123</sup>. Inflammation might cause the increased lipolysis by

decreasing insulin sensitivity in the organ, in the attempt to avoid further expansion of the adipocytes<sup>62,89,100</sup>. But increased lipid turnover in both adipocytes and immune cells induce ER stress and the generation of ROS and toxic lipids, similar to the scenario in hepatic steatosis<sup>89,106</sup>. ROS<sup>124</sup>, ceramide<sup>125</sup>, cholesterol<sup>126</sup> and saturated fatty acids<sup>124</sup> can activate the NLRP3 inflammasome, which produces IL-1 $\beta$  and IL-18 by caspase-1 activity that decreases insulin signalling in the tissue<sup>64,127</sup>. IL-1 $\beta$  is capable of activating the NF- $\kappa$ B and JNK signalling pathways, which upregulate the expression of various pro-inflammatory cytokines (TNF $\alpha$ , IL-1 $\beta$  and IL-6)<sup>5,128</sup>. Fatty acids might also be capable of activating the NF- $\kappa$ B pathway via the toll like receptors (TLRs)<sup>64</sup>, but Erridge and Samani planted a seed of doubt regarding this matter, when they published a study in 2009 showing that bovine serum albumin (BSA), that is used to get fatty acids into dilution, often is contaminated with lipopolysaccharide (LPS), a potent activator of TLRs<sup>129</sup>. When they used a LPS-free BSA, they discovered that saturated fatty acids did not directly stimulate TLR-dependent signalling, as previously thought<sup>129</sup>. LPS has also been suggested to play a role in the initiation of obesity-induced inflammation, since the intake of a high fat meal induces increased levels of circulating LPS (endotoxemia)<sup>130-132</sup>. Cani and colleagues have shown that endotoxemia cause obesity and adipose tissue inflammation in mice<sup>133</sup>. However, the endotoxemia hypothesis only deals with the increase in monocyte/macrophage recruitment to the adipose tissue and does not explain other alterations in the immune cell population. Moreover, the LAL assay, which are generally used to measure LPS, does not discriminate between various LPS types, which may be problematic since hexaacyl LPS has a higher inflammatory potential than pentaacyl and tetraacyl LPS<sup>134</sup>.

To summarize, lean healthy adipose tissue is characterized by M2 macrophages, eosinophils, Treg, Th2 and natural helper cells, which during normal circumstances maintain tissue homeostasis. During development of obesity and insulin resistance, the adipose tissue becomes pro-inflammatory probably mediated by lipotoxicity, ER stress, hypoxia, ROS formation and activation of the inflammasome as a result of the increased lipid turn over. The inflammatory adipose tissue is characterized by the presence of M1 macrophages, CD8<sup>+</sup> T cells, Th1, B cells, neutrophils, mast cells and dendritic cells (Figure 7).

## **Animal experiments included in the thesis**

In the literature, a range of animal models have been used to investigate perinatal programming of metabolic syndrome (non-human primates, sheep and rodents)<sup>135</sup>. Rodents are extensively used in physiologic and metabolic research<sup>136</sup>, however, normal rodents have limitations; they have a strict appetite regulation, so it can be difficult to get them to overeat and become obese; they cannot develop cardiovascular disease and atherosclerosis; pups are born in litters and are very immature at birth compared to humans<sup>135,136</sup>. Today a range of obese prone rodent strains, which also develops hyperglycemia and whole body insulin resistance, have been developed commercially, so this limitation can be overcome strain selection<sup>137</sup>. The immature nature of the offspring at birth can be taken into account, when interpreting results related to perinatal programming in rodent models. Development of adipose tissue is important to point out, since it plays a critical role in the development of obesity and metabolic dysfunction; White adipose tissue develops in rodents in the postnatal period, while it develops in the third trimester in human<sup>138</sup>. Thus, postnatal effects on adipose tissue development in rodents lie within the prenatal development in humans.

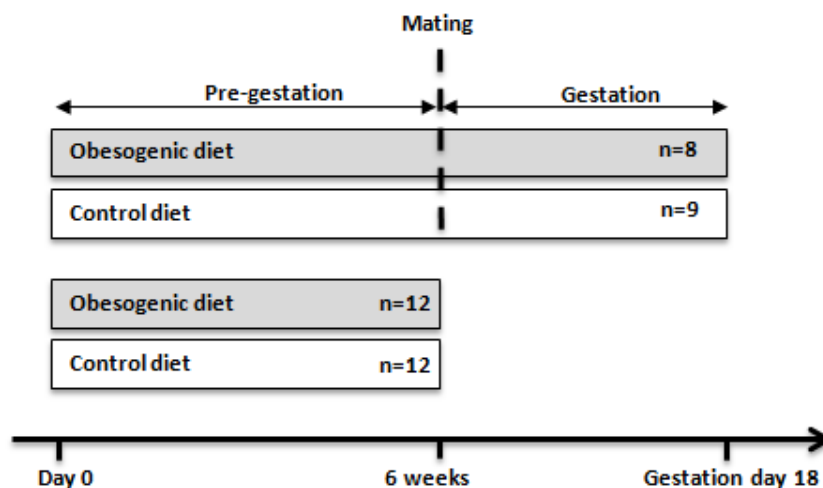
We chose to use rodents as a model, since they can develop the relevant metabolic phenotype for our studies (obesity, impaired glucose homeostasis, impaired lipid homeostasis and obesity-induced inflammation), concurrent with a short life span which allow us to finalize the studies within the time frame of a PhD project. This thesis includes one mouse study conducted at Institute of Metabolic Sciences at University of Cambridge, UK, in collaboration with Dr. Susan E. Ozanne and two rat studies conducted at the Technical University of Denmark, DK. The common denominator for the animal studies is maternal high fat feeding and fetal programming of obesity and metabolic dysfunction. However, the aim of the mouse study was to phenotype the dams, while the rat studies investigate metabolic and immunological parameters in the offspring.

## **Mouse study**

When studying immunological phenotypes, mouse models are the ideal choice. They have been extensively used in immunological research, so murine immune cell subsets are better characterized in the literature compared to rats. At Institute of Metabolic Science at University of Cambridge (UK), Dr. Susan E. Ozanne and colleagues have developed a maternal obesity mouse model in C57BL/6 mice, which leaves a metabolic imprint in the offspring, making them more

susceptible to obesity and metabolic dysfunction<sup>25</sup>. We were interested in studying if obesity-associated inflammation was present in the dams of Dr. Ozannes model at the time of mating, and therefore potentially could cause some of the effects they have reported in the offspring. Additionally, we wanted to investigate how obesity-induced inflammation, if present before mating, was affected by pregnancy, since we hypothesized that a Th1 skewed obesity-induced inflammation would conflict with a Treg/Th2 skewed pregnancy (Chapter 2).

Female mice were randomly assigned to a control or an obesogenic diet. After 6 weeks of feeding, blood was collected and the animals were left to recover for 2 days before 12 mice from each group were euthanized (blood and tissues were collected), while the remaining were mated. The pregnant dams and the unborn fetuses were euthanized on gestation day 18 (GD18), since we wanted to collect the placenta, which normally gets eaten by the dams straight after delivery (Figure 8). We also chose not to collect blood from the pregnant dams before termination, since stress from the blood sampling potentially could induce spontaneous labor and therefore prevent collection of the placenta. The outcomes of the study were an immunological phenotyping of monocyte activation in the blood and monocyte recruitment to various tissues in the dams before mating and on GD18. Additionally, we evaluated the animal's adiposity, levels of circulating metabolic markers and ectopic lipid accumulation at the two time points (Chapter 3).



**Figure 8. Experimental setup of mice study**

## Rat studies

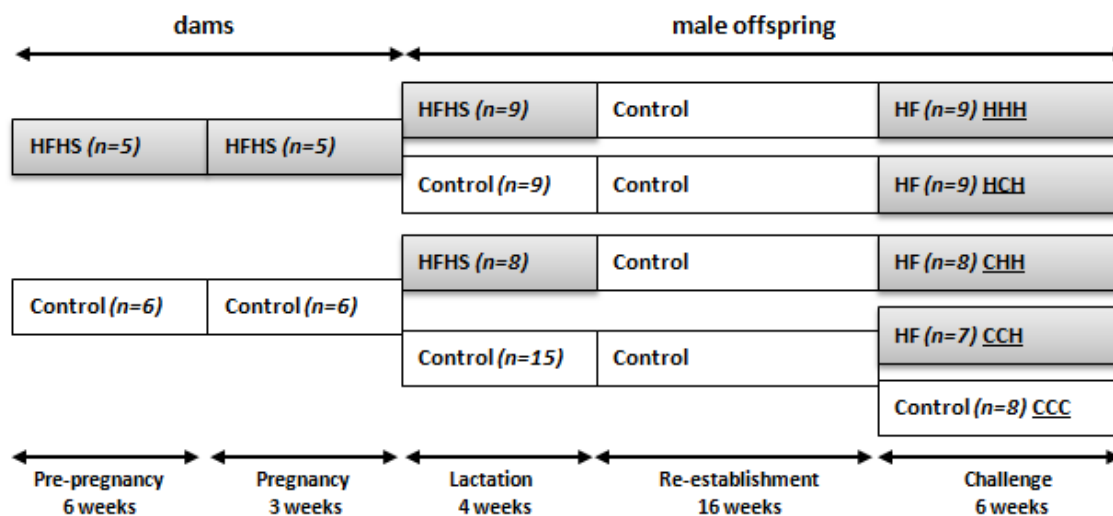
Within Centre for Fetal Programming, who has partly founded this PhD stipend, we have tried to standardize our animal experiments to enable closer comparison of results from different studies. Rats are our preferred rodent model, but there are large individual variation in the outbred rat strains<sup>137</sup> and, as previously mentioned, normally rats do not become obese. So we chose an obese prone rat model from Charles River (CrI:OP-CD), hoping that the animals would be less restricted in their appetite when fed a high fat diet.

### *Rat study 1*

Female obese prone rats were randomly assigned to a control diet or a high fat diet (60E%) supplemented with 15% sucrose water (HFHS). Since the typical human junk food diet consists of a combination of high fat food and sucrose sweetened beverage, and some data indicate that intake of sucrose as beverage has a more adipogenic effect, than sucrose in solid food<sup>139</sup>, we have chosen a model mimicking these conditions. Female rats were maintained on the diets for 6 weeks before they were mated. All pregnant dams had normal deliveries, and all pups were weighed the day after birth. Female pups were euthanized and male pups were give identification marks and subsequently cross-fostered among the dams; control born pups were lactated by control dams (CC) or HFHS dams (CH) and HFHS pups were lactated by control (HC) or HFHS dams (HH). We chose to cross-foster the offspring after birth to be able to discriminate between prenatal and postnatal effects. Originally we wanted to euthanize half of the male offspring at weaning, and continue the remaining half on a control diet. However, the group sizes were smaller than expected (CC=15, CH=8, HC=9, HH=9), so we had to wean all male offspring on to the control diet at 4 weeks of age. The offspring were kept on the control diet for 16 weeks, a so called “re-establishment” period, where we wanted to study if the altered metabolic phenotype, observed in the HFHS exposed offspring at weaning, could be recovered. Finally, the offspring were dietary challenged with a high fat (45E%) diet in adulthood from 20-26 weeks of age, which makes this experimental setup unique from previously published studies<sup>26,32,37</sup>. The purpose of our study was not only to investigate metabolic adaptability to a high fat diet in adulthood after perinatal HFHS exposure compared to the control group (Figure 9) The parametric outcomes of the study were levels of circulating metabolic markers over time and also adiposity and hepatic lipid accumulation



in the male offspring after the dietary challenge (Chapter 5). Additionally, we studied monocyte activation in the blood and monocyte recruitment to adipose tissue (Chapter 6).



**Figure 9. Experimental setup of rat study 1**

### *Rat study 2*

The offspring yield after the first round of mating was sparse, so we decided to re-mate the dams and repeat the cross-fostering experiment with the purpose of studying all male offspring in the second litters at weaning (Figure 10). After the first litters were weaned, the dams were left to recover before they were re-mated. Thus, the dams received the respective diets for approximately 17 weeks before they were mated the second time. It is therefore not possible to make an exact comparison between the two rat studies, since the length of the maternal dietary intervention might be important for the magnitude of the metabolic imprinting in the offspring. The parametric outcomes of the study were levels of circulating metabolic markers, adiposity, hepatic lipid accumulation and composition in the male offspring at weaning (Chapter 4). Additionally, we studied monocyte activation in the blood and monocyte recruitment to adipose tissue (Chapter 6).

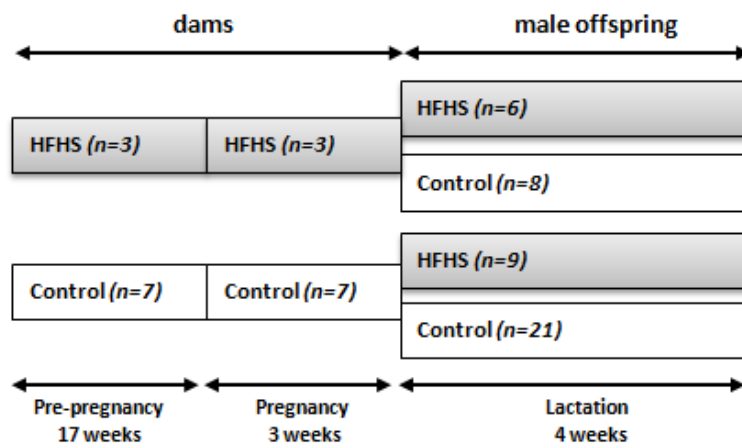


Figure 10. Experimental setup of rat study 2.





## **2. The effect of maternal inflammation on fetal development and the risk of metabolic disease later in life.**

**Camilla Ingvorsen, Susanne Brix, Susan E. Ozanne, Lars I. Hellgren**

### **Abstract.**

Maternal obesity during pregnancy is associated with increased risk of impaired metabolic function in the offspring. However, the obesity phenotype is complex and many factors can potentially affect the metabolic imprinting in the offspring. Many studies have focused on elucidating the role of elevated metabolic markers in obese mothers, but diet-induced obesity is associated with chronic low-grade inflammation, suggesting that fetal programming due to maternal obesity, also could be causally linked to this inflammatory phenotype. Therefore, we have surveyed the literature to set up a hypothesis regarding the role of maternal obesity-induced inflammation in fetal programming of the metabolic syndrome. Nobody has yet investigated the role of obesity-induced inflammation in programming of metabolic syndrome in the offspring, so we are therefore restricted to the studies of acute inflammation from LPS injections, or, alternatively, we can be inspired from studies that investigate how other chronic low-grade inflammatory diseases, like autoimmune diseases, are affected by pregnancy. However, obesity-induced inflammation is characterised by a Th1-driven immune response, a process that normally is suppressed via a Treg/Th2-skewed immune response during a normal pregnancy to avoid rejection of fetus. Thus, we speculated that there might be a conflict between maintaining a Th1 obesity-induced inflammatory response during a Treg/Th2-skewed pregnancy.

## Introduction

Obesity and metabolic disorders have become a global health concern in the 21<sup>st</sup> century, no longer only affecting the western world. Today we are experiencing a paradigm shift towards obesity being the biggest threat to human health, since 65% of the world population lives in countries where overweight kills more people than under-nutrition<sup>2</sup>. In 1992, Hales and Barker were the first to describe a hypothesis regarding programming of metabolic disease during fetal life<sup>11</sup>. Fetal programming is defined as maternal stimuli during pregnancy, which can have an effect on the offspring's physiology, structure or metabolism that is maintained into adulthood. Many studies have shown that maternal obesity can lead to increased risk of adiposity<sup>26,30,140</sup>, glucose intolerance<sup>30,140</sup>, reduced beta-cell function<sup>28</sup>, hypertension<sup>140,141</sup>, non-alcoholic fatty liver disease (NAFLD)<sup>26,41,142</sup> and altered innate immunity<sup>27,143</sup> in the offspring later in life. Obesity and metabolic dysfunction coexist with systemic low-grade inflammation and local inflammation in adipose tissue, liver<sup>144,145</sup>, muscle<sup>78,79</sup> and hypothalamus<sup>80</sup>, suggesting that fetal programming due to maternal obesity, could be causally linked to this inflammatory phenotype. Metabolic and immunological regulations are closely linked together, since an efficient immune response requires that fluxes of energy and metabolites are directed towards leukocytes combat of pathogens<sup>75</sup>, in a manner so that metabolism can optimize nutrient availability and tissue remodelling via an inflammatory response<sup>100</sup>. Kosteli and colleagues<sup>123</sup> showed that increased lipolysis in adipose tissue during weight loss promoted infiltration of adipose tissue macrophages probably via a concurrent secretion of monocyte chemoattractant protein 1 (MCP-1). Lipolysis is also increased during obesity development due to elevated basal lipolysis<sup>123</sup>, but the adipose tissue also has to expand and remodel to accommodate the need of storing excess nutrition<sup>146</sup>. As a consequence of the obesity-induced hypertrophy and hyperplasia, the extracellular matrix must be broken down and rebuilt, and a pronounced angiogenesis is required to avoid hypoxia, while dead adipocytes need to be removed by macrophages<sup>147,148</sup>. This complex inflammation-induced tissue remodelling is partially coordinated through signalling via pro-inflammatory cytokines<sup>148</sup>. These local inflammatory events are part of a normal adaptation of the adipose tissue structure to situations of energy surplus, but, if the metabolic overload continues, the inflammatory phenotype will no longer only be local and transient, but instead become systemic and chronic. The inflammatory phenotype associated with obesity has been hypothesised to be the link

between obesity and metabolic diseases, such as development of type 2 diabetes and atherosclerosis. However, it is unclear how obesity triggers the chronic systemic low-grade inflammation, but several hypothesis have been suggested including lipotoxicity and ectopic lipid accumulation<sup>147</sup>, endotoxemia involving activation of monocytes due to enhanced lipopolysaccharide (LPS) leaking from the intestine<sup>133</sup>, ceramide-mediated activation of NLRP3 in the inflammasome<sup>125</sup>, foam cell formation like in cardiovascular disease as well as production of reactive oxygen species due to a stress response in the endoplasmic reticulum (In depth reviews have previously been published on these hypothesis<sup>5,65,149-151</sup>). Regardless of the etiology of the chronic low-grade inflammation, a skew towards a type 1-driven inflammatory immunity is a core component. Th1-driven immunity is characterized by CD4<sup>+</sup> Th1 cells, CD8<sup>+</sup> effector T cells and recruitment of inflammatory macrophages (M1-type)<sup>150</sup> into the tissue, all being promoted via an activated innate immune system, involving dendritic cells and inflammatory monocytes. In the lean state, the adipose tissue has an anti-inflammatory phenotype dominated by the presence of regulatory T (Treg) cells, T helper cell (Th)2 cells, eosinophils and resident macrophages (M2-type)<sup>106</sup>. This phenotype is maintained by IL-4 and IL-13 production from eosinophils and IL-10 production from Tregs and resident macrophages<sup>106</sup>. But as obesity develops, these regulatory immune cells that normally maintain tissue homeostasis are gradually depleted, and the cytokine profile is skewed towards the production of pro-inflammatory cytokines (MCP-1, TNF $\alpha$ , IL-6, IL-1b, IL-18). Altogether, this profile comes along with the Type 1-driven inflammatory phenotype (CD4<sup>+</sup> Th1 cells, CD8<sup>+</sup> effector T cells and inflammatory macrophages (M1-type))<sup>150</sup> where the effector T-cells are primed by IL-12p70-producing dendritic cells. How the switch to IL-12p70 production in dendritic cells is promoted in adipose tissues is still unresolved, but it is likely that it could involve endotoxemia, as endotoxins represent one agonist that triggers IL-12p70 production in dendritic cells via specific signalling pathways. IL-12p70 production is tightly controlled in dendritic cells, and therefore only very specific conditions will lead to amplification of the non-microbial/sterile promoted pro-inflammatory phenotype associated with obesity development.

For the obesity induced maternal inflammation to affect the fetus, this can only occur if; 1) Maternal-derived inflammatory components such as cytokines or activated immune cells are transferred across the placenta, representing the maternal-fetal interface, and enters the fetal circulation (direct impact) or 2) Maternal inflammatory-derived components affect the placenta's

capability of transferring other components such as nutrients or oxygen to the fetus (indirect impact). It is known that cytokines<sup>152</sup> and maternal immune cells<sup>153</sup> can be transferred across the placenta, and that cytokine exposure can alter placental perfusion rate<sup>154</sup> (see below), but obesity-induced inflammation is characterised by a Th1-driven immune response, as previously described, and this process is suppressed during a normal pregnancy<sup>155</sup> (see below). Therefore, we speculate that there might be a conflict between a Treg/Th2 skewed pregnancy status in the mother and fetal transfer of a Th1-driven maternal obesity-induced inflammation that would, attenuate obesity-induced inflammation during pregnancy, and, hence, it's impact on the fetus.

### **Maternal immunity during pregnancy**

The immunological profile of pregnancy is firmly regulated from conception to delivery: Initially, implantation of the blastocyte in the earliest phase of pregnancy needs a strong activation of the innate immune system to repair the damaging events caused by breakage of the endothelial lining, invasion of the endometrial tissue and establishment of a placental/fetal blood supply<sup>156</sup>. From early on, the semi-allogeneic fetus (paternal DNA and antigens) will also promote changes in the immunological phenotype towards a pro-inflammatory status, demonstrated by an increased leukocyte count in the blood and increased levels of C-reactive protein throughout pregnancy<sup>157</sup>. Further subtyping of the leukocytes present, shows that innate immune cells (monocytes, neutrophils and plasmacytoid dendritic cells) are increased, while cells of the adaptive immune system are decreased (B-cells and T cells)<sup>158,159</sup> upon conception. In order to avoid rejection of the embryo, the maternal immune system needs to adapt the pro-inflammatory response to the pregnant state. This is achieved by skewing the immune response, initially via a Treg response and later on via promotion of a Th2 response (Figure 1A). Immediately after fertilization, human chorionic gonadotropin (hCG) is secreted, which will recruit Treg to the site of implantation<sup>160</sup>. After around 12 weeks of pregnancy, the hCG secretion decreases and the Th2 phenotype is then orchestrated by enhanced estrogen and progesterone levels that remain high until short before delivery. Both estrogen and progesterone have profound regulatory functions on immunity. Progesterone is produced by the placenta and the ovaries and progesterone receptors are found on mast cells, eosinophils, macrophages, dendritic cells and lymphocytes. Increased progesterone levels will induce the production of progesterone-induced binding factor (PIBF) in lymphocytes,

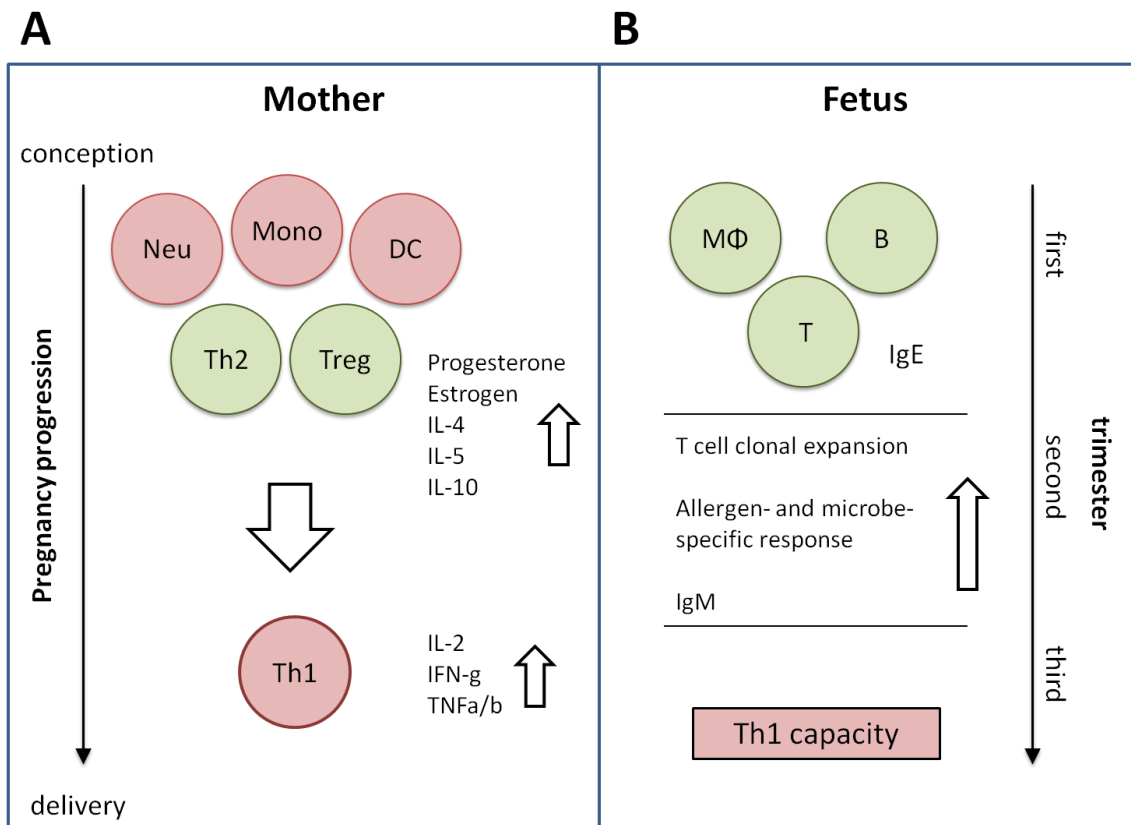


which will allow newly developed CD4+ T cells to differentiate into Th2 cells, secreting common Th2-derived cytokines (IL-4, IL-5 (and IL-10))<sup>155</sup>. This will decrease the Th1 response both at the maternal/fetal interface, but also systemically, as demonstrated by peripheral blood mononuclear cells (PBMCs) from pregnant woman showing increased secretion of IL-4 (Th2) and IL-10 (Treg), but decreased production of IL-2 (proliferation enhancer) and IFN-g (Th1)<sup>161</sup>. Estriol constitutes 90% of the estrogens produced during pregnancy, and it interacts with T cells, B cells, neutrophils, monocytes/macrophages, NK cells and dendritic cells through estrogen receptors<sup>155,159</sup>. Upon binding, estrogen can reduce B-cell lymphopoiesis, which will decrease antibody production during infections<sup>159</sup>. Furthermore, estriol inhibits T cell migration and interacts with the NF-κB pathway by inhibiting the degradation of IκBα, which will decrease e.g. TNFα production and thereby result in diminished Th1 immunity. The Th1 phenotype and a pro-inflammatory environment are suppressed in the uterus until delivery, since spontaneous labor at term is dependent on a pro-inflammatory Th1 switch to induce uterine contraction and expulsion of the fetus and placenta, however, presently the underlying mechanism behind this switch in immunity is incomplete<sup>156,162</sup>.

## Fetal immunity

The maternal and fetal immune systems are closely linked, although the inter-linkage between the two seems to differ dependent on the time from conception. Maternal cells can cross the placenta and infiltrate fetal tissues and in response, the fetus develops tolerance to maternal antigens via generation of Treg<sup>153</sup>. Thus, although the fetal immune system is considered immature at birth, due to limited microbial exposures during fetal life, it starts to develop in the first trimester of human pregnancies<sup>163</sup> (Figure 1B). Production of B cells, T cells and macrophages are already initiated a few weeks after implantation and IgE production begins in the liver in the middle of the first trimester (8 weeks) of human pregnancy followed by lungs and the spleen (week 11). By the end of the first trimester the thymus cortex are also being formed and the bone marrow lymphopoiesis and T-cell clonal expansion is initiated. In the middle of second trimester (week 19), IgM production is activated and by the end of the second trimester the differentiation and maturation of the immune cell compartment is complete. The immune system is capable of inducing an allergen- and microbe-specific antibody response already in the middle of the second trimester (week 23), which makes the fetus vulnerable to maternal inflammatory stimuli from this

time point onwards. However, the fetus is not capable of inducing a Th1-driven immune response before the very end of the third trimester (week 37)<sup>163</sup>, possibly due to an anti-inflammatory impact from maternal factors crossing the placenta.



**Figure 1. Maternal and fetal immunity.** A. Implantation of the embryo causes an inflammatory response mediated via the innate immune system. Dendritic cells (DC), neutrophils (Neu) and monocytes (Mono) are increased during pregnancy, but to avoid rejection of the fetus, tolerance are induced via CD4<sup>+</sup> Th2 cells (Th2) and regulatory T cells (Treg). The Treg/Th2 phenotype suppresses a pro-inflammatory Th1 phenotype until the end of gestation. A Th1 response is induced at term to initiate contractions of the uterus and expulsion of the fetus and placenta, which is essential for a natural spontaneous labor. B. The fetal adaptive immune system is considered largely immature at birth, due to limited exposure to pathogens during pregnancy. However, the fetal immune system starts to develop already in the first trimester where macrophages (MΦ), B cells (B) and T cells (T) are the first to differentiate. At the end of the first trimester, the thymus cortex develops and T cell clonal expansion is initiated. During the second trimester, the fetus is capable of responding towards microbes, but the Th1 capacity is not acquired before the end of the third trimester.

## The Placenta - the maternal-fetal interface

The placenta constitutes the maternal-fetal interface and functions to protect the fetus from rejection and exposure to maternal pathogens, concurrent with ensuring the fetal need for nutrients and oxygen<sup>164,165</sup>. Oxygen can diffuse across the placenta, and the supply is regulated by a decreasing gradient from maternal to fetal side, a process highly dependent on the thickness of

the placenta along with placental blood flow and metabolism<sup>164</sup>. Other essential components such as ions, amino acids and fatty acids reach the fetal circulation via protein-mediated transport across the maternal-fetal barrier<sup>164</sup>. The secretome of the placenta is very similar to that of adipose tissue and helps accommodate nutrient transfer to the fetus. Leptin and IL-6 are produced by placental cells while TNF $\alpha$  is produced by Hofbauer cells, the resident macrophages within the placenta<sup>166</sup>. During a normal pregnancy, the placenta produces TNF $\alpha$  during the last trimester with the formed TNF $\alpha$  being asymmetrically distributed to the maternal side<sup>167</sup>. It is hypothesized that TNF $\alpha$  can attenuate insulin sensitivity in maternal insulin-sensing organs, hereby shifting the flux of glucose and fatty acid to increased availability in the maternal blood stream<sup>166</sup>. Simultaneously, IL-6 produced by placental cells induce fatty acid uptake and accumulation within the placental cells<sup>168</sup>. Thus, placental cytokine production diverts the nutrient flow away from the maternal storage compartments towards the fetus, presumably, to accommodate the increased fetal nutritional needs in later gestation.

Several studies have focused on placental transfer of maternal cytokines to the fetus, but with inconclusive results. A study by Ashdown et al<sup>169</sup> showed no TNF $\alpha$ , IL-1 $\beta$  or IL-6 in the fetal brain upon maternal LPS exposure on gestation day 18 in rats, whereas Oskvig et al<sup>170</sup> showed elevated levels of all three cytokine in the fetal brain after maternal LPS exposure on gestation day 15 also in rats. The differences in the two findings might rely on different time points for maternal LPS exposure, as for instance IL-6 is transferred across rat placenta to a greater extent at mid-gestation compared to late gestation<sup>152</sup>. A minimal transfer of IL-1 $\alpha$  and TNF $\alpha$  across the placenta has also been shown in human placentas at term, while IL-6 seems to be transferred to a greater extent<sup>171</sup>. These data shows that the placenta prevents and enhances transport of components differently during the early, mid and late trimester, perhaps due to a fluctuating need for provision of different nutritional and regulatory components during fetal development. This also implies that maternal inflammation affects fetal development differently at the different stages of pregnancy.

### **Fetal outcome after exposure to maternal inflammation**

Maternal inflammation occurs naturally during pregnancy if bacterial and viral infections arise. During pregnancy, the mother is more susceptible to infections and the mortality and morbidity

rate is higher in the second and third trimester than in the first<sup>159</sup>. Preterm delivery can be a consequence of maternal infections<sup>172</sup>, and it has been shown that PBMCs from aborting mothers produce more Th1 cytokines (IL-2, IFN $\gamma$ , TNF $\alpha$  and TNF $\beta$ ) and less Th2/Treg cytokines (IL-4, IL-6 and IL-10) when stimulated with PHA (a mitogen that activates T-cells non-specifically) than PBMCs from normal pregnant mothers<sup>173</sup>.

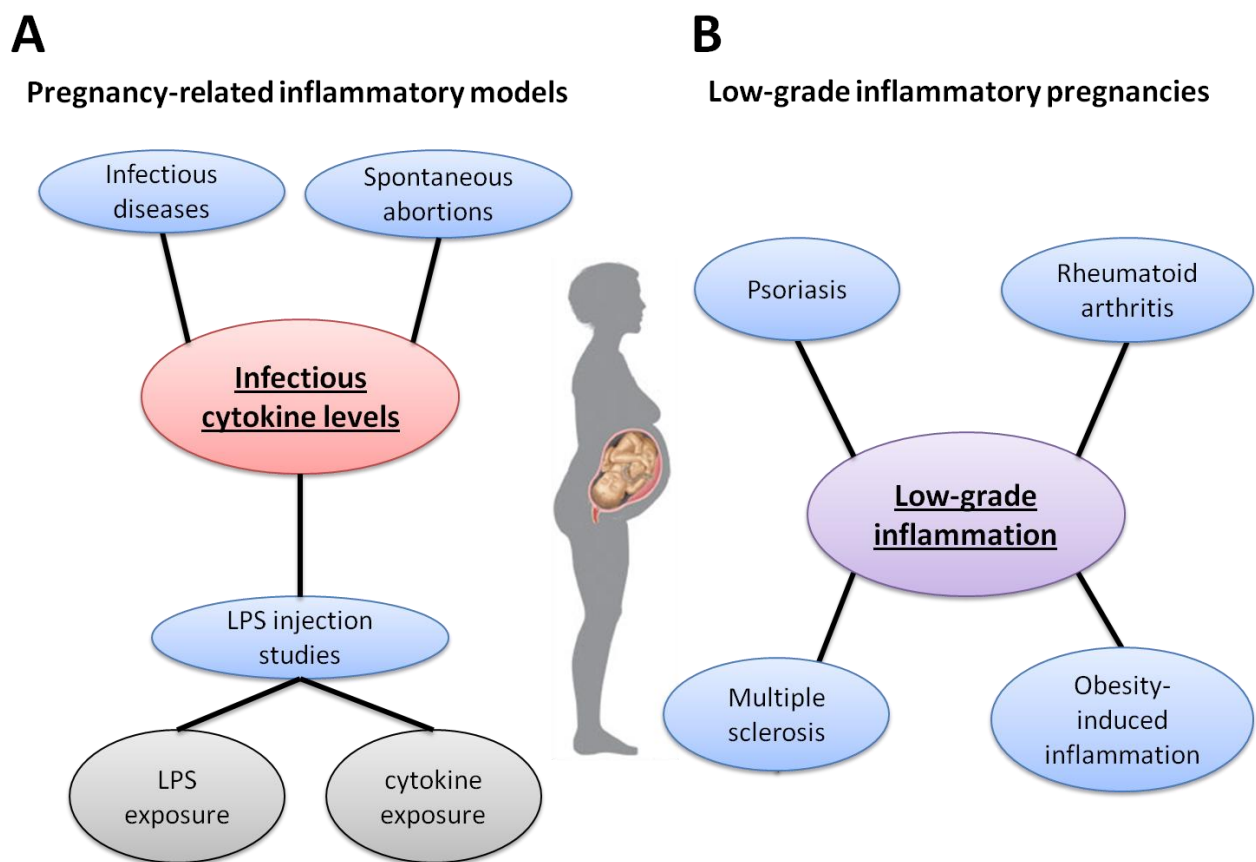
Injections of LPS in animal models (single or repetitive) have been used to investigate the effect of maternal inflammation on disease-risk in the offspring. Repetitive LPS injections in mice induce immune tolerance, meaning that repeated LPS challenge will induce a lower cytokine response and a down regulation of TLR4 expression on leukocytes compared to the initial challenge<sup>174</sup>. This also seems to be the case with prenatal exposure to LPS and repeated LPS challenges during postnatal life. Several studies have shown that maternal LPS exposure during late gestation in rats induces lower TNF $\alpha$ , IL-1 $\beta$  and IL-6 response in the offspring when challenged with LPS later in life<sup>175-178</sup>. But it also induced a decreased secretion of the anti-inflammatory cytokine IL-10<sup>176</sup>, which could indicate that maternal endotoxemia (high level LPS in blood circulation) results in an overall decreased reactivity towards LPS in offspring. A similar result was reported in a study done by Williams and co-workers<sup>179</sup>, where they investigated maternal LPS exposure during the periconceptional period where the zygote is formed. They showed that LPS tolerance in the offspring developed in a maternal dose-dependent manner. Increasing the maternal LPS dose resulted in progressively decreased immune response to LPS exposure in the adult offspring<sup>179</sup>. As previously described endotoxemia has been suggested to be important for the development of obesity, adipose tissue inflammation and type 2 diabetes<sup>133</sup>. Since offspring of LPS-exposed dams have a decreased endotoxemia response, the endotoxemia hypothesis suggests that offspring display decreased risk of obesity development and metabolic dysfunction in adult life. This is however not the case. Nilsson et al<sup>180</sup> and Wei et al.<sup>181</sup> reported that injection of LPS in pregnant rats during mid-gestation resulted in normal birth weight but increased body weight in the offspring at 11 weeks of age due to increased adiposity which correlated positively with circulating leptin levels. Food intake was also increased among the pups in both studies despite the elevated leptin level, which could indicate leptin resistance. The offspring exposed to LPS had normal glucose and insulin levels, but insulin sensitivity was decreased when estimated by euglycemic hyperinsulinemic clamp<sup>180</sup> and also systemic arterial blood pressure was increased when

compared to the control born offspring<sup>181</sup>. What might be more important, than the actual LPS tolerance, is the decreased number of circulating neutrophils, monocytes and eosinophils also observed in the offspring exposed to pre-natal endotoxemia<sup>178</sup>. As earlier described, the insulin sensitive, non-inflamed adipose tissue is characterized by a large number of eosinophils that produce IL-4, a cytokine that is necessary to sustain the anti-inflammatory M2-phenotype in tissue-resident macrophages. Contrarily, loss of eosinophils leads to infiltration with inflammatory macrophages, and reduced insulin sensitivity<sup>76,182</sup>. It is therefore noteworthy, that maternal LPS-induced inflammation during pregnancy has been observed to cause reduced number of tissue-resident eosinophils in the airways of the adult offspring<sup>182</sup>, while no data exist on adipose tissues. If this also occurs in adipose tissue in the offspring from mothers suffering from sub-clinical inflammation, they could be primed to a higher risk of adipose inflammation and, hence, metabolic dysfunction.

Endotoxemia may also impact on the function of the placenta, since LPS injections every 12 hour during late gestation in rats increased placental macrophage infiltration along with decreased placental perfusion rate<sup>154</sup>. Placental macrophage infiltration and reduced placental function were hypothesized to be caused by an increase in IL-1 levels, since administration of the IL-1 receptor antagonist half an hour prior to LPS injection protected the animals from an LPS-induced increase in numbers of infiltrating macrophages and a reduction in placental perfusion capacity<sup>154</sup>. Some of the characteristics in prenatally LPS-exposed offspring are also reported to be imprinted by prenatal exposure to cytokines alone. Prenatal exposure to TNF $\alpha$  or IL-6 (cardinal cytokines also produced after LPS exposure) results in increased body weight and adiposity in the offspring, and IL-6 exposure by itself, furthermore, results in decreased glucose uptake (i.e. decreased glucose tolerance), while no change in food intake was observed between cytokine-exposed and -control offspring<sup>183</sup>. Interestingly, a lack of pre-natal IL-6 also results in increased body weight and adiposity, increased leptin levels and decreased glucose tolerance<sup>184,185</sup>.

All these studies using animal models of LPS injections induce maternal cytokine levels that mimic that of infections, and are therefore substantially higher than the cytokine levels present during obesity-induced inflammation. Obesity-associated low grade inflammation might affect the fetus

during pregnancy, but to our knowledge nobody has yet investigated if chronic low-grade inflammation associated with obesity will affect fetal development or how a maternal Th1-skewed phenotype is affected by pregnancy. To fill the gap, we are therefore restricted to the studies of acute inflammation from LPS injections, cited above, or, alternatively, we can be inspired from studies that investigate how other chronic low-grade inflammatory diseases, like autoimmune diseases, are affected by pregnancy (Figure 2).



**Figure 2. Impact of pregnancy on inflammatory status.** Maternal inflammation happens naturally during infections and preterm labor. Besides the naturally-occurring inflammation, various animals models of maternal inflammation (A) have been developed that are mainly based on injections with LPS from Gram-negative bacteria, or injection with specific pro-inflammatory cytokines (IL-6, TNFa). The common denominator for these studies are that they focus on maternal inflammation levels that are normally present during infections. Obesity-induced inflammation is characterized by lower levels of inflammatory cytokines compared to infectious levels, so alternatively we might get a better idea of the fetal programming potential from maternal obesity-induced inflammation by studying other low grade inflammatory diseases like psoriasis, rheumatoid arthritis and multiple sclerosis (B) and the effect on neonatal immunity.

## Pregnancy has a positive effect on low-grade inflammatory diseases

Although we lack data on the impact of pregnancy on obesity-induced inflammation, data exists that describes the maternal inflammatory profiles in chronic low-grade inflammatory diseases. From these data, it is evident that the disease burden is reduced during pregnancy in several of them. In multiple sclerosis patients, relapse rates are decreased during pregnancy, with a following increase in rates after birth<sup>186</sup>. The same is seen in psoriasis patients<sup>187,188</sup>, and in patients with rheumatoid arthritis<sup>189</sup>. Based on this, we hypothesize that obesity-induced inflammation may improve during gestation, as observed for other low-grade inflammatory diseases, and that the programming of metabolic disease in offspring, observed with maternal obesity, might be mediated by changes in nutritional or hormonal components rather than being due to placental transfer of pro-inflammatory mediators. To address this issue, we recently investigated how gestation in an obese mouse model, with known effect of metabolic outcome in the offspring, affected obesity-induced inflammation at gestational day 18 (Ingvorsen et al, submitted). The study showed that macrophage infiltration in adipose tissue and liver was reversed in obese dams after 18 days of gestation to the levels seen in controls, concomitant with a normalization of pro-inflammatory cytokines in the blood. Additionally the obese dams had a normal macrophage count in the placenta. However, local tissue inflammation, like we know it from adipose tissue, liver and muscle, has been reported in the placenta of obese mothers in humans<sup>190</sup> and non-human primates<sup>191</sup>. The placental inflammation is characterized by increased infiltration of inflammatory macrophages with maternal origin and increased expression levels of pro-inflammatory cytokines (TNF- $\alpha$ , IL-6 and IL-1 $\beta$ )<sup>190-192</sup>. Additionally, increased plasma levels of C reactive protein (CRP) and IL-6 has also been reported in obese pregnant women<sup>190,192,193</sup> alongside with the presence of adipose tissue inflammation<sup>192</sup>. Thus, the obesity-induced inflammation is not completely reverse by pregnancy in obese women, like we have reported in the obese mice model. However, based on the immune-skewing that take place during pregnancy and the improvement of clinical symptoms observed in other inflammatory-diseases during pregnancy, we hypothesize that the inflammation is less pronounced during pregnancy and that a direct transfer of inflammatory mediators might not play a strong direct role in programming of metabolic dysfunction in the offspring. Instead, we can speculate that increased cytokine levels and the increased cytokine production and secretion from the placenta would result in increased

insulin resistance in the maternal insulin sensing organs via TNFa<sup>166,167</sup> and an increased fatty acid uptake via IL-6<sup>168</sup>, like described above. The inflammation would therefore have an indirect effect on the fetus by increasing the flow of lipids to the fetus. In human neonates the size of abdominal adipose tissue and hepatocellular lipid content is strongly correlated to maternal BMI<sup>194</sup> and it has also been shown in non-human primates and rodents that offspring from obese mothers have increased hepatic triglyceride and ceramide accumulation<sup>41</sup> (Ingvorsen et al, submitted, Chapter 3), which is the hepatic manifestation of metabolic syndrome<sup>195</sup>. Because hepatic lipid metabolism plays a central role in development of metabolic syndrome, an altered metabolix flux of lipids in the liver already at birth could initiate a long-time metabolic trajectory that makes the offspring more vulnerable to dyslipidemia and metabolic dysfunction later on in life.

### Concluding Remarks

Although we lack experimental data on whether chronic exposure to sub-clinical levels of inflammatory mediators in the mother are involved in metabolic programming of the offspring of obese mothers, the available literature on symptoms of other inflammatory diseases during pregnancy, data on exposure to acute inflammation through LPS injection and observations of increased macrophage infiltration of the placenta in obese mother give us some leads on the role of maternal inflammation. Pregnancy reduce the clinical manifestation of chronic-inflammatory diseases, indicating that pregnancy *per se*, can counteract an inflammatory phenotype, whereas exposure to an inflammatory environment (i.e. LPS-injections) during fetal development will program the offspring towards a metabolic phenotype similar to what is observed in offspring from obese mother. In this context, the observed reduction in tissue eosinophils in the offspring warrants particular attention, since this might be a mechanism that increases the susceptibility to future development of adipose inflammation. Future research in this area should also focus on the role of cytokines produced by infiltrating macrophages in the placenta on regulation of fatty acid flux to the fetus and whether the observed increased ectopic lipid load in the fetus/newborn leads to a persistent programming of lipid metabolism that can drive future development of metabolic diseases.







### 3. Gestation reverses obesity-induced inflammation in a mouse model of fetal programming

Camilla Ingvorsen, Anna Hammerich Thysen, Denise Fernandez-Twinn, Pernille Nordby, Kristian Fog Nielsen, Susan E Ozanne, Susanne Brix, Lars I. Hellgren

#### Abstract

**Objective.** Maternal obesity is associated with increased risk of metabolic dysfunction in the offspring. It is not clear which physiological aspects of the obese state causes this metabolic programming. Obesity causes many metabolic changes but also chronic low-grade inflammation. We therefore investigated if low-grade inflammation was present in obese dams compared to controls dams at gestation day 18.

**Methods.** Female mice were fed either a standard chow diet or a highly palatable obesogenic diet for 6 weeks prior to conception. Mice were either euthanized before mating (n=12 in each group), or euthanized on gestation day 18 (n=8 in each group). Blood and tissues were collected for analysis.

**Results** The consumption of the obesogenic diet increased body weight and decreased insulin sensitivity prior to conception, while there was no difference between the groups at gestation day 18. Local inflammation was assayed by macrophage count in adipose tissue and liver. Macrophage count in the adipose tissue was increased significantly by the obesogenic diet, and the hepatic count also showed a tendency to increased macrophage infiltration prior to gestation. This was further supported by a decreased population of monocytes in the blood of the obese animals, which suggested that monocytes are being recruited from the blood to the liver and adipose tissue in the obese animals. Gestation reversed this infiltration such that obese dams showed a lower adipose tissue macrophage count at the end of gestation compared to pre-pregnancy obese mice and there was also no longer a tendency to increased hepatic macrophage count. Placental macrophage count was also similar in the two groups.

**Conclusion** At gestation day 18, obese dams were found not to have increased macrophage infiltration in placenta, adipose tissue and liver compared to lean dams, despite an incipient infiltration before gestation. Thus, the obesity-induced inflammation was reversed during gestation

## Introduction

Maternal obesity before and during pregnancy increases the risk of metabolic disorders, such as obesity, hyperphagia, hypertension, non-alcoholic fatty liver disease and insulin resistance in the offspring<sup>25,26</sup>. The maternal cues programming offspring's metabolism are still unknown, but inflammatory mediators produced in the mother due to the obesity-induced low-grade chronic inflammation could be causative agents. This inflammatory state is characterized by increased infiltration of immune cells into white adipose tissue and the liver, as well as enhanced levels of pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-6<sup>196</sup>. Administration of inflammatory cytokines to pregnant rodents, at levels reflecting infections, such as prenatal exposure to TNF- $\alpha$  and IL-6, results in increased bodyweight and adiposity in the offspring and exposure to IL-6 furthermore resulted in decreased glucose uptake<sup>175-177,183</sup>. However, there is yet no data on the effects of fetal exposure to the sub-acute levels seen in obesity-induced low-grade inflammation. Although it is well established that excessive adiposity induces low-grade inflammation, pregnancy is characterized by local anti-inflammatory skewing of the immune response, to avoid rejection of the fetus<sup>197,198</sup>. Therefore, extrapolating the interpretation of experimental data from non-pregnant subjects to the situation during pregnancy might be misleading.

Various hypotheses have been raised to explain why excess adiposity is associated with sub-clinical inflammation; two recent models are the endotoxemia- and the lipotoxicity hypotheses. According to the endotoxemia hypothesis, recruitment of monocytes to adipose tissues is initiated through lipopolysaccharide (LPS)-mediated activation of circulating monocytes, via a TLR4- and CD14-dependent mechanism, that propagates the inflammatory phenotype<sup>131-133</sup>. This hypothesis is supported by observations that obese subjects have increased levels of circulating LPS<sup>133,199</sup>. However, it has been shown that neutrophils<sup>70,200</sup>, mast cells<sup>101</sup> and also T-cells<sup>111</sup> infiltrate the adipose tissue, coincident with a depletion of eosinophils, prior to monocyte infiltration. Due to these drastic alterations of the tissue-localized leukocyte compartment during obesity development, the endotoxemia hypothesis is unlikely to explain all these changes. The lipotoxicity hypothesis alternatively suggests that the triglyceride storage capacity of adipocytes becomes limiting, causing ectopic lipid accumulation and formation of lipotoxic intermediates that impairs metabolic function and induces an inflammatory phenotype in the tissue<sup>147</sup>. Furthermore, adipose tissue expansion alters the adipokine expression profile, which, among other effects, results in

increased leptin and monocyte chemoattractant protein-1 (MCP-1) production, which initiate migration and cytokine release of monocytes and neutrophils<sup>109,201</sup>.

Regardless of the aetiology of obesity-induced inflammation, maternal obesity-induced inflammation during gestation might cause programming effects in the offspring, leading to metabolic impairment, but it requires that the low-grade inflammation is not counter-balanced by the gestationally-induced anti-inflammatory response. To gain insight into the gestational influence on obesity-induced inflammation and its impact on the fully-matured fetus, we have first determined the level of obesity-associated inflammation in a commonly used mouse model of obesity-induced fetal-programming of metabolic dysfunction<sup>26,140</sup>, and then focused on the effect of inflammation and maternal obesity on ectopic lipid deposition in the offspring at gestational day 18.

## Materials and methods

### *Animal experiment*

All studies were approved by the local Ethics Committee and conducted according to the Home Office Animals (Scientific Procedures, UK) Act 1986. The animal model used has been described in detail by Samuelsson et al. 2008. However it should be noted in this previous study offspring of second pregnancies were studied where in the current study it is the first pregnancy that is being characterized. In brief, female C57BL/6 mice at weaning were fed a regular chow diet (3% fat (w/w), RM1, Special Diets Services, Witham, UK) (n=20) or a highly palatable obesogenic diet consisting of a high fat pellet diet (22% fat (w/w), 824053, Special Diets Services, Witham, UK) supplemented with sweetened condensed milk (Nestle, Croydon, UK) (n=21) (Supplementary figure 1). After 6 weeks on the diets, 12 mice in each group were euthanized by rising CO<sub>2</sub> concentrations, followed by immediate cardiac puncture; tissues were collected and treated as further described below. Blood plasma samples were stored at -80°C until analysed. The remaining mice were mated and euthanized on gestation day 18 (GD18), along with the unborn fetuses, followed by tissue and blood plasma sampling (lean n=8, obese n=9). Before and during gestation the dams were weighed weekly and body fat was evaluated by time-domain nuclear magnetic resonance (Bruker minispec LF series, Bruker Optik GmbH, Ettlingen, Germany).

#### *Blood stimulations and identification of monocytes in blood by flow cytometry*

Blood for profiling of monocyte numbers, TLR4 and CD14 expression levels and for stimulation with LPS was collected 2 days before the animals were euthanized. It was collected from the saphenous vein in heparin coated tubes and stained with CD45-PE, CD11b-FITC, CD14-PerCP-Cy5.5 and TLR4-APC (eBiosciences) for 30 minutes at 4°C followed by lysis of red blood cells with FACS lysing solution (BD Biosciences). Samples were subsequently analysed by flow cytometry on a FACS Calibur (BD Biosciences). The remaining blood was diluted 10 fold in complete media (RPMI-1640 with 1% penicillin and streptomycin and 1% L-glutamine) and stimulated for 24h with 500 ng/mL LPS (*E. coli* O111:B4, InvivoGen) or complete media. After stimulation, supernatants were collected for TNF- $\alpha$  analysis (Meso Scale Discovery TNF- $\alpha$  kits and Meso Scale Selector Imager 6000) according to manufacturer's instructions. The cells were collected for flow cytometry analysis, and analysed as described for the whole blood. Monocytes were gated as shown in supplementary figure 2.

#### *Blood glucose and plasma parameters*

Blood glucose was analysed by a glucometer (AlphaTRAK, Abbott Animal Health, Illinois, USA) on blood collected from the tail vein after 4 hours of fasting. Plasma triglycerides were analysed on a Cobas Mira Plus with a Horiba ABX Pentra kit (Montpellier, France). Plasma MCP-1, insulin and leptin were analysed on a Meso Scale Selector Imager 6000 with Meso Scale Discovery kits according to manufacturer's instructions.

#### *Adipocyte size*

Parametrial adipose tissue was fixed in 4% PFA immediately after sacrifice, dehydrated in industrial methylated spirit (IMS), cleared in xylene and embedded in paraffin before being cut into 5  $\mu\text{m}$  sections and stained in Mayer's Haematoxylin (Pioneer Research Chemicals) and 1% Eosin (Pioneer Research Chemicals). Slides were evaluated on a light microscope using Cell<sup>^</sup>P software program to calculate adipocyte area. All adipocytes in one image were measured and 3 images from each animal were analysed. On average 445 adipocytes from control animals and 279 from obese were quantified. Areas smaller than 200  $\mu\text{m}^2$  were not considered adipocytes.

### *Lipid analysis*

The livers were snap-frozen in liquid N<sub>2</sub> and stored at -80°C until analysed. Total lipids were extracted from maternal livers, fetal livers and placentas, as earlier described in Pedersen et al<sup>202</sup> with the exception that the following internal standards were added prior to extraction: <sup>2</sup>H-ceramide (N-palmitoyl(d31)-D-erythro-sphingosine (Avanti Polar Lipids, Alabaster, AL, US) and sphingomyelin (N-palmitoyl(d31)-D-erythro-sphingosylphosphorylcholine (Avanti Polar Lipids)), pentadecanoic acid, di-pentadecanoy-sn-glycerol, tri-pentadecanoylglycerol (all Larodan Fine Chemicals) and β-sitostanol (Sigma-Aldrich). The total lipid extract was fractionated on Strata NH2 cartridges (Phenomenex). The cartridges were rinsed and activated with 2x 1000 µL heptane, samples were applied in 200 µl CHCl<sub>3</sub> and consecutively eluted with 2x 2.0 ml CHCl<sub>3</sub>:2-propanol (2:1, v:v) (elutes neutral lipids) and 5.0 ml 2% acetic acid in diethylether (elutes FFA). The neutral-lipid fraction was dried down, re-dissolved in 200 µl CHCl<sub>3</sub> and applied to a new NH2 cartridge and further fractionated into cholesterol esters, TAG and a fraction containing cholesterol, DAG and ceramide through elution with 2x 2.0 ml hexane (cholesterolesters), 2x 1.0 ml hexane:CHCl<sub>3</sub>:ethylacetate (100:5:5 (TAG) and 2x 2.0 ml CHCl<sub>3</sub>:2-propanol (2:1). The fractionation method is a combination of the methods of Kaluzny<sup>203</sup>, Burdge et al.<sup>204</sup> and Bodennec et al<sup>205</sup> and have been validated in-house through spiking of authentic samples with synthetic lipids. Triacylglycerols were analysed as earlier described<sup>202</sup>, using GC-FID. The fraction containing cholesterol and ceramide was analysed using LC-MS as described in Supplemental Materials.

### *Histology*

Placenta and liver from female mice and fetuses were fixed in 4% PFA and incubated in 30% sucrose over night before being cut in 7 µm frozen sections. Slides were washed in 60% Isopropanol, stained with Oil Red O (Sigma, St. Louis, MO, US) and counterstained with Mayer's Haematoxylin (Sigma, St. Louis, MO, US) to detect lipid accumulation within the various tissues.

### *Immunohistochemistry*

Liver, Parametrial adipose tissue, placenta and fetal livers were fixed in 4% PFA, dehydrated in ethanol, cleared in xylene and embedded in paraffin before being cut into 5 µm sections. All

sections were dewaxed in xylene and rehydrated in ethanol. Antigens were retrieved by proteinase K (Qiagen, Hilden, DE), followed by blocking of endogenous peroxidases with 0.3% hydrogen peroxide for 30 minutes. Slides were then incubated with 10% rabbit serum (Vector laboratories, Peterborough, UK) added avidin (Vector laboratories) to block endogenous biotin for 40 minutes, followed by incubation with a primary rat anti-mouse F4/80 antibody (liver, placenta and fetal liver: BM8, Abcam, 1:200 dilution; adipose tissue: Cl:A3, AbD Serotec, 1:150 dilution) added biotin (Vector laboratories) and incubated for 2 hours. Slides were then incubated with a biotinylated rabbit anti-rat IgG antibody (1:200 dilution) for 1 hour (Vector laboratories) followed by treatment with ABC reagent (Vector laboratories) for 30 minutes and developed with a DAB reagent (Vector laboratories). Slides were finally counterstained with Mayer's haematoxylin (Sigma). Liver macrophage infiltration was measured by counting all macrophages in six images obtained at 10x magnification. Macrophage infiltration in adipose tissue was measured by counting all adipocytes and macrophages in six images taken at 10x magnifications and a macrophage-to-adipocyte ratio was calculated. Placenta samples were analysed by counting all F4/80 positive cells in a placental cross-section. The liver is involved in formation and development of blood cells during the perinatal period, where resident macrophages form erythroblastic islets with other blood cells<sup>206</sup>. This complicates macrophage count in the fetal liver, so instead macrophage accumulation was quantified by estimating the area with F4/80-positive stain. Images were obtained at 20x magnification and 4 images from each fetal liver were quantified by the "color segmentation" plug-in in ImageJ (<http://imagej.en.softonic.com/>)

### *Statistical analysis*

Data are presented in boxplots with whiskers that represent 5-95 percentiles. Not all data followed a Gaussian distribution or had equal variance among groups, therefore the gestation and diet effects were analysed by a permuted 2-way ANOVA in the Imperm package in R (<http://www.r-project.org/>), as described by Anderson, 2001<sup>207</sup>. Post hoc-tests were performed as a pair-wise comparison between groups by a two sample permutation test in the coin package in R<sup>207</sup>. Effects were considered significant when  $p < 0.05$ .



## Results

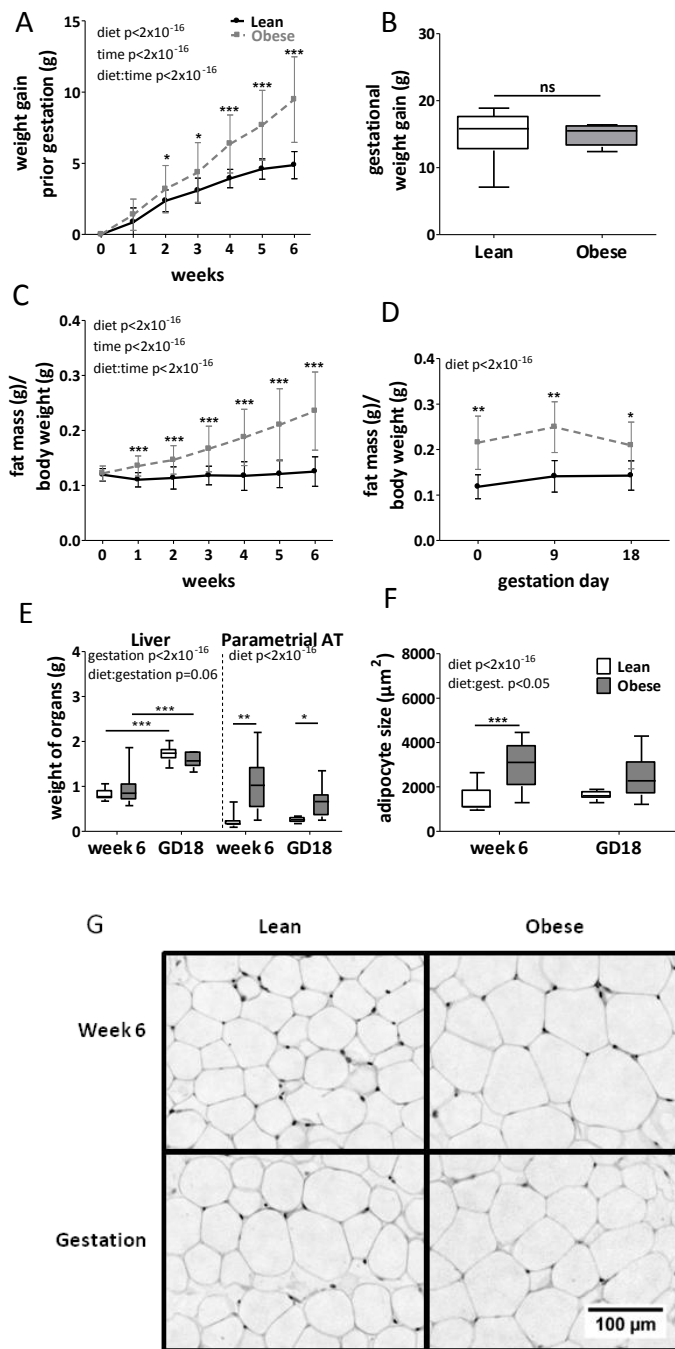
We characterized the metabolic status of non-pregnant female mice after 6 weeks feeding of an obesogenic diet and then on gestation day 18 (GD18).

Weight gain and adiposity upon 6 weeks of obesogenic diet and effects of gestation.

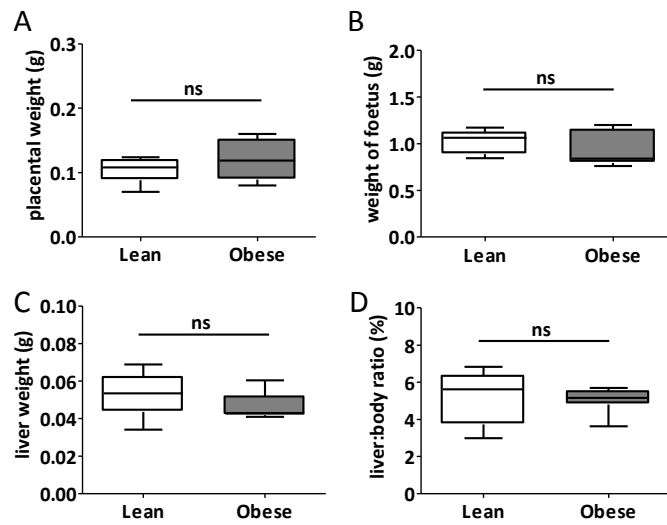
Female mice on an obesogenic diet gained more weight and had higher adiposity than mice on the control diet from 2 weeks of feeding (Fig. 1A, 1C). After 6 weeks, the obese mice had higher body weights (lean:  $22.9\text{g}\pm 1.57$ , obese:  $26.7\text{g}\pm 3.5$ ,  $p<0.001$ ), increased adiposity (Fig 1C,  $p<0.001$ ), larger parametrial adipose tissue depots (Fig 1E,  $p<0.01$ ), and larger adipocytes compared to the lean mice (Fig 1F-G,  $p<0.001$ ). The two groups had similar weight gain from conception (Fig. 1B). On GD18, the obese mice still had increased total adiposity ( $p<0.05$ ) and larger parametrial adipose tissue depots ( $p<0.05$ ) (Fig 1D-E). Gestation had no effect on adipocyte size. The obesogenic diet did not affect liver weight after 6 weeks of feeding nor at GD18, however, the liver almost doubled in weight in both diet groups during gestation (control: from  $0.81\text{g}\pm 0.11$  to  $1.73\text{g}\pm 0.19$ , obese: from  $0.94\text{g}\pm 0.35$  to  $1.58\text{g}\pm 0.17$ ) (Fig 1E). We observed no effect of maternal obesity on placental weight, fetal body weights, fetal liver weights or the liver-to-body weight ratios (Fig 2A-D).

### *Maternal metabolic parameters in blood*

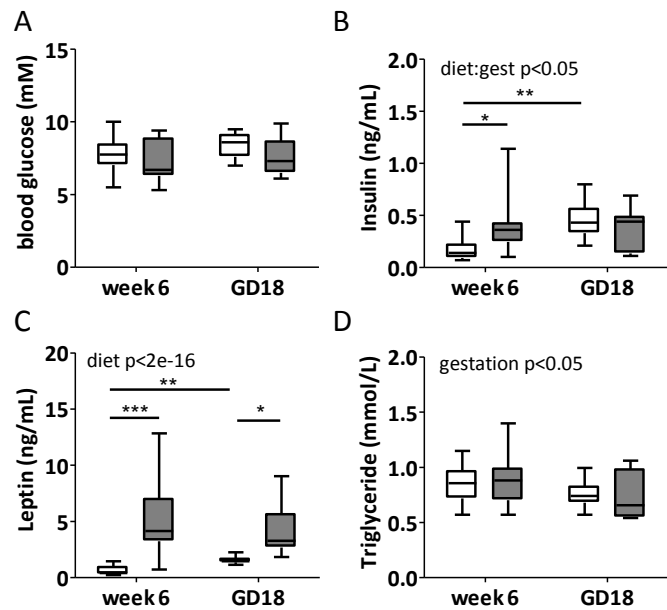
Fasting blood glucose levels were not different between diet groups after 6 weeks or at GD18 (Fig 3A). However, the obesogenic diet resulted in increased fasting insulin levels after 6 weeks on the diet ( $p<0.05$ ). Fasting insulin levels were increased as an effect of gestation only in lean dams ( $p<0.01$ ), and, notably, there was therefore no significant difference between insulin levels in the two groups at GD18 (Fig 3B,  $p=0.34$ ). Leptin was elevated in the obese animals both at week 6 and on GD18, although leptin levels also increased in the control mice with gestation (Fig 3C,  $p<0.05$ ). The levels of triglycerides in the plasma were unaffected by diet, but gestation had a significant lowering effect in both groups (Fig 3D,  $p<0.05$ )



**Figure 1. Maternal characteristics.** Maternal weight gain prior to gestation (A) and at Day 18 of gestation (B) in lean (solid line and open box) and obese (broken line and grey box) female mice. Their percentage fat mass was determined by TD-NMR prior to (C) and during gestation (D) and parametrial fat pads and livers were weighed at post-mortem (E). Finally the parametrial adipose tissue was sectioned and H&E stained (G) to evaluate the adipocyte size (F). Open boxes/broken lines : lean dams, shaded boxes/solid lines: dams fed obesogenic diet. Boxes show the 25-75 percentile and whiskers the 5-95 percentile. In A, C and D, data are given as mean  $\pm$  std. dev. At week 6, prior to conception  $n=12$ , and on gestation day 18  $n=8$  in lean and  $n=9$  in the obese group. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , ns- non significant.



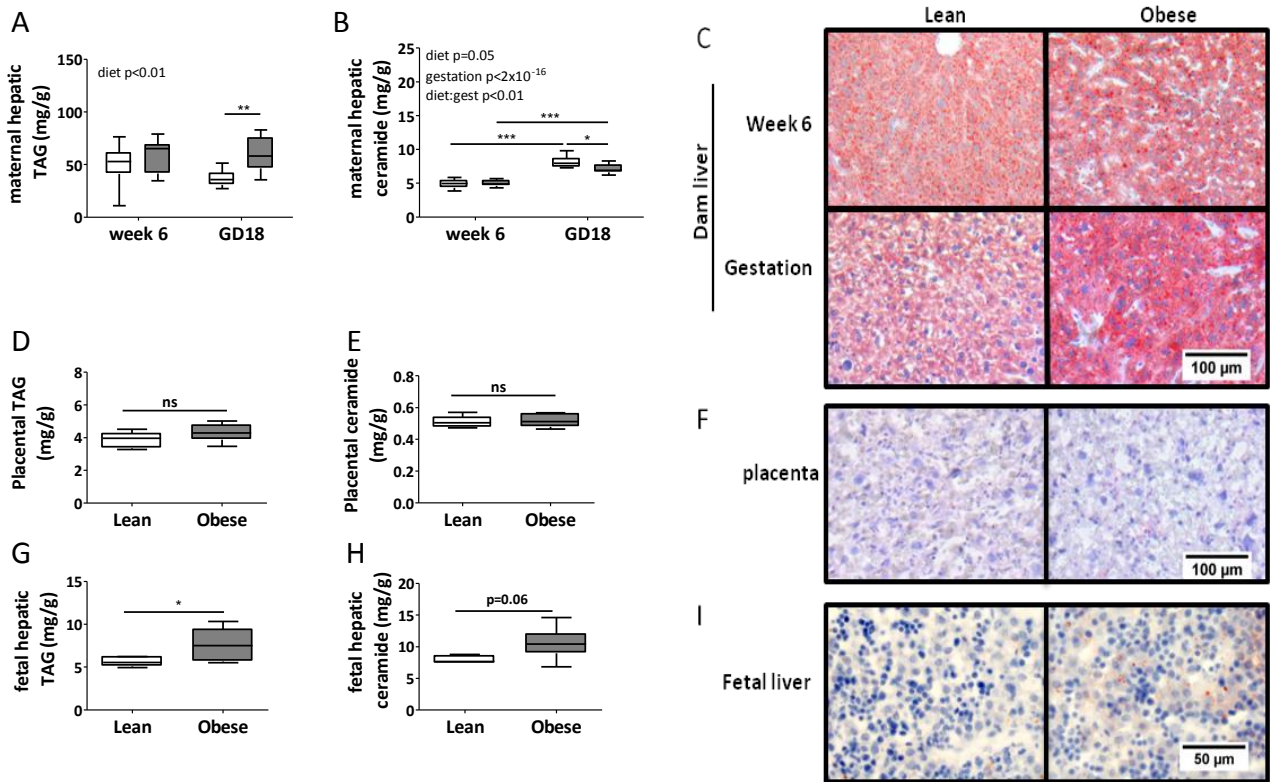
**Figure 2. Fetal characteristics.** On gestation day 18, dams were euthanized and placenta and fetuses was removed and weighed (A and B respectively). Livers from foetuses were also removed and weighed (C) and a liver to body ratio was calculated (D). Open boxes lean, grey boxes obese. All pups from a single dam were considered as a single natural replicates, so the average weight of placenta, fetuses, fetal livers and the average fetal liver to body ratio from each dam are presented (lean n=8, obese n=9). ns non significant.



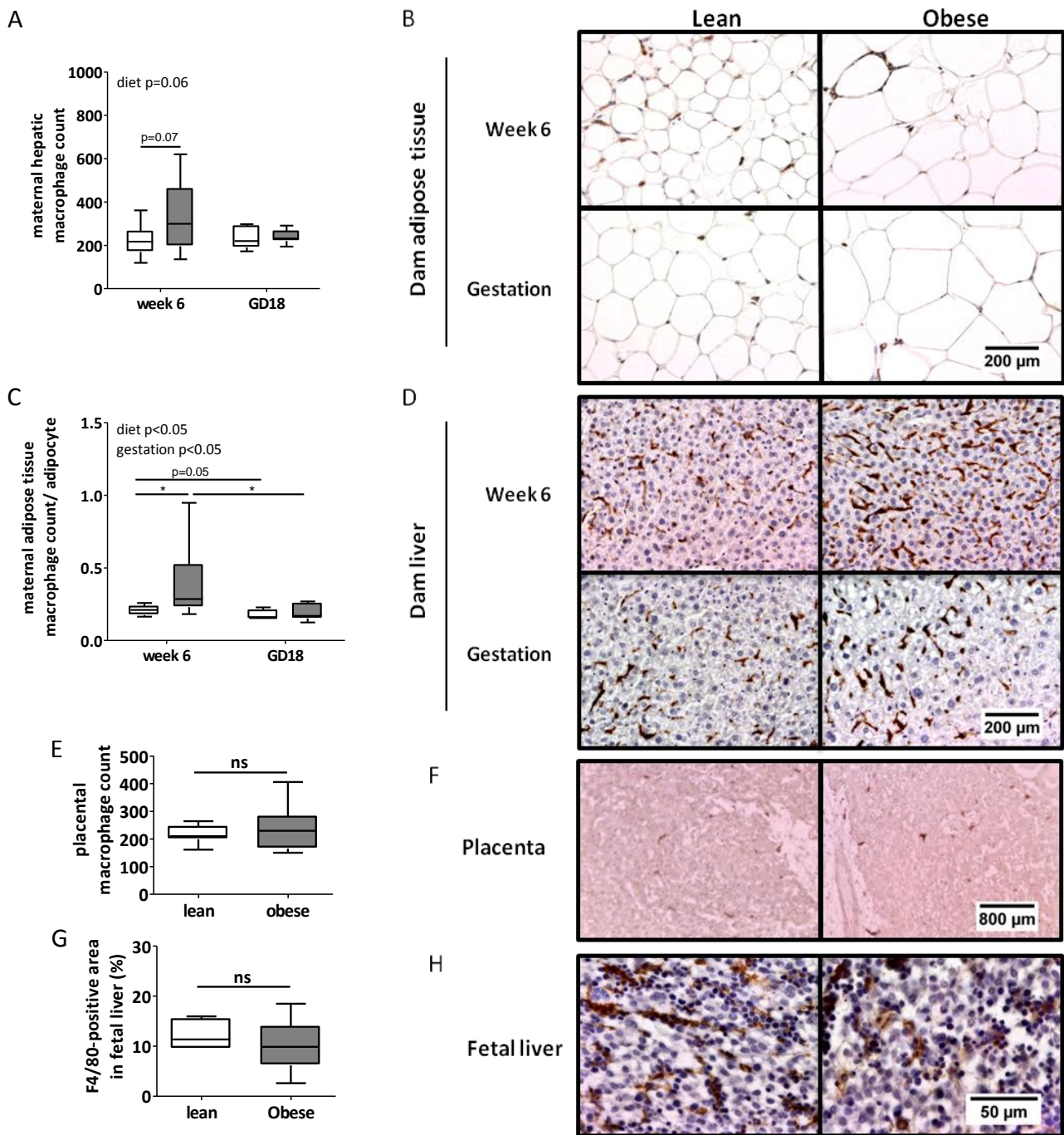
**Figure 3. Metabolic markers in plasma.** At week 6 and on gestation day 18, blood was collected after a 4-hour fast from animals fed chow (white boxes) or an obesogenic diet (grey boxes). Blood glucose levels (A) were measured in whole blood from the tail vein and insulin (B), leptin (C) and triglycerides (D) were analysed in plasma obtained from blood collected by cardiac puncture. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001

*Lipid accumulation in maternal liver, placenta and fetal liver.*

The obesogenic diet had no effect on liver weight or on hepatic lipids after 6 weeks of feeding. However, at GD18 the diet significantly increased hepatic TAG accumulation compared to lean dams ( $p < 0.01$ ) (Fig 4A, 4C accumulation). Hepatic ceramide level was also not affected by the obesogenic diet at week 6, while gestation increased the levels in both groups ( $p < 0.0001$  for 6 week vs. gestation day 18). At GD18 this increase was attenuated in obese dams, which now had lower hepatic ceramide than lean dams (Fig 4B,  $p < 0.05$ ). Interestingly, both diet and gestation altered the distribution between different ceramide species. Gestation tended to increase the concentration of C16 and C18-species, while the obesogenic diet increased the amount of ceramide-species with sphinganine as long-chained base (PCA analysis in Supplemental Fig. 3). We found no differences in triglyceride or ceramide concentrations in the placenta (Fig 4D-F), however the fetuses from obese dams had higher hepatic triglyceride ( $p < 0.05$ ) and borderline increased ceramide levels (Fig 4G-I,  $p = 0.06$ ), but no difference in ceramide-species composition (not shown). Based on all metabolic parameters, it was apparent that the obesogenic diet used in this model of fetal programming induced significant changes in overall metabolic parameters prior to mating, but also in fetuses analysed at GD18. We proceeded thereafter to characterize the level and influence of gestation on inflammation.



**Figure 4. Hepatic and placental lipid accumulation.** Lipids were extracted from maternal and fetal liver, as well as the placenta, and TAG content was analysed. A) TAG content in maternal liver; B) Ceramide content in maternal liver; C) Lipid accumulation in maternal liver determined Oil-Red O staining; D) Placental TAG content; E) Placental ceramide content; F) Lipid accumulation in placenta determined with Oil Red O staining G) TAG content in fetal livers; H) Hepatic ceramide levels in foetuses; I) Lipid accumulation in fetal liver stained with OilRed O. p<0.05, \*\*p<0.01, \*\*\*p<0.001, ns non significant



**Figure 5. Macrophage infiltration in adipose tissue, liver and placenta.** Mice were euthanized and adipose tissue (A & B), liver (C & D), placenta (E& F) and fetal liver (H) were collected, fixed and stained with an antibody targeting a macrophage surface marker (F4/80). Macrophages were counted in adipose tissue (A), liver (C) and placenta (E), but during the perinatal period macrophages form erythroblastic islets with other blood cells in the liver, which obstruct a macrophage count. It was therefore only possible to estimate the F4/80 positive stained area in the fetal livers (G), which were done by the “color segmentation” plugin in ImageJ. p<0.05, ns non significant

#### *Levels of macrophage infiltration in maternal liver and adipose tissue, placenta and fetal liver*

Adipose tissue macrophages were enhanced in obese mice prior to gestation ( $p < 0.05$ , Fig 5A, B), while liver macrophage counts tended to be higher upon 6 weeks on the obesogenic diet ( $p = 0.07$ ) (Fig 5C, D). Interestingly, macrophage counts were equalized in obese and lean dams at GD18 both in adipose tissue (Fig 5A, D) and the liver (Fig 5C, D) and there were no difference between the groups in placenta (Fig 5E, F) and fetal livers (Fig 5G, H).

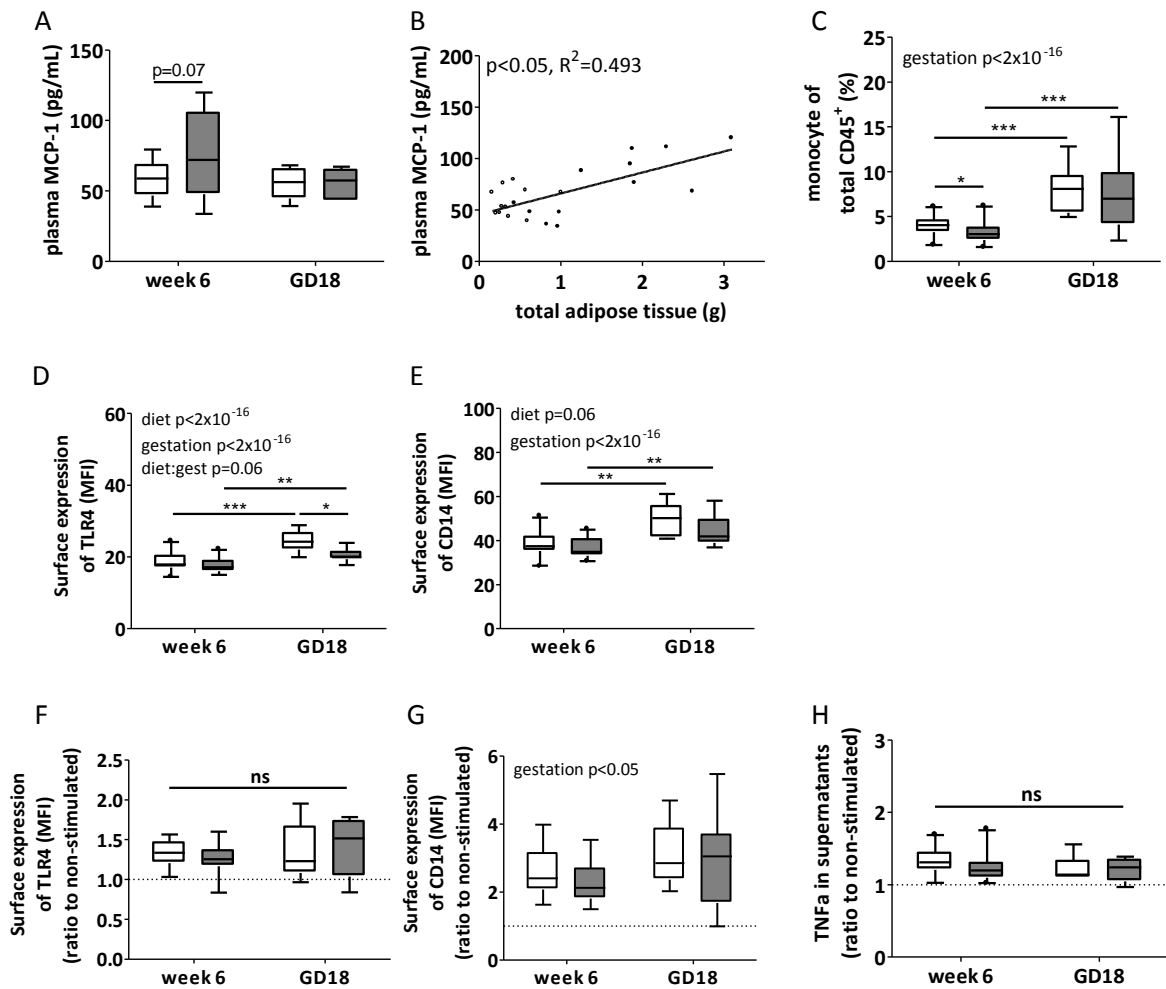
#### *Gestational effects on monocyte recruitment and activation propensity*

Since we found increased macrophage infiltration in adipose tissue (and tendencies in liver) following 6 weeks of obesogenic diet consumption, we studied the general recruitment of blood monocytes into tissues by MCP-1. MCP-1 was elevated in the obese mice at week 6, although only borderline significant ( $p = 0.07$ ), but like the hepatic macrophage count, this tendency was not observed at GD18 (Fig 6A). The levels of plasma MCP-1 correlated significantly with parametrial adipose tissue mass (Fig. 6B) implying a link between circulating MCP-1 levels and adiposity. Concomitant with the enhanced plasma MCP-1, there was a decrease in the number of blood monocytes in obese mice at week 6 (Fig 6C,  $p < 0.05$ ) supporting the idea that monocytes were being recruited to the tissues at this time-point (Fig 5A and C). At GD18, no differences were observed in plasma MCP-1 levels (Fig. 6A), blood monocyte counts (Fig 6C) or adipose tissue macrophage counts (Fig 5A) between obese and lean dams. However, blood monocyte numbers increased two-fold during gestation independent of diet (Fig 6C).

To investigate if blood monocytes were pre-activated by endotoxemia, we measured the expression of CD14 and TLR4 on monocytes from freshly drawn blood. The results show that the obesogenic diet resulted in a highly significant reduction in the expression of TLR4 (Fig 6C;  $p < 2 \times 10^{-16}$ ) and tended to decrease CD14 (Fig 6D,  $p = 0.06$ ) when analysed by 2-way ANOVA. These effects were most pronounced at GD18, where there were significant differences ( $p < 0.05$ ) between control and obese dams. This implies that monocyte function might be regulated by obesity during gestation.

To test whether the lower TLR4 expression level was indicative of LPS-tolerance due to chronic endotoxemia, we stimulated the blood with LPS for 24 hours. If LPS-tolerance is induced, we would expect TLR4 expression levels and also secreted TNF- $\alpha$  to be less up-regulated upon *ex vivo*

LPS stimulation<sup>208</sup>. Notably, the LPS stimulation resulted in an equal up-regulation of TLR4 and CD14 in both diet groups (Fig. 6F, G,  $p=0.27$  and  $p=0.80$ , respectively), and TNF- $\alpha$  secretion was also similar (Fig 6H), suggesting no apparent LPS pre-activation of blood monocytes *in vivo*. The lower TLR4 expression in gestating obese mice (Fig 6D) did not influence their ability to become activated by LPS.



**Figure 6. Monocyte recruitment and activation in blood.** Blood was collected and monocyte recruitment investigated by analyses of plasma MCP-1 levels (A). Plasma MCP-1 levels were correlated with adiposity among the obese animals at week 6 (B,  $p<0.05$ ), which could indicate increased monocyte recruitment to the tissues. Monocytes in the blood were identified by flow cytometry (CD45<sup>+</sup> CD11b<sup>high</sup>) and the abundance of monocytes (C) along with their surface expression of CD14 (D) and TLR4 (E) were analysed. Blood was also stimulated with 500ng/mL LPS for 24 hours to investigate the change in surface expression of CD14 (F) and TLR4 (G) and secretion of TNF- $\alpha$  into the supernatants (H). \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , ns non significant. Lean, week 6  $n=21$ ; obese, week 6  $n=22$ ; lean, gestation day 18  $n=8$ , obese, gestation day 18  $n=9$



## Discussion

Maternal obesity is a risk factor for metabolic dysfunction in the offspring<sup>25,26</sup>. Obesity and adipose tissue expansion induces many metabolic changes, but also a low-grade inflammatory phenotype. Both metabolic and inflammatory components might play a key role in the impaired metabolic programming in offspring. However, since gestation induces an anti-inflammatory state in order to avoid rejection of the fetus, both pro- and anti-inflammatory cues are in play in the obese mother. In order to understand the potential role of inflammatory-signals in this metabolic programming, it is important to determine to what extent metabolic inflammation is attenuated by gestation in models of offspring metabolic programming by maternal obesity. Therefore, we determined both the inflammatory and metabolic state in the mothers prior to pregnancy and at the end of gestation using a mouse model of maternal obesity with well-described negative metabolic implications for the offspring<sup>140,209</sup>.

### *Obesity-associated inflammation and metabolic impairment is induced after 6 weeks of obesogenic feeding*

In this model, 6 weeks of feeding on a high-fat diet supplemented with condensed milk prior to mating induced an obese phenotype in the dams with increased body weight, increased adiposity and decreased insulin sensitivity, as reflected by an elevated insulin level to maintain a normal glycemia. Obesity development was associated with increased macrophage infiltration in the parametrial adipose tissue and a tendency to increased macrophage infiltration in the liver. Hepatic macrophage infiltration has previously been reported to be associated with hepatic lipid accumulation and ceramide is known to induce an inflammatory phenotype<sup>195</sup>, however our data indicate that obesity-induced hepatic inflammation can occur prior to changes in the lipid content, since no significant increases in liver triglycerides or ceramide were observed at this time point. The data from the macrophage count was supported by a tendency to increased levels of MCP-1 in the blood. This cytokine is produced as a result of an early adipocyte response to high fat intake<sup>210</sup>, which activates circulating monocytes and mobilizes them to a site of inflammation<sup>211</sup>. Furthermore, the number of circulating monocytes was also reduced in the obese dams prior gestation, indicating that the increased concentration of MCP-1 provoked recruitment of circulating monocytes from the blood into tissues. Altogether, our data indicates that obesity-

induced tissue inflammation is initiated upon 6 weeks on obesogenic diet, and thus prior to mating for first pregnancy in this model.

Since the metabolic-endotoxemia hypothesis proposes that obesity leads to a chronic LPS-exposure, and thereby induces relative tolerance in target cells<sup>208</sup>, we investigated if the LPS-driven activation of monocytes differed between the two groups. In the blood, circulating LPS is bound to LPS-binding protein (LBP) and activates monocytes through a TLR4/MD2/CD14-dependent mechanism. CD14 is secreted from the monocytes and binds to LBP, which allows LPS to interact with MD2 on the monocyte surface, and then form a complex with TLR4. LPS first binds the MD2 receptor and then finally the TLR4 receptor to activate the production of inflammatory cytokines via the NF- $\kappa$ B and IRF3-signalling pathway<sup>212-214</sup>. Although our data showed a strongly significant reduction in TLR4 expression and a tendency ( $p=0.06$ ) to reduced CD14 expression on the blood monocytes of obese dams, their response to stimulation with LPS for 24 hours was similar to that of the lean dams, therefore suggesting there was no endotoxemia/LPS tolerance<sup>215</sup>. Thus, although the reduced TLR4 and CD 14 expression could be interpreted as an adaptation to chronic LPS-exposure, this did not result in functional LPS-tolerance.

#### *Effect of gestation on obesity-induced inflammation and metabolic impairment.*

The main objective of our study was to investigate how obesity-induced inflammation is affected by gestation in a well-described model of metabolic programming<sup>25</sup>. Dams fed the obesogenic diets had higher fat mass and circulating leptin levels at GD18, but despite this, our data showed that all tendencies towards obesity-induced inflammation were normalized during gestation. We found no change in hepatic or placental macrophage count on GD18. This is in contrast to previous findings by Challier et al 2008, who reported increased macrophage accumulation in human placenta from obese mothers<sup>190</sup>. However, our data is consistent with other studies, which have reported that pro-inflammatory-related diseases such as multiple sclerosis, rheumatoid arthritis and psoriasis are improved during pregnancy. Additionally, pregnant women have been shown to be more susceptible to infections such as influenza, due to the suppression of the pro-inflammatory immune system, and that persistent infections, like malaria, during pregnancy increases the risk of abortion and preterm delivery, because the body is no longer capable of sustaining the anti-inflammatory state (reviewed in Robinson and Klein<sup>155</sup>). Despite the anti-

inflammatory properties of gestation, this study cannot rule out that the inflammatory state at the time of embryonic implantation potentially could have programming effects on offspring.

However, while gestation skews the systemic immune-response towards an anti-inflammatory direction, it has been shown that maternal obesity dramatically increases fetal exposure to lipids. Zhu and colleagues reported increase lipid transport to the fetus in obese ewes<sup>216</sup> and McCurdy and co-workers have reported that a maternal high fat diet resulted in lipid accumulation in fetal liver of non-human primates<sup>41</sup>. Although we found no lipid accumulation or any sign of increased inflammation in the placenta after the relatively short pre-gestational time on the obesogenic diet, we did observe increased accumulation of triglycerides and ceramides in fetal livers. Gestation increased ceramide levels in maternal livers in both groups, but, unexpectedly, this increase was less pronounced in livers from obese mothers, resulting in a significantly higher hepatic ceramide level in lean dams compared to obese dams. These data could indicate that maternal obesity is associated with increased transfer of ceramides from the mother to the fetus. Since it is well established that ceramides inhibit signalling from the insulin receptor<sup>217</sup>, it is also noteworthy, that ceramide accumulation in the livers of the lean dams occurred concomitant with reduced systemic insulin sensitivity during pregnancy, manifested as increased fasting insulin. In order to optimize nutrient flow to the fetus, maternal insulin sensitivity is normally reduced during pregnancy<sup>218</sup>. Whether the observed hepatic ceramide accumulation is part of this normal metabolic adaption to pregnancy requires further study. Besides attenuating insulin receptor signalling, ceramide is also a highly potent inducer of apoptosis<sup>219-220</sup>. Therefore, ceramide accumulation in skeletal muscles, the liver or the heart, has been linked to development of type 2 diabetes and increased risks for cardiac failure<sup>219-221</sup>.

The model of developmental programming used in this study is more susceptible to development of metabolic diseases and cardiac dysfunctions later in life<sup>140,209,222</sup>. If the higher tissue ceramide content in the fetuses reflects persistent alterations in ceramide metabolism, this could explain the observed increased incidence and earlier onset of metabolic diseases in pups from the obese mothers.

In conclusion, in this study we found no evidence that fetal programming of metabolic dysfunction in mice born by obese dams is driven by sub-clinical inflammation during gestation. On the contrary, data supports a model in which gestation induces an anti-inflammatory skewing of the

immune system and an attenuation of the metabolic inflammation induced by the obesogenic diet. Since the mice in our study were in an early phase of the inflammatory development prior to mating, this model will not answer if gestational anti-inflammatory effects are able to reverse more pronounced pro-inflammatory states. However, the obesogenic diet caused increased ectopic deposition of TAG and ceramide in the fetal liver prior to birth. Hence the results indicate that ectopic lipid deposition, rather than maternal inflammation, may provide a mechanism for the increased susceptibility to metabolic diseases later in life in this model of developmental programming.

## Supplemental materials

### Materials and methods

#### Liver and placenta lipid analysis

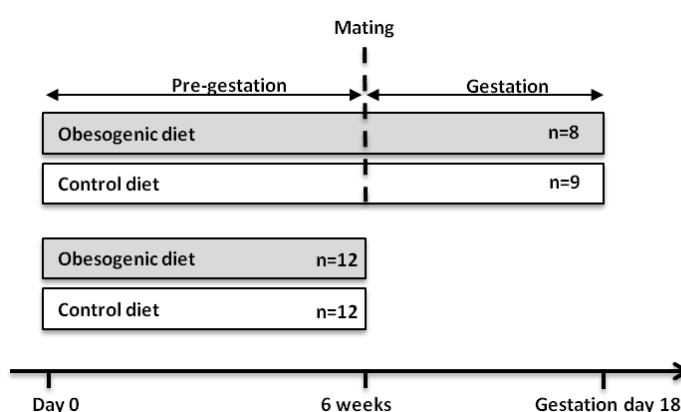
Total lipids was extracted from maternal and fetal livers as well as from the placentas, as earlier described<sup>204</sup> with the exception that following internal standards was added prior to extraction: <sup>2</sup>H-labelled ceramide (N-palmitoyl(d31)-D-erythro-sphingosine (Avanti Polar Lipids, Alabaster, AL, US) and sphingomyelin (N-palmitoyl(d31)-D-erythro-sphingosylphosphorylcholine (Avanti Polar Lipids, Alabaster, AL, US)), pentadecanoic acid, di-pentadecanoy-sn-glycerol, tri-pentadecanoylglycerol (all Larodan Fine Chemicals, Malmö, SE) and  $\beta$ -sitostanol (Sigma-Aldrich, St. Louis MO, US). The total lipid extract was fractionated into four fractions: 1) triacylglycerols (TAG), 2) diacylglycerols, ceramides and cholesterol 3) free fatty acids, and 4) phospholipids, on 500 mg Strata NH<sub>2</sub> cartridges (Phenomenex, Torrance, CA). The cartridges were activated with 2 × 1000  $\mu$ L heptane, samples was applied in 200  $\mu$ L CHCl<sub>3</sub> and consecutively eluted with 2 × 2.0 mL CHCl<sub>3</sub>:2-propanol (2:1, v:v) (elutes neutral lipids), 5.0 mL 2% acetic acid in diethylether (elutes FFA) and 4.0 mL methanol (elutes phospholipids). The neutral-lipid fraction was dried down with N<sub>2</sub> flow, re-dissolved in 200  $\mu$ L CHCl<sub>3</sub> and applied to a new NH<sub>2</sub> cartridge and further fractionated into cholesterol esters, TAG and a fraction containing cholesterol, DAG and ceramide through elution with 2 × 2.0 mL hexane (cholesterol esters), 2 × 1.0 mL hexane:CHCl<sub>3</sub>:ethylacetate (100:5:5) (TAG) and 2 × 2.0 mL CHCl<sub>3</sub>:2-propanol (2:1). The fractionation method is combination of the methods of Kaluzny<sup>203</sup>, Burdge et al.<sup>204</sup> and Bodennec et al.<sup>205</sup> and have been validated in-house through spiking of authentic samples with synthetic lipids, and analyzed on GC-FID and UHPLC-HRMS as described below. The FFA, TAG and PL fractions was analyzed by GC-FID as earlier described<sup>204</sup>.

#### UHPLC-HRMS

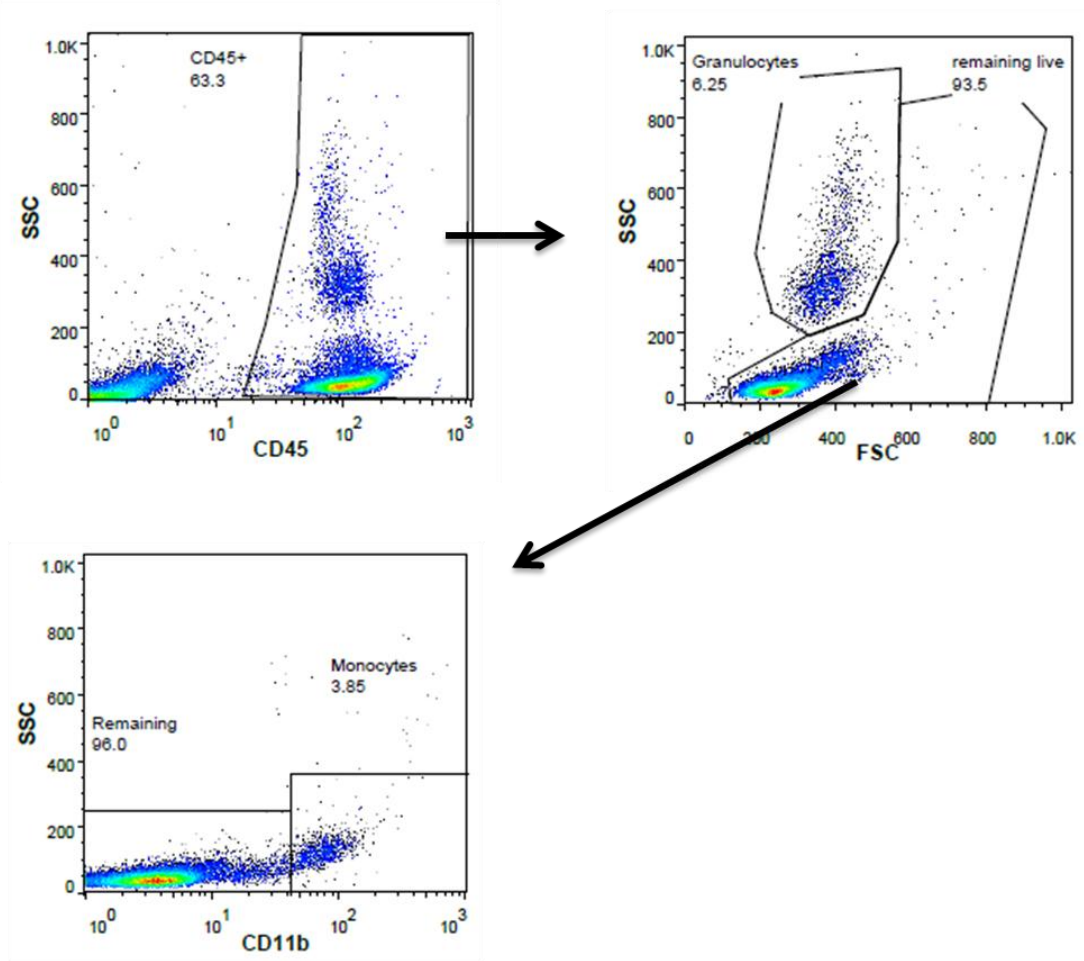
Samples were analysed by Ultra High Performance Liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) on a maXis G3 quadrupole time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) ion source. The mass spectrometer was connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, CA). Separation of 1  $\mu$ L samples were performed at 70°C on a Agilent ZORBAX Rapid Resolution HD 300SB-C3 100 × 2.1 mm ID, 1.8  $\mu$ m column (Agilent Technologies, Santa Clara, CA) using a linear

gradient of (A) water (buffered with 20 mM formic acid) and (B) iso-propanol/acetonitrile (20:80 v:v) at a flow of 0.4 mL min<sup>-1</sup> starting from 35% B and increasing to 100% in 15 min, remaining at 100% B for 4 min. MS was performed in ESI<sup>+</sup> with a data acquisition range of 10 scans s<sup>-1</sup> at *m/z* 100–1000. The MS was calibrated using the HPC algorithm on sodium formate automatically infused before each run, providing a mass accuracy of <2 ppm.

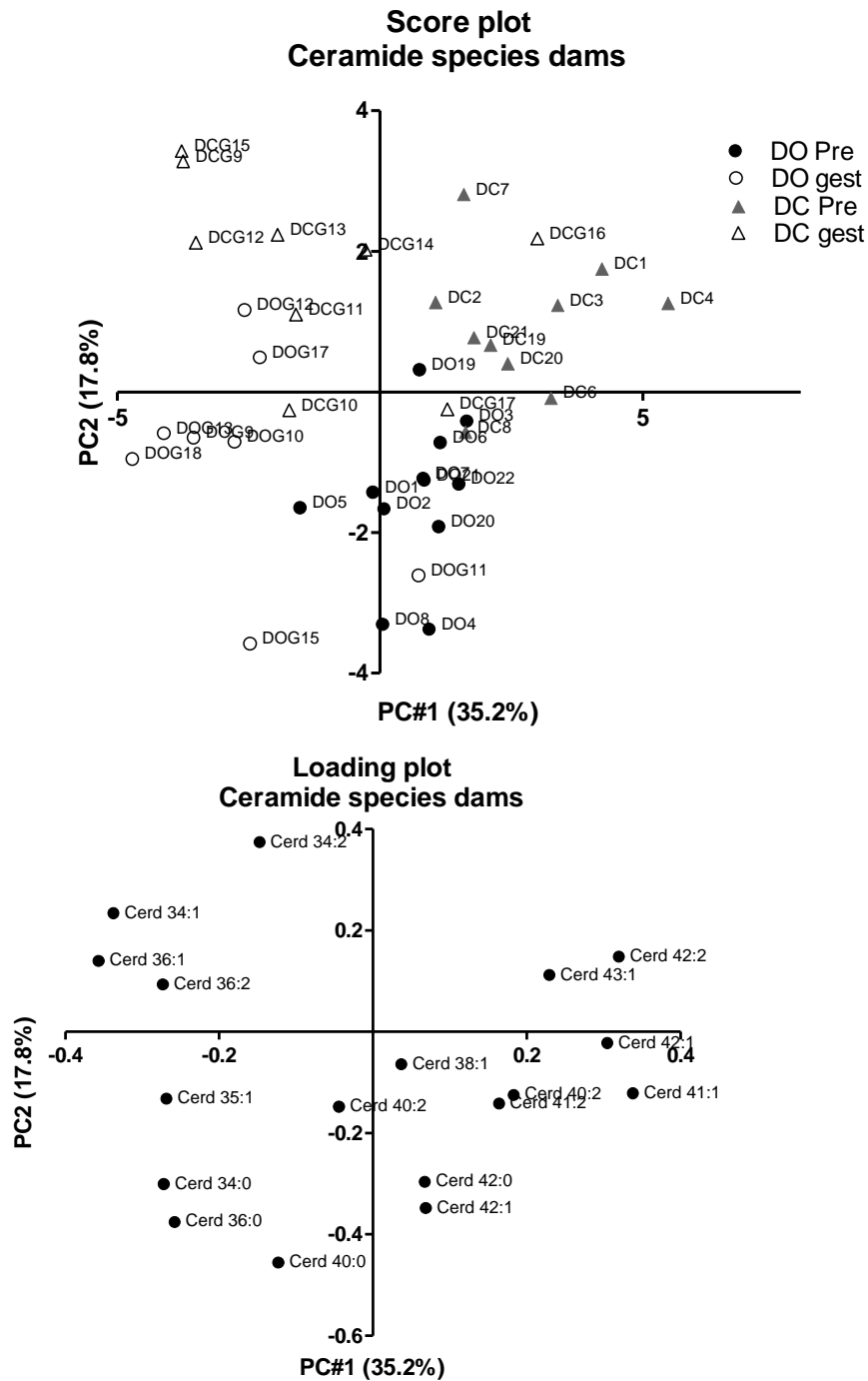
Extracted ion chromatograms of the [M+H]<sup>+</sup> ions ( $\pm 0.001$  *m/z*) for all target peaks were constructed using Target Analysis (Bruker). The target lipid compounds were identified by the retention time and accurate masses ( $\pm 1.2$  ppm). The sum of the peak areas of the [M+H]<sup>+</sup>, [M+Na]<sup>+</sup> and [M+H-H<sub>2</sub>O]<sup>+</sup> ions were used for quantification. Ceramide masses were determined based on standard-curves constructed from N-palmitoyl-D-erythro-sphingosine (C16:0 ceramide), N-lignoceroyl-D-erythro-sphingosine (C24:0 ceramide) and N-nervonoyl-D-erythro-sphingosine (C24:1 ceramide). Response factors for other ceramide species were interpolated based on the difference in response to these three ceramide species.



**Supplementary figure 1. Experimental setup.** Female mice at 5 weeks of age was assigned to either a chow diet or a highly palatable obesogenic diet consisting of a high fat pellet diet supplemented with sweetened condensed milk. After 6 weeks on the diets, 12 mice from each group were euthanized and the remaining was mated up and euthanized on gestation day 18.



**Supplementary figure 2. Gating strategy to identify monocytes in fresh blood by flow cytometry.** Monocytes were identified first as CD45<sup>+</sup>, then according to the forward scatter and side scatter and finally as CD11b<sup>high</sup>.



**Supplementary figure 3. Effects of pregnancy and the obesogenic diet on hepatic ceramide composition.** Hepatic ceramides were analysed as described in material and methods and data were analysed using principal component analysis. In the names of the ceramide species in the loading plot is the first number describing the number of carbons in the molecule and the number after the colon the number of double bonds.







## 4. Effect of maternal high fat/high sucrose intake during gestation and/or lactation on metabolic markers of hepatic lipid metabolism

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### Abstract

**Background:** Maternal high fat intake or obesity predisposes the offspring to a higher risk of developing metabolic diseases. It is however not clear if pre- and postnatal exposures have similar effects on adiposity and hepatic lipid metabolism in the young offspring.

**Method:** Rat dams were exposed to a high fat/high sucrose (HFHS) diet for 17 weeks prior to mating and during pregnancy. After birth was the male offspring cross-fostered, creating four groups differing in dietary exposure *in utero* or during lactation; control diet during both periods (CC), HFHS diet during both periods (HH), control diet during pregnancy but HFHS during lactation (CH) or viceversa (HC). At weaning was the pups euthanized, and metabolic markers in plasma were assayed, together with hepatic lipid composition and gene expression related to hepatic fatty acid metabolism.

**Results:** Pups born by control-fed dams but exposed to HFHS-diet during developed most pronounced adiposity, while pups born by HFHS-fed dams seemed to be partially protected from the effect of the high energy diet during lactation. A mismatch between maternal diet during pregnancy and lactation also led to accumulation of free fatty acids in the liver. The prenatal HFHS exposure did cause a very strong inhibition of FADS1 and FADS 2 gene expression, indicating a decreased capacity of synthesis of long chained polyunsaturated fatty acids from the dietary C18 polyunsaturated fatty acids.

**Conclusion:** Intake of energy dense diet during lactation has most severe effects in offspring that have not been exposed to this diet *in utero*. The prenatal exposure had however very pronounced effects on expression of genes involved in synthesis of polyunsaturated fatty acids.

## Introduction

Obesity and the associated metabolic disorders have become a worldwide epidemic and as a consequence, an increasing number of children are born by obese mothers<sup>4,223</sup>. From different animal models, as well as from human epidemiological studies, we know that maternal obesity and/ or high fat intake during pregnancy leads to increased risk of obesity, type-2 diabetes, hyperlipaemia, hypertension and non-alcoholic fatty liver disease (NAFLD)<sup>4</sup>. Exposure to maternal obesity and/or high fat diet during fetal life have been shown to cause very early dysfunctions in lipid metabolism manifested as increased hepatic fat accumulation in fetuses from mice and Japanese macaques (Ingvorsen et al submitted<sup>41</sup>), as well as in human neonates<sup>194,224</sup>. In human neonates, hepatocellular lipid content was shown to be significantly correlated to maternal pre-pregnancy BMI<sup>194</sup>. Thus, already at the time of birth do these children suffer from aberrant hepatic lipid metabolism, leading to increased hepatic steatosis, with potential trajectories towards metabolic diseases later in life. In most studies the maternal diets during pregnancy have also been maintained throughout lactation, but it is known that exposures during various critical windows during fetal and post-natal development results in different phenotypic outcomes<sup>14,225</sup>. In 2007, Gluckman, Hanson and Beedle described the predictive adaptive response hypothesis, which states that offspring are adapted to an expected environment during fetal life to ensure optimal survival for the offspring after birth. But if the environment turns out differently than predicted, then a mismatched phenotype occur, that can lead to increased risk of metabolic diseases<sup>19</sup>. Although some studies have addressed the issue on whether mismatch between high energy diet exposure in fetal and neonate life might have particular negative impact<sup>26,226</sup>, as suggested by the hypothesis on predictive adaptive response<sup>227</sup>, we lack information on how such a mismatch affects hepatic lipid composition and metabolism.

Hepatic steatosis is *per se* considered a benign and reversible condition, but it is often linked to accumulation of lipotoxic intermediates, such as ceramide and free fatty acids (FFA)<sup>228</sup>. Since ceramides attenuate insulin signaling, induce apoptosis, can cause formation of reactive oxygen species and are involved in inflammatory development in the liver<sup>229</sup>, increased ceramide accumulation has been suggested to be a major factor involved in the progress from the benign NAFLD, to the much more malign state, non-alcoholic steatohepatitis (NASH)<sup>229</sup>. Increased hepatic concentration of both saturated and monounsaturated free fatty acids also attenuate hepatic

insulin-receptor signaling<sup>230</sup>, indicating that early metabolic programming of the balance between FFA and TAG concentrations in the liver might affect hepatic insulin sensitivity. Thus, only considering effects on hepatic TAG levels, but not these bioactive intermediates can be misleading in evaluating metabolic risks of the offspring to nutritionally challenged mothers. The aim of this study is therefore to determine how a maternal intake of a cafeteria type diet, high in fat and sucrose, prior to and during gestation, as well as during lactation affects accumulation and composition of hepatic lipids, the expression of genes involved in hepatic lipid and fatty acid metabolism and how these factors are related to the metabolic outcome at weaning. By using a cross-fostering model, we are able to distinguish between effects induced during gestation and lactation. Because of the problems in distinguishing between effects of difference in adiposity and direct dietary effects in obese rodent-models, we have developed a model in which the high fat/high sucrose intake do not lead to increased obesity in the dams, compared to intake of chow. Since the typical human junk food diet consists of a combination of high fat food and sucrose sweetened beverage, and some data indicate that intake of sucrose as beverage has a more adipogenic effect, than sucrose in solid food<sup>139</sup>, we have chosen a model mimicking these conditions.

## Materials and methods

### *Animal experiment*

Female obese prone rats (strain: OP-CD, Charles River, Kingston, United States) 8 weeks of age, were fed a high fat/high sucrose (60E% fat, D12492, Research Diets, Brogaarden, Denmark, supplemented with 15% sucrose water) or a control diet (10E% fat, D12450B, Research Diets, Brogaarden, Denmark) for 17 weeks. After 17 weeks of feeding, the female rats were mated by strain-matched male rats, what were fed a standard maintenance diet prior to mating (Altromin, Brogaarden, Denmark). At the end of gestation all the dams had natural deliveries. Female pups were euthanized the day after birth and blood and tissues were collected and treated as further described below. The male pups were cross-fostered the day after birth among dams on either control or HFHS diet, which resulted in 4 male offspring groups; born by control dams and lactated by control dams (CC), born by control dams and lactated by HFHS dams (CH), born by HFHS dams and lactated by control dams (HC) and born by HFHS dams and lactated by HFHS dams (HH). Litters

were standardized to 4-6 pups pr dam and dams were maintained on the respective diets during mating, gestation and lactation. After 4 weeks of lactation the male offspring were euthanized along with the dams. Blood and tissues were collected and treated as further described below.

#### *Weighing and blood sampling*

Dams were weighed and blood was collected from the tail vein after an overnight fast before gestation and after weaning. Offspring was weighted at birth and weekly throughout gestation. Blood was collected from the offspring non-fasted at day 1 by decapitation, and blood from 2-3 pups was pooled for plasma generation. At 4 weeks of age, the male offspring was fasted for 4 hours before blood from the tail vein were collected for blood glucose analysis. Then the offspring were anaesthetized with a ketamin/xylazine cocktail (200mg/kg body weight ketamin and 10mg/kg body weight xylazine) and blood was collected by cardiac puncture for generation of plasma.

#### *Blood glucose and plasma parameters*

Blood glucose was analysed by a glucometer (On Call Plus, ACON laboratories, San Diego, United States). Plasma triglycerides were analysed on a Cobas Mira Plus with a Horiba ABX Pentra kit (Montpellier, France). Plasma insulin and leptin were analysed on a Meso Scale Selector Imager 6000 with Meso Scale Discovery kits according to manufacturer's instructions (Meso Scale Discovery, Rockville, United States). Insulin and blood glucose measurements were used to calculate a qualitative insulin sensitivity check index (QUICKI),  $1/(\log(\text{insulin}) + \log(\text{glucose}))$ <sup>231</sup>.

#### *Lipid analysis of mother's milk and the offspring's liver*

Stomachs from the female offspring at day 1, containing mother's milk, were collected and snap-frozen in liquid nitrogen and stored in -80° until analysis. The stomachs were divided into 2 pieces, one piece for protein extraction and 1 piece for lipid extraction.

Proteins were extracted from mother's milk by homogenizing half the content from a stomach into 100µL of water containing a protease inhibitor, followed by 2 minutes of centrifugation at 20.000xg. The supernatants were analyzed for leptin content on a Meso Scale Selector Imager 6000 with a Meso Scale Discovery kit according to manufacturer's instructions (Meso Scale

Discovery, Rockville, United States)

Lipid were extracted from the milk-bolus by the Folch method <sup>232</sup>, after addition of trionadecanoyl as internal standard and mQ-H<sub>2</sub>O to obtain the required ratio between CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O (8:4:3) in the final extract. Fatty acid content and composition in the final lipid extract was analyzed as have been described <sup>202</sup>.

The livers were snap-frozen in liquid N<sub>2</sub> and stored in -80° until analysed. Lipids were extracted using the Folch procedure, in the presence of internal standards for FFA, TAG and PL as has been described <sup>202</sup>, in addition ceramide with C17:1 long-chained base (Avanti Polar Lipids, Alabaster, AL, US) was added as internal standard for ceramide quantification, prior to extraction. One aliquot of the lipid extract was used for PL, TAG and FFA analysis as described <sup>202</sup>, and one aliquot used for quantification of ceramide, as described in Drachmann *et al.* <sup>233</sup>, but using the above mentioned C17:1 ceramide as internal standard..

### *Histology*

Liver from the offspring were immediately fixed in 4% PFA until usage. Before the tissues were cut into 7µm frozen sections, they were left in 30% sucrose over night. Slides were washed in 60% Isopropanol, stained with Oil Red O (Sigma, St. Louis, MO, US) and counterstained with Mayer's Haematoxylin (Sigma, St. Louis, MO, US) to detect lipid accumulation.

### *RT-qPCR*

Liver samples was collected from female offspring at day 1 and male offspring after weaning and stored in RNAlater at 4°C for 24 hours and then at -80 until RNA extractions. RNA was extracted by a RNeasy kit (Qiagen) according to manufacturer's instructions and converted to cDNA by a High Capacity cDNA Reverse Transcription kit (Invitrogen). Quantitative PCR was run on an 7900HT Fast Real-time PCR system (Applied Biosystem) with a Taqman Fast Universal PCR master mix (Applied Biosystems) and primers and probers for target genes from Integrated DNA Technologies (Iowa, United States) (Table 1).

**Table 1:** Primers for qPCR, 5' FAM is the reporter signal from all the probes and 3' Iowa Black FQ/ZEN is quencher

Gene	Forward primer	Reverse primer	Probe
lpl	GCTTGTCTTCTCAGTTCAGTA	CCAAGAGAAGCAGTAAGATGTACC	CGCTCTCAG ATGCCCTACAAAGTATTCC
atgl	CCAACGCCACTCACATCTAC	GCTTCCGAGCCTCCTTG	ATAATGTTGGCACCCGCTTCGC
hsl	CTACAGGACTATGTCACGCTA	CTCGTTGCGTTTGTAGTGTC	CTTCCAGTTCACACCTGCCATCCG
chrebp	CCAGTAATTACCCTCCAAGACAA	GAGAGCCTGGTACATTCAAGTATG	CAGATGAGCACCCGAAACCTGAGG
sreb1	GAAGCGGATGTAGTCGATGG	GCTACCGTTCCTCTATCAATGAC	TTATTCACTTTGCCTCAGTGCCCA
ucp1	GTCCTAAGACACCTTTATACCT	CTAGCAGACATCATCACCTTCC	CCTGGCCTTCACCTTGGATCTGA
cpt1a	AAAGTCAAAGAGCTCCACCTG	TGTTCAAGTATCTTGACAGTCG	CCTCCATGGCTCAGACAATACCTCC
fasn	GCTGATACAGAGAACGGATGAG	GCGAGTCTATGCCACTATTCTG	AATGTCACGCCTTGCTCCTTGC
cd36	CAATGAGTAGGTCTCCAACCG	TGCTCTTCTTGATTCTGCTG	TGAGAATGCCTCCAACACAGCCA
ccaca	CATCTCTAACTTCCTTCACTCCTT	TGCCGAGTCACCTTAAGTACA	AGTCCTTCTGCTCACACACTTCTG
acox	GAAGTCTGATTCTGAACGACCCA	CACTCTTCTTAACGCCACCT	TTCCTCACTCGAAGCCAGCGTT
fads1	TGCTGCCTCTCTACTTCCA	GAAGACACGAACATAGAAGCTGA	AAGTCCACCCATTTCTTCCGCTGA
fads 2	AAGACTGCTGAGGACATGAAC	TGCCGAAGTACGAGAGGAT	AGGCGATGCTTTCCATGACGATGA
scd1	GATCTTCTCATCATTGCCAAC	CGTGTGTCTCAGAGAACTTGT	TCCAGAACGATGTGTATGAATGGGCC
elov15	CCTGCTGTCTCTACATGTTC	CACCAGAGGACACGAATAACC	TTCCACACACCTGTCACCAACTC
b2m	CCAGATGATTCAGAGCTCCATAG	ACCGAGACCGATGTATATGCT	CCTGGGACCGAGACATGTAATCAAGC
gapdh	GTAACCAGGCGTCCGATAC	GTTCTAGAGACAGCCGCATC	ATCCGTTACACCCGACCTTCACC

### Statistical analysis

Not all data followed a Gaussian distribution or had equal variance among groups, so pre-natal effects at day 1, was evaluated by a two-sided two sample permutation test in the “coin” package in R (<http://www.r-project.org/>)<sup>207</sup>. Pre-natal and post-natal effects at weaning were analyzed by a permuted 2-way ANOVA in the “Imperm” package in R as described by Anderson (2001)<sup>207</sup>. Post test were performed as a pair-wise comparison between groups by a two-sided two sample permutation test and the p-values adjusted for multiple testing by the Holm method. Effects were considered significant when  $p < 0.05$ .



## Results

### *Maternal metabolic phenotype*

Female rats were fed at control or a HFHS diet for 17 weeks prior to mating. During the 17 weeks of feeding, the rats on the HFHS diet had no increased weight gain when compared to the control fed rats, despite having a net energy intake that was 1.25 fold higher than the control diet (438 kJ/d vs. 342 kJ/d, Table 2). Also blood glucose, plasma insulin, leptin, TAG and insulin sensitivity, estimated by the QUICKI index, were similar between the two groups prior to mating (Table 3). There was neither difference in weight gain during gestation or litter size among the two groups. After lactation, the dams on the HFHS diet had similar body weight and weight of total fat pads when compared to the control fed dams. Also blood glucose, plasma insulin, leptin, TAG and insulin sensitivity (QUICKI) were similar between the groups after lactation, although there was a tendency toward increased leptin ( $p=0.06$ ) and decreased plasma TAG ( $p=0.07$ ) in the HFHS group. There was neither any difference in the total fat (Table 4) nor leptin content in the milk from the two groups (data not shown). There were however, significant differences in the fatty acid composition of the milk. Thus, while the endogenously synthesized monounsaturated palmitoleic (C16:1n-7) and *cis*-vaccenic acid (C18:1n-7) constituted 10 percent of total fatty acid in the control dams, they only constituted 5 percent in the HFHS fed dams ( $p<0.05$ ). The milk-bolus from HFHS fed dams also contained considerably less fatty acids with a chain length of 8 to 14 C ( $p<0.05$ ), but more stearic acid (C18:0) ( $p<0.05$ ) (Figure 1A). To determine if these differences could be explained by the fatty acid composition in the two diets, we calculated the difference in percental distribution between the fatty acids in the milk-bolus and the diet (Figure 1B). These data show that the reduction in the monounsaturated n-7 FA and myristic acid, is due to reduced bio-enrichment in the HFHS-dams, but that there is no difference in bio-enrichment (the difference between the percental contribution of the specific fatty acid in the milk fatty acid fraction, compared to the diet) of stearic acid between the two groups. Due to the extremely low levels of medium chained fatty acids in the diet ( $<0.3\%$  of total FA), bioenrichment could not be calculated. Figure 1A and B also show that there were no differences in the PUFA concentration in the milk, despite the relative higher level of both n-6 and n-3 PUFA in the control diet, but a larger intake in mass of PUFA, in the HFHS-diet.

**Table 2:** Macronutrient and fatty acid composition of the two diets (high fat/high sucrose is based on both food and sucrose-sweetened drinking water) .

	Control	High fat/ high sucrose
Protein E%	20	11
Carbohydrate E%*	70	57
Sucrose E%*	35	50
Fat E%	10	32
Fat intake (g/d)	0.65 ± 0.08	3.8 ± 0.6
Carbohydrate intake (g/d)	10.4 ± 1.5	16.7 ± 1.8
Protein intake (g/d)	2.96 ± 0.4	2.81 ± 0.5
Energy intake (kJ/d)	342 ± 49	438 ± 39
Myristic acid (C14:0)	0.9 ± 0.0	1.2 ± 0.0
Palmitic acid (C16:0)	15.9 ± 0.0	20.2 ± 0.0
Palmitoleic acid (C16:1D <sup>9</sup> )	0.8 ± 0.0	1.5 ± 0.0
Stearic acid (C18:0)	7.6 ± 0.0	10.8 ± 0.0
Oleic acid (C18:1D <sup>9</sup> )	27.6 ± 0.0	34.8 ± 0.0
<i>cis</i> -Vaccenic acid ((C18:1D <sup>7</sup> )	1.7 ± 0.0	2.1 ± 0.0
Linoleic acid (C18:2D <sup>9,12</sup> )	39.3 ± 0.0	25.0 ± 0.0
α-Linolenic acid (C18:3D <sup>9,12,15</sup> )	4.3 ± 0.0	1.8 ± 0.0
w-6/w-3 ratio	9.2 ± 0.0	14.6 ± 0.0
Ratio C16:0/C18:0	2.1 ± 0.0	1.9 ± 0.0

\* In HFHS the sum of consumed food and water with sucrose are reported. Fatty acid data are given as percent of total fatty acids and are average ± range of duplicate determination.

**Table 3.** Physiological data on the dams before mating and when euthized after lactation. Data are given as average ± stdev. N for control = 7 n for HFHS = 3

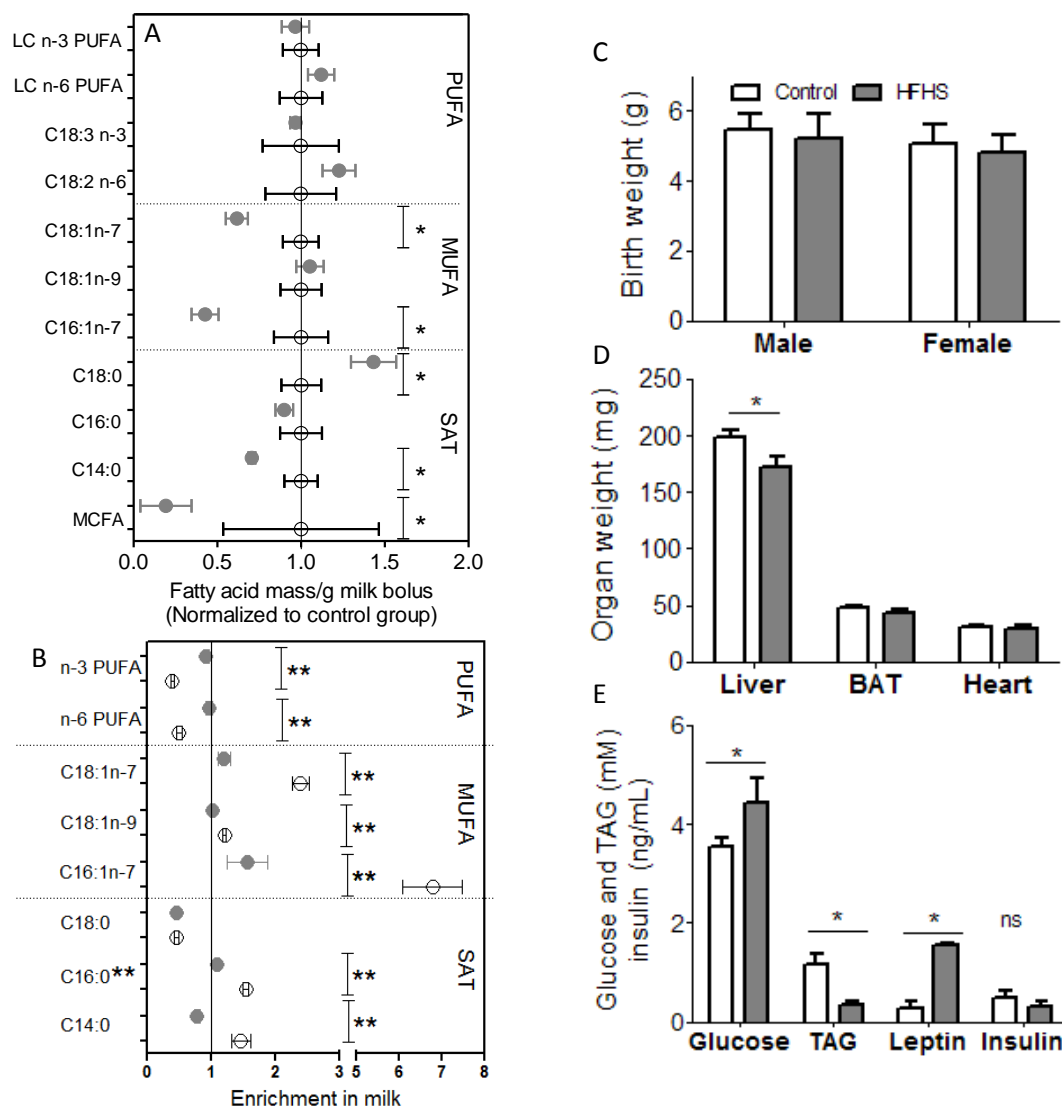
	Control	High fat/ high sucrose	p-value
Weight gain before mating (g)	124 ± 9	126 ± 16	ns
Weight gain during gestation (g)	132 ± 16	119 ± 8	ns
Litter size	11.3 ± 1.0	10.0 ± 1.4	ns
Body weight when euthanized (g)	352 ± 12	351 ± 15	ns
Total weight of fat pads when euthanized (g)	26.6 ± 5.0	30.9 ± 9.1	ns
Glucose before gestation (mM)	4.63 ± 0.77	4.13 ± 0.68	ns
Glucose when euthanized (mM)	3.74 ± 0.78	4.37 ± 0.74	ns
TAG before gestation (mM)	1.05 ± 0.24	1.07 ± 0.30	ns
TAG when euthanized (mM)	0.97 ± 0.23	0.66 ± 0.15	p= 0.07
Insulin before gestation	1.46 ± 0.58	1.12 ± 0.19	ns
Insulin when euthanized	1.36 ± 0.32	2.23 ± 1.08	ns
QUICKI index before gestation	0.29 ± 0.02	0.30 ± 0.01	ns
QUICKI index when euthanized	0.30 ± 0.02	0.28 ± 0.01	ns
Leptin before gestation	3.12 ± 0.97	4.05 ± 1.36	ns
Leptin when euthanized	4.54 ± 1.43	7.07 ± 2.19	p= 0.06

### Metabolic phenotype at birth

All pups were weighted the day after birth and the female pups were euthanized. Pups from HFHS dams tended to be slightly lighter ( $p = 0.06$ ) than the control-pups, and female pups were significantly smaller than male ( $p < 0.01$ ) (Figure 1C). The female pups born by HFHS dams had significantly smaller livers than the pups from control fed dams ( $p < 0.05$ , Figure 1D), and the liver to body-weight ratio was borderline significant ( $p = 0.052$ , data not shown). There was no difference in the mass of brown adipose tissue (BAT) or heart (Figure 1D). Although the maternal HFHS intake only had very small effects on maternal weight, and no significant effect on fat pad size, it had substantial impact on metabolic markers at birth (Figure 1E). Hence, HFHS pups had 1.25-fold increased blood glucose ( $p < 0.05$ ), although there were no differences in insulin level, five-fold increased plasma leptin ( $p < 0.05$ ) and plasma TAG concentration in the HFHS group was only 28 percent of the concentration in control pups ( $p < 0.05$ ) (Figure 1E).

**Table 4.** Fatty acid composition in milk bolus received from the stomach of the euthanized female pups. Data are given as average  $\pm$  stdev  $\text{mg} \cdot \text{g}^{-1}$ . N for control = 6 (each n is average of two pups from one dam), N for HFHS = 3.

	Control	High fat/ high sucrose	p-value
Medium chained-fatty acids ( C8:0+C10:0+C12:0)	<b>0.46 <math>\pm</math> 0.22</b>	<b>0.09 <math>\pm</math> 0.07</b>	<b>P&lt;0.05</b>
Myristic acid (C14:0)	<b>3.36 <math>\pm</math> 0.33</b>	<b>2.38 <math>\pm</math> 0.07</b>	<b>P&lt;0.05</b>
Palmitic acid (C16:0)	62.6 $\pm$ 7.8	56.3 $\pm$ 3.1	ns
Palmitoleic acid (C16:1D <sup>9</sup> )	<b>13.9 <math>\pm</math> 2.2</b>	<b>5.91 <math>\pm</math> 1.08</b>	<b>P&lt; 0.05</b>
Stearic acid (C18:0)	<b>8.86 <math>\pm</math> 1.07</b>	<b>12.7 <math>\pm</math> 1.2</b>	<b>P&lt; 0.05</b>
Oleic acid (C18:1D <sup>9</sup> )	85.5 $\pm$ 10.6	89.9 $\pm$ 7.2	ns
<i>Cis</i> -vaccenic acid (C18:1D <sup>7</sup> )	<b>10.4 <math>\pm</math> 1.1</b>	<b>6.41 <math>\pm</math> 0.69</b>	<b>P&lt;0.05</b>
Linoleic acid (C18:2D <sup>9,12</sup> )	31.8 $\pm$ 6.7	39.0 $\pm$ 3.25	ns
$\alpha$ -linolenic acid (C18:3D <sup>9,12,15</sup> )	<b>1.07 <math>\pm</math> 0.03</b>	<b>1.10 <math>\pm</math> 0.25</b>	ns
Arachidonic acid (C20:4D <sup>5,8,11,14</sup> )	12.1 $\pm$ 1.5	13.8 $\pm$ 0.8	ns
Eicosapentanoic acid (C20:5D <sup>5,8,11,14,17</sup> )	<b>0.83 <math>\pm</math> 0.11</b>	<b>0.84 <math>\pm</math> 0.01</b>	ns
Docosapentanoic acid (C22:5D <sup>4,7,10,13,16</sup> )	0.78 $\pm$ 0.15	0.52 $\pm$ 0.17	$p = 0.06$
Docosapentanoic acid (C22:5D <sup>7,10,13,16,19</sup> )	<b>0.94 <math>\pm</math> 0.13</b>	<b>0.95 <math>\pm</math> 0.04</b>	ns
Docosahexaenoic acid (C22:6D <sup>4,7,10,13,16,19</sup> )	1.49 $\pm$ 0.14	1.36 $\pm$ 0.28	ns
Total fatty acids	243 $\pm$ 34	242 $\pm$ 19	ns

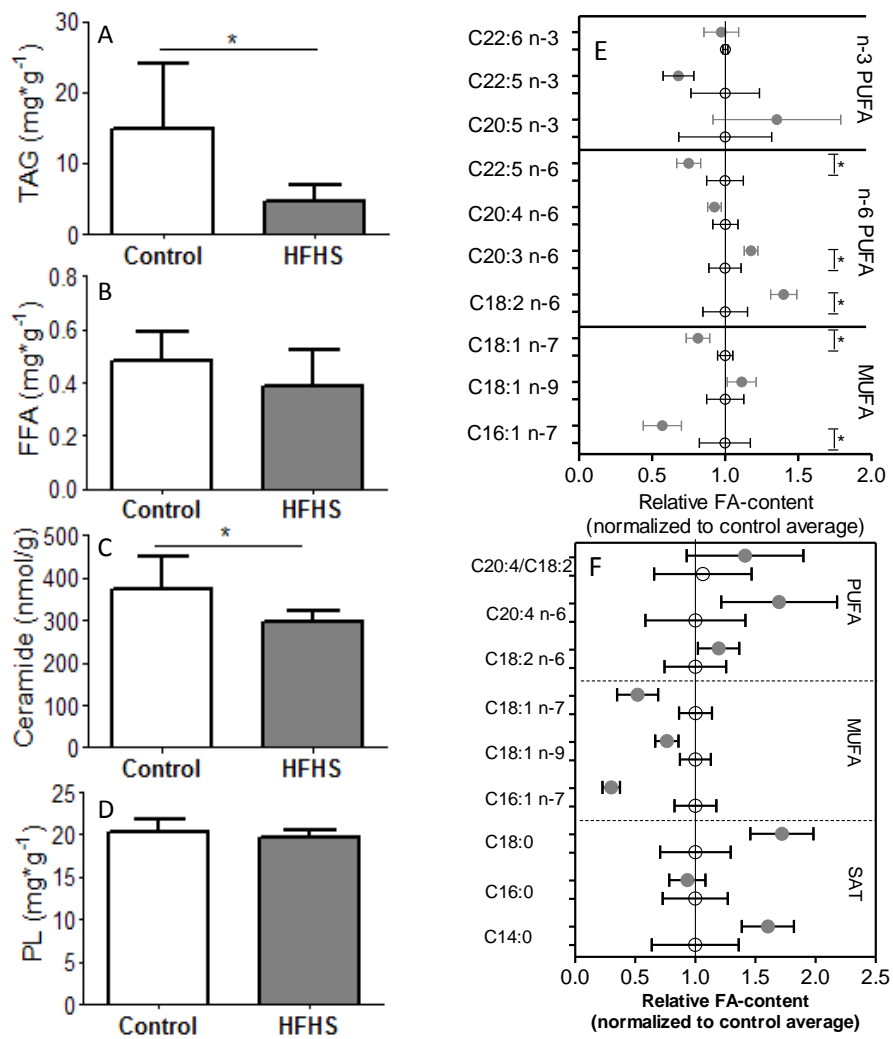


**Figure 1. Weight, metabolic parameters at the day after birth and fatty acid composition in mother's milk. A)** Weight of male and female pups from dams fed control-diet (open bars) and high fat/high sucrose diet (HFHS) (closed bars). Data are given as average  $\pm$  std. dev. N for male control pups were 27 and female control pups 32, n for male HFHS were 19 and females 12. **B)** Organ weight in female pups at birth. Otherwise as in A. **C)** Metabolic plasma parameters in the pups at birth, otherwise as in A. **D)** Fatty acid composition in the milk bolus retrieved from the stomach of female pups the day after birth. Data are normalized to the concentration of each fatty acid in milk from the control mothers and are given as average  $\pm$  std. **E)** Enrichment of the different fatty acids in mother's milk, compared to the percentage of each fatty acid in the two diets. A value of 1, represents that the percental contribution of the fatty acid in mother's milk equal its percental contribution in the diets. \* =  $p < 0.05$ , \*\* =  $p < 0.01$

### *Hepatic lipid metabolism in female offspring at birth*

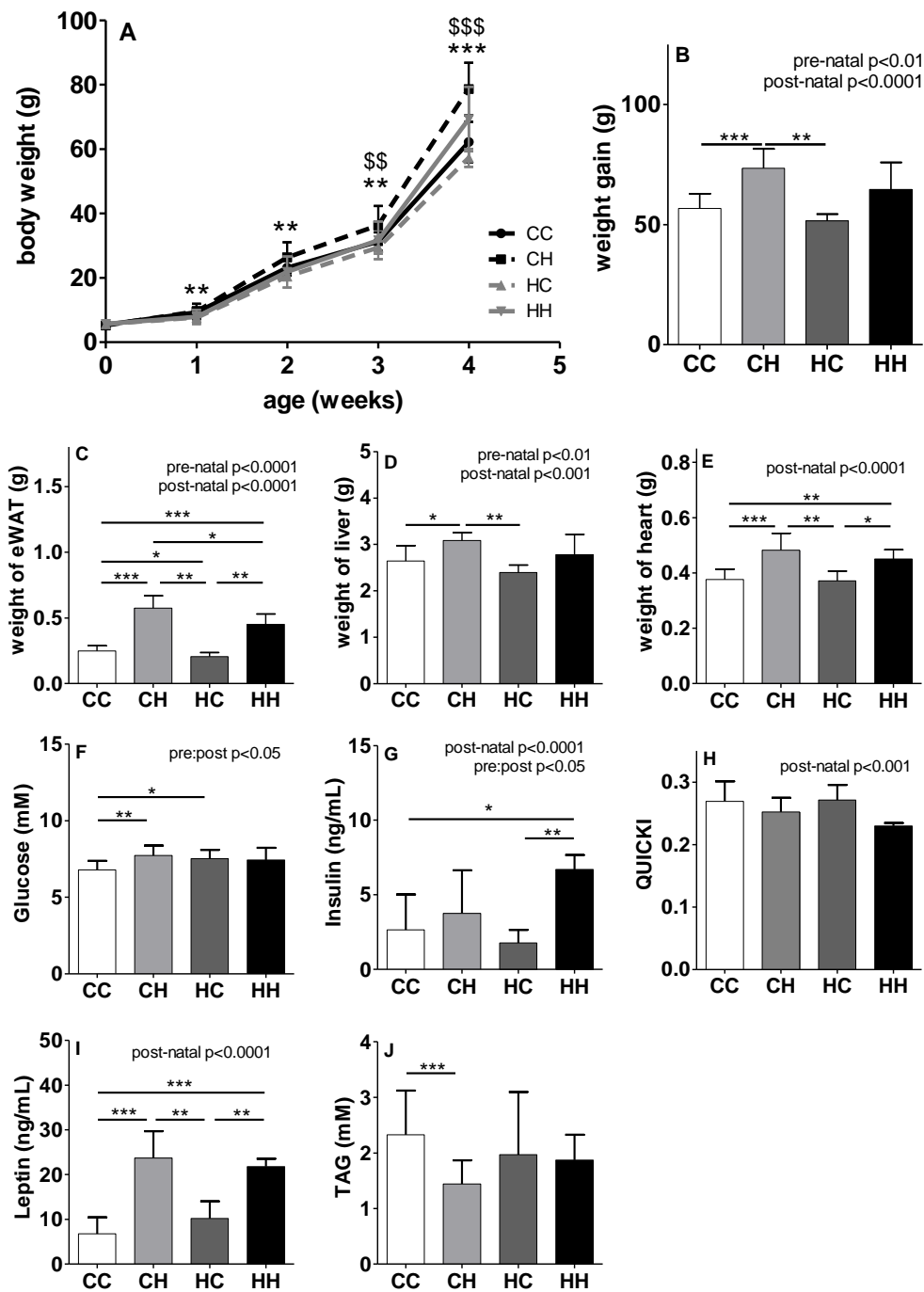
The pups from HSHF-fed dams had on average only one third as high hepatic TAG concentration as the control pups (4.62 vs. 14.94 mg/g) at birth (Figure 2A). The reduced hepatic lipid levels was also manifested in a less pronounced reduction in ceramide, while there were no differences in FFA or PL content (Figure 2 B-D). The maternal HFHS diet caused substantial alterations in the FA-composition of the hepatic lipids in the newborn pups, compared to the control diet. Figure 2E show that hepatic phospholipids from the HFHS-pups were depleted in palmitoleic (almost 50% decrease,  $p < 0.05$ ) and *cis*-vaccenic acid (20% decrease,  $p < 0.05$ ) compared to the control pups, thus a similar pattern to what we observed in the milk. In the PUFA fraction, there was a skewing towards the initial steps in the biosynthesis of the n-6 long-chained PUFA (LC-PUFA) in the HFHS-pups. Thus, they had significantly higher levels of linoleic acid (C18:2n-6) (1.4 fold increase,  $p < 0.05$ ) and di-homo- $\omega$ -linoleic acid (C20:3n-6) (1.2 fold increase) but reduced levels of the end product in this pathway, docosapentaenoic acid (C22:5n-6) (25 % reduction,  $p < 0.05$ ), while there were no difference in the concentration of arachidonic acid (C20:4 n-6). A similar tendency was seen in the n-3 LC-PUFA, were an average reduction in n-3 docosapentaenoic acid with 30 % was borderline significant ( $p = 0.07$ ) (Figure 2E).

The increase in the FA representing the earlier steps and decrease in the final product of the transformation of the dietary linoleic acid to LC-PUFA, could either be due to the composition of the FA transferred over the placenta, or to altered hepatic FA metabolism in the fetuses. Therefore, we analyzed the FA-composition in plasma from the dams after they were euthanized (Figure 2F), as well as the gene expression for the enzymes involved in n-7 MUFA (stearoyl-CoA desaturase I (SCDI) and LC-PUFA synthesis (FADS1, FADS2, ELOVL5) (Supplementary figure 1), in fetal livers. The maternal plasma fatty acid composition, showed the same pronounced decrease in n-7 MUFA (Figure 2F), as we observed in the milk bolus (Figure 1A) and the fetal hepatic PL (Figure 2E). There was no significant difference between the two groups in the relative content of PUFA, in the maternal plasma (Figure 2F). None of the assayed genes was statistically significantly differently expressed in the HSHS born pups, although SCD1 was borderline increased ( $p = 0.06$ ) (Supplemental figure 1).



**Figure 2. Hepatic lipids and fatty acid composition in the female pups the day after birth.** A) Hepatic lipid composition (A- D) as well as the fatty acid composition in hepatic phospholipids (E) and fatty acid composition of total lipids in maternal plasma at weaning (F). In E and F data are normalized to the percental contribution of each fatty acid to the total fatty acid pool in the pups born by control mothers.

N for control pups were 12 and for HFHS pups 6. N for dams in figure D was 4.



**Figure 3. Body weight, organ weight and metabolic plasma markers at 4-weeks of age.** A-B) Growth curves (A) and weight-gain (B) for the pups during lactation. \*\* = pre-natal effect  $p < 0.01$ , \*\*\* = prenatal effect  $p < 0.001$ , \$\$ = post-natal effect  $p < 0.01$ , \$\$\$ = post-natal effect  $p < 0.001$  C-E) Organ weights at 4-weeks of age. F-H) Fasting blood glucose, fasting plasma insulin and the derived QUICKI values,; I) Plasma leptin levels and J) Plasma TAG concentrations. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$  between the connected groups.

### *Weight of offspring during lactation*

After birth, the male offspring were cross-fostered, meaning that control- and HFHS-born pups were lactated by either control (CC, HC) or HFHS dams (CH, HH). The pups were weighted weekly and at 1 week of age, pups born by HFHS dams (HC, HH) had lower body weight than the control-born groups (CC, CH) (pre-natal effect,  $p < 0.01$ ), independent of the diet of the lactating dam (Figure 3A). This pre-natal effect was maintained throughout lactation ( $p < 0.01$  at week 2,  $p < 0.01$  at week 3,  $p < 0.001$  at week 4), but at 3 and 4 weeks of age the pups lactated by HFHS dams had increased body weights compared to the control lactated pups (post-natal,  $p < 0.01$  and  $p < 0.001$  respectively). The total weight gain during lactation was decreased in the HFHS-born pups (HC, HH) compared to the control born (CC, CH) (pre-natal,  $p < 0.01$ ), but the weight gain were increased in HFHS-lactated pups (CH, HH) compared to control-lactated pups (CC, HC) (post-natal,  $p < 0.0001$ ) (Figure 3B). Hence only weight-gain in the CH group was significantly higher than in the groups lactated by control dams.

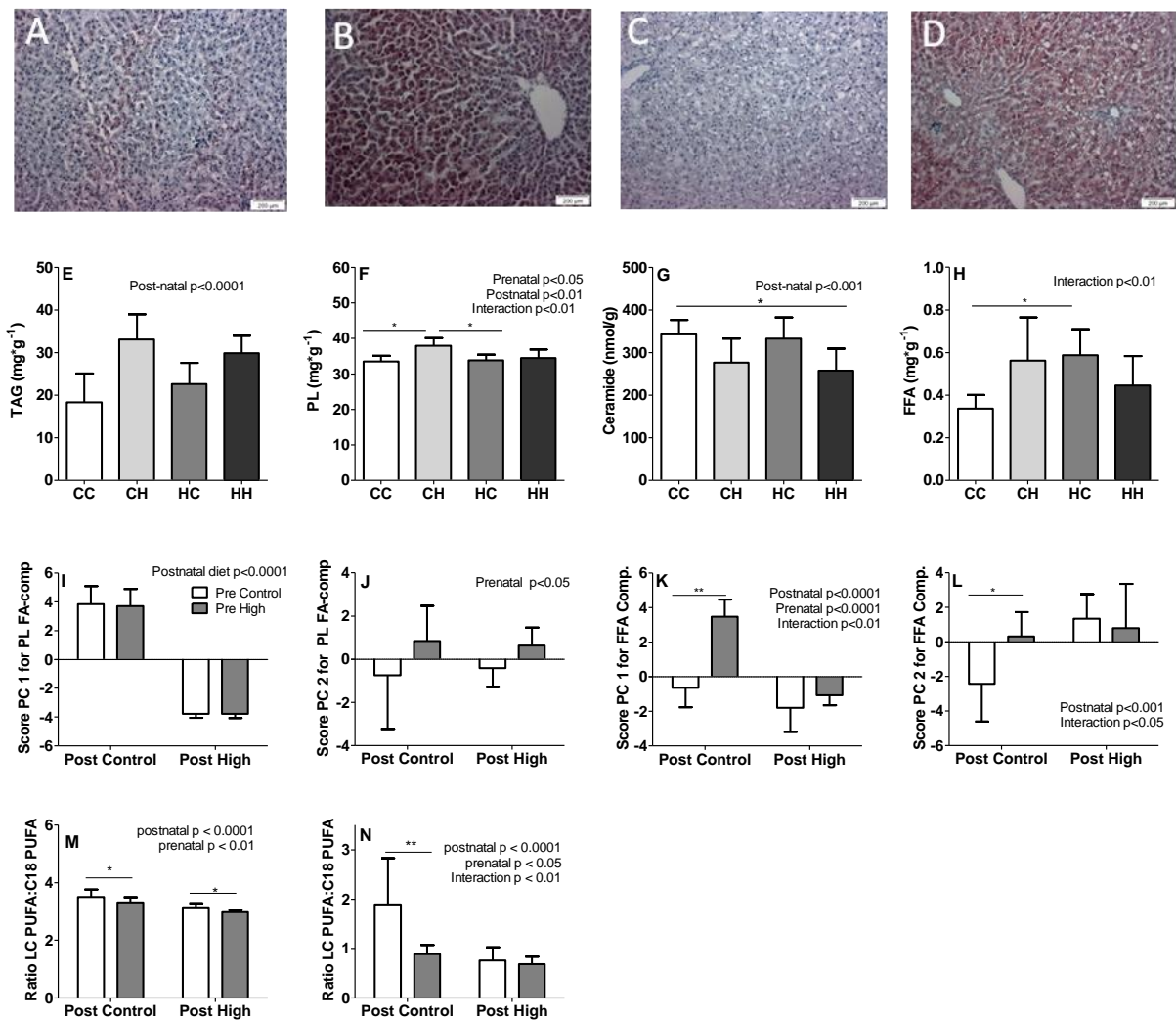
### *Metabolic status at weaning*

HFHS born pups had decreased adipose tissue mass compared to the control-born pups (pre-natal,  $p < 0.0001$ ), but lactation by HFHS dams increased adiposity to a larger extent than lactation by control dams (post-natal,  $p < 0.0001$ ) (Figure 3C). Thus, epididymal fat pads were largest in the CH group ( $0.574\text{g} \pm 0.09$ ), followed by the HH group ( $0.451\text{g} \pm 0.08$ ), then the CC group ( $0.248\text{g} \pm 0.04$ ) and finally the HC group ( $0.205\text{g} \pm 0.03$ ) (Figure 3C). The same pattern was seen on the weight of the liver; pre-natal exposure to HFHS diet decreased the weight ( $p < 0.01$ ), while post-natal exposure to HFHS during lactation increased the weight ( $p < 0.001$ ), resulting in significantly larger livers in the CH pups compared to CC pups (Figure 3D), also when calculated as percent of bodyweight (data now shown). There were no pre-natal effect on the weight of the hearts, but pups lactated by HFHS dams had increased heart weights compared to the control lactated pups ( $p < 0.0001$ ) (Figure 3E).

Blood glucose was measured in blood from the tail vein before the animals were anaesthetized. Glucose levels were significantly increased in the groups representing a mismatch between pregnancy and lactation, the CH and the HC groups ( $p < 0.01$  and  $p < 0.05$ , respectively), when compared to the control (CC) group (Figure 3F). Plasma insulin levels were increased in the HFHS



lactated pups (post-natal,  $p < 0.0001$ , Figure 3G). It is noteworthy that this was not the case in the HC group, despite the increased glucose level. HFHS-lactated pups also had a decreased insulin sensitivity, shown by a decrease in QUICKI value (post-natal,  $p < 0.001$ ) (Figure 3H), and increased plasma leptin levels (post-natal,  $p < 0.0001$ ) (Figure 3I). Plasma TAG was decreased in the CH group compared to the control (CC) group ( $p < 0.001$ ), this was the only effect seen on plasma TAG (Figure 3J).



**Figure 4. Hepatic lipid accumulation and composition.** The livers from the animals sacrificed at 4 weeks of age were Oil Red O stained (A=CC, B=CH, C=HC, D=HH) to visualize lipid accumulation and the concentration of specific lipid classes were chemically analyzed (E-H). The fatty acid composition of hepatic phospholipids and free fatty acids were analyzed by principal component analysis, and the derived principal component 1 (PC 1) and 2 (PC 2) were tested for significant variation between the treatments (I-L). Since the PCA analysis indicated an altered production of long-chained PUFA from C18-PUFA, we calculated their ratio in phospholipids and free fatty acids (M-N). Otherwise as in figure 3

### *Lipid composition and lipid metabolism in male offspring at weaning*

At four weeks of age, HFHS-lactated pups have increased hepatic lipid-levels, as seen in the oil-red O staining (Figure 4 A-D) and in the results from the chemical analysis of hepatic TAG (post-natal,  $p < 0.0001$ ) (Figure 4E). The hepatic phospholipid (PL) content was also significantly affected by the dietary treatments, the CH pups had an increased concentration of PL compared to CC and HC ( $p < 0.05$  vs. CC and HC, and  $p < 0.08$  vs. HH) (Figure 4F). Unexpectedly, hepatic ceramide levels were decreased with about 20 % (post-natal,  $p < 0.001$ ) in animals lactated by HFHS mothers, compared to the control lactated pups (Figure 4G). There was an interaction between the pre-natal and post-natal exposure in the effect on hepatic free fatty acid concentration, with a tendency to increased free fatty acids in the CH-group ( $p = 0.07$ ) and a significant increase in the HC groups  $p < 0.05$ ) (Figure 4H).

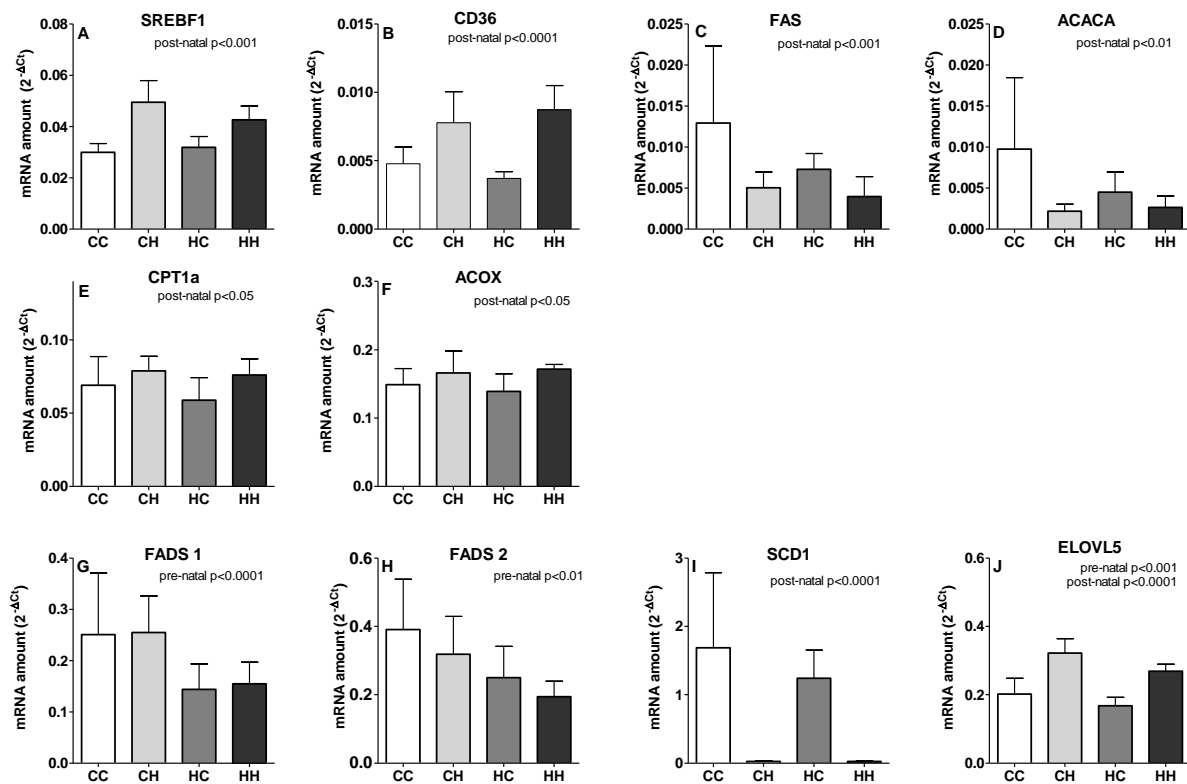
In order to detect potential changes in the total fatty acid composition of the glycerolipids, we performed principal component analysis on these data sets (Supplemental figure 2 for score and loading plots), and tested the derived principal components for treatment effects. For PL and TAG, separation along the first principal component, which explained 62 (PL, supplementary 2A-B) and 78 (TAG, supplementary 2C-D) percent of the variation in the data, was driven by the postnatal treatment ( $p < 0.0001$ ). However, separation along the second principal component, explaining 11 (PL) and 12 (TAG) percent of the variation, was significantly affected by the prenatal treatment ( $p < 0.05$  and  $p < 0.01$  for PL and TAG, respectively) (Figure 4I-J for PL, data not shown for TAG). While the postnatal intervention had a dominating role in determining the TAG and PL fatty acid composition, this was not the case for the free fatty acids. For the free fatty acids, both pre- and postnatal treatments had strongly significant effects ( $p < 0.0001$  for both) effects on PC 1, as well as a significant interaction ( $p < 0.01$ , Figure 4K). This was in particular driven by the separation of the HC group, from the other three groups along PC 1 (Supplemental figure 2 E-F). Only the post-natal treatment had significant effects on PC 2, although a significant treatment interaction effect and a significant difference between CC and HC ( $p < 0.05$ ) indicates that the pre-natal exposure to the HFHS-diet affected the response to the post-natal control diet s (Figure 4L). From the loading plots for PL and TAG, it is evident that the first endogenous product in the n-6 pathway,  $\alpha$ -linolenic acid (C18:3n-6), have strong impact on the distribution along PC 2, and among the major fatty acids, C18:2 n-6 and C20:4 pulls the samples apart along this axis. Since this indicate that the

biotransformation of the dietary derived C18-PUFA to C20 and C22 was affected by the prenatal treatment, we calculated the ratios between the long-chained (C20 and C22) and C18 PUFAs, as a measure of the efficacy in the PUFA biosynthetic pathway. The efficacy was decreased by both pre- and post-natal exposure to the HFHS diet, as seen in Figure 4M for PL (a similar result was seen in TAG, data not shown) and Figure 4N for free fatty acids.

To assay whether the increased amount of hepatic FFA in the pups that had been exposed to a dietary mismatch (HC & CH), was due to transcriptional up-regulation of hepatic fatty acid uptake, fatty acid synthesis or reduced  $\beta$ -oxidation, we analyzed mRNA levels for the rate-limiting enzymes, as well as the transcription factors, controlling these processes (Figure 5A-F). The results show that the hepatic metabolism was transcriptionally adapted to the increased fatty acid load in the HFHS-lactated pups, but dietary mismatch did not alter transcriptional regulation of these genes. Thus, postnatal HFHS-exposure caused increased expression of the fatty acid transporter CD36 ( $p < 0.0001$ ), reduced expression of genes involved in fatty acid synthesis (FAS,  $p < 0.001$ ) and acetyl-CoA carboxylase (ACACA,  $p < 0.01$ ) and a slight increased expression of genes for enzymes controlling mitochondrial and peroxisomal  $\beta$ -oxidation (Cpt1a,  $p < 0.05$  and ACOX,  $p < 0.05$ ). In the pups lactated by HFHS-dams, Srebf1 was also increased ( $p < 0.001$ ).

We also determined the expression of the genes involved in synthesis of LC-PUFA, the desaturases FADS1 and FADS 2 as well as the elongase ELOVL5. Expression of both FADS1 and FADS2 were significantly reduced by the pre-natal, but not by the post-natal HFHS exposure (Figure 5G-H). However, ELOVL5 was up-regulated by the postnatal HFHS exposure and, strikingly, the gene expression of SCD1, was almost totally inhibited in animals lactated by HFHS-fed dams (Figure 4I-J). Hence, pups lactated by control dams had 60-fold higher expression of SCD1 (Figure 4I).

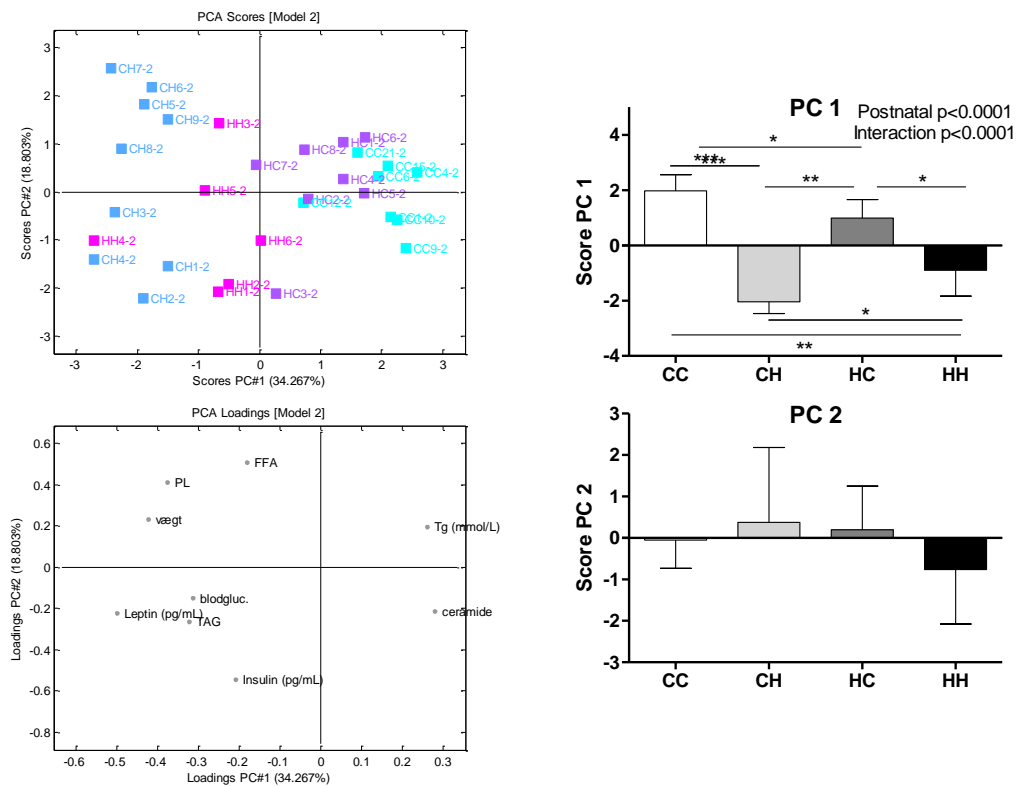
To get an integrated quantification of the parameters related to metabolism and hepatic lipids, we performed a PCA analysis in which we combined the general metabolic data and hepatic liver masses (Figure 6). The resulting score-plot show that the postnatal effect is driving the separation along PC1 ( $p < 0.0001$ ), but that there are a strong interaction factor ( $p < 0.0001$ ), caused by the separation of that the CH-group from the HH group (CH vs HH  $p < 0.05$ ), showing that the metabolic parameter in CH group deviates most from the control-animal (Figure 6).



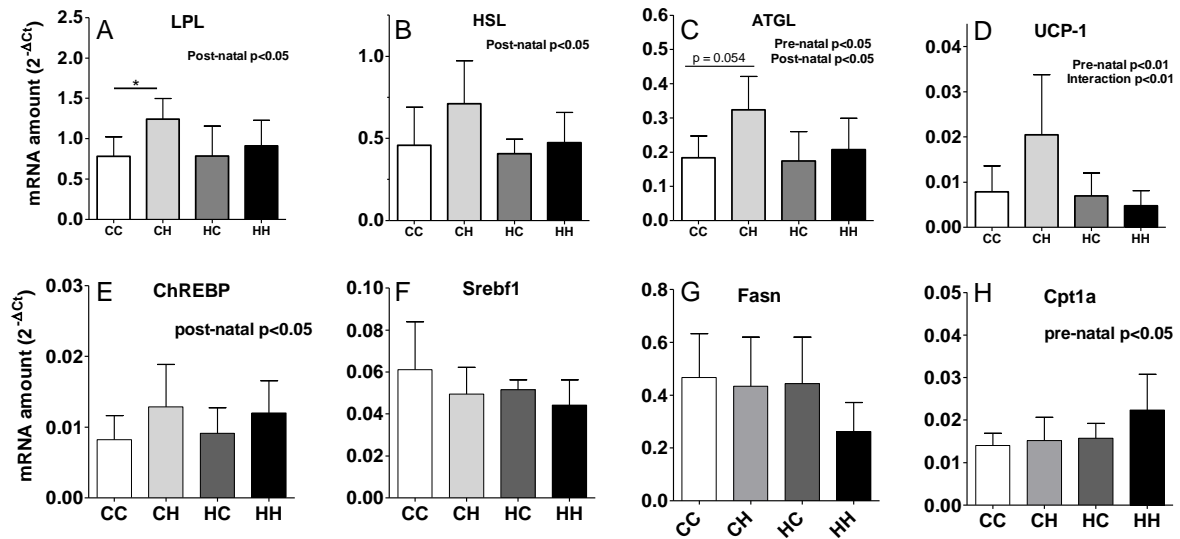
**Figure 5. Expression of genes related to hepatic fatty acid metabolism.** mRNA level of related to fatty acid uptake (A), fatty acid synthesis (B-D),  $\beta$ -oxidation (E-F), PUFA desaturation (G-H), MUFA synthesis (I) and elongation of PUFA (J) was quantified using RT-qPCR. Otherwise as in figure 3.

### *Lipid metabolism in adipose tissue at weaning*

Altered dynamics in adipose fatty acid metabolism and release of fatty acids could explain the increased level of FFA in the mismatched groups, therefore we investigated expression of genes coding for the enzymes responsible for fatty acid flux in the epididymal adipose tissue (Figure 7). The pups that had been exposed to HFHS during lactation had increased mRNA levels of lipoprotein lipase (LPL), adipose triglyceride-lipase (ATGL) and hormone-sensitive lipase (HSL) compared to control exposed pups (post-natal,  $p < 0.05$  for all three genes). The level of mRNA for ATGL was furthermore decreased in epididymal adipose tissue in pups born by HFHS-fed dams (pre-natal,  $p < 0.05$ ), while no effects of the pre-natal exposure were seen on LPL and HSL. ChREBP was also increased in HFHS lactating pups (post-natal,  $p < 0.05$ ), but no effect were seen of the mRNA level of Srebf1. UCP-1 mRNA levels were decreased in HFHS born pups (pre-natal,  $p < 0.01$ ), whom also showed increased mRNA level of Cpt1a (pre-natal,  $p < 0.05$ ). There were no pre-natal or post-natal effects on the mRNA level of Fasn.



**Figure 6. Principal component analysis of the combined phenotypic parameters.** A PCA was performed, loading body weight, metabolic plasma parameters and the hepatic concentration of the different lipids. A) Score-plot, B) Loading plot. C-D) PC1 and 2 for the different groups. Otherwise as in figure 3.



**Figure 7. Gene expression of genes related to fatty acid flux in epididymal white adipose tissue.** mRNA for genes involved in adipose fatty acid uptake (A), TAG hydrolysis (B-C), fatty acid oxidation (D and E) and fatty acid synthesis (F-H) were analyzed using RT-qPCR.

## Discussion

Exposure to a maternal high energy diets during gestation and lactation increases the risk of metabolic dysfunction in the offspring later in life and causes changes in hepatic lipid metabolism in the offspring that are evident already at birth. However, less is known on whether changes in maternal diets during lactation can reverse these changes, or whether a dietary mismatch between pregnancy and lactation will worsen the metabolic consequences. This study uses a maternal HFHS dietary rat model and reports acute effects in the male offspring at weaning. Furthermore, the offspring has been cross-fostered during lactation, which made it possible to investigate matched (CC and HH) and mismatched (CH and HC) predictive adaptive responses in the offspring.

The maternal HFHS dams used in this study had similar body weight, blood glucose, plasma insulin levels, insulin sensitivity, leptin and TAG levels as the control-fed dams. Thus, the effects reported in the offspring were primary caused by dietary pre- and post-natal exposure, and not maternal metabolic changes or obesity, which can develop during HFHS feeding.

At birth, the male and female pups born by HFHS dams tended ( $p=0.06$ ) to have decreased body weight as well as reduced liver weight. Along with a tendency to growth retardation, the HFHS offspring had increased blood glucose and plasma leptin levels, while no effect on insulin levels were seen. It is important to note that control and HFHS dams had almost identical protein intakes, the tendency to lower weight in the HFHS-born pups can therefore not be explained by protein restriction. Additionally the HFHS pups had decreased plasma TAG levels and decreased levels of hepatic TAG and ceramide.

HFHS-weaned pups had an increased body weight compared to the control-lactating offspring at 3 weeks of age. We suspect that this weight gain was caused by the pup's natural dietary changes around 2-3 weeks of age, where their primary nutritional intake no longer is dominated by the mother's milk. At 3 and 4 weeks of age the CH group was significantly heavier than the control (CC) group, potentially due to the combination of exposure to a control diet during fetal life and HFHS diet during lactating, which both increased the offspring's body weight. The increased body weight was caused by elevated adiposity in the HFHS weaned offspring, but the CH offspring had

larger epididymal fat depots than any of the other groups, including the HH group. This result indicates that adiposity is controlled by a predictive adaptation in fetal life, potentially through a higher energy expenditure in the HH group than in the CH group. Additionally, the HC group also had decreased epididymal fat depots compared to the CC group. This data support that a mismatch between the predicted adaptive response during fetal life and the actual post-natal environment are important in determining adiposity.

The observed reduced birth and liver weight as well as the reduced hepatic and plasma lipid levels at birth in pups from high energy-fed dams is in accordance with the finding by Zhang *et al* (2011) in pups born by rat dams fed a chocolate and high fructose beverage diet<sup>39</sup> and Krasnow *et al* (2011) in pups born by high fat fed C57/bl5 mice dams<sup>234</sup>. In the latter study, the authors also analyzed body composition of the neonates, showing that pups from HF-fed dams had more body fat, than the control pups. This indicate that, at least in a mouse-model, the observed effects on hepatic and plasma lipids is not due to reduced placental energy transfer, but rather altered fat distribution and metabolism, pointing toward a reduced capacity for lipid storage in the fetal liver as a target for this programming.

A comparison of the maternal plasma fatty acid composition with the fatty acid composition in hepatic phospholipids in the newborn pups (Figure 2E and F), also indicates that the fetal fatty acid metabolism is affected by the maternal HFHS-diet. Thus, while there were no difference in plasma PUFA composition between the dams, there is a very pronounced skewing toward incorporation of the shorter chained, less unsaturated, PUFA (i.e increased C18:2 n-6 and decreased incorporation of C22:5 n-6) in the HFHS-born pups. This indicates that synthesis of the long-chained PUFA from the dietary C18-precursors is hampered in the HFHS-born pups. Synthesis of long-chained PUFA takes place through desaturation and elongation of the dietary essential fatty acids, linoleic and  $\alpha$ -linolenic acid. The desaturation is catalyzed by the  $\Delta^5$ - and  $\Delta^6$ -desaturase (FADS1 and FADS2, respectively), and elongation by the elongase ELOVL5<sup>235,236</sup>. In the rodents, as well as in humans, FADS1 and 2 are localized in close proximity in a gene-cluster, and gene expression is to a large extent controlled by the same factors, were in particular dietary PUFA intake have strong inhibitory effects on their expression<sup>237</sup>. Analyses of mRNA levels for these enzymes in the liver at birth did not show any significant differences in their expression, although

average FADS1 mRNA level was substantially lower than for the control animals (Supplemental figure 1), but at weaning, a very strong inhibitory effect of the prenatal high energy diet was evident on the FADS-genes. Thus in both the HC and HH groups was average mRNA level for FADS1 only 50% of what was found in pups born by chow-fed dams, and the expression of FADS2 was similarly down-regulated in the HFHS-born pups, although not to the same extent (figure 5G and H). This is partly in accordance with recent findings, that in rat mothers, increasing intake of butter during pregnancy and lactation, programs a reduced expression of FADS2 but not FADS1 in the offspring at 2.5 month of age, through increased methylation of several sites in the promoter region of the FADS2 gene <sup>238</sup>. However, in addition to the findings by Hoile and co-workers, our data shows that it is the prenatal, but not post-natal exposure to the high energy-diet that programs FADS expression-pattern. It should however be noted, that interpretation of these results is not straightforward. Although the HFHS-diet in present study contained a lower concentration of linoleic and  $\alpha$ -linolenic acid, than the control diet, the high fat fed dams in our study, as well as in the by Hoile *et al*, have had a higher intake of PUFA than the low fat fed controls. Hence, in our study was the average PUFA intake among the HFHS-fed dams 1.0 g/day and in the control dams 0.27 g/day and in the study by Hoile *et al*. had the HF fed dams 6 times higher absolute PUFA intake, than the low fat fed. Thus, we cannot exclude that it is the higher absolute PUFA intake, rather than high fat intake, that cause the programming. In our study, the changes in expression of FADS and EVOVL5 only resulted in minor changes in the fatty acid composition of hepatic lipids at weaning, but it cannot be excluded that the *in utero* programming of hepatic FADS expression will lead to lower PUFA supply to the tissues later in life, as for example seen as a reduced C20:4 n-6/C18:2 n-6 ratio in hepatic lipid at 90 days of age in pups born and lactated by cafeteria-diet fed dams in a study by Bouanane *et al*. <sup>239</sup> and a reduced C20:4 n-6 and C22:6 n-3 concentrations in hepatic PL in the earlier mentioned study by Hoile and co-workers <sup>238</sup>.

In all analyzed samples; mother's plasma mother's milk, neonate livers and livers at 4 week of age, the concentration of the n-7 unsaturated monounsaturated fatty acids were downregulated. (figure 1A-B, figure 2E-F and supplemental figure 2). The n-7 monounsaturated fatty acids are synthesized from palmitic acid by stearoyl-CoA desaturase I (SCDI), and while this expression of its



gene tended to be up-regulated in the liver of the newborn dams, it was almost fully inhibited in the livers of the 4 week old pups that had been lactated by HFHS-fed dams. Hepatic SCD1 expression is down-regulated by increased PUFA-load and by leptin, but up-regulated by insulin<sup>240</sup>. Thus, the most likely explanation for the observed 60-fold decrease in SCD1 expression in the HFHS-lactating pups is their increased leptin levels. As further discussed below, the reduction in synthesis of palmitoleic acid (C16:1 n-7) in the HFHS-lactated pups, might have metabolic implications.

It is also noteworthy that the phenotype with increased blood glucose without a concomitant compensatory rise in insulin seen at birth in the HFHS-born female pups (Figure 1E), also is evident at 4 weeks of age in pups that have been cross-fostered to control mothers during lactation (HC group in Figure 3F and G). This suggests that fetal exposure to a high fat/high sucrose diet, without maternal obesity, cause relatively persistent alteration of glucose homeostasis, which is not reversed by a normal diet during lactation. This could indicate altered  $\beta$ -cell function already at birth, manifested as reduced glucose-induced insulin secretion. It was earlier shown that islets isolated from 9-month old rats born by dams fed high fat diet had reduced glucose-induced insulin secretion, although this was not manifested as an altered fasting glucose:insulin ratio, as we see in present study<sup>241</sup>, and in a recent study, newborn mice born by high fat fed dams had almost 50 % reduced  $\beta$ -cell mass at birth, compared to pups born by control dams<sup>242</sup>. However, as seen by the raised fasting insulin (figure 3G) and the QUICKI data in figure 3H, exposure to the high energy diet in both pre- and post-natal life cause most severe effects on insulin sensitivity in the pups at weaning.

While there were only subtle differences in the PUFA-composition of the hepatic lipids at 4 weeks of age, the effects on the content of the different lipid classes were more pronounced. Thus, as seen in many other studies, lactation from the HFHS-fed dams, cause pronounced increase in hepatic TAG, but also a decrease in ceramide levels that have not been reported elsewhere. *In utero* exposure to the HFHS-diet did not have any impact on these effects. While the post-natal effect on TAG concentrations was expected, the reduced ceramide levels was surprising, since ceramide synthesis is induced by excess palmitic acid<sup>243</sup>, which the HFHS-diet is expected to

supply. However, in a recent study, it was suggested that the hepatocytes rapidly secrete excess ceramide, when ceramide synthesis is induced by fatty acid oversupply, as a protective mechanism against its lipotoxic effects<sup>244</sup>. Thus, an up-regulation of hepatic ceramide secretion, could potentially explain the observed decrease in hepatic ceramide in the pups-lactating from HFHS-fed dams. The mismatch between maternal diet during gestation and lactation altered hepatic lipid composition, in a way that has not been earlier reported. Thus, being born by control-fed and being lactated by HFHS-fed dams, caused an increase in total PL per mass liver in the CH group and both HC and CH pups had increased FFA, although only the effect in the HC-group reached significance. Since the amount of PL is a proxy for membrane area, this indicates that ultrastructural changes leading to a larger membrane area per mass liver (for example smaller cell-size or increased area of intra-cellular membrane systems) have taken place. Based on the available data, we cannot explain this effect, and to the best of our knowledge, it has not been reported elsewhere. The increase in hepatic FFA is potentially important, since in the liver, FFA reduce signaling from the insulin receptor<sup>230</sup>, potentially by inducing hyperactivity of protein phosphatase 2A<sup>245</sup>. Additionally, saturated fatty acids induce endoplasmic reticulum stress and they function as precursor for synthesis of other lipotoxic intermediates such as acyl-CoA, diacylglycerol and ceramides<sup>246</sup>. The gene expression pattern for the assayed genes responsible for hepatic fatty acid uptake (CD36), synthesis (FAS, ACACA and SREBF1) or fatty acid oxidation (Cpt1a and ACOX), did not reveal any transcriptional regulation that could explain the increased FFA concentration in the mismatched groups, all these genes were regulated by the postnatal HFHS-exposure, in a way that would decrease hepatic fatty acid load. The gene-expression analysis of the enzymes involved in fatty acid flux in the adipose tissue did show that the CH group had increased expression of lipoprotein lipase, and tended ( $p=0.054$ ) to have an increased expression of the adipose triglyceride lipase (ATGL). Since LPL catalyzes the extracellular hydrolysis of TAG that is required for uptake of fatty acids to adipose tissue, and ATGL catalyzes the rate-limiting step in hydrolysis of TAG required for release of fatty acid from adipose tissue, this could indicate that HC-pups had an increased fatty acid turn-over in the epididymal adipose tissue (figure 7), but these data do not explain the increased FFA concentration in the liver of pups exposed to dietary mismatch.

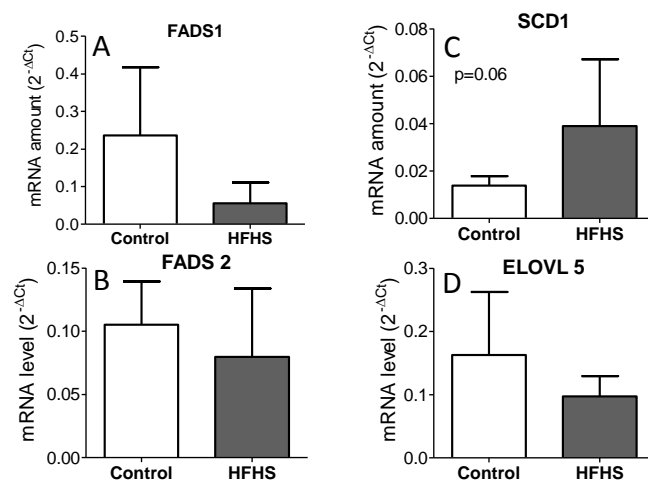
Although we found pronounced effects of the post-natal maternal diets, there was no difference in fat or leptin content in the mother's milk from HFHS or chow-fed dams. This is in contrast to finding in other studies <sup>247</sup>. It shall however be noted, that dams in present study had similar adiposity, while the dams in the study by Franco *et al.* were more obese than the control dams, which might explain the discrepancies in results. Furthermore, in present study we were only able to sample colostrum, we can therefore not conclude anything on the milk composition later on in lactation. There were however substantial differences in the milk fatty acid composition that might be relevant for the metabolic effects in pups lactated by HFHS dams, which cannot be explained by the maternal dietary FA-composition. As seen in figure 1A, milk from HFHS-fed dams contained substantially less n-7 monounsaturated and medium-chained (C8 – C14) fatty acids. The medium-chained FA is synthesized in the mammary gland and the mammary fatty acid synthesis is down-regulated when the mother has high levels of circulating lipids <sup>248</sup>. Medium chained fatty acids, in contrast to longer fatty acids, are to a large extent absorbed via the portal veins directly to the liver, where they are oxidized by a carnithine-independent pathway <sup>249</sup>. This is in contrast to the longer fatty acids, that are absorbed via the chylomicron-pathway and are systemic circulated before being taken –up by the liver as chylomicron remnants. Thus, the reduced levels of MCFA in the milk from HFHS-dams will lead to reduced hepatic exposure to fatty acids in the very early life in pups lactating from these mothers. Although speculative, this might mean that these pups are less primed for hepatic fatty acid oxidation during the postnatal developmental window which could cause reduced fatty oxidation later in life. The decrease in palmitoleic acid observed not only in mother's milk, but in all lipid samples in this study, could also have consequences for metabolic regulation in the pups. In rodents, it has been suggested that palmitoleic acid function as lipokine, increasing muscle insulin sensitivity and suppressing hepatic steatosis in mice <sup>250</sup>. Furthermore, chronic administration to palmitoleate reduces insulin resistance and hepatic lipid accumulation, as well as increases satiety and increase the release of appetite-reducing hormones in rats, when compared to other similar FA <sup>251,252</sup>. Thus, the drastic reduction in its intake in the pups from the HFHS-dams, as well as the reduced synthesis from palmitic acid due to the strong inhibition of SCD1 during lactation, could skew the metabolic regulation toward a phenotype with reduced insulin sensitivity and increased hepatic lipid load, and could therefore partly explain the observed reduced insulin sensitivity, measured as decrease

QUICKI value, and increased hepatic TAG levels, in the HFHS-lactating pups.

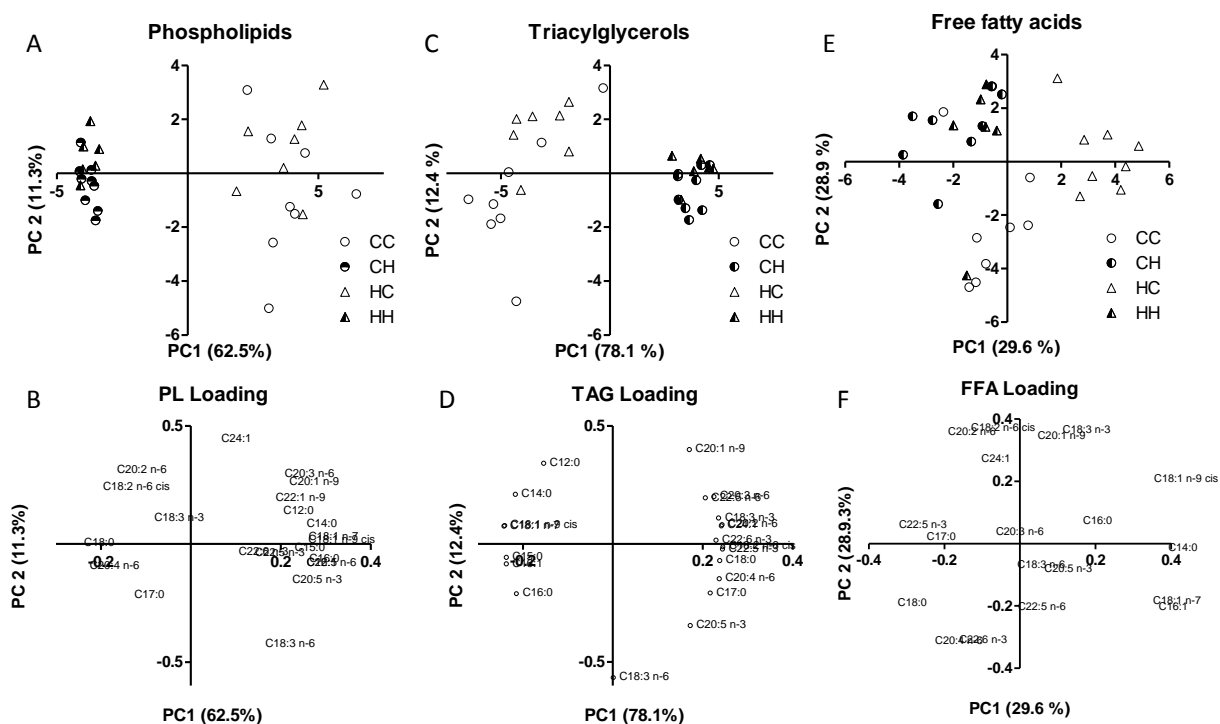
In figure 6, we summarize the results using a PCA analyzing, and the most important result from this is that a dietary mismatch between the control diet *in utero* and the maternal high fat/high sucrose intake during lactation, cause more severe phenotypic alteration in the studied metabolic parameters at weaning than when the pups have been exposed to the HFHS-diet both *in utero* and during lactation, as seen in figure 6. This is in good accordance with the predictive adaptability hypothesis, indicating that epigenetic adoptions occur *in utero*, that predisposes the fetuses to, in this case, the exposure to a diet with high energy diet after birth. The loading plot shows that it is the increase in weight, leptin and phospholipid level that are the primary drivers for this separation.

To conclude, we have in this study shown that that maternal high fat/high sucrose feeding during gestation and lactation have partly different effects on metabolic parameters and on hepatic lipid status at weaning in rat pups. The prenatal exposure has a very strong inhibitory effect on expression on FADS1 and 2, while a mismatch between pre- and postnatal diet cause increased level of hepatic free fatty acids.

## Supplementary material



**Supplementary figure 1. Fatty acid composition of triacylglycerols in the liver at the day after birth.** Data are normalized to the percental contribution of each fatty acid to the total fatty acid pool in the pups. Open symbols = control pups, closed symbols = pups born by HFHS-mothers. N for control pups were 12 and for HFHS pups 6.



**Supplementary figure 2. Principal component analysis of the fatty acid composition in hepatic phospholipids, triacylglycerols and free fatty acids at four weeks of age.** Upper panel show score plots and the lower panel the loading plots.



## 5. The impact of maternal pre- and post-natal intake of high fat/high sucrose diet on metabolic adaptability to a high fat diet in adult rat offspring

Camilla Ingvorsen, Lars I. Hellgren

### Abstract

**Objective** Maternal high fat feeding during gestation and lactation is associated with increased adiposity, decreased insulin sensitivity and elevated hepatic lipid accumulation. The phenotype is particularly pronounced when the offspring have been exposed to a control diet during pregnancy and then transferred to an obesogenic diet after birth, possibly due to the mismatch between the pre- and post-natal environment. Low birth weight is associated with reduced metabolic flexibility and adaptation when challenged with a high fat diet, but it is not known whether exposure to high energy diets during gestation and/or lactation have similar effects, or whether this will improve their capacity to metabolically cope with the high fat challenge, in accordance with the predictive adaptability hypothesis. Therefore we have investigated if rats exposed to maternal high fat/high sucrose diet during gestation and/or lactation have impaired metabolic adaptation when challenged with a high fat diet in adulthood.

**Methods** Female obese prone rats were fed a control (C) or a high fat/high sucrose diet (HFHS) for 6 weeks prior to mating. Female offspring were euthanized and characterized at birth. Male offspring were cross-fostered on complementary diet during lactation, after which they were weaned on to a control diet from 4-20 weeks of age followed by 6 weeks of high fat feeding, and subsequently euthanized and characterized.

**Results** The dietary challenge increased body weight in all offspring, however HFHS lactated offspring had elevated adiposity and plasma leptin levels compared to C lactated offspring. Additionally, the leptin level was decreased in the HFHS born offspring, which indicates that they might be protected against hyperleptinemia during the dietary challenge. Insulin sensitivity was altered to the same extent in all groups during the dietary challenge and there were found no alterations in plasma TAG. The weight of the liver was elevated during the challenge probably due to accumulation of hepatic triacylglycerides. Prenatal HFHS exposure increased the hepatic concentration of free fatty acids, and if the offspring additionally were postnatal HFHS exposed the accumulation was even higher. These alterations could not be explained by changes in mRNA levels of genes involved with hepatic lipid metabolism. Hepatic ceramide content was surprisingly decreased by the dietary challenge. Hepatic phospholipid content was not affected by the dietary challenge but HFHS lactated offspring showed elevated levels which indicated ultrastructural changes in the hepatocytes.

**Conclusion** Maternal intake of a HFHS diet during pregnancy and lactation alters the metabolic response to a high fat diet in the adult offspring. In particular leptin secretion and accumulation of hepatic free fatty acids was affected

## Introduction

Obesity and metabolic dysfunction are rapidly increasing in children, which later in life enhances the risk of severe metabolic disease<sup>253</sup>. Maternal obesity during pregnancy, which also is an escalating problem, might account for a part of the explanation, since it is known to enhance the risk of obesity, type-2 diabetes, cardio-vascular disease and non-alcoholic fatty liver disease in the offspring later in life<sup>4,254,255</sup>. Animal studies have shown that pups exposed to maternal obesity during gestation and lactation have increased adiposity, decrease insulin sensitivity and elevated hepatic lipid accumulation, which can develop into non-alcoholic fatty liver disease (NAFLD). Additionally, increased hepatic mRNA levels of inflammatory cytokines and fibrogenesis have been reported<sup>26,32,36,140</sup>. This impaired metabolic phenotype is particularly pronounced, if the offspring has been prenatally exposed to a control diet and then transferred to an obesogenic environment after birth<sup>26,32</sup>. It has been suggested that maternal obesity is necessary for metabolic imprinting in the offspring<sup>256</sup>, but several studies have subsequently contradicted this finding by showing that maternal high fat feeding alone can affect the offspring<sup>37,39,41</sup>.

Maternal obesity or high fat feeding during pregnancy alter lipid metabolism in the offspring and increases hepatic lipid accumulation, which can develop into non-alcoholic fatty liver disease (NAFLD), already at birth in mice (Ingvorsen et al, submitted, Chapter 3), non-human primates<sup>41</sup> and human neonates<sup>194,224</sup>. Thus, these children suffer from impaired hepatic lipid metabolism from perinatal life, leading to increased hepatic fat accumulation, with potential trajectories towards metabolic diseases later in life. NAFLD is considered benign, but it is often linked to accumulation of toxic lipids, such as ceramide and free fatty acids (FFA)<sup>228</sup>. Ceramide is known to attenuate insulin signaling, induce apoptosis, cause formation of reactive oxygen species (ROS) and induced inflammation in the liver<sup>229</sup>, while in particular saturated FFA also reduce signaling from the insulin receptor and cause endoplasmic reticulum stress in hepatocytes<sup>230,246</sup>. Thus, both ceramide and free fatty acids have been suggested to play a pivotal role in the progression from benign NAFLD towards the more malignant state, non-alcoholic steatohepatitis (NASH)<sup>229,246</sup>. The early effects on hepatic lipid accumulation persist in the young adult rodents<sup>26,257</sup> alongside with increased hepatic inflammation and hyperinsulinemia, even when they have been fed a control diet after weaning<sup>26</sup>. While studies in human subjects by Brøns and colleagues have shown that healthy young males born with low birth weight has less metabolic flexibility when challenged



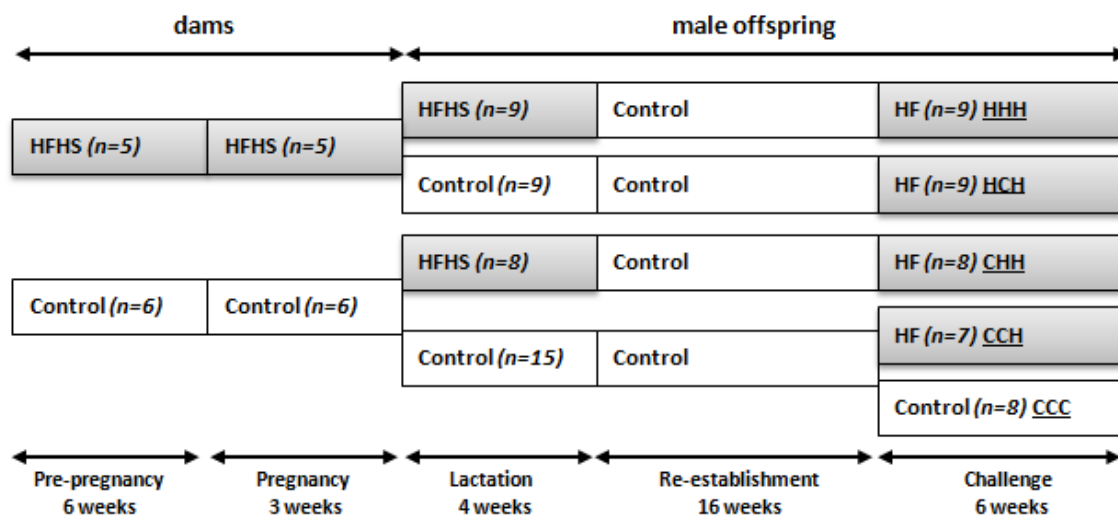
with a high fat diet <sup>258</sup>, and therefore display limited metabolic adaptations in response to dietary challenges <sup>57</sup>, nothing is known on whether similar reduced metabolic adaptability are manifested in subjects exposed to overnutrition *in utero* and/or during lactation. Therefore we have investigated if perinatal maternal intake of a high fat/high sucrose diet during gestation and/or lactation influences the capability of the offspring to deal with a high fat load as adults, with particular focus on hepatic lipid metabolism. Since the typical human junk food diet consists of a combination of high fat food and sucrose sweetened beverage, we have chosen a rat model mimicking these conditions.

## Materials and methods

### *Animal experiment*

Female obesity-prone rats (strain: OP-CD, Charles River, Kingston, United States) 8 weeks of age, were fed a high fat/high sucrose (60E% fat, D12492, Research Diets, Brogaarden, Denmark, supplemented with 15% sucrose in the drinking water) or a control diet (10E% fat, D12450B, Research Diets, Brogaarden, Denmark) for 6 weeks prior to conception. After 6 weeks of feeding, the female rats were mated by strain-matched male rats, what were fed a standard maintenance diet prior to mating (Altromin, Brogaarden, Denmark). At the end of pregnancy all the dams had natural deliveries. Female pups were euthanized the day after birth. Blood glucose was analysed on all female pups, but plasma were generated by pooled blood from 2-3 pups. Tissues were collected and treated as further described below. The male pups were cross-fostered at day 1 among dams on either control or HFHS diet, which resulted in 4 male offspring groups; born by control dams and lactated by control dams (CC), born by control dams and lactated by HFHS dams (CH), born by HFHS dams and lactated by control dams (HC) and born by HFHS dams and lactated by HFHS dams (HH), (Figure 1). The dams were maintained on the respective diets during mating, gestation and lactation. After 4 weeks of lactation the male offspring were weaned onto a chow diet (Altromin, Broggarden, Denmark) until 20 weeks of age, after which they were challenged with a high fat diet (45E% fat, D12451, Research diets, Brogaarden, Denmark) for 6 weeks before they were euthanized. Only half of the CC group were transferred to the dietary challenge, so a pure control was maintained (control diet during gestation, lactation and the challenge period). This resulted in 5 groups of male offspring after the challenge; CCC, a pure control and 4 groups

challenged with the high fat diet (CCH, CHH, HCH, HHH). Tissues and blood was collected and treated as further described below.



**Figure 1. Experimental setup.** Female obese prone rats were fed a conventional (control) diet or a high fat/high sucrose (HFHS) diet for 6 weeks prior to mating (N as displayed in the figure). All dams had spontaneous deliveries and female offspring were euthanized at weaning. Male offspring were cross-fostered so control born pups were lactated by control (CC) or HFHS dams (CH), while HFHS born pups were lactated by control (HC) or HFHS (HH) dams. All offspring were weaned onto a control diet for 4-20 weeks age, after which they were challenged with a high fat diet for 6 weeks and subsequently euthanized (N as displayed in the figure).

### *Weighing and blood sampling*

Dams were weighed weekly and blood was collected from the tail vein before gestation after an overnight fast. Offspring was weighted at birth and every week from 2 weeks of age. Blood was collected from female pups the day after birth by decapitation. Additionally blood was collected from the tail vein of male offspring at weaning after 4 hours of fasting and again at 11, 20 (before challenge) and 26 weeks of age (after challenge) after overnight fasting.

### *Blood glucose and plasma parameters*

Blood glucose was analysed by a glucometer (On Call Plus, ACON laboratories, San Diego, United States). Plasma triglycerides were analysed on a Cobas Mira Plus with a Horiba ABX Pentra kit (Montpellier, France). Plasma insulin and leptin were analysed on a Meso Scale Selector Imager 6000 with Meso Scale Discovery kits according to manufacturer's instructions (Meso Scale Discovery, Rockville, United States). Insulin and blood glucose measurements were used to

calculate a qualitative insulin sensitivity check index (QUICKI),  $1/(\log(\text{insulin}) + \log(\text{glucose}))$  <sup>231</sup>.

#### *Liver lipid analysis*

The livers were snap-frozen in liquid N<sub>2</sub> and stored in -80° until analysed. Lipids were extracted using the Folch procedure, in the presence of internal standards for FFA, TAG and PL as has been described <sup>259</sup>, in addition ceramide with C17:1 long-chained base (Avanti Polar Lipids, Alabaster, AL, US) was added as internal standard for ceramide quantification, prior to extraction. One aliquot of the lipid extract was used for PL, TAG and FFA analysis as described <sup>259</sup>, and one aliquot used for quantification of ceramide, as described in Drachmann *et al.* <sup>233</sup>, but using the above mentioned C17:1 ceramide as internal standard.

#### *RT-qPCR*

Liver samples was collected from female offspring at day 1 and male offspring at 26 weeks of age and stored in RNeasy kit at -80 until RNA extractions. RNA was extracted by a RNeasy kit (Qiagen) according to manufacturer's instructions and converted to cDNA by a High Capacity cDNA Reverse Transcription kit (Invitrogen). For quantitative PCR, target genes were amplified with Taqman Fast Universal PCR master mix (Applied Biosystem) and primer and probe from Integrated DNA Technologies (Iowa, United States) (Suppl. Table 1) on a 7900HT Fast Real-time PCR system (Applied Biosystem).

#### *Statistical analysis*

Not all data followed a Gaussian distribution or had equal variance among groups, so the pre-natal, post-natal and challenge effects were analyzed by a permuted 2way (before the dietary challenge) or 3way ANOVA (after the dietary challenge) in the "Imperm" package in R (<http://www.r-project.org/>), as described by Anderson (2001)<sup>207</sup>. Post test were performed as a pair-wise comparison between groups by a two sample permutation test in the "coin" package in R <sup>207</sup> and the p-values from the two sample tests were adjusted for multiple testing by the Holm method. Effects were considered significant when  $p < 0.05$ .

## Results

### Maternal metabolic phenotype

Female obese prone rats were fed a HFHS or control diet for 6 weeks prior to conception. During the 17 weeks of feeding, the rats on the HFHS diet had no increased weight gain when compared to the control fed rats, despite having a net energy intake that was 1.25 fold higher than the control diet (438 kJ/d vs. 342 kJ/d, Table 1). Before the dams were mated, there were found no differences in weight gain, blood glucose levels, plasma insulin, leptin, triglyceride or insulin sensitivity (estimated by the QUICKI index) between the two groups (Table 2). The two groups also had similar gestational weight gain and litter size.

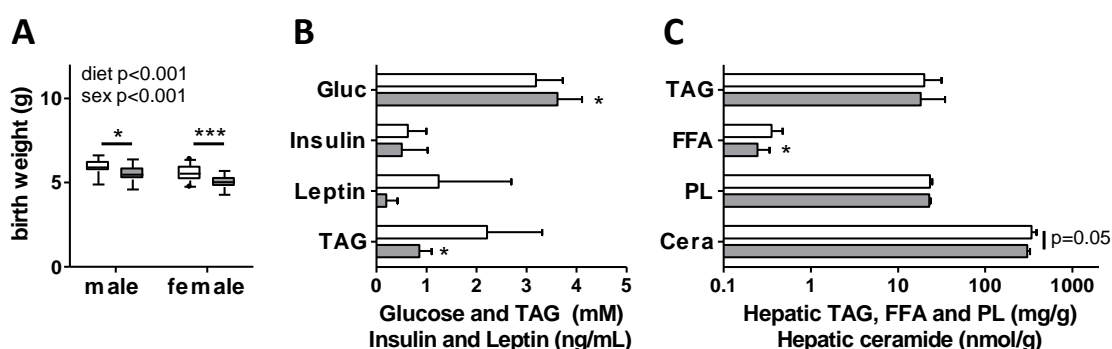
**Table 1:** Macronutrient and fatty acid composition of the two diets (high fat/high sucrose is based on both food and sucrose-sweetened drinking water) .

	Control	High fat/ high sucrose
Protein E%	20	11
Carbohydrate E%*	70	57
Sucrose E%*	35	50
Fat E%	10	32
Fat intake (g/d)	0.65 ± 0.08	3.8 ± 0.6
Carbohydrate intake (g/d)	10.4 ± 1.5	16.7 ± 1.8
Protein intake (g/d)	2.96 ± 0.4	2.81 ± 0.5
Energy intake (kJ/d)	342 ± 49	438 ± 39
Myristic acid (C14:0)	0.9 ± 0.0	1.2 ± 0.0
Palmitic acid (C16:0)	15.9 ± 0.0	20.2 ± 0.0
Palmitoleic acid (C16:1D <sup>9</sup> )	0.8 ± 0.0	1.5 ± 0.0
Stearic acid (C18:0)	7.6 ± 0.0	10.8 ± 0.0
Oleic acid (C18:1D <sup>9</sup> )	27.6 ± 0.0	34.8 ± 0.0
<i>cis</i> -Vaccenic acid ((C18:1D <sup>7</sup> )	1.7 ± 0.0	2.1 ± 0.0
Linoleic acid (C18:2D <sup>9,12</sup> )	39.3 ± 0.0	25.0 ± 0.0
α-Linolenic acid (C18:3D <sup>9,12,15</sup> )	4.3 ± 0.0	1.8 ± 0.0
w-6/w-3 ratio	9.2 ± 0.0	14.6 ± 0.0
Ratio C16:0/C18:0	2.1 ± 0.0	1.9 ± 0.0

\* In HFHS the sum of consumed food and water with sucrose are reported. Fatty acid data are given as percent of total fatty acids and are average ± range of duplicate determination.

**Table 2.** Physiological data on the dams before mating. Data are given as average  $\pm$  stdev. N for control = 6; n for HFHS = 5

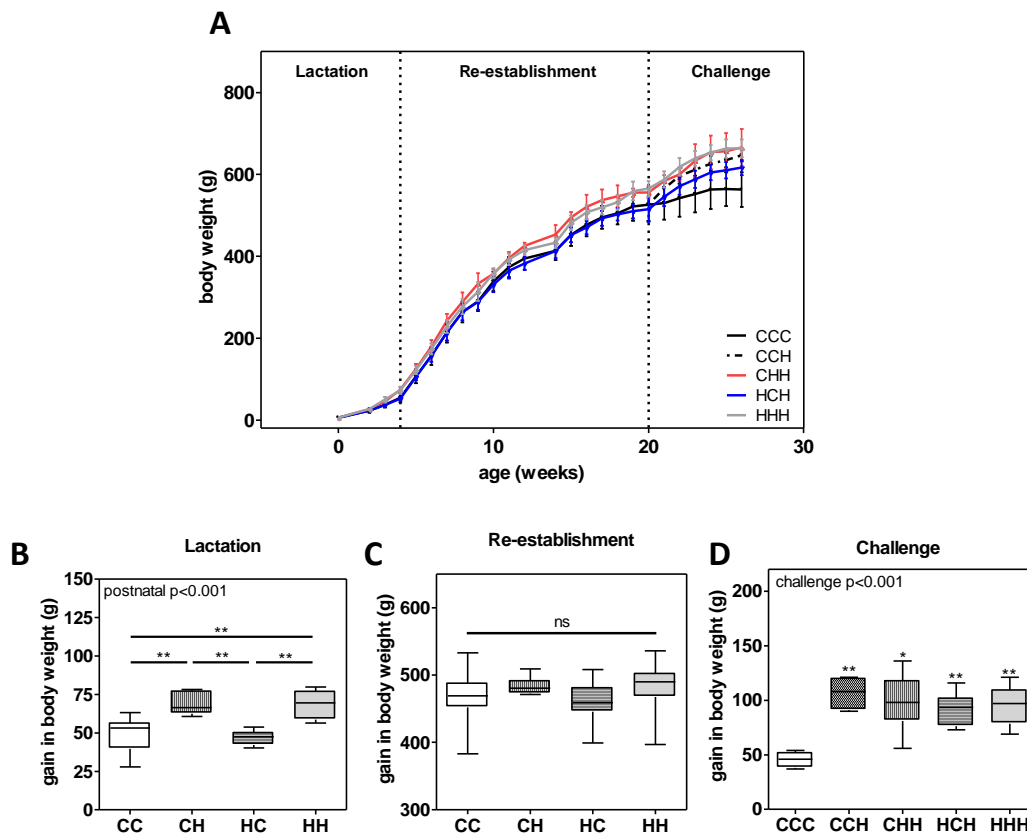
	Control	High fat/	p-value
Weight gain before mating (g)	70 $\pm$ 14	77 $\pm$ 19	ns
Weight gain during gestation (g)	72 $\pm$ 12	68 $\pm$ 8	ns
Litter size	8.3 $\pm$ 3.1	8.0 $\pm$ 2.5	ns
Glucose (mM)	4.25 $\pm$ 0.73	4.34 $\pm$ 0.86	ns
Insulin (ng/mL)	0.58 $\pm$ 0.19	0.67 $\pm$ 0.23	ns
QUICKI (AU)	0.33 $\pm$ 0.02	0.32 $\pm$ 0.02	ns
Leptin (ng/mL)	5.78 $\pm$ 2.20	8.16 $\pm$ 6.07	ns
TAG (mM)	0.96 $\pm$ 0.38	1.31 $\pm$ 0.44	ns



**Figure 2. Metabolic phenotype of female offspring at birth.** A. The birth weight of male and female offspring. B. Blood glucose and plasma levels of insulin, leptin and TAG in female offspring at birth. C. Hepatic lipid concentrations of triacylglycerides (TAG), free fatty acids (FFA), phospholipids (PL) and ceramide (cera) in neonate female offspring. \*  $p < 0.05$ , \*\*\*  $p < 0.001$

### Metabolic phenotype of female offspring at birth

All pups were weighed the day after birth and the female pups were euthanized. Female pups weighed less than males ( $p < 0.001$ ), but both male and female pups born by HFHS fed dams had a lower birth weight than pups born by control fed dams ( $p < 0.001$ ), (Figure 2A). Tissues from the female pups were harvested and weighed and the organ to body ratio was calculated to account for the lower birth weight in the HFHS pups. The weight of the liver as percent of body weight did not differ between the groups ( $C = 3.73\% \pm 0.45$ ,  $H = 3.48\% \pm 0.52$ ,  $p = 0.10$ ). Blood glucose were elevated in the HFHS pups ( $p < 0.05$ ) and plasma TAG were decreased ( $p < 0.05$ ) compared to the control pups, while no changes were found in insulin and leptin levels, (Figure 2B). Lipids were extracted from fetal livers, and pups born by HFHS dams had decreased hepatic FFA ( $p < 0.05$ ) and tended to decreased ceramide concentrations ( $p = 0.05$ ) compared to control born pups, while no effect were seen on TAG and PL content (Figure 2C).



**Figure 3. Body weight and weight gain of male offspring.** A. Growth curve of male offspring during lactation, re-establishment and the challenge period. B. Weight gain during lactation, where the offspring were cross-fostered to dams on complementary diets. C. Weight gain during the re-establishment period from 4-20 weeks of age, where the offspring were fed a conventional diet. D. Weight gain during the challenge period, where the offspring were fed a high fat diet. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , ns non significant.

### *Body weight of male offspring*

Male pups were cross-fostered the day after birth, so that control born pups were lactated by control dams (CC) or HFHS dams (CH) and HFHS pups were lactated by control dams (HC) or HFHS dams (HH). Pups lactated by HFHS dams gained more weight than the control lactated dams until weaning (postnatal effect  $p < 0.001$ ) (Figure 3A and 3B). During the re-establishment period from 4-20 weeks of age, when all offspring were fed a control diet, there were no difference in weight gain between the groups, (Figure 3A and 3C). However, since the HFHS-lactated offspring had elevated body weights compared to control-lactated at weaning (CC=  $54 \pm 1$  g ; CH=  $74 \pm 7$  g ; HC=  $52 \pm 4$  g ; HH=  $73 \pm 9$  g ; postnatal effect  $p < 0.001$ ), the HFHS lactated pups remained heavier until 20 weeks of age (CC=  $526 \pm 40$  g ; CH=  $556 \pm 15$  g ; HC=  $514 \pm 31$  g, HH=  $565 \pm 19$  g ; postnatal effect  $p < 0.01$ ). The same pattern were seen when the offspring were challenged with a 45E% fat diet

from 20-26 weeks of age; all groups receiving the dietary challenge (CCH, CHH, HCH, HHH) had similar elevated weight gain (challenge effect  $p < 0.001$ ) (Figure 3A and 3D). Thus, the offspring exposed to the HFHS diet during postnatal life (CHH and HHH) were heavier at 26 weeks (CCC=  $563 \pm 43$  g; CCH=  $646 \pm 15$  g; CHH=  $666 \pm 45$  g; HCH=  $617 \pm 18$  g; HHH=  $664 \pm 22$  g, postnatal  $p < 0.01$ ), due to a persistent elevated body weight from weaning.

#### *Plasma parameters in male offspring*

Blood samples were collected after weaning (4 weeks of age), during the re-establishment period (11 weeks of age) and also before (20 weeks of age) and after (26 weeks of age) the dietary challenge. Blood glucose and plasma insulin were analysed and a QUICKI value were calculated to evaluate insulin sensitivity. There were no difference in insulin sensitivity among the groups before the dietary challenge, which decreased the insulin sensitivity to the same degree in all groups compared to the control (CCC) group (challenge effect  $p < 0.01$ ) (Table 3). Plasma leptin was increased in the HFHS lactating offspring at weaning (postnatal effect  $p < 0.001$ ), which was maintained until 11 weeks of age (postnatal effect  $p < 0.001$ ). At 20 weeks of age, the leptin levels were re-established in the HH groups and therefore equivalent to the control group (CC), but there was a tendency to a remained elevated leptin level in the CH groups ( $p = 0.07$ ). The dietary challenge increased leptin levels in all groups (challenge effect  $p < 0.01$ ). Additionally, offspring lactated by HFHS dams had an elevated leptin level after the challenge compared to control lactated offspring (postnatal  $p < 0.05$ ) and control born offspring also displayed increased leptin levels compared to HFHS born offspring at this time point (prenatal  $p < 0.05$ ). Hence, the HCH group did not reach a significant higher leptin level compared to the CCC group at 26 weeks. (CCC vs CCH  $p < 0.05$ , CCC vs CHH  $p < 0.05$ , CCC vs. HHH  $p < 0.05$ , Table 3), since exposure to HFHS prenatally and control diet postnatally both caused decreased leptin levels after the dietary challenge. Plasma TAG was also elevated in the HFHS lactated pups at weaning (postnatal effect  $p < 0.01$ ) and the HC group had the lowest concentration, which was significantly lower than for the HH-group ( $p < 0.05$ , Table 3). At 11 weeks of age, there were no differences in TAG levels between the groups. At 20 weeks of age, plasma TAG level were again significantly higher in the HFHS-lactated offspring (postnatal  $p < 0.01$ ), but only the HH group had a significant increased elevated TAG level compared to CC ( $p < 0.05$ ). The dietary challenge had no overall effect on plasma TAG concentration

(challenge effect  $p=0.76$ ), but after the challenge HFHS lactated offspring had higher TAG concentrations (postnatal effect  $p<0.01$ ), this was in particular evident in the CHH group, (pre:postnatal  $p<0.01$ ), despite normalisation of the TAG level in the HHH group after the challenge (Table 3).

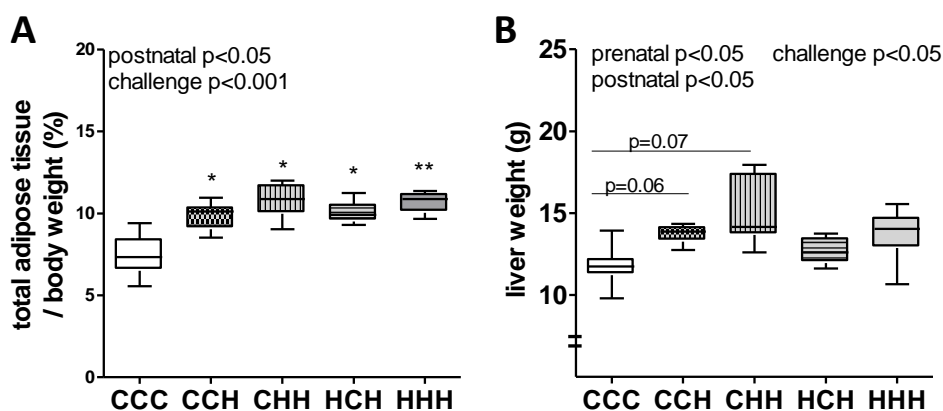
**Table 3.** Circulating metabolic markers in male offspring at weaning (4 weeks of age), at 11 weeks of age and before (20 weeks of age) and after (26 weeks of age) a high fat dietary challenge. Data are given as average  $\pm$  stdev. N for the groups as in Figure 1.

		CCC	CCH	CHH	HCH	HHH	Pre-natal	Post-natal	Inter-action	Challenge
Blood glucose	Weaning	1.00 $\pm$ 0.10		1.01 $\pm$ 0.11	1.06 $\pm$ 0.16	0.97 $\pm$ 0.05	$p=0.75$	$p=0.18$	$p=0.10$	
	Week 11	1.00 $\pm$ 0.10		0.99 $\pm$ 0.12	1.05 $\pm$ 0.23	0.98 $\pm$ 0.13	$p=0.43$	$p=0.36$	$p=0.60$	
	Before challenge	1.00 $\pm$ 0.13		0.93 $\pm$ 0.06	1.00 $\pm$ 0.13	1.00 $\pm$ 0.17	$p=0.39$	$p=0.69$	$p=0.49$	
	After challenge	1.00 $\pm$ 0.17	1.19 $\pm$ 0.17	1.06 $\pm$ 0.10	1.15 $\pm$ 0.19	1.09 $\pm$ 0.11	$p=0.78$	$p=0.12$	$p=0.50$	<b><math>p&lt;0.05</math></b>
Insulin	Weaning	1.00 $\pm$ 0.58		0.68 $\pm$ 0.25	0.83 $\pm$ 0.33	1.03 $\pm$ 0.32	$p=0.48$	$p=0.71$	$p=0.11$	
	Week 11	1.00 $\pm$ 0.31		0.78 $\pm$ 0.27	1.20 $\pm$ 0.92	1.00 $\pm$ 0.33	$p=0.28$	$p=0.75$	$p=1.00$	
	Before challenge	1.00 $\pm$ 0.56		1.11 $\pm$ 0.86	0.89 $\pm$ 0.70	1.16 $\pm$ 0.49	$p=0.96$	$p=0.31$	$p=0.84$	
	After challenge	1.00 $\pm$ 0.31	2.22 $\pm$ 0.72	2.16 $\pm$ 1.14	2.49 $\pm$ 1.34	3.69 $\pm$ 2.64	$p=0.27$	$p=0.31$	$p=0.21$	$p=0.18$
QUICKI	Weaning	1.00 $\pm$ 0.10		1.03 $\pm$ 0.04	1.00 $\pm$ 0.07	0.98 $\pm$ 0.04	$p=0.32$	$p=1.00$	$p=0.34$	
	Week 11	1.00 $\pm$ 0.04		1.04 $\pm$ 0.06	1.01 $\pm$ 0.12	0.99 $\pm$ 0.03	$p=0.51$	$p=0.96$	$p=0.64$	
	Before challenge	1.00 $\pm$ 0.06		1.02 $\pm$ 0.08	1.07 $\pm$ 0.14	0.99 $\pm$ 0.06	$p=0.64$	$p=0.71$	$p=0.22$	
	After challenge	1.00 $\pm$ 0.06	0.89 $\pm$ 0.05	0.91 $\pm$ 0.07	0.89 $\pm$ 0.06	0.86 $\pm$ 0.06	$p=0.47$	$p=0.61$	$p=0.28$	<b><math>p&lt;0.01</math></b>
Leptin	Weaning	1.00 $\pm$ 0.33		1.40 $\pm$ 0.42	1.17 $\pm$ 0.46	1.65 $\pm$ 0.62	$p=0.12$	<b><math>p&lt;0.001</math></b>	$p=0.75$	
	Week 11	1.00 $\pm$ 0.20		1.43 $\pm$ 0.31	0.89 $\pm$ 0.21	1.43 $\pm$ 0.35	$p=0.78$	<b><math>p&lt;0.001</math></b>	$p=0.38$	
	Before challenge	1.00 $\pm$ 0.19		1.40 $\pm$ 0.41	1.15 $\pm$ 0.28	1.05 $\pm$ 0.11	$p=0.55$	$p=0.10$	<b><math>p&lt;0.01</math></b>	
	After challenge	1.00 $\pm$ 0.30	1.73 $\pm$ 0.38	2.35 $\pm$ 0.67	1.61 $\pm$ 0.61	1.77 $\pm$ 0.33	<b><math>p&lt;0.05</math></b>	<b><math>p&lt;0.05</math></b>	$p=0.17$	<b><math>p&lt;0.01</math></b>
TAG	Weaning	1.00 $\pm$ 0.38		1.07 $\pm$ 0.41	0.65 $\pm$ 0.16	1.28 $\pm$ 0.39	$p=0.40$	<b><math>p&lt;0.01</math></b>	<b><math>p&lt;0.05</math></b>	
	Week 11	1.00 $\pm$ 0.19		1.03 $\pm$ 0.17	1.01 $\pm$ 0.21	1.11 $\pm$ 0.29	$p=0.58$	$p=0.36$	$p=0.84$	
	Before challenge	1.00 $\pm$ 0.21		1.24 $\pm$ 0.23	1.10 $\pm$ 0.28	1.46 $\pm$ 0.34	$p=0.11$	<b><math>p&lt;0.01</math></b>	$p=0.29$	
	After challenge	1.00 $\pm$ 0.26	0.86 $\pm$ 0.19	1.34 $\pm$ 0.39	1.01 $\pm$ 0.23	1.01 $\pm$ 0.11	$p=0.84$	<b><math>p&lt;0.01</math></b>	<b><math>p&lt;0.01</math></b>	$p=0.76$



### *Organ weight and adiposity after 6 weeks challenged with a high fat diet.*

All animals were euthanized at 26 weeks of age, after the 6 week dietary challenge. Epididymal, visceral and perirenal adipose tissue depots were removed and weighed and adiposity was evaluated by calculating the total adipose tissue mass to body weight ratio. The dietary challenge increased adiposity in all groups (challenge effect  $p < 0.001$ ), but HFHS lactated offspring had a higher degree of adiposity than the control lactated pups (postnatal effect  $p < 0.05$ , Figure 4A). The liver weight was increased after the dietary challenge (challenge effect  $p < 0.05$ ), but prenatally HFHS-exposed offspring had smaller livers (prenatal effect  $p < 0.05$ ) while postnatally HFHS-exposed had larger livers (postnatal effect  $p < 0.05$ , Figure 4B).



**Figure 4. Organ weight in male offspring after the dietary challenge.** A Weight of total adipose tissue pads (Visceral, epididymal and perirenal) as percentage of body weight. B Weight of liver. \* $p < 0.05$ , \*\*  $p < 0.01$ .

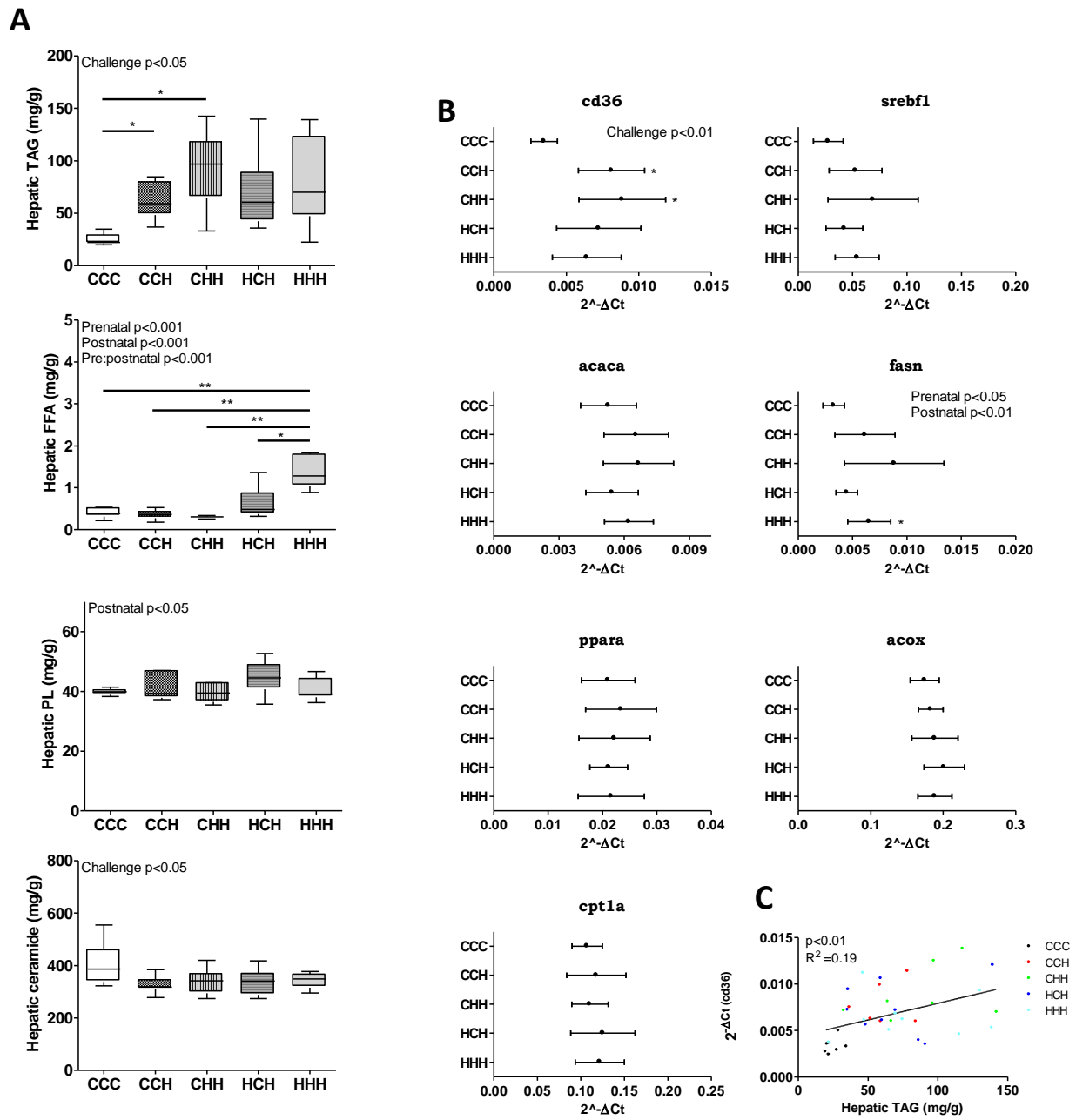
### *Lipid composition and lipid metabolism after dietary challenge in adulthood*

The dietary challenge increased TAG accumulation in the liver (challenge  $p < 0.05$ ), in particular in CCH and CHH, which had significant higher hepatic TAG concentration compared to the CCC group ( $p < 0.05$  for both, Figure 5A). Hepatic FFA content was elevated in HFHS-born offspring (prenatal  $p < 0.001$ ) and most so in the rats that both were born and lactated by HFHS-dams. Thus, the HHH group had in average 3.5 fold higher FFA concentration in the liver than the CCC group and almost 5-fold higher than the CHH group ( $p < 0.01$  vs CCC, CCH and CHH and  $p < 0.05$  vs HCH) in pair wise comparison, Figure 5A). Offspring lactated by HFHS dams also had decreased levels of hepatic phospholipids (postnatal  $p < 0.05$ ), which could indicate structural alterations in the hepatocytes (Figure 5A). Finally, hepatic ceramide concentrations were reduced after the dietary challenge

(challenge  $p < 0.05$ , Figure 5A). To determine whether the increased hepatic FFA in HFHS-born rats was explained by transcriptional regulation of genes involved in fatty acid metabolism, we examined hepatic mRNA levels of genes involved with fatty acid uptake (CD36), fatty acid synthesis (srebf1, acaca, fasn) and fatty acid oxidation (ppara, acox, cpt1a), Figure 5B. The mRNA level of CD36 was elevated by the dietary challenge, but no effects were seen on srebf1 and ppara expression, which are the major transcription factors controlling of fatty acid synthesis and oxidation, respectively. Expression of the gene for acetyl-CoA carboxylase (acaca), the rate-limiting step in fatty acid synthesis was neither affected, but mRNA levels of the fatty acid synthase (fasn) was elevated in HFHS lactated offspring compared to control lactated (postnatal  $p < 0.05$ ), while the HFHS-born offspring had decreased level compared to control born (prenatal  $p < 0.05$ ). Expression of the genes coding for the  $\beta$ -oxidation enzymes acyl-CoA oxidase (acox) and carnithine-palmitoyl transferase 1 (cpt1) were not affected by any of the perinatal interventions or the dietary challenge. It was noticeable that the mRNA level of cd36 displayed a pattern similar to hepatic TAG among the groups and a correlation analysis (Figure 5C) showed a significant association between the two parameters ( $p < 0.01$ ), indicating that hepatic fatty acid uptake through CD 36 was limiting the hepatic TAG accumulation.

## Discussion

We have earlier shown that male rats, which have been exposed to a high fat/high sucrose diet during gestation and/or lactation in the absence of obesity and altered maternal metabolic function (Chapter 4), showed increased adiposity and plasma leptin levels, impaired glucose homeostasis, increased hepatic lipid accumulation and an altered hepatic lipid composition at four weeks of age. Knowing this, it is important to determine whether the metabolic impairments that are induced in early life can be reversed by a transfer to normal low fat diet and whether the early life exposure to the high-energy diet compromise the ability to adapt to metabolic challenges, such as high fat load, later in life, as have been observed in small for gestational age humans<sup>260</sup>. Male offspring from rat mothers fed a HFHS-diet during gestation and/or lactation was therefore transferred to a conventional diet for 16 weeks after weaning at four weeks of age, and at 20 weeks of age challenged with a high fat (45 E%) diet for six weeks.



**Figure 5. Hepatic lipid composition and lipid metabolism after the dietary challenge.** A Hepatic lipid concentrations of triacylglycerides (TAG), free fatty acids (FFA), phospholipids (PL) and ceramide in male offspring after the dietary challenge. B mRNA levels of genes involved with fatty acid uptake (*cd36*), fatty acid synthesis (*srebf1*, *acaca*, *acox*) and fatty acid oxidation (*ppara*, *acox*, *cpt1a*) in the liver. C Correlation analysis on hepatic TAG concentration and hepatic mRNA levels of *cd36*. \*  $p < 0.05$ , \*\*  $p < 0.01$

The six weeks intervention with a HFHS diet in the dams before conception did not affect body weight, insulin sensitivity, plasma leptin or TAG levels in the dams (table 2), which shows that these general physiological parameters did not cause the altered metabolic response in the offspring. Both female and male offspring from HFHS-fed dams had a reduced weight compared to the control born pup the day after birth. In our study, the female offspring from HFHS dams also displayed smaller BAT depots and decreased hearts compared to offspring from control dams, while no effect was seen on liver size. Additionally, they had a modest increase in blood glucose but a 2 fold decrease in plasma triglycerides the day after birth, while no effects were seen on plasma insulin and leptin. Decreased birth-weight have previously been reported from other studies of offspring born by high fat fed rodents <sup>39,261,262</sup>. However, the literature also reports unaffected <sup>37,256,263</sup> or increased birth weights <sup>140</sup> after prenatal exposure to maternal obesity or high fat diets. Akyol and colleagues hypothesised that the pre-pregnancy diet might play a key role in the programming of birth weight <sup>264</sup>, since they reported a decreased body weight at birth only in offspring were the dams had been fed a cafeteria diet prior to gestation. However, a study by Zhang and colleagues <sup>39</sup> shows conflicting data, since their dietary intervention started on gestation day 2 and resulted in decreased birth weight in the pups. Thus, it seems as the effect of high energy diet interventions during pregnancy on birth weight is determined of a yet undetermined parameter. A reduced protein intake during pregnancy in the high fat groups could be one reason. In both the studies of Zhang et al and Akyol and co-workers, the animals given the cafeteria diet had a reduced protein intake, this was however not the case in the present study, were the percental higher protein content in the high fat diet, compensated for a reduced intake. Thus, in present study, this is neither the explanation.

Male offspring were cross-fostered the day after birth, hereby generating four different groups. Male pups lactated by a HFHS dams gained more weight during the lactation period independent of the prenatal diet, which resulted in an increased body weight at weaning. We have previously published a similar result, were we found that the increased body weight reflected increased adiposity, which gave rise to elevated plasma leptin level (Chapter 4). Since we also in this study found increased plasma leptin levels in these pups, we find it likely that the increased body weight is due to increased adiposity. Plasma triglycerides were also elevated in the HFHS suckled pups, but this effect was mainly driven by a significant difference between the HC and HH group. <sup>265,266</sup>.

All groups had normal insulin sensitivity (estimated by QUICKI value) at weaning despite the increase body weight and increased plasma TAG levels in the CH and HH groups.

All male pups were transferred to a conventional diet (10E% fat) after weaning and until 20 weeks of age. During this time all groups had similar weight gains, why the elevated body weights in the CH and HH offspring were sustained throughout the re-establishment period. The increased body weight did not give rise to altered insulin sensitivity in the groups, and the chow-diet normalised the leptin levels in the HH group over time, to a larger extent than in the CH-group. At 11 weeks of age, leptin-levels were still increased in both groups of HFHS-suckled pups, but at 20 weeks of age, only the CH group showed a tendency to increased leptin level compared to the CC group ( $p=0.07$ ). Increased leptin levels can be a sign of increased adiposity and/or decreased leptin sensitivity in the CH group, which might have root in the mismatch between prenatal and postnatal diet. It has previously been shown in humans, that children born with a low birth weight have an impaired leptin response when challenge with a high fat over-feeding in adulthood, due to an elevated basal leptin level prior to the overfeeding experiment <sup>260</sup> Our data suggest that this might also be the case, when there is a mismatch from prenatal to postnatal life. The more rapid re-establishment of a normal leptin level in the HH-group indicate that this group might have a metabolic advantage when transferred to a healthy diet, due to a greater flexibility to adapt to dietary changes, while this was not the case in the CH group.

Plasma TAG levels were increased in the HFHS lactated offspring at weaning mainly due to a significant difference between the HC and HH group, due to a decreased level in the HC-group, this effect were also maintained in the pups at 20 weeks of age, although it was not evident in the data at 11-weeks of age.

To test the offspring's capability of adapting to metabolic changes, we challenged their metabolic flexibility with a high fat diet (45 E% fat) for 6 weeks. The dietary challenge increased the body weight to a similar degree in all groups, so the elevated body weight in the HFHS suckled pups persisted through the challenge. The increased bodyweight were a sign of increased adiposity in the animals, since the ratio of adipose tissue mass to body weight was highest in the HFHS lactated offspring. The dietary challenge decreased the insulin sensitivity to the same amount in all four groups, but leptin levels did not follow this pattern. Leptin was, as expected, increased with the dietary challenge and the increased adiposity, however, HFHS exposure during fetal life

decreased the leptin level after the dietary challenge compared to control lactated offspring ( $p < 0.05$ ), while HFHS exposure during postnatal life increased it ( $p < 0.05$ ). Thus, among the high fat challenged animals, the CHH group had the highest and the HCH group had the lowest leptin level. The latter group was the only one that did not respond to the adult exposure to the high fat diet with significantly increased leptin levels. Hence, a mismatch were the animals was been born by HFHS-fed dams but lactated by control mothers seems to give some protection against hyperleptinemia at a later high fat challenge, while the reversed mismatch increases susceptibility. This is in accordance with earlier finding, that have showed fetal development in mothers given a cafeteria diet, combined with lactation from chow-fed dams, decreased plasma leptin level in in the offspring compared to animals born by control mothers but lactated by cafeteria-diet fed mothers<sup>39</sup>. We therefore suggest that the leptin-gene promoter is the target for different epigenetic changes due to maternal high fat/high sucrose intake during gestation and lactation, *in utero* exposure will in this scenario, induce a predictive adaptive response that attenuates hyperleptinemia when exposed to high energy-diets later in life. This response seems however to be limited to a developmental window *in utero*, while exposure after birth no longer can cause this adaption. It is known that that leptin gene expression is under control by promoter DNA and histone methylation<sup>56,267</sup>, and both impaired glucose metabolism and maternal high fat intake during pregnancy and lactation alters the epigenetic pattern of the promoter in rodents<sup>56,267</sup>. However, to our knowledge no data are available on epigenetic DNA and histone modifications in cross-fostered offspring from high fat fed mothers.

There was no effect of the dietary challenge on plasma TAG levels. The higher plasma TAG level the CHH-group, reflected the difference also observed prior to the challenge, at 20 weeks of age. Thus, we can conclude that post-, but not prenatal, exposure to the high energy diet programs an increased plasma TAG concentration in the offspring, and this was most pronounced in the CHH-group. .

Looking at figure 4B and 5A, it is evident that liver weight and hepatic TAG concentration followed a similar pattern, were the challenge increased both outcomes. There was however only minor effects of the pre- and postnatal treatments on the concentration on these parameters. The prenatal exposure to the HFHS-diet did however cause very pronounced effects on the level of

free fatty acids in the liver (Figure 5A). Thus, while all animals born by control-fed dams had an average concentrations of FFA, between 0.3 and 0.4 mg\*g<sup>-1</sup> liver weight, which is normal in rodent liver, the average concentration for the HCH group was doubled (0.7 mg\*g<sup>-1</sup>) and the concentration in the HHH-group (1.4 mg\*g<sup>-1</sup>) was almost five-fold higher than in the CHH group and significantly higher than in all other groups as well (p<0.05 vs HCH, otherwise p<0.01). Thus, this suggests that the animals born by dams fed a HFHS-diet during pregnancy had reduced metabolic flexibility, with impaired ability to metabolize the increased hepatic influx of fatty acids during a high fat challenge as adults. This ability was further impaired, when the pups also were exposed to the HFHS-treatment during lactation. An increased hepatic concentration of free fatty acids are strongly associated to insulin resistance<sup>268</sup>, as well as to hepatocyte injury and apoptosis through its lipotoxic activity<sup>269,270</sup>. In hepatocytes, free fatty acids induce mitochondrial and endoplasmic reticulum stress, lysosomal disintegration and sensitizes the cells TNF-related apoptosis inducing ligand (TRAIL)-mediated cytotoxicity<sup>269-271</sup>. Thus, the increased concentration of free fatty acids in rats born by HFHS-dams represent an increased risk for development of hepatic insulin resistance as well as serious pathologies associated to the fatty liver, i.e. non-alcoholic steatohepatitis (NASH). Furthermore, we have recently shown that rapid weight-loss is associated to accumulation of free fatty acids in the liver and the heart in mice<sup>259</sup>, this could indicate that offspring from mothers that have had high intake of fat and sucrose during the pregnancy are more susceptible to lipotoxic effects if they go through a rapid weight-loss. Under normal conditions, free fatty acids are rapidly condensed to CoA, forming acyl-CoA, by the acyl-CoA synthase and these are metabolized either via the oxidative or lipid synthesis pathway, the latter causing formation of TAG and PL<sup>268</sup>. When the influx of free fatty acids are larger than the flux through these pathways, formation of other lipotoxic intermediates occur, concomitant with accumulation of free fatty acids<sup>268</sup>. In this study, we do however not see accumulation of ceramide as an effect of the high fat challenge, on the contrary, in all groups that were fed the high fat diet in adulthood, this caused a decrease in hepatic ceramide concentration. However, in a recent study, it was suggested that the hepatocytes rapidly secretes excess ceramide when ceramide synthesis is induced by fatty acid oversupply, as a protective mechanism against its lipotoxic effects<sup>244</sup>. Thus, an up regulation of hepatic ceramide secretion, could potentially explain the observed decrease in hepatic ceramide in the animals exposed to the high fat challenge. The

challenge also caused an increase in hepatic phospholipids, in animals lactated by control- but not HFHS-fed dams. This indicates that cell-size is decreased, intracellular membrane area increased or that the storage of TAG in the hepatocytes of these animals occurs in smaller lipid-droplets, leading to a larger area of the phospholipid monolayer to volume of the droplets. Future ultrastructural studies are required to determine which of these interpretations that is correct.

To determine whether the altered concentration of free fatty acids can be explained through changes in the transcriptional regulation of fatty acid uptake,  $\beta$ -oxidation or fatty acid synthesis, we assayed mRNA levels of the relevant genes (Figure 5B). The results showed that expression of the fatty acid synthase gene (*fasn*) are slightly up-regulated in animals lactated by high energy diet fed dams. Although, we cannot say whether this also means that free fatty acid synthesis are increased in these animals, it could potentially mean that they face a larger risk for hepatic lipid accumulation and that this could be involved in the large increase in free fatty acids in the HH-group. This is however speculative and require more detailed studies of fatty acid metabolism in the offspring.

It should be noticed, that the prenatal HFHS-exposed rats had a lower birth-weight, than the pups born from conventional fed animals, and human low-birth weight subjects are known to have a higher risk of developing metabolic syndrome and type-2 diabetes, as well as having an altered metabolic adaptability to a high fat challenge <sup>260,272</sup>. Thus, in the present study we cannot distinguish the effect from the low birth weight, from direct effects of the maternal intake of the high energy diet. The pronounced effect of the *in utero* exposure to HFHS-diet on hepatic free fatty acid accumulation might therefore also be a consequence of *in utero* growth retardation.

In summary, we have in this study showed that maternal intake of a high fat/high sucrose diet during pregnancy and lactation do alter the response to a metabolic challenge with a high fat diet in the adult offspring. In particular leptin secretion and accumulation of hepatic free fatty acids was affected. The increase in circulating leptin levels, induced by the high fat challenge, was attenuated in offspring from dams fed the high energy diet during pregnancy, while this treatment increased accumulation of free fatty acids. Exposure to the HFHS-diet during lactation increased



both plasma leptin levels and free fatty acid accumulation in the liver. Thus, while the gestational effect on leptin secretion supported the predictive adaptability hypothesis, the effect on hepatic free fatty acids levels contradict it, since animals that have been exposed to a high fat diet *in utero*, are less able to cope with the same environmental challenge in adult life.

## Supplementary material

**Supplementary table 1.** Primers for qPCR, 5' FAM is the reporter signal from all the probes and 3' Iowa Black FQ/ZEN is quencher

Gene	Forward primer	Reverse primer	Probe
sreb1	GAAGCGGATGTAGTCGATGG	GCTACCGTTCCTCTATCAATGAC	TTATTCAGCTTTGCCTCAGTGCCCA
cpt1a	AAAGTCAAAGAGCTCCACCTG	TGTCCAAGTATCTTGCACTCG	CCTCCATGGCTCAGACAATACCTCC
fasn	GCTGATACAGAGAACGGATGAG	GCGAGTCTATGCCACTATTCTG	AATGTCACGCCTTGCTCCTTGC
cd36	CAATGAGTAGGTCTCCAACCG	TGCTCTTCCTTGATTCTGCTG	TGAGAATGCCTCAAACACAGCCA
acaca	CATCTCTAACTTCCTTCACTCCTT	TGCCGAGTCACCTTAAGTACA	AGTCCTTCTGCTCACACACTTCTG
acox	GAACTTGATTCTGAACGACCCA	CACTCTTCTTAACAGCCACCT	TTCCTCACTCGAAGCCAGCGTT
ppara	CGAGCCAAGACTGAAGTTCAAG	CTCTCTGTGTCCACCATGTTG	AGCACCAATCTGTGATGACAACGTCT
b2m	CCAGATGATTCAGAGCTCCATAG	ACCGAGACCGATGTATATGCT	CCTGGGACCGAGACATGTAATCAAGC
gapdh	GTAACCAGGCGTCCGATAC	GTTCTAGAGACAGCCGCATC	ATCCGTTACACCCGACCTTCACC





## 6. Maternal high fat/high sucrose feeding during lactation alters systemic and adipose tissue inflammation in young and adult rat offspring

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### Abstract

**Objective** Maternal high fat feeding during gestation and lactation is associated with increased risk of obesity and metabolic dysfunction in the offspring, while its impact on chronic low grade inflammation is less well documented. Here, we examined immune profiles in young rat offspring after maternal high fat/high sucrose (HFHS) diet during gestation and/or lactation, and again after a high fat challenge during adulthood.

**Methods** Female obese prone rats were fed conventional (C) or HFHS diet before mating and offspring were cross-fostered on complementary diets during lactation. At weaning, offspring was administered with C diet until week 20, where they were challenged with a 45% high fat diet for 6 weeks. Offspring was profiled for immune markers in blood at day 1, week 4, 20 and 26, and within epididymal adipose tissue at week 4 and 26. Immune profiles were correlated to metabolic markers at each time point.

**Results** Offspring born by C dams and lactated by HFHS dams display a distinct immunological phenotype at weaning, characterized by decreased pro-inflammatory gene expression in blood and increased expression of the resident M2 macrophage marker, CD163, in adipose tissue. This immune phenotype was associated with increased adiposity and lipolysis in adipose tissue. Offspring lactated by HFHS dams also displayed altered immunity after high fat challenge in adulthood; characterised by decreased pro-inflammatory gene expression in blood, and increased tumor necrosis factor- $\alpha$  secretion after *in vitro* LPS challenge. In all groups, high fat challenge decreased Toll-Like Receptor 4 display on blood monocytes, and increased adipose tissue expression of the generic macrophage marker CD68 along with decreased cd163 (M2 macrophage marker) expression.

**Conclusion** Exposure to maternal HFHS diet during lactation alters systemic and adipose tissue immune expression profiles at weaning and systemic immunity in adulthood both before and after challenge with a high fat diet. However, associations between immune profiles and metabolic markers were only present in early life.

## Introduction

Maternal obesity is an escalating problem worldwide, since the abundance of obese women in the childbearing age is increasing<sup>24</sup>. Evidence from epidemiological and animal studies show that maternal high fat intake and obesity during pregnancy are associated with increased risk of obesity, type 2 diabetes, cardiovascular disease and non-alcoholic fatty liver disease<sup>4</sup>. Development of obesity and metabolic disease is associated with chronic low grade inflammation<sup>1</sup>, but despite innumerable studies which report alterations in the metabolic phenotype of the offspring, only a sparse number of studies have investigated the immunological profile in offspring after pre- and postnatal exposure to maternal high fat intake. Adipose tissue expandability and ectopic lipid distribution might be important for the onset of the low-grade inflammatory phenotype<sup>147</sup>, however the responsiveness of the immune system and the capability to maintain adipose tissue homeostasis might also be important for the development of obesity-induced inflammation. A Th2 skewed immune system, like seen in Balb/c mice, protects to a large extent development of metabolic disease<sup>64,273</sup>.

Lipid accumulation in the liver and development of non-alcoholic steatohepatitis (inflammation) is the hepatic manifestation of metabolic syndrome<sup>60</sup>. Studies have shown increased activation of the inflammatory signalling pathways JNK and NF- $\kappa$ B in liver after exposure to maternal high fat feeding during pregnancy and lactation in mice and non-human primates<sup>41,274</sup>. Similar result was published by Muralidarane and colleagues, which showed increased TNF- $\alpha$  mRNA expression in the liver of offspring derived from a mouse model for maternal obesity. However, the maternal diet had no effect on hepatic IL-6 mRNA expression or the number of Kupffer cells (hepatic resident macrophages) and their immunological function (ROS production and phagocytosis)<sup>27</sup>. Vascular inflammation plays a pivotal role in the development of atherosclerosis, and a study in non-human primates reported increased mRNA levels of TNF- $\alpha$  in abdominal aorta tissue after perinatal exposure to maternal obesity<sup>275</sup>. Inflammation in hypothalamus interferes with leptin and insulin sensing in the brain, which is important to maintain appetite regulation and glucose homeostasis<sup>1</sup>. Rother and colleagues studied hypothalamic inflammation in mice offspring at weaning after exposure to maternal perinatal high fat feeding and found increased mRNA levels of IL-6 and TLR4 in hypothalamus along with increased expression of pJNK and pIKK. Contrary to the previously mentioned studies, they found no increase in mRNA levels of either TNF- $\alpha$  or IL-6 in

liver or pancreas<sup>276</sup>. Microglia (resident macrophages in the brain) showed increased expression of CD11b and TLR4 in hippocampus already at birth as a consequence of fetal exposure to maternal obesity<sup>38</sup>. Additionally, the IL-1 $\beta$  production was elevated in hippocampus and liver after an *in vivo* LPS challenge in offspring born by obese dams compared to control born, which makes us speculate that the immune system might be hyper-responsive after exposure to maternal obesity during perinatal life, although a similar result was not seen in relation to IL-1 $\beta$  expression in adipose tissue and serum<sup>38</sup>. Contrarily, other studies report increased serum levels of IL-1 $\beta$ , but also TNF- $\alpha$  and CRP in offspring from obese mothers<sup>143,274</sup>.

Adipose tissue inflammation is a hallmark of obesity-induced inflammation, but to our knowledge nobody has studied the effect of maternal high fat feeding on adipose tissue inflammation in the offspring. We have therefore determined if maternal high fat feeding during pregnancy and/or lactation affect the level of macrophage and pro-inflammatory markers in the adipose tissue and activation of monocytes both in young rats at weaning and in adulthood after a dietary challenge. The purpose of the challenge was to elucidate differences in the robustness of the immune response to a metabolic load in offspring exposed to maternal high fat feeding during gestation and/or lactation.

## Materials and methods

### *Animal experiment*

The experimental setups of the rat studies included in this chapter are described in Chapter 4 and 5, along with the metabolic phenotype of dams and offspring. Thus, the experimental setup will only briefly be described here.

*Study 1:* 8 weeks old female obese prone rats (strain: OP-CD, Charles River, Kingston, United States) were fed HFHS (60E% fat, D12492, Research Diets, Brogaarden, Denmark, supplemented with 15% sucrose water) or control (C) diet (10E% fat, D12450B, Research Diets, Brogaarden, Denmark) for 17 weeks prior to mating. The dams were maintained on their respective diets throughout mating, gestation and At the end of gestation, female pups were euthanized the day after birth and male pups born by control dams were cross-fostered among C (CC, n=11) or HFHS dams (CH, n=9), while male pups born by HFHS dams were cross-fostered among C (HC, n=8) or HFHS dams (HH, n=6). All male pups were euthanized at weaning and blood and epididymal

adipose tissue was collected and analysed as described below.

*Study 2:* 8 weeks old female obese prone rats were fed HFHS or C diet for 6 weeks prior to mating, and then maintained on their respective diet throughout mating, gestation and lactation (rat strain and diets were the same as in study 1) (C: n= 7, H: n=3). At delivery, male pups born by C dams were cross-fostered among C (CC, n=15) or HFHS dams (CH, n=7), while male pups born by HFHS dams were cross-fostered among C (HC, n=9) or HFHS dams (HH, n=9). After 4 weeks of lactation the male offspring were weaned onto a chow diet (Altromin, Broggaarden, Denmark) until 20 weeks of age after which they were challenged with a medium HF diet (45E% fat, D12451, Research diets, Broggaarden, Denmark) for 6 weeks before they were sacrificed. Half of the CC group was maintained on the chow diet during the challenge period, which resulted in 5 offspring groups after the challenge; CCC (n=8), representing a pure control, and 4 groups challenged with the medium fat diet (CCH: n=7, CHH: n=7, HCH: n=9, HHH: n=9). Blood and epididymal adipose tissue was collected and analysed as described below.

#### *RNA extractions from blood and epididymal adipose tissue from the offspring*

Epididymal adipose tissue from the offspring were collected and stored in RNAlater (Invitrogen) at -80°C until usage. Adipose tissue was first homogenised in TriZol (Invitrogen) and then centrifuged at 12.000xg for 10 min at 4°C. The layer between the floating lipids in the top and the cell debris pellet was transferred to a new tube and mixed with chloroform before being centrifuged at 12.000xg for 15 minutes at 4°C. The upper aqueous phase is mixed with 70% ethanol and added to a RNA purification column from an RNeasy kit (Qiagen). The following procedures were according to manufacturer's instructions. Blood was collected in an EDTA-coated tube and RNA was extracted with a PerfectPure RNA blood kit (5PRIME). Red blood cells were lysed with a lysing buffer and centrifuged at 2.000xg for 2 minutes. The supernatant was discarded and the pellet, containing the leukocytes, was resuspended in RNA cell protect (Qiagen) and stored at -80°C until usage. For RNA extractions the leucocytes were centrifuged and the RNA cell protect was discarded. The following procedures were according to manufacturer's instructions. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent) with a Agilent RNA 6000 Nano chip (Agilent).



### RT-qPCR

RNA was reverse transcribed into cDNA by a High Capacity cDNA Reverse Transcription kit (Invitrogen). Quantitative PCR was performed with a TaqMan Fast Universal PCR master mix (Applied Biosystems) and primers and probes purchased from IDT-DNA (Leuven, Belgium) (please refer to table 1). The probes were conjugated with a FAM reporter dye and an Iowa Black FQ/ZEN double quenching dye. PCR was run on a 7500HT Fast Real-Time PCR System (Applied Biosystems). mRNA levels for RelA/NF- $\kappa$ B, TNF $\alpha$ , TLR4, Ccr2, IL-1 $\beta$  were analysed in blood and CCL2, cd163, cd68, TLR4, arg1, Nos2, TNF $\alpha$  and IL-1 $\beta$  were analysed in adipose tissue. Data are plotted as  $2^{-\Delta\Delta Ct}$  values and reference Ct value was calculated as an average Ct value for the two reference genes (B2M and GAPDH).

**Table 1:** Primers for qPCR, 5' FAM is the reporter signal from all the probes and 3' Iowa Black FQ/ZEN is quencher

Gene	Forward primer	Reverse primer	Probe
Rela	GTCTCGCTTCTTCACACT	GCCTCATCCACATGAACCTGT	ATGCATCCACAGCTTCCAGAACCT
TNF- $\alpha$	GTCTTTGAGATCCATGCCATTG	AGACCCTCACACTCAGATCA	CACGTCGTAGCAAACCACCAAGC
TLR4	CCATGCCTTGTCTTCAATTGTC	AACCTAGTTCTGAGCTTCAACC	CAGCCACTGAAGTTGTGAGAAATTGGTG
CCR2	CCACAAAACAAAGATGAATACCA	CCACCACACCGTATGACTATG	ATCCTGCCCCACTCTACTCCC
IL-1 $\beta$	TCGTTGCTTGTCTCTCCTTG	TGACCCATGTGAGCTGAAAG	ACCACTTGTGGCTTATGTTCTGTCCA
CCL2	ATCTCTCTCCTCCACCACTA	GAATGAGTAGCAGCAGGTGAG	TGTCTCAGCCAGATGCAGTTAATGCC
CD163	TCTCATTGCCTTCTCTTGTG	TCATCCGTCTTGAATCCATC	ACCCAACGGCTTACAGTTTCTCAA
CD68	CAT TCCCTTACGGACAGCTTAC	TGAGAATGTCCACTGTGCTG	CTCTGATGTCGGTCTGTTTGAATCCA
Arg1	TCCACCCAAATGACGCATAG	GTAGCAGAGACCCAGAAGAATG	TGGAGACCACAGTATGGCAATTGGAA
Nos2	CGAAGGTGAGTTGAACAAGGA	AGCATCCACGCCAAGAAC	CAGAGTGAGAAGTCCAGCCGCA
Gapdh	GTAACCAGGCGTCCGATAC	GTTCTAGAGACAGCCGCATC	ATCCGTTACACCGACCTTCACC
B2m	CCAGATGATTGAGGCTCCATAG	ACCGAGACCGATGTATATGCT	CCTGGGACCGAGACATGTAATCAAGC

### Flow cytometry and stimulation of blood

Blood from the adult offspring in study 2 was collected from the tail vein after the dietary challenge. It was stained with v450 conjugated anti-rat CD45 antibody (BD Biosciences) and a PE-konjugated anti-rat TLR4 antibody (Abcam) for 30 minutes at 4°C followed by lysis of red blood cells with FACS lysing solution (BD Biosciences). The stained blood samples were analysed on a BD FACSCanto II flow cytometer (BD Biosciences). Monocytes were identified as CD45<sup>+</sup> cells and subsequently divided into lymphocytes, monocytes and granulocytes based on size and granularity on a forward vs. side scatter plot (Supplementary figure 1). TLR4 expression levels on monocytes

were identified. The remaining blood was diluted 10x in complete media (RPMI-1640 with 1% penicillin and streptomycin and 1% L-glutamine) and stimulated with media or lipopolysaccharide (LPS) at 100ng/mL. Supernatants were collected after 24 hours and analysed for TNF $\alpha$  production by a Meso Scale Discovery TNF $\alpha$  kit on a Meso Scale Selector Imager 6000 according to manufacturer's instructions (Meso Scale Discovery, Rockville, Maryland, US)

### *Statistics*

26 weeks data: we used a blocked design (10 animals pr day for four days, 2 animals per group per day) to enable analysis by flow cytometry and blood stimulations. The data were adjusted for day to day variation. Not all data followed a Gaussian distribution or had equal variance among groups, so the pre-natal, post-natal and challenge effects were analysed by a permuted 2way (before the dietary challenge) or 3way ANOVA (after the dietary challenge) in the "Imperm" package in R (<http://www.r-project.org/>), as described by Anderson (2001)<sup>207</sup>. Post-hoc tests were performed as a pair-wise comparison between groups by a two sample permutation test in the "coin" package in R<sup>207</sup> and the p-values from the two sample tests were adjusted for multiple testing by the Holm-Bonferroni method. Effects were considered significant when  $p < 0.05$ .

## **Results**

### *Inflammatory mRNA expression profiles in blood and adipose tissue in young offspring*

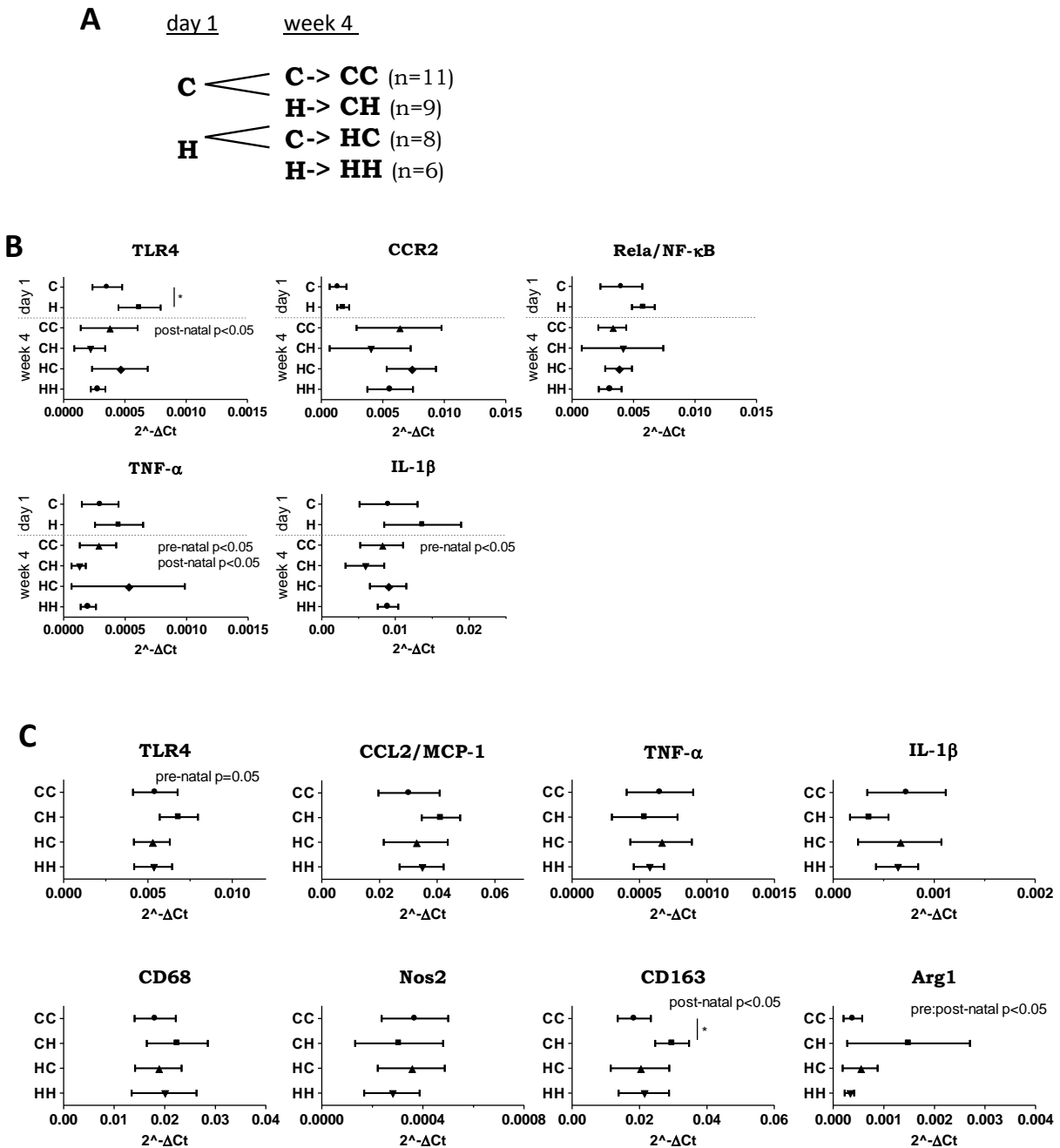
We characterised the inflammatory status at day 1 and at 4 weeks of age in rat offspring from mothers exposed to maternal HFHS or control diet during gestation and/or lactation to examine early immunological effects of a maternal high-fat/high sucrose (HFHS) diet on offspring (Figure 1A). Female dams were fed the HFHS or control diet for 17 weeks prior to mating, however, the dietary intervention did not significantly alter body weight, gestational weight gain, litter size or the circulating levels of glucose, insulin, leptin and triacylglyceride in the dams (Chapter 4).

Inflammatory gene expression was determined in blood the day after birth to study the effect of HFHS exposure during gestation on the immune status in newborns. We analysed: tlr4, the LPS receptor; ccr2, mainly expressed on inflammatory monocytes that migrate to CCL2-expressing tissues; TNF- $\alpha$  and IL-1b, pro-inflammatory cytokines; and Rela/NF- $\kappa$ B, a transcription factor that regulates the expression of pro-inflammatory genes. TLR4 expression was found increased

( $p < 0.05$ ) in the HFHS born pups (H) compared to the control born (C) offspring, whereas no changes in CCR2, TNF- $\alpha$ , IL-1b or Rela/NF- $\kappa$ B expression were observed at birth (Figure 1B).

We then examined the effect of cross-fostering the C- and HFHS- born pups on complementary diets, so that C-pups were lactated by C dams (CC) or HFHS dams (CH) and HFHS-pups were lactated by C dams (HC) or HFHS dams (HH), to study pre-natal and post-natal effects of maternal HFHS intake on immunity in the offspring at weaning (Figure 1A). Pre-natal refers to the maternal HFHS diet during pregnancy and therefore compares the response in the CC and CH groups with the response in the HC and HH groups. Post-natal denotes the effect of maternal HFHS diet during lactation and therefore compares the response in the CC and HC groups with the response in the CH and HH groups. At 4 weeks of age, TLR4 and TNF- $\alpha$  mRNA levels in blood cells were decreased in the offspring lactated by HFHS dams compared to the control offspring (postnatal  $p < 0.05$ , Figure 1B, d28). Moreover, we observed an increase in TNF- $\alpha$  and IL-1b in HFHS born pups compared to C born (prenatal  $p < 0.05$ , Figure 1B: d28). No effects were seen in CCR2 or Rela/NF- $\kappa$ B expression levels in blood from 4 weeks old offspring (Figure 1B, d28).

In epididymal adipose tissue, we analysed mRNA levels of *tlr4*, *tnfa*, *il-1b* as in blood, and also included CCL2 (the chemokine for CCR2), *cd68* (overall macrophage marker), *nos2*, (M1 macrophage marker), CD163 and Arg1 (M2 macrophage markers) in week 4 offspring (Figure 1C). The level of *tlr4* was decreased in the HFHS born pups compared to C born offspring (prenatal  $p < 0.05$ , Figure 1C), but we found no changes in the expression of the chemokine CCL2 nor the pro-inflammatory cytokines *tnfa* and *il-1b* in adipose tissue. The markers related to general macrophages (*cd68*) and M1 macrophages (*nos2*) were not affected. However, the M2 macrophage marker CD163 was elevated in the HFHS lactated offspring (post-natal  $p < 0.05$ ) and an interaction between the pre-natal and post-natal HFHS exposure was found in *arg1* (pre:post-natal  $p < 0.05$ , Figure 1C). The result for CD163 and Arg1 appears to be driven by an increase in mRNA levels of CD163 and Arg1 in the CH group compared to the CC group, although this did not reach a statistically significant level for Arg1 (*cd163*  $p < 0.05$  for CC vs CH, *arg1*  $p = 0.15$  for CC vs CH).

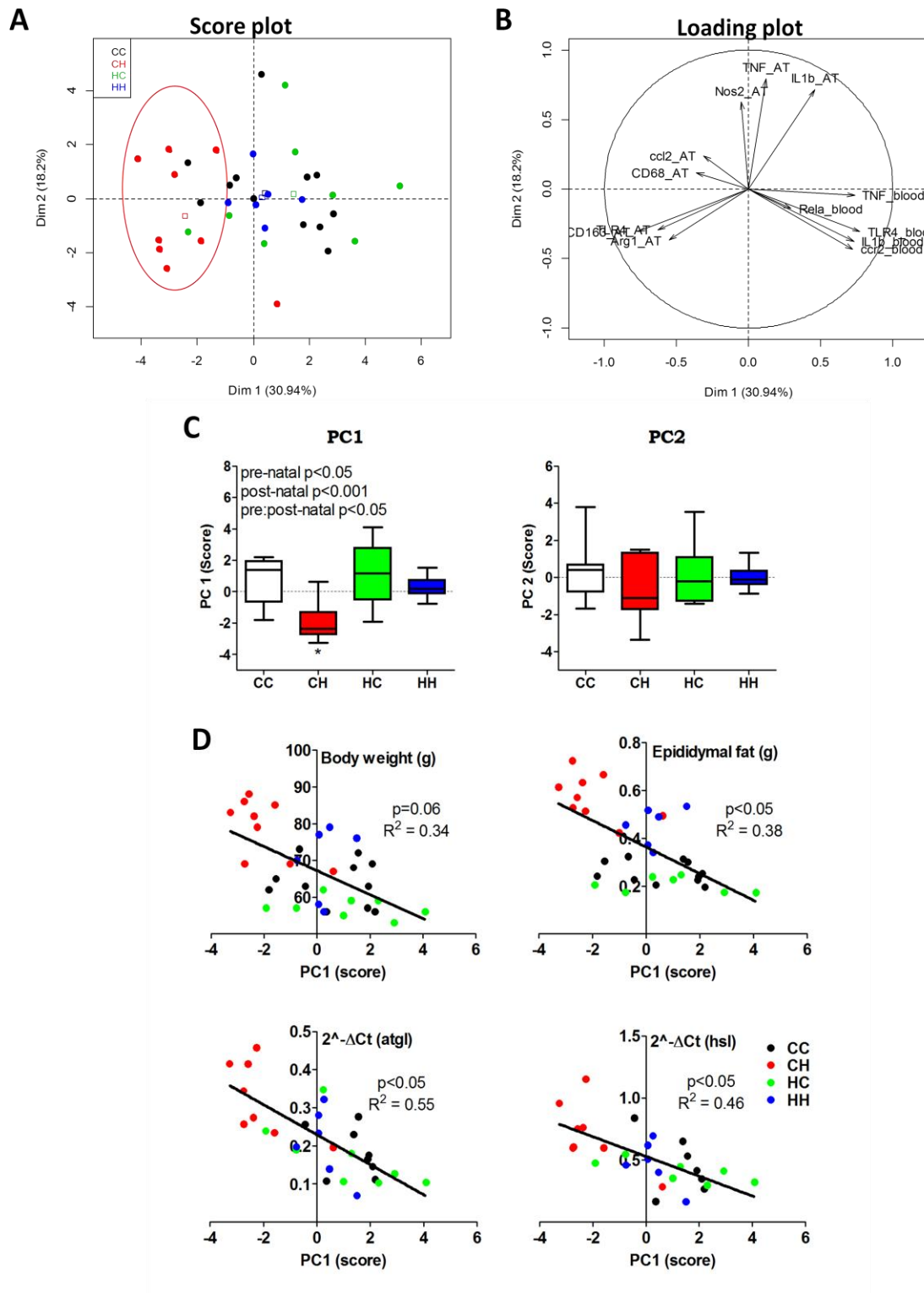


**Figure 1. Gene expression of inflammatory markers in blood and adipose tissue of young offspring at weaning.** A) Experimental setup. Dams were fed a conventional (C) or high fat/high sucrose (H) diet for 17 weeks prior to mating. Female offspring were euthanized the day after birth. Male offspring were cross-fostered on complementary diets during lactation and euthanized at weaning. The number of offspring in each group is given as n. B) Blood was collected at day 1 and week 4, followed by measurement of mRNA expression levels of TLR4 and CCR2, the transcription factor NF- $\kappa$ B and the pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  by qRT-PCR. C) Epididymal adipose tissue was collected at weaning, and subjected to analysis of mRNA expression of TLR4, the chemokine CCL2 (ligand for CCR2 on the blood cells), the cytokines TNF- $\alpha$  and IL-1 $\beta$ , the generic macrophage markers CD68, the M1 (inflammatory) macrophage marker Nos2 and the two M2 (resident) macrophage markers CD163 and Arg1 by qRT-PCR. Data are presented as mean $\pm$ stdev, with n as shown in A). Pre- and post-natal effects are evaluated by a permuted 2-way ANOVA. \* p<0.05

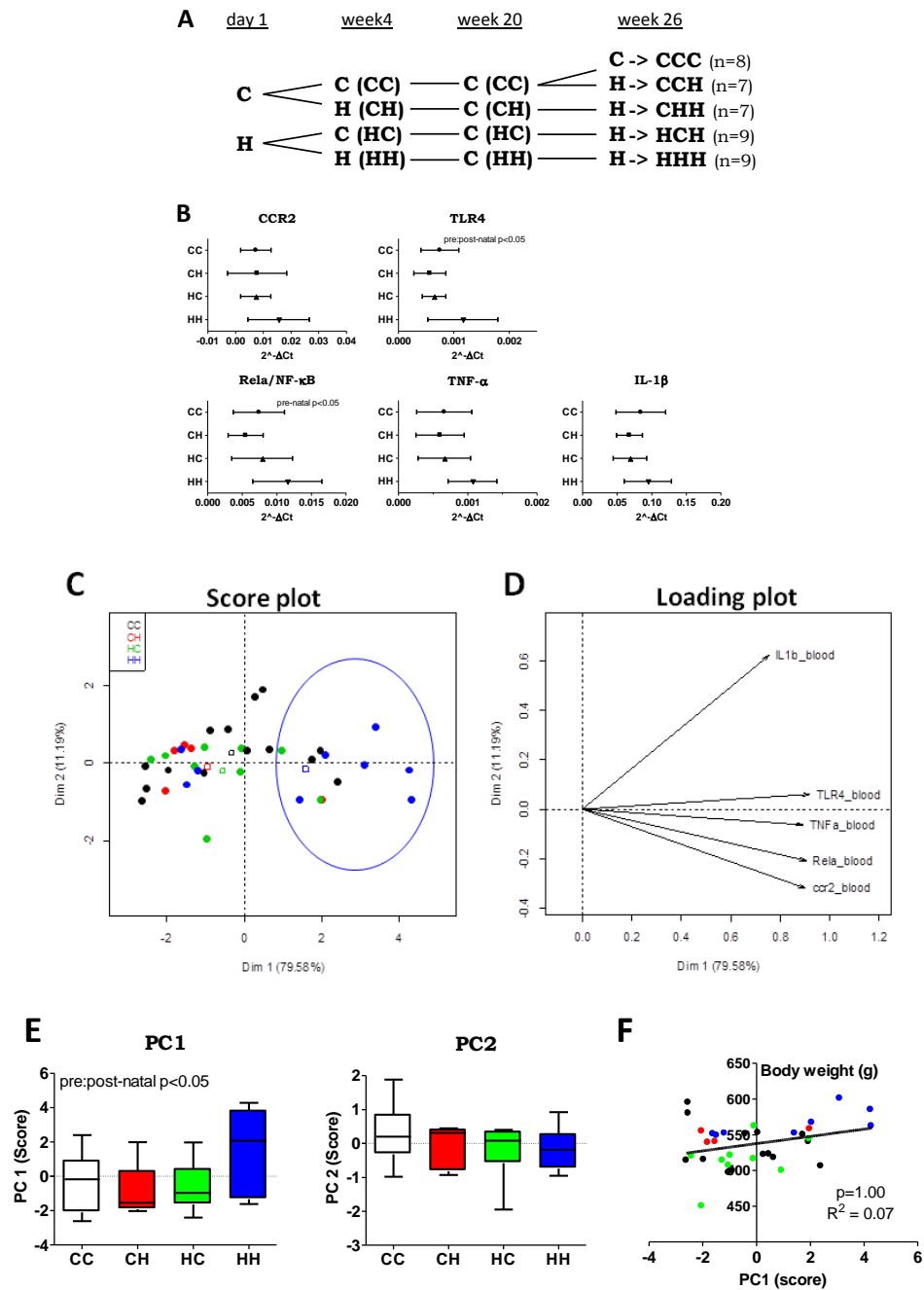
*The combined inflammatory phenotype in 4-weeks old offspring is affected by both prenatal and postnatal diet and link to adiposity and lipolysis in adipose tissue.*

To evaluate the overall inflammatory phenotype in the offspring at weaning, we performed a multivariate principal component analysis (PCA) on the mRNA profiles at week 4 from blood and adipose tissue. Score and loading plots are shown in figure 2A and B, respectively, where principal component 1 (PC1) makes up 31% of the variation between animals, while PC2 explains 18%. The score plot separates individual animals based on overall differences within the groups CC, CH, HC and HH (Figure 2A). We found an overall separation of CH offspring from other groups in PC1, while no apparent clusters were seen in PC2 (Figure 2A) and PC3 (data not shown). PC1 was found to be explained by all 5 inflammatory markers in blood (TLR4, TNF- $\alpha$ , IL-1 $\beta$ , CCR2 and Rela/NF- $\kappa$ B) and 5 out of 8 markers from adipose tissue (CCL2, CD68, CD163, TLR4 and arg1, Figure 2B). When extracting the coordinates of PC1 and PC2 for all animals (Figure 2C), we observed a post-natal effect on early immunity ( $p < 0.001$ , PC1). Particular cross-fostering from the control diet during prenatal life onto the HFHS diet during postnatal life (CH) resulted in an altered immune response in offspring at 4 weeks of age ( $p < 0.05$  compared to the other groups), characterized by a decrease in expression of inflammatory genes in blood and an increase in M2 macrophage markers (Arg1 and CD163) in adipose tissue. Moreover, we identified a slight effect of the pre-natal diet on early immunity in offspring (Figure 2C, PC1). There were no differences among the groups in PC2 coordinates (Figure 2C) or PC3 (data not shown).

In order to identify if the changes in inflammatory profiles were linked to metabolic variations, PC1 immune coordinates were correlated by linear regression analysis to common metabolic markers determined simultaneously in the offspring (Chapter 4). PC1 immune coordinates associated with weight of epididymal adipose tissue ( $p < 0.05$ ), mRNA levels of adipose triglyceride lipase (atgl), and hormone sensitive lipase (hsl) in adipose tissue, both involved in triglyceride lipolysis, and they were, moreover, borderline associated to whole body weight ( $p = 0.06$ ) (Figure 2D). Thus, an increase in adiposity and lipolysis were linked to decreased gene expression of inflammatory genes in the blood and increased expression of M2 macrophage markers in adipose tissue (Figure 2D). Notably, it is evident that the CH group, which also displayed a distinct elevated adiposity compared to all other groups (Chapter 4), showed a divergent immunological profile (Figure 2C).



**Figure 2. Principal component analysis of the immune profile in offspring at weaning.** The mRNA levels of immune genes in blood and adipose tissue were loaded into a principal component analysis (PCA), where A) shows the score plot, and B) the loading plot. Each dot displays one animal, color-coded according to groups. C) The coordinates for principal component (PC) 1 and 2 were extracted for each animal. Data are presented in boxplot with whiskers representing 5-95 percentiles. Pre- and post-natal effects are evaluated by a permuted 2-way ANOVA. \*  $p < 0.05$ . D) Correlations of PC1 with metabolic parameters determined simultaneously in the offspring. N in each group is displayed in Figure 1A.



**Figure 3. Gene expression of inflammatory markers in blood in adult offspring at 20 weeks of age.** A. Experimental setup. Dams were fed a conventional or high fat/high sucrose diet for 6 weeks prior to mating. Male offspring were cross-fostered on complementary diets during lactation and weaned onto a conventional diet at 4 weeks of age. Until 20 weeks of age, all offspring were administered a control diet. At 20 weeks of age, offspring was challenged with high fat diet for 6 weeks and subsequently euthanized at 26 weeks of age. B) Blood was analysed for mRNA levels of TLR4 and CCR2, the transcription factor NF-κB and the pro-inflammatory cytokines TNF-α and IL-1β by qRT-PCR. Data are presented as mean±stdev with n for each group as displayed in A). The combined mRNA immune profile in blood was evaluated by PCA, where C) displays the score plot, and D) the loading plot. Each dot represents one animal, color-coded according to groups. E) The coordinates for principal component (PC) 1 and 2 were extracted for each animal. Data are presented in boxplot with whiskers representing 5-95 percentiles. F) Correlations of PC1 with body weight at 20 weeks of age. Pre- and post-natal effects are evaluated by a permuted 2-way ANOVA

We then went on to study if these early immunological changes in the offspring due to maternal HFHS intake would be affected by feeding the offspring control diet for 16 weeks post-weaning. Subsequently, we applied a dietary challenge to elucidate if the maternal diet during perinatal life influenced the offspring's immunological response during adulthood.

*Immunological profiling in cross-fostered rats after intake of a control diet for 16 weeks post-weaning followed by 6 weeks HF challenge*

In this follow-up study, the offspring was treated as previously (Figure 1A), and then weaned onto a control diet for 16 weeks, followed by a challenge with a HF diet for 6 weeks during adulthood (week 20 to 26, Figure 3A). The control group (CCC) remained on the control diet for the whole period. One important difference from the previous study was that female dams were fed the HFHS or control diet for 6 weeks prior to mating, in contrast to 17 weeks. Again, the prenatal diet did not alter body weight, gestational weight gain, litter size or the circulating levels of glucose, insulin, leptin and triacylglyceride in mothers (Chapter 5).

After ingestion of control diet for 16 weeks post-weaning, blood was analysed for mRNA expression of inflammatory markers (Figure 3B, week 20). A significant interaction was found on *tlr4* indicating that prenatal HFHS exposure affects the response to a HFHS diet during lactation ( $p < 0.05$ ). Additionally, *Rela* was increased in HFHS born pups compared to control born ( $p < 0.05$ ), while no effects were seen on *CCR2*, *TNF- $\alpha$*  and *IL-1 $\beta$* . The overall variation in inflammatory markers between groups was tested in a PCA, where PC1 coordinates made up 79% of the variation and PC2 11% (Figure 3C, D). The CH group showed a unique immunological profile at 4 weeks of age in the first study, however in this study at 20 weeks of age, the immunity in the HH group described by PC1 differed from the other groups (pre:postnatal  $p < 0.05$ , Figure 3E), although this effect did not reach statistically significant levels in the pairwise comparison among the groups. The combined inflammatory profile in PC1, characterized by expression of inflammatory genes in blood, did not correlate to overall body weight at week 20 (Figure 3F), or any metabolic markers collected at this time point (Chapter 5).

After 6 weeks of HF challenge during adulthood (week 20 to 26), we observed decreased levels of *TLR4*, *IL-1 $\beta$*  and *Rela* in blood (postnatal  $p < 0.05$ ,  $p < 0.05$  and  $p < 0.01$ , respectively) from HFHS lactated offspring compared to C lactated offspring, while no effects were seen on *TNF- $\alpha$*  and



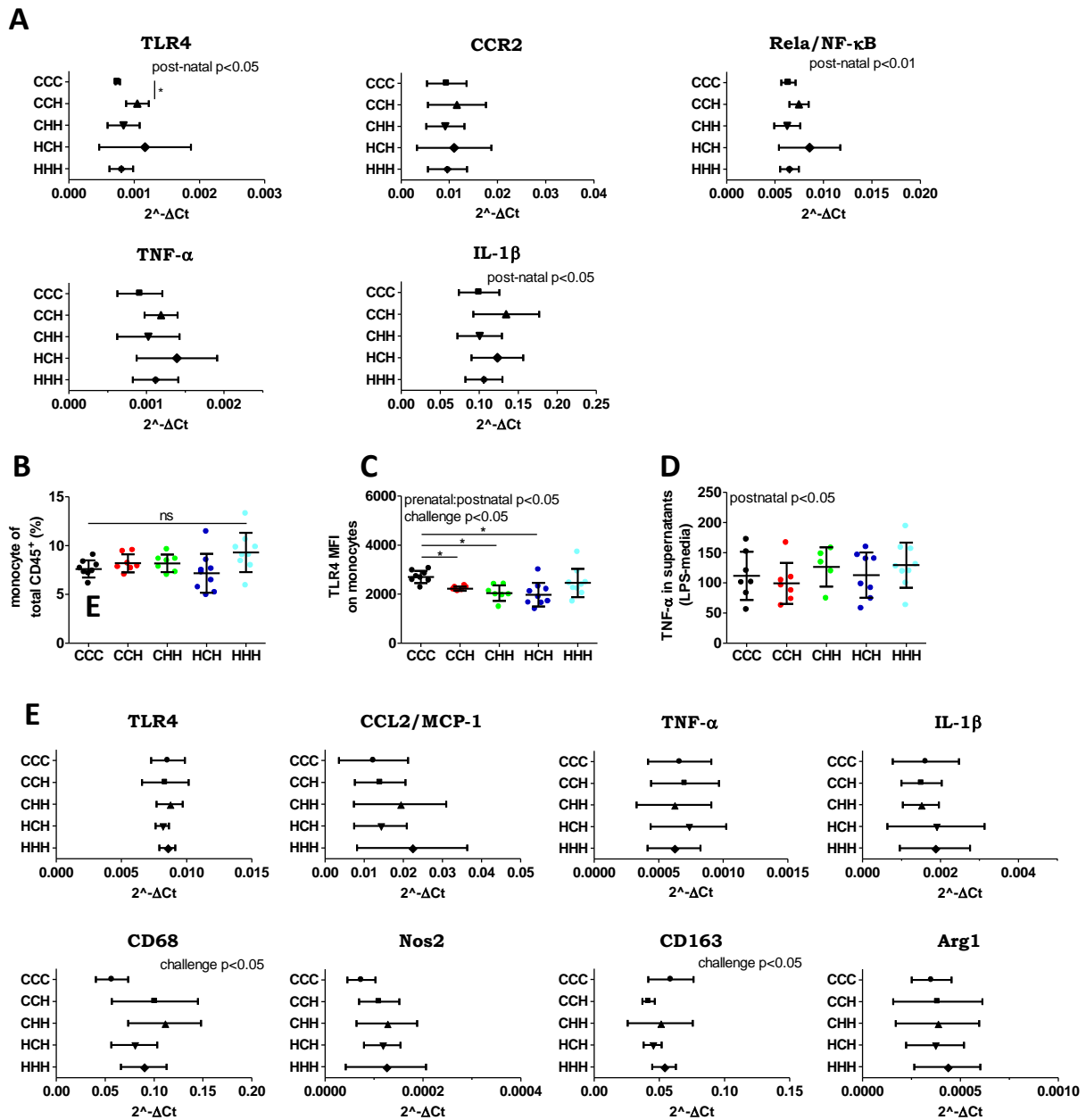
CCR2 (Figure 4A). The mRNA level of TLR4 seemed to be particularly affected by maternal diet, since it was affected by pre-natal or post-natal HFHS exposure at d1 and d28, respectively, in the first study. Additionally, in the second study, we found an interaction effect on TLR4 before the dietary challenge and a post-natal effect after the challenge. Therefore, we examined surface TLR4 expression on fresh blood monocytes drawn at the day of termination at week 26. Monocytes express high levels of TLR4 and they are known to play an important role in macrophage infiltration of adipose tissue, liver and pancreas in obesity-induced inflammation<sup>277</sup>. We found no changes in the abundance of monocytes within the circulating blood leukocytes (CD45+ cells, Figure 4B), but surface TLR4 expression on monocytes were overall decreased in HF-challenged animals (challenge  $p < 0.05$ , Figure 4C). However, a functional effect of the decreased surface TLR4 expression was not apparent in the HFHS lactated offspring, which was demonstrated by slightly increased secretion of TNF $\alpha$  in the supernatants from CHH- and HHH-derived whole blood upon stimulation with the TLR4-ligand lipopolysaccharide (LPS) at 100 ng/mL for 24 hours (post-natal  $p < 0.05$ , Figure 4D).

Epididymal adipose tissue was collected from adult offspring after the dietary challenge (week 26) and subjected to analysis of expression of immune-related markers by qPCR (Figure 4E). Decreased expression of the M2 macrophage marker CD163 and increased overall cd68 expression indicate that M1 might accumulate in adipose tissue during the HF challenge (Figure 4E). There were no apparent effects of the pre- and post-natal diet on other immunological markers assessed in adipose tissue after the dietary challenge.

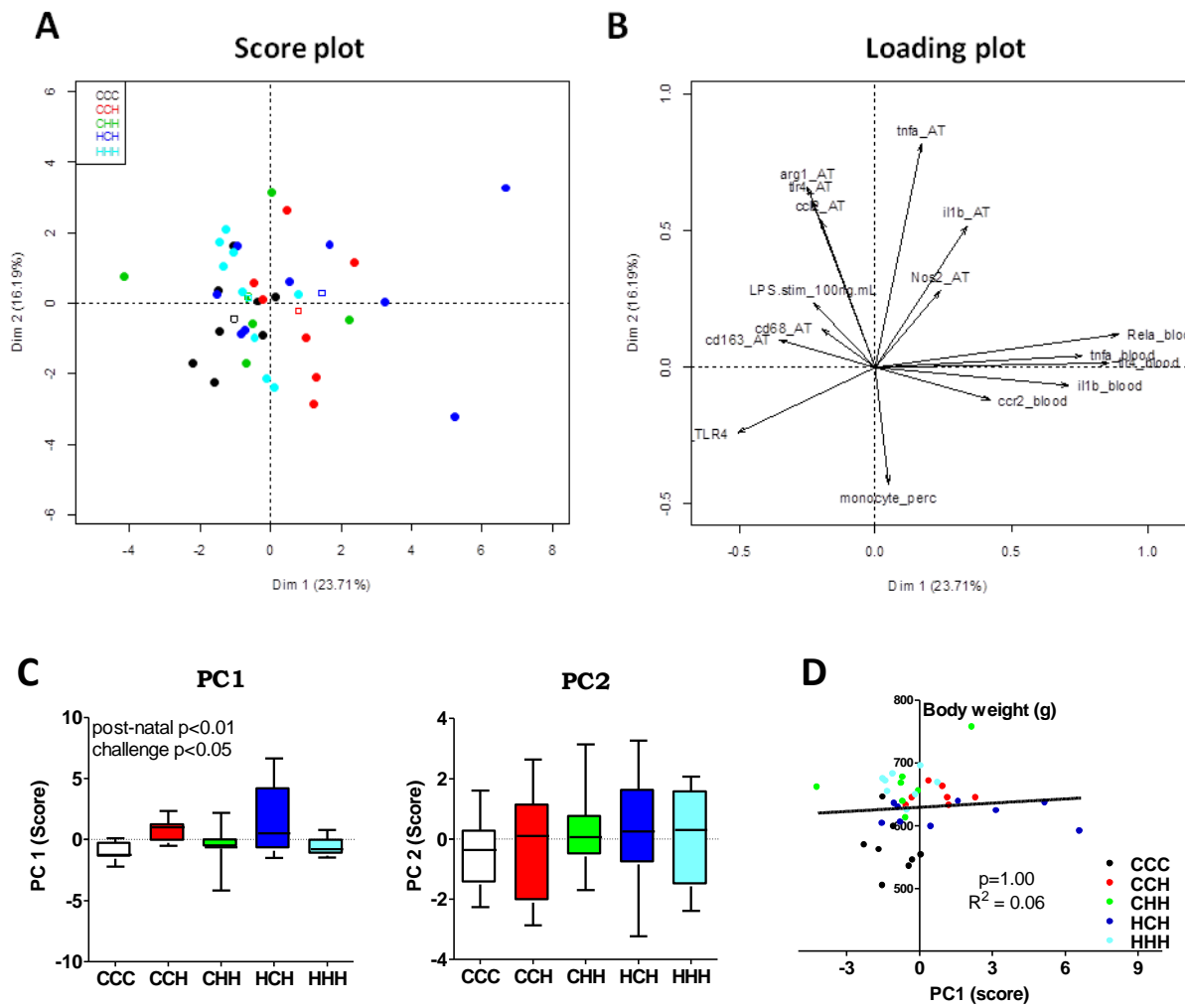
#### *The inflammatory phenotype in the adult rat is affected by the post-natal diet upon later HFHS challenge*

The overall inflammatory phenotype in the offspring after the HF challenge was evaluated in a PCA (Figure 5A, B). PC1 explained 24% and PC2 16% of the variation between individual animals at week 26. PC1 was found to separate the immunological profile according to post-natal exposure (Figure 5C, postnatal  $p < 0.01$ ). The PC1 coordinates for the HFHS lactated offspring were characterised by increased TNF $\alpha$  secretion in response to LPS-stimulation in blood immune cells, despite decreased mRNA levels of inflammatory markers in fresh blood, concurrent with decreased CD163 expression in adipose tissue. The combined inflammatory profile in PC1 did not

correlate to overall body weight at week 26 (Figure 5E), or any other marker of metabolic function (Chapter 5).



**Figure 4. Gene expression of inflammatory markers in blood and epididymal adipose tissue in adult offspring at 26 weeks of age after 6 weeks of high fat feeding.** A) Blood was analysed for mRNA expression of TLR4 and CCR2, the transcription factor NF- $\kappa$ B and the pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  by qRT-PCR. B) Monocyte abundance in blood as identified by flow cytometry. C) TLR4 expression on the surface of blood monocytes, determined by flow cytometry. D) TNF- $\alpha$  secretion from whole blood upon stimulation with 100 ng/mL LPS for 24 hours. E) Epididymal adipose tissue was analysed for mRNA expression of TLR4, the chemokine CCL2 (ligand for CCR2 on the blood cells), the cytokines TNF- $\alpha$  and IL-1 $\beta$ , the generic macrophage markers CD68, the M1 (inflammatory) macrophage marker Nos2 and the two M2 (resident) macrophage markers CD163 and Arg1 by qRT-PCR. Data are presented as mean $\pm$ stdev; with n for each group as shown in Figure 3A. Pre-natal, post-natal and challenge effects are evaluated by a permuted 3-way ANOVA. \*  $p < 0.05$



**Figure 5. Principal component analysis of the immunologic profile of the adult offspring at 26 weeks of age after 6 weeks of high fat feeding.** Immunological parameters from blood and adipose tissue were evaluated by PCA where one dot displays the combined profile from one animal, distributed on groups by colors. A) Score plot, B) Loading plot. C) The coordinates for principal component (PC) 1 and 2 were extracted for each animal. Data are presented in boxplot with whiskers representing 5-95 percentiles. Pre-natal, post-natal and challenge effects are evaluated by a permuted 3-way ANOVA. D) Correlations of PC1 with body weight at 26 weeks of age after 6 weeks of high fat feeding.

## Discussion

Maternal high fat feeding during pregnancy and lactation increases markers of obesity-induced inflammation in circulation<sup>143,274</sup>, liver<sup>27,41,274</sup>, vasculature<sup>275</sup> and hypothalamus<sup>38,276</sup>. However, the general characterization of obesity-induced inflammation have initially focused on inflammation and macrophage infiltration of adipose tissue<sup>1</sup>, so in this study markers of adipose tissue inflammation have been mapped in young and adult rat offspring exposed to maternal high fat/high sucrose feeding during gestation and/or lactation.

Our data demonstrate that both pre-natal and post-natal HFHS feeding impact the immune profile in offspring, but with varying effects at different ages and following HF challenge during adulthood. The statistically significant findings on the immune profiles collected at different ages and interventions are displayed in an overview (Figure 6). From the study in the young offspring (Figure 6A), it was apparent that prenatal HFHS resulted in increased expression of TLR4 in blood at birth, and also in enhanced blood expression of TNF- $\alpha$  and IL-1b at 4 weeks of age, whereas tlr4 was reduced in adipose tissue. Contrarily, maternal postnatal HFHS during lactation resulted in reduced blood TLR4 and TNF- $\alpha$ , and increased M2 macrophage marker expression at 4 weeks of age. We were able to identify correlations between the combined immune profile and specific metabolic parameters. At this stage, it was striking that elevated adiposity were linked to reduced mRNA expression of pro-inflammatory genes in blood and increased markers of macrophage influx into adipose tissue (CD163, and borderline CD68, arg1, CCL2 and TLR4). This immunological profile were particular significant in the offspring born by control dams and lactated by HFHS dams (CH). Kosteli and colleagues have previously shown that increased lipolysis in adipose tissue during weight loss result in macrophage infiltration of the M2 type presumably to enhance lipid clearance in adipose tissue<sup>123</sup>. We found a correlation between the immunological profile and mRNA levels of the lipolysis genes atgl and hsl in adipose tissue. This could imply that the elevated levels of cd163 in adipose tissue from HFHS lactated offspring might relate to increased lipolysis resulting in the need for improved lipid clearance by macrophages. Additionally, it has also been shown that the first macrophages that enter the adipose tissue after just a few days of high fat feeding have a M2 phenotype<sup>278</sup>, so the elevated CD163 marker in adipose tissue might also be a very early sign of obesity-induced adipose tissue inflammation in the offspring.

A	
<u>Prenatal-HFHS</u>	<u>Postnatal-HFHS</u>
D0: Blood-tlr4↑ D28: Blood-tnfa/il1b↑ AT-tlr4↓	D28: Blood-tlr4/tnfa↓ AT-cd163↑

B	
<u>Prenatal-HFHS</u>	<u>Postnatal-HFHS</u>
W20: Blood-rela↑ W26: none	W20: none W26: Blood-tlr4/il1b/rela↓ Blood-TNF-stim.↑ AT: none
<u>Challenge - HF</u>	
W26: Blood-monocytes TLR4↓ AT-cd163↓, AT-cd68↑	

**Figure 6. Summary of the significant pre- and post-natal effects found in the young offspring at weaning (A) and after a high fat dietary challenge in adulthood (B).**

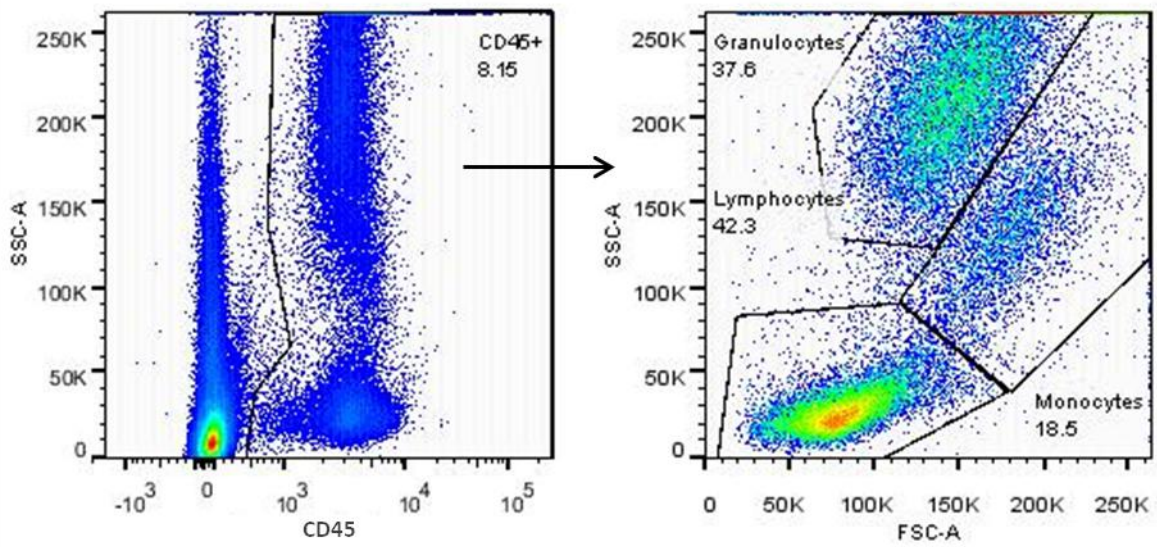
In the adult offspring that was fed a conventional diet for 16 weeks post-weaning (20 weeks of age), we found that HFHS exposure during fetal life increased the TLR4 response to a HFHS environment after birth and Rela/NF- $\kappa$ B was elevated in HFHS in blood from offspring born by HFHS dams. The overall immunological profile at 20 weeks of age showed general elevated levels of inflammatory genes in the HH group only. After 6 weeks of HF challenge in adulthood (from 20-26 weeks of age), we observed reduced TLR4, IL-1b and Rela in blood of offspring lactated by HFHS dams, resembling that of CCC, along with enhanced TNF- $\alpha$  secretion upon blood stimulation with LPS. In the present study, we were not able to perform functional analysis of immune responsiveness at every time point, besides in 26 weeks old animals. However, the analysis at 26 weeks of age showed that TNFa secretion was enhanced in the postnatal HFHS groups upon stimulation with the TLR4 agonist LPS, although TLR4 surface expression on monocytes were reduced to the same level in all HF challenge offspring in whole blood. The tlr4 mRNA expression in the blood was also reduced by the HF challenge. This disparity in expression and functionality of TLR4 might reflect that HFHS during lactation programs offspring to respond more strongly towards TLR4 triggering with LPS when rechallenged with HF later in life or that the functional

response is somewhat independent on surface TLR4 display. This is in conflict with the endotoxemia hypothesis, which states that insulin resistance is associated with activation of monocytes by elevated circulating levels of lipopolysaccharide via TLR4 dependent mechanism<sup>133</sup>

Despite the alterations in immunity in the blood from HFHS lactated offspring, we identified no post-natal effects on markers of inflammation in adipose tissue. The HF challenge, however, reduced the mRNA levels of the M2 macrophage marker CD163 and increased the generic macrophage marker CD68 independent of perinatal HFHS exposure. A skew in macrophage markers away from CD163 and towards CD68, might indicate an elevated infiltration of M1 macrophage in the adipose tissue, although no effects were seen on the pro-inflammatory cytokine mRNA expression levels. It was somewhat surprising that 6 weeks of HF challenge had so little effect on the immunity in the blood and only affected few markers in the adipose tissue.

Collectively, the study revealed that exposure to maternal HFHS diet during lactation alters systemic and adipose tissue immune expression profiles at weaning which could be linked to metabolic alterations in the offspring. Additionally, post-natal HFHS exposure also altered the systemic immunological profile in adult offspring both before and after challenge with a HF diet. These results demonstrate that especially maternal HFHS intake during lactation leaves an immunological imprinting in blood cells in both young and adult offspring.

## Supplementary material



**Supplementary figure 1. Gating strategy to identify monocytes in fresh blood by flow cytometry.** Monocytes were identified first as CD45<sup>+</sup>, then according to the forward scatter and side scatter





“Nature is beautiful but not correct”

- Wilhelm Johannsen, 1911

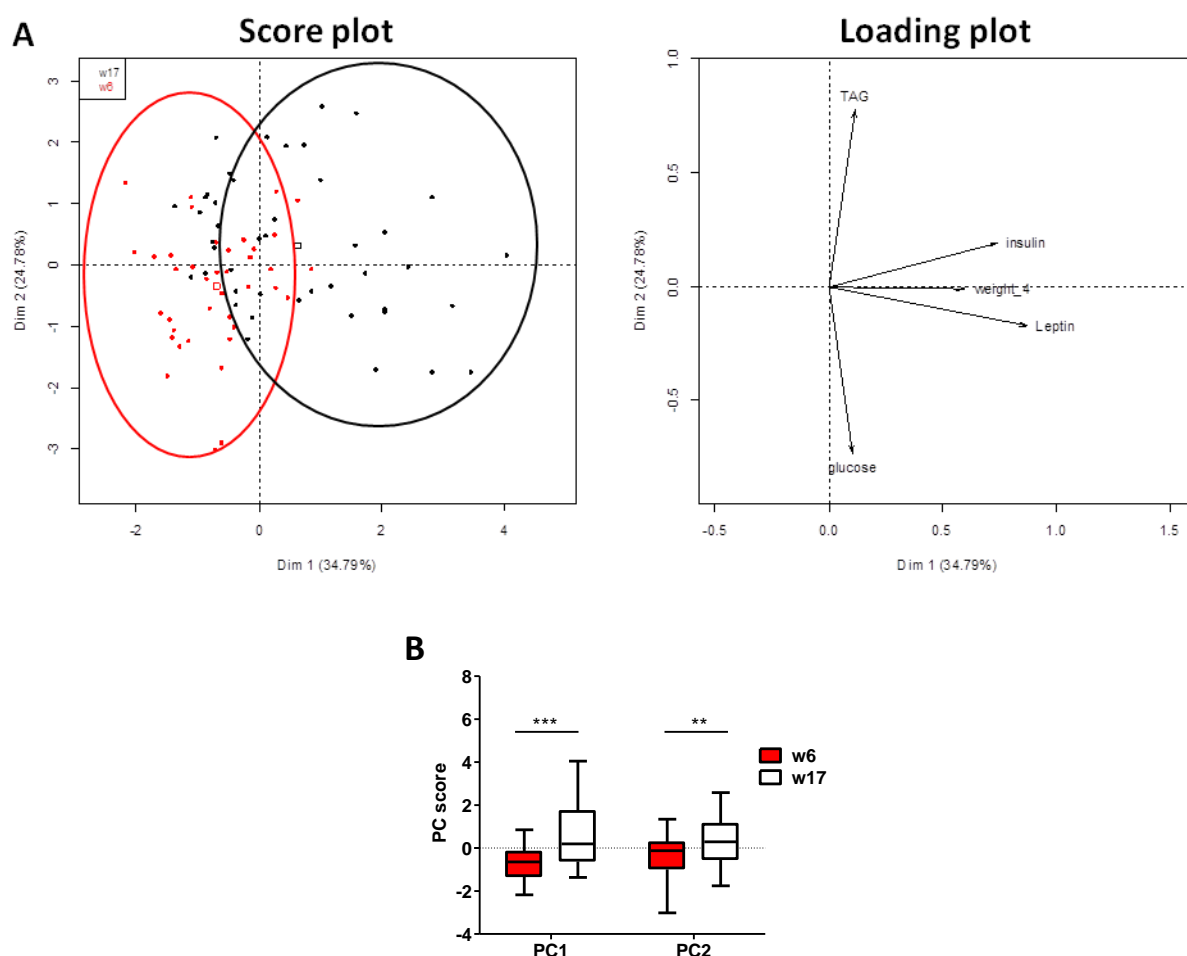
## 7. Summarizing discussion

Maternal obesity or high fat feeding during gestation and lactation is associated with increased risk of obesity and metabolic dysfunction in the offspring<sup>4</sup>. However, diet induced obesity in the mother is associated with a range of alterations in the composition of nutrients, hormones and inflammatory markers which all potentially can affect the metabolic imprinting in the offspring<sup>4,279</sup>. Prepregnancy BMI alone has been associated with elevated birth weight in neonates<sup>280</sup> and maternal obesity might be a stronger predictor for childhood obesity than maternal hyperglycemia<sup>281</sup>, although conflicting studies have shown that maternal sucrose intake and glucose concentrations increases obesity risk in the children independent of prepregnancy BMI<sup>282,283</sup>. The Pedersen hypothesis suggests that maternal hyperglycemia also leads to hyperinsulinemia in the offspring<sup>284</sup>. Studies in rats have additionally shown that maternal fructose intake induced hyperglycemia in the pups at birth,<sup>285</sup> and exposure to a sucrose –rich diet during gestation and lactation impair glucose and lipid metabolism in the adult offspring<sup>226</sup>. But again, results on this matter are conflicting since other studies have shown that gestation diabetes alone is not a predictor for metabolic dysfunction in the children, if they are born with an average birth weight<sup>6</sup>. Maternal intake of saturated fatty acids and maternal triglycerides levels have also been associated with elevated birth weight of neonates and childhood obesity<sup>280,282</sup>, while maternal PUFA intake during pregnancy decreases adiposity in the children<sup>286</sup>. The main problem, about trying to elucidate mono causative effects from each of the factors, is that the factors intertwine in obesity; Hyperleptinemia occurs naturally in obesity due to elevated adiposity, hyperglycemia is counteracted by hyperinsulinemia, and these symptoms are often associated with dyslipidemia. Additionally obesity is associated with a chronic low grade inflammatory phenotype, characterizes by local inflammation in adipose tissue, liver, pancreas, hypothalamus and muscles along side with increased levels of circulating cytokines<sup>1</sup>. A few studies have brought attention to obesity induced inflammation as a potential maternal stimulus during pregnancy that might affect the offspring. Challier and colleagues showed that macrophages infiltrate the human placenta, like we know it from adipose tissue and liver, in obese mothers during pregnancy<sup>190</sup>, and subsequently the results have also been confirmed in non-human primates<sup>41</sup>. These studies made us speculate which role maternal obesity-induced inflammation might play in programming of metabolic function in the

offspring (Chapter 2). To our knowledge, nobody have yet address obesity-induced inflammation in fetal programming of metabolic syndrome, so we can only speculate which role it might play based on animal models with acute inflammation from LPS injections<sup>180-182</sup> and other chronic low grade inflammatory diseases, like psoriasis<sup>187,188</sup>, multiple sclerosis<sup>186</sup> and rheumatoid arthritis<sup>189</sup>. In the review (Chapter 2) we hypothesized that pregnancy, a Treg/Th2 skewed immunological phenotype, can reduce obesity induced inflammation, which is characterized by a Th1 driven phenotype. Subsequently, we tested this hypothesis in a commonly used maternal obesity mice model, where metabolic imprinting in the offspring is known<sup>25,26</sup>. We studied if the low grade inflammation were reduced during pregnancy and therefore less likely to play a role in fetal programming of obesity and metabolic dysfunction (Chapter 3). The study demonstrated that obesity-induced inflammation, characterized by macrophage infiltration in adipose tissue and liver along side with decreased abundance of blood monocytes and a tendency to elevated levels of circulating MCP-1, was present prior to mating. However, all parameters were similar to the control group on gestation day 18 and we found no macrophage infiltration in the placenta of the obese dams. This indicated that the obesity-induced inflammation, present before mating, was reversed during gestation. This result conflicts with previous findings, which have shown not only placental macrophage infiltration in obese women but also elevated circulating levels of C reactive protein (CRP) and IL-6 along side with macrophage infiltration in the adipose tissue<sup>190,192,193,279</sup>. However it is important to emphasize, like we did in the review (Chapter 2), that we do not suggest a complete reversal of obesity-induced inflammation during pregnancy, but the results from the maternal obesity mouse model demonstrate that the inflammation is reduced during pregnancy, similar to other low grade inflammatory diseases. Instead, we suggest that the early elevated lipid exposure caused by maternal obesity might be more important for long term metabolic effect in the offspring, since previous studies have shown increased lipid transport to the fetus in obese ewes<sup>216</sup> and elevated hepatic lipid accumulation in offspring of obese mothers at birth in non-human primates<sup>41</sup>. The mouse fetuses from obese dams is our study, also showed increased accumulation of TAG, but more interestingly hepatic ceramide also tended to accumulate. Ceramide can inhibit insulin signaling and induced inflammation which important in the etiology of non-alcoholic steatohepatitis (NASH)<sup>229</sup>. Thus, if the elevation of hepatic ceramide is maintained into adulthood, the offspring born by obese dams would have increased risk of

developing type 2 diabetes and NASH later in life. Based on these data, we believe that the maternal high fat or obesogenic diet and the increased lipid exposure during fetal life are important for programming of metabolic dysfunction in the offspring.

White and colleagues published a study in 2009 showing that maternal obesity was necessary for programming effects in rat offspring<sup>256</sup>, however conflicting studies have proven otherwise both in rats<sup>37</sup> and non-human primates<sup>41</sup>. In the two rat studies included in this thesis, we neither observed a difference in body weight or circulating metabolic markers at the time of mating among dams fed a pre-gestational conventional or a high fat /high sucrose (HFHS) diet for 6 or 17 weeks. However, the offspring perinatally exposed to the HFHS diet still showed distinct metabolic and immunological phenotypes both at weaning and in adulthood. The metabolic phenotype of the dams was surprising, since the obese dams on average had a 1.25 fold higher caloric intake (Chapter 4), but many metabolic markers are not being evaluated in our setup. The length of the intervention period prior to mating might be important for the outcome in the offspring<sup>262,264</sup>. Therefore, we cannot assume that the maternal phenotypes in the two rat studies are comparable. The metabolic profile of the first (6 weeks of pre-gestational intervention, w6) and second litter (17 weeks of pre-gestational intervention, w17) were therefore described by body weight and circulating metabolic markers at weaning and compared by principal component analysis, to elucidate if the two litters displayed similar metabolic patterns (Figure 1). The score plot (Figure 1A) showed that length of the pre-gestational intervention of the dams separates the metabolic phenotype in the male offspring at weaning both along PC1 and PC2 ( $p < 0.001$  and  $p < 0.01$ , respectively, Figure 1B). PC1 separates the litters according to body weight and plasma insulin and leptin levels, which demonstrates that an extended pre-gestational intervention increases the metabolic dysfunction in the offspring at weaning. Thus comparison of the two studies therefore has to be done with caution.

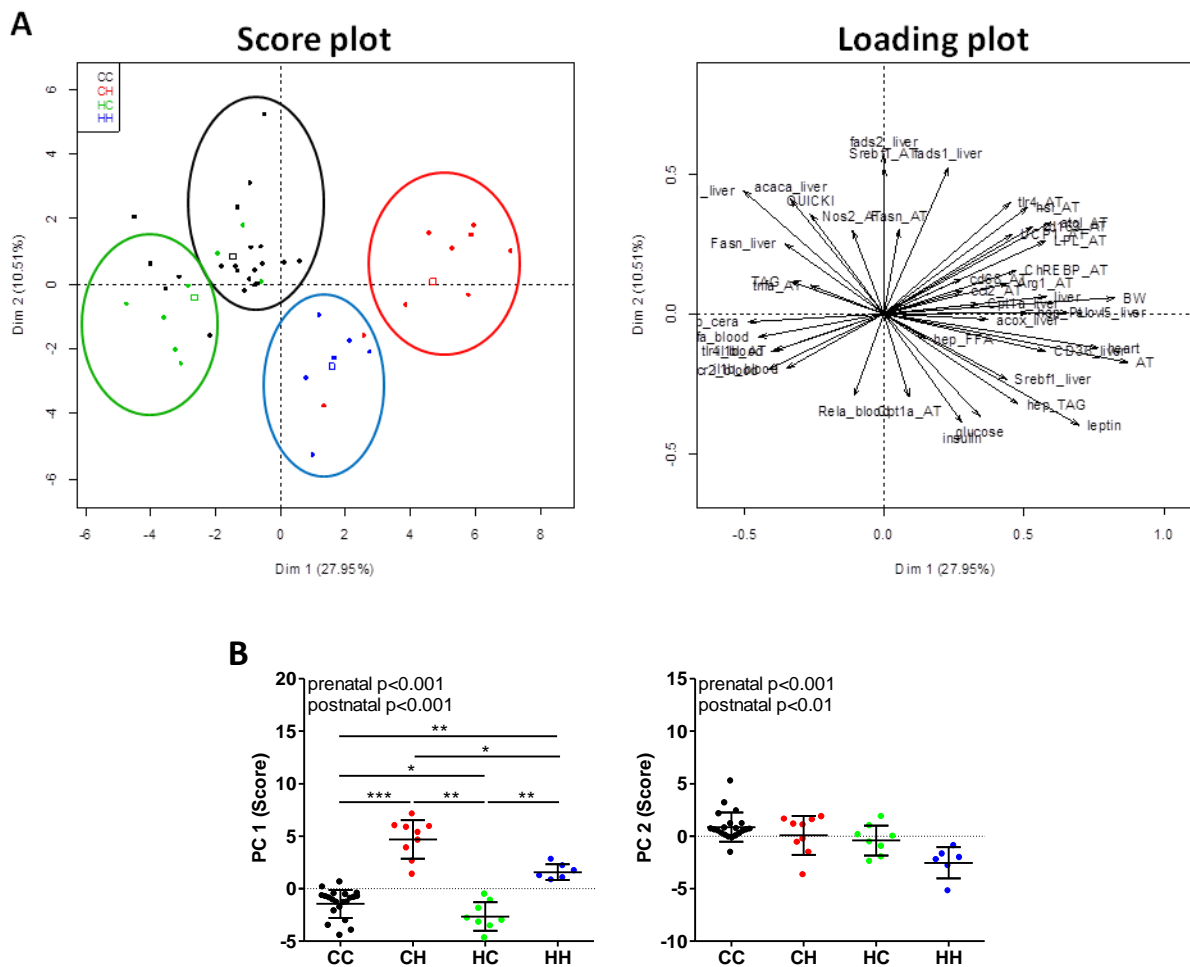


**Figure 1. Principal component analysis of metabolic markers in male offspring at weaning according to length pre-gestational intervention.** Body weight and circulating metabolic markers were used to describe the metabolic phenotype. w6 (red), offspring born by dams with a 6 weeks intervention period prior to mating, w17 (black) offspring born by dams with a 17 weeks intervention period prior to mating. A. Scoring and loading plots for the PCA. The square symbol is the average phenotype of the group. B. Extracted scores for principal component (PC) 1 and 2. Data are presented as mean  $\pm$  stdev. Effects between w6 and w17 offspring were analysed by a permuted t-test. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . TAG triacylglyceride.

Offspring exposed to a maternal HFHS diet during gestation (HC), lactation (CH) or both (HH) have been metabolically and immunologically phenotyped at weaning. These results are separately reported in Chapter 4 and 6. However, as mentioned previously, the obesogenic phenotype is complex and many of the factors intertwine, so a multivariate principal component analysis (PCA) was performed on both metabolic and immunologic parameters to elucidate overall phenotypical changes between the groups (Figure 2). Score and loading plots are shown in Figure 2A. The offspring clustered according to the four different groups (CC = black circle, CH = red circle, HC = green circle, HH = blue circle, Figure 2A, scoring plot). However, no apparent clustering was visible

when plotting PC2 vs PC3 (data not shown). To analyze if the clustering was significant, the scores of principal components 1 (Dim 1, PC1) and 2 (Dim 2, PC2) were extracted and the statistical analysis showed that all groups were significantly different from the other along the PC1 axis (Figure 2B). PC1 explained 28% of the variation between the animals and was characterized by an increase in: adiposity, lipolysis in adipose tissue (indicated by mRNA levels of *atgl* and *hsl*) and hepatic accumulation of triacylglycerides and phospholipids, in combination with a decrease in: expression of inflammatory genes in blood (indicated by mRNA levels of *tnfa*, *ccr2* and *tlr4*) and hepatic ceramide accumulation. It is surprising that lipolysis in adipose tissue and hepatic triacylglyceride accumulation were clustered opposite from ceramide concentration along PC1. Hepatic steatosis is normally linked to accumulation of lipotoxic intermediate like ceramide and FFA<sup>228</sup>, however, ceramide might be rapid secreted to avoid the lipotoxic effects<sup>244</sup>. Ceramide can interfere with insulin signaling<sup>229</sup> and it was therefore not surprising that hepatic ceramide content clustered with mRNA levels on inflammatory genes in the blood along PC1, since obesity-induced inflammation operates locally in various tissue but also systemic. Additionally it was surprising that inflammatory parameters clustered opposite from the obesity parameters indicating that the obese CH group (Chapter 4), might have a less tendency to develop obesity-induced inflammation and subsequently metabolic disease, while the HC offspring might have a higher risk despite their lean phenotype (HC). However, as mentioned in Chapter 6, the decreased pro-inflammatory phenotype is the CH group could potentially also indicate the very early stage of obesity-induced inflammation, since M2 macrophages are recruited to the adipose tissue after short term high fat feeding<sup>278</sup> probably due to an increased lipid turn over in the tissue<sup>123</sup>. Further research is needed to elucidate if dietary mismatch between prenatal and postnatal life can affect the offspring's immunity. The HFHS lactated offspring (CH and HH) grouped on one side of the CC group along the PC1 axis while the HC clustered on the other, thus the mismatch groups (CH and HC) were phenotypically furthest from each other leaving the match phenotypes (CC and HH) in the intermediate area. The CC were closer related to the HC and HH group than the CH group, demonstrating that a dietary mismatch between the control diet *in utero* and the maternal HFHS intake during lactation, cause more severe phenotypic alteration in the offspring at weaning than when the pups that have been exposed to the HFHS diet both *in utero* and during lactation. This is in good accordance with the predictive adaptability hypothesis<sup>19</sup>, which suggest that epigenetic

adaptions occur *in utero* to predisposes the fetuses, in this case, to a high energy diet after birth. PC2 explained 10.5% of the variation between the animals and was characterized by an increase in overall hepatic lipid synthesis (indicated by mRNA levels of *fads1*, *fads2*, *scd1* and *acaca*) and a decrease in circulating metabolic markers (leptin, insulin and glucose). Both prenatal and postnatal HFHS exposure affected the PC2 score ( $p < 0.001$  and  $p < 0.01$  respectively, Figure 2B), however the separation between the groups along PC2 was much smaller compared to PC1.



**Figure 2. Principal component analysis of immunity and metabolism in male offspring at weaning.** A. Scoring and loading plots for the PCA. The squares are the average phenotype of the group. B. Extracted scores for principal component (PC) 1 and 2. Data were analysed by a permuted 2-way ANOVA for prenatal and postnatal effects and presented as mean  $\pm$  stdev. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

Then we studied the phenotype of male offspring exposed to maternal HFHS intake during gestation (HC), lactation (CH) or both (HH) after they were challenge with a high fat diet in

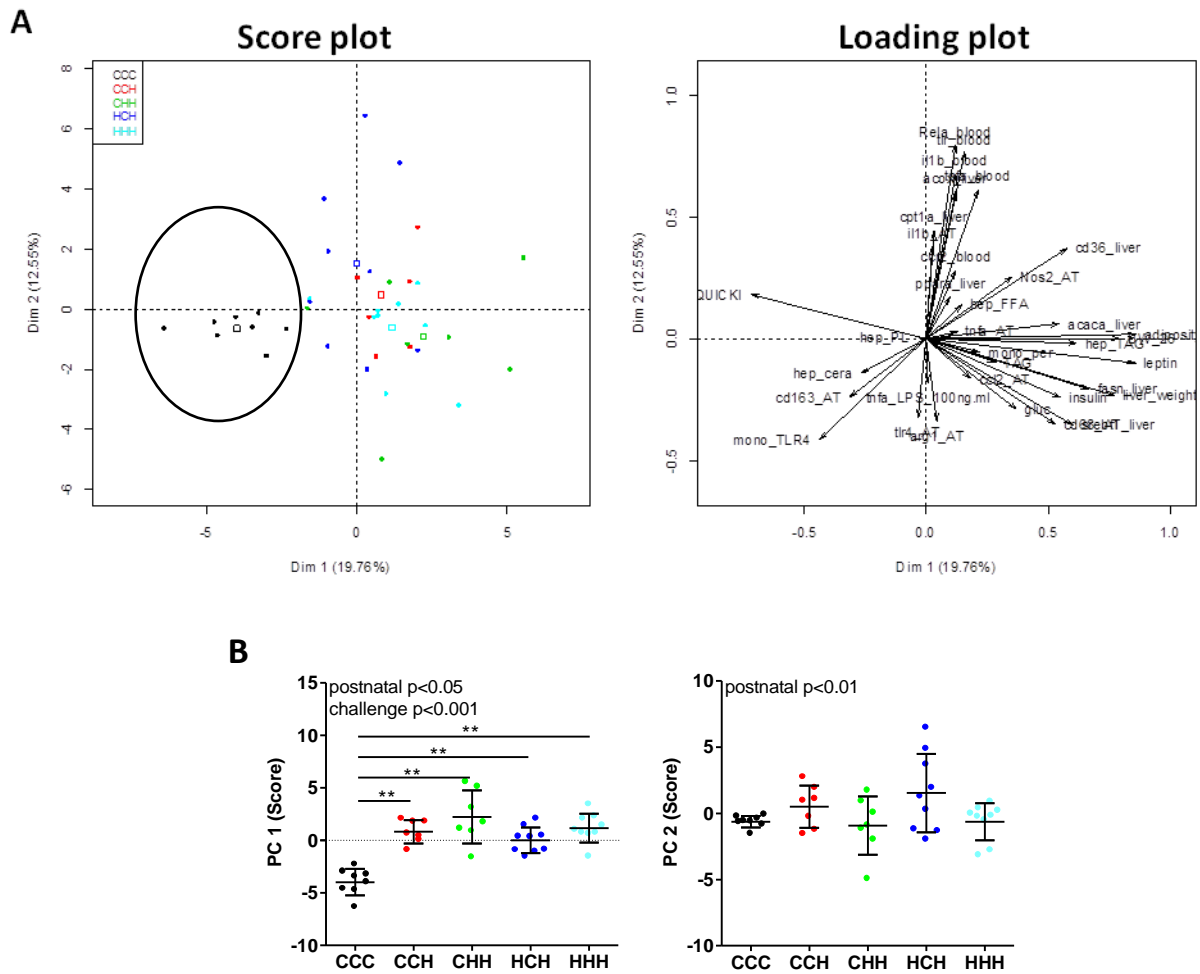
adulthood, to elucidate if the maternal HFHS diet influenced the offspring's capability to deal with a high fat load as adults. Similar to the previous study in young offspring at weaning, we were interested in changes in the overall phenotype describes by both metabolic (Chapter 5) and immunologic (Chapter 6) parameters between the groups, so an PCA were performed (Figure 3). Score and loading plots are shown in figure 3A. As expected, the control group that were exposed to conventional diet throughout the experiment clustered along PC1 (challenge  $p < 0.001$ , Figure 3B), which explained 20% of the variation between the animals. PC1 was characterized by an increase in: adiposity and plasma leptin, hepatic de novo lipogenesis (indicated by mRNA levels of *fasn* and *acaca*), triacylglyceride accumulation in the liver and plasma insulin levels and a decrease in: TLR4 expression on the surface of blood monocytes, expression of the M2 macrophage marker *cd163* in adipose tissue and hepatic ceramide accumulation. Again, the disparity between hepatic triacylglyceride content and hepatic ceramide content was observed along PC1, similar to the young offspring at weaning. However, as discussed in Chapter 5, this was very surprising, not only due to the elevation in hepatic triacylglyceride, but in particular due to a dramatic increase in hepatic free fatty acids in the HFHS born offspring (HCH and HHH) .When free fatty acids accumulate in the liver, due to limited capacity in the synthesis of complex lipids and the oxidation pathways, they are shuttled into alternative pathways, which creates lipotoxic intermediates like ceramide<sup>268</sup>. Based on the data reported in chapter 5, we are not capable of concluding if ceramide is actually produced to a larger extend in the HFHS born offspring, due to the elevated level of hepatic free fatty acids, but just rapidly secreted resulting in a ceramide accumulating similar to the C born offspring<sup>244</sup>.

Postnatal effects were also affecting the separation of PC1, so the phenotype in the HFHS lactated offspring diverged more from the CCC group than the control lactated offspring (postnatal  $p < 0.05$ , Figure 3B). Similar to the overall phenotype observed in the young offspring at weaning, the HC and CH groups were furthest apart along the PC1 axis with the matched phenotypes (CCH and HHH) in between (Figure 3B). Plasma leptin levels might be one of the factors explaining this separation. The CHH groups had the highest leptin concentration after the dietary challenge and HCH had the lowest, so in Chapter 5 we suggested that offspring born by HFHS dams but lactated by control mothers seems to protect against hyperleptinemia during high fat feeding in adulthood, while the reversed mismatch increase the susceptibility, which is in accordance with earlier



finding<sup>39</sup>. We proposed that the leptin-gene promotor was the target for epigenetic changes due to maternal high fat/high sucrose intake during gestation and lactation, since maternal high fat feeding during pregnancy and lactation alters the epigenetic pattern on the leptin promoter<sup>56</sup>. Thus, *in utero* exposure would induce a predictive adaptive response that attenuates hyperleptinemia in our scenario, when exposed to high energy-diets later in adulthood. However, this response seemed to be limited to a developmental window *in utero*, while exposure after birth did not cause this adaptation.

PC2 explained 13% of the variation between animals and was characterized by an increase in expression of inflammatory markers in blood (indicated by mRNA levels of *tnfa*, *il1b*, *tlr4* and *Rela*) concurrent with a decrease in TLR4 expression on the surface of blood monocytes. This opposite clustering of TLR4 on monocytes and expression of inflammatory genes indicates that the monocytes are activated by a TLR4 independent mechanism. This is in conflict with the endotoxemia hypothesis, which states that insulin resistance is associated with activation of monocytes by elevated circulating levels of lipopolysaccharide via TLR4 dependent mechanism<sup>133</sup>. In this study, TLR4 expression cluster with insulin sensitivity along PC1 and opposite from the expression of inflammatory genes, indicating that insulin resistance might develop via a TLR4 independent mechanism. Offspring exposed to a HFHS diet during lactation had a profile similar to the CCC group along PC2, but the profile of the control lactated offspring were skewed towards higher expression of inflammatory markers in the blood after the dietary challenge (postnatal  $p < 0.01$ , figure 3B). This indicates that the HFHS lactated offspring might display as smaller tendency to develop obesity-induced inflammation despite increased adiposity, and therefore more capable of dealing with a high fat diet in adulthood without developing metabolic disease. Further research is needed to elucidate this matter.



**Figure 3. Principal component analysis of metabolic and inflammatory markers in male offspring at 26 weeks of age after a metabolic load.** Dams were fed a conventional or a HFHS diet for 6 weeks prior to mating. Male offspring were cross-fostered on complementary dams during lactation and weaned onto a conventional diet from 4-20 weeks of age. A. Scoring and loading plots for the PCA. The squares are the average phenotype of the group. B. Extracted scores for principal component (PC) 1-2. Data were analysed by a permuted 2-way ANOVA for prenatal and postnatal effects and presented as mean  $\pm$  stdev. \*\*  $p < 0.01$

## 8. Conclusion and future perspectives

In this thesis we have demonstrated that obesity induced inflammation is reversed during pregnancy in mice, and is therefore less likely to affect the fetal programming of metabolic dysfunction. However, macrophage infiltration has been found in the human placenta from obese mothers along with elevated levels of circulating. Further research is therefore needed, to first elucidate how obesity-induced inflammation is affected by pregnancy in humans, and secondly, investigate if pro-inflammatory cytokines can cross the placenta and directly affect the fetus, or alternatively alter the placental function and transfer of nutrients and hereby indirectly affect have a effect. If obesity-induced inflammation plays a role in metabolic imprinting in the offspring, then anti-inflammatory treatment during pregnancy might improve the metabolic outcome in the offspring. However, we suggest that the early elevated lipid exposure caused by maternal high fat feeding might be more important for long term metabolic effect in the offspring. Thus, we have demonstrated that a dietary mismatch between pre- and post-natal life alters the phenotype at weaning, so that exposure to a control diet *in utero* and a high fat/high sucrose diet during lactation cause more severe phenotypic alteration in the offspring at weaning than pups exposed to the high fat/high sucrose diet both *in utero* and during lactation. The same pattern was seen in the adult offspring after being challenged with a high fat diet for 6 weeks. However, HFHS exposure during fetal life protected against hyperleptinemia in the adult offspring during the challenge. Since the epigenetic regulation of the leptin-gene promoter is known to be altered by maternal high fat feeding, it could be interesting to investigate if this attenuation of the leptin response is linked to changes in the epigenome. HFHS lactated offspring displayed a decrease level of inflammatory genes in the blood after the dietary challenge, which could indicate that perinatal HFHS exposure protect against the detrimental effects of high fat feeding leading to metabolic disease, which is in accordance with the predictive adaptive response hypothesis. LPS injections in mothers induce tolerance in the offspring in a mouse model, so it could also be interesting to investigate if maternal high fat feeding also can induce tolerance towards high fat intake in the offspring. However, a more thorough phenotyping of the immunity in blood and tissues from the offspring is needed to resolve this question.



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