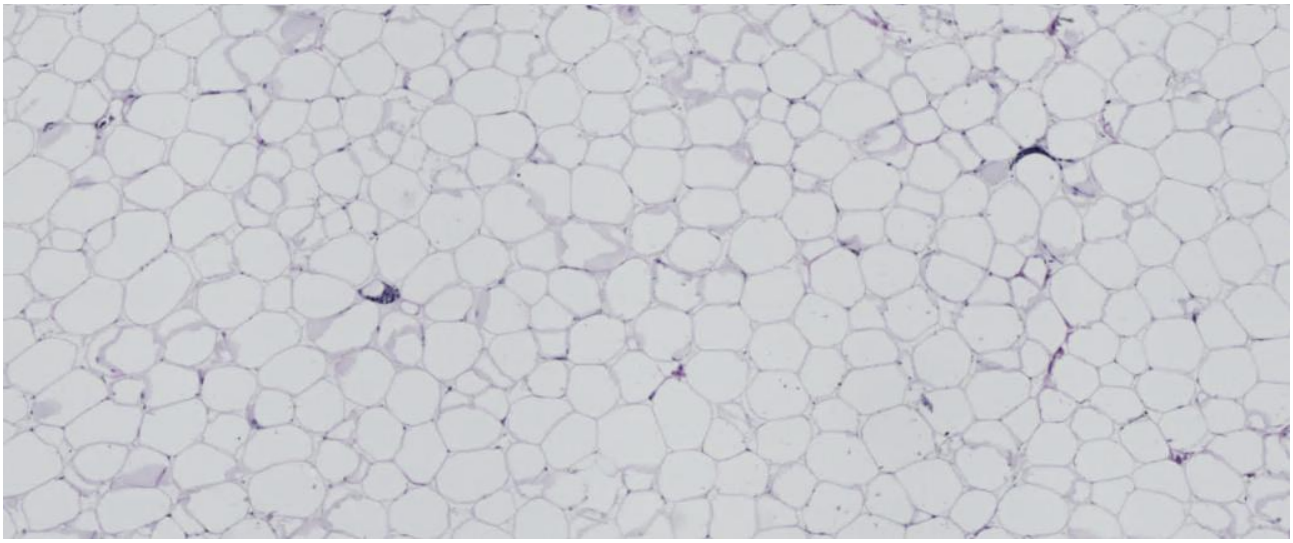
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

Effects of late gestational under- and overnutrition and early postnatal overnutrition on long-term development and function of adipose tissue in a sheep model



MSc Thesis, Animal Science, 45 ECTS

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Preface

This Master's thesis is made to fulfil the requirements of a 45 ECTS thesis as a completion of the Mater's education in Animal Science at the University of Copenhagen. The project has been completed in the period September 2016 - August 2017 at the Department of Veterinary and Animal Sciences, Section for Production, Nutrition and Health at University of Copenhagen, Denmark.

The thesis was written with guidance from my supervisor Professor Mette Olaf Nielsen and consists of two parts. The first part provides a theoretical background on the topic of foetal programming and effects of pre- and early postnatal malnutrition on adipose tissue development and function. The second part presents the methods used and results procured from the experimental work done in relation to this thesis, as well as a discussion of the results. The data procured for this thesis is part of an ongoing study using the Copenhagen sheep model.

I wish to express my gratitude towards the people who have helped and supported me throughout this process. I first and foremost wish to thank my supervisor Mette Olaf Nielsen for invaluable guidance, and constructive criticism of the thesis. Furthermore I would like to thank PhD fellow Morteza Mansouryar for teaching me the laboratory techniques required to perform the experimental work relating to qPCR, and for providing the qPCR results for two of the four adipose tissues that I analyse in this thesis. I am also thankful to laboratory technician Anni Christiansen for kind assistance and guidance with the qPCR work and for agreeing to share her office with me for a large part of the year. Finally, a large thanks to Rajan Dhakal for valuable support in setting up the statistical models and making the statistical analysis work as well as PhD student Sharmila Binti Ahmad for collaboration on working with the histological data.

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Abstract

Foetal programming is a term that refers to how environmental influences *in utero* can impose long-term effects of on health later in life. Nutritional insults such as under- or overnutrition during foetal and/or early postnatal life have been found to increase the risk of metabolic and cardiovascular diseases in later life. Adipose tissue is both a storage organ for excess nutrients and an endocrine organ. Obesity and dysfunctional adipose tissue is linked to the development of metabolic and cardiovascular disorders, making it relevant to investigate whether the adverse effects of foetal programming are linked to programming of adipose tissue.

This study aimed at investigating long-term effects of late gestation under- and overnutrition on adipose tissue development and function. Furthermore, it was aimed at investigating if late gestation malnutrition altered the ability of adipose tissue cope with obesity in early postnatal life. This was investigated partly through a study of literature, which provided a theoretical background on foetal programming, and adipose tissue development and function. Additionally, an experimental study was performed in order to assess how gene expression was affected in four different adipose tissue depots (subcutaneous, perirenal, mesenteric and epicardial) as a consequence of nutritional foetal programming. Laboratory analyses were performed on samples of adipose tissue from adult sheep that had been either under-, over- or normally nourished in late gestation and subsequently provided with a conventional or an obesogenic diet for the first six months of life, followed by two years on a conventional diet. Furthermore, histological analyses were performed to investigate adipose tissue morphology, but an analysis of those results are not included in the current thesis.

The overall findings were that altered gene expression in the subcutaneous adipose tissue indicated reduced ability to expand. Perirenal adipose tissue gene expression reacted differently to prenatal under- and overnutrition, while mesenteric adipose tissue showed very few programming effects. Epicardial adipose tissue was targeted to a greater extend by the postnatal obesogenic diet. An additional finding was that males and females showed great variation both in overall gene expression and in their reactions to foetal programming.

In conclusion, the study showed that gene expression of adipose tissue was susceptible to long-term programming effects of both prenatal under- and overnutrition as well as early postnatal overnutriton in a depot and gender specific manner.

Sammendrag

Føtal programmering er et term der refererer til hvordan miljømæssige påvirkninger *in utero* kan medføre langsigtede påvirkninger af helbred senere i livet. Det er blevet fundet at fejlernæring, så som under- og overernæring i løbet af det føtale og/eller tidlige postnatale liv kan øge risikoen for metabolske og hjertekarsygdomme senere i livet. Fedtvæv fungerer både som et lager for overskydende energi og som et endokrint organ. Fedme og dysfunktionelt fedtvæv kan medføre udvikling af metabolske og hjertekarsygdomme, hvilket gør det relevant at undersøge om negative effekter af føtal programmering er forbundet med programmering af fedtvæv.
Formlet med dette studie var at undersøge langsigtede effekter af under- og overernæring i sen drægtighed for udvilking og funtion af fedtvæv. Derudover var formålet at undersøge om fejlernæring i den sene drægtighed ændrede fedtvævets evne til at håndtere overvægt i det tidlige postnatale liv. Dette blev undersøgt til dels gennem et litteraturstudie, som skulle give en teoretisk baggrund for at forstå føtal programmering og fedtvævs udvikling og funktion. Dernæst blev der udført et eksperimentelt studie for at undersøge hvordan genekspression blev påvirket i fire forskellige fedtdepoter (subkutant, perirenalt, mesenterielt og epikardialt) som konsekvens af føtal programmering. Laboratorieanalyser blev udført på fedtvævsprøver fra voksne får, som enten havde været under-, over-, eller normalt ernærede i sen drægtighed og derefter var blevet entern normalt eller overernærede i de første seks måneder efter fødslen, efterfulgt af to år på en konventionel diæt. Derudover blev der udført histologiske analyser for at undersøge fedtvævets morfologi, men en analyse af resultater for dette er ikke inkluderet i denne opgave.
Overordnet blev det fundet at ændret ændret genekspression i det subkutane fedtvæv indikerede at vævets evne til at ekspandere var reduceret. Genekspression i det perirenale fedtvæv reagerede forskelligt efter om dyret havde været under- eller overernæret prænatalt, mens det mesenteriske fedtvæv var stort set upåvirket. Epikardialt fedtvæv blev i højere grad påvirket af den postnatale overernæring. Et yderligere resultat var at hanner og hunner udviste stor variation både i genekspression generelt og i deres reaktioner på føtal programmering.
Det kunne dermed konkluderes udfra studiet at genekspression i fedtvæv var modtagelig for langsigtet programmering forårsaget af både prænatal under- og overernæring, og tidlig postnatal overernæring, på en måde der varierede mellem depoter og køn.

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Abbreviations

ACTB (β-actin)

AdipoQ (Adiponectin)

ADRα1 (Alpha-1 adrenergic receptor)

ADRβ1 (Beta-1 adrenergic receptor)

ATGL (Adipose triglyceride lipase)

CD34 (Cluster of differentiation 34)

CD44 (Cluster of differentiation 44)

CD68 (Cluster of differentiation 68)

cDNA (Complementary DNA)

C/EBPβ (CCAAT/enhancer binding proteinβ)

CGI-58 (Comparative gene identification-58)

CONV (Conventional diet)

EC (External control)

FABP4 (Fatty acid binding protein 4)

FAS (Fatty acid synthase)

FBPase (Fructose 1,6-bisphosphatase)

FA (Fatty acid)

FFA (Free fatty acids)

FTO (Fat mass and obesity-associated protein)

GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)

GcR (Glucocorticoid receptor)

GLUT-1 (Glucose transporter 1)

GLUT-4 (Glucose transporter 4)

HCHF (High-carbohydrate-high-fat diet)

HIGH (150% of recommended daily digestible energy and 110% of recommended daily protein)

HSL (Hormone-sensitive lipase)

IGF1R (Insulin-like growth factor I receptor)

IL6 (Interleukin-6)

IRS1 (Insulin receptor substrate 1)

LEPR (Leptin receptor)

LOW (50% of recommended daily digestible energy and protein)

LPL (Lipoprotein lipase)

MCP-1 (Monocyte chemotactic protein-1)

miRNA (micro-RNA)

NORM (Recommended daily allowance diet)

PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator alpha isoform)

PLIN-1 (Perilipin-1)

PPARα (Peroxisome proliferator-activated receptor alpha)

PPARγ (Peroxisome proliferator-activated receptor gamma)

Pref-1 (Preadipocyte factor 1)

qPCR (Quantitative real-time PCR)

TG (triglyceride)

TGF-β1 (Transforming growth factor beta-1)

TLR-4 (Toll-like receptor 4)

TNFα (Tumor necrosis factor-alpha)

UCP-2 (Uncoupling protein 2)

VEGF (Vascular endothelial growth factor)

VEGF-A (Vascular endothelial growth factor-A)

Wnt5α (Wingless-related integration site 5α)

1 Introduction

1.1 Background

The global prevalence of obesity has been increasing rapidly over the past decades. It was estimated, that 36.9% of adult men and 38.0% of adult women worldwide could be classified as overweight or obese in 2013, reflecting an increase of 27.5% since 1980. The prevalence of overweight and obesity in children rose by 47.1% in the same period (Ng *et al.*, 2014).

Obesity is associated with the development of disorders, such as cardiovascular disease, type 2 diabetes and hypertension (Kopelman, 2000). The increase in the prevalence of obesity as well as related metabolic disorders has been linked to environmental factors, such as a more sedentary lifestyle and a greater availability of foods with a high content of energy and fat. However, some individuals are more susceptible to developing obesity related metabolic disorders than others (Primeau *et al.*, 2011).

Ample evidence from human epidemiological studies as well as experimental animal studies demonstrate that maternal undernutrition during pregnancy predisposes for later development of obesity, cardiovascular disease, glucose intolerance and other metabolic disorders in the offspring (Hales & Barker 1992; Ravelli et al. 1999; Torrens et al. 2009; Cleal et al. 2007). Hales & Barker (1992) formulated a hypothesis stating that foetal malnutrition leads to the development of a 'thrifty phenotype' that improves the chance of survival of the foetus *in utero*, by prioritising allocation of nutrients to organs that are critical for immediate survival. This can become a disadvantage in later life if conditions change, as the metabolism of the individual will then be adapted to a different environment.

It has also been shown that foetal overnutrition, like undernutrition, can increase the risk of obesity and related disorders (Grattan, 2008; Long *et al.*, 2010; McMillen *et al.*, 2009), and a 'U'-shaped relation has been found between birth weight and development of obesity in adulthood (Pettitt and Jovanovic, 2001).

Furthermore, overnutrition during early postnatal life has been found to have a programming effect, which is most clearly seen when combined with adverse nutritional programming in foetal life (Cleal *et al.*, 2007; Vickers *et al.*, 2000). These studies using animal models showed that mismatching pre- and postnatal nutrition in the form of prenatal undernutrition and postnatal overnutrition was associated with increased risk of metabolic and cardiovascular abnormalities (Cleal *et al.*, 2007; Vickers *et al.*, 2000). It has also been shown that an increased risk of obesity as well as alterations in appetite regulation and lipid and glucose metabolism caused by prenatal

overnutrition can be exaggerated by postnatal overnutrition (Chen *et al.*, 2008).

Adipose tissue not only acts as a storage of excess energy, but is also an important endocrine organ that affects several systems including metabolism (Sethi and Vidal-Puig, 2007). A permanently decreased ability of subcutaneous adipose tissue to expand, associated with increased development of visceral adiposity has been found in prenatally undernourished sheep (Nielsen *et al.*, 2016) and in adolescent lambs that had been either under- or overnourished prenatally (Khanal *et al.*, unpublished). Visceral obesity in particular, rather than whole body obesity, has been linked to development of insulin resistance, increased risk of developing certain cancers and impaired glucose and lipid metabolism (Shuster *et al.*, 2012). It is therefore relevant to investigate programming effects on adipose tissues in relation to the development of metabolic disorders later in life.

Following this evidence, effects of nutrition in the foetal and early postnatal life appear to be of great importance from the point of view of human health and as such, many studies on foetal programming are made from the point of view of examining human health and disease. However knowledge on foetal programming also has relevance in other areas, such as animal production. Growth performance and meat quality is affected through programming of muscle-, skeletal and adipose tissue (Du *et al.*, 2015) and furthermore, metabolic disorders may cause problems in animals used for breeding purposes.

1.2 Objectives of the thesis

The overall objectives of this MSc thesis were to investigate if late gestation under- and overnutrition affect adipose tissue in a similar, but depot-specific manner, leading to altered morphology and expression of genes involved in adipocyte development (hyperplasic and hypertrophic growth), metabolism and inflammation in adult sheep. An additional objective was to investigate if late gestation over- or undernutrition affects the ability to cope with exposure to an obesogenic diet in early postnatal life.

1.3 Hypotheses

- Both late gestation under- and overnutrition reduces the ability of subcutaneous adipose tissue to expand in adult animals, thus predisposing for visceral adiposity.

- Late gestation under- and overnutrition interferes with mechanisms controlling hyperplasic and hypertrophic growth and metabolism in adipocytes in a depot specific manner.

- Effects of obesity development in early postnatal life can be alleviated through dietary interventions in individuals who have been nourished normally in foetal life, but not in individuals that have been exposed to prenatal malnutrition.

1.4 Methods

These objectives and hypotheses will be addressed firstly through a study of literature, providing a theoretical background on foetal programming and adipose tissue development and function, and secondly, through an experimental study.

The literature study is based on peer reviewed scientific literature, found through a systematic search of electronic databases (mainly Ovid CAB Abstracts) using search words including: "f?etal programming", " f?etal nutrition*", "prenatal nutrition*", " adiposity", "adipose tissue*", "adipocyte*", "gene expression", "sheep", " intrauterine", "late gestation", etc. Furthermore the snowball method was employed, using the references of an article to find other articles.

The experimental part of the study consisted of laboratory analyses of samples of adipose tissue from adult sheep that had been either under- or overnourished in late gestation and subsequently provided with an obesogenic diet for the first six months of life, followed by two years on a conventional diet. Gene expression was investigated using quantitative real-time PCR (qPCR) and histological analyses were performed to investigate adipose tissue morphology.

2 Theoretical background

2.1 What is foetal programming?

The purpose of this section is to provide an overview of the concept of foetal programming. Additionally, evidence that nutritional insults can have programming effects on health later in life is outlined, to emphasise the relevance of this research area.

2.1.1 The concept of foetal programming

Foetal programming, also referred to as 'the developmental origins of health and disease', is a term used to describe long-term effects of environmental influences *in utero* on later life health and productivity (Chavatte-Palmer *et al.*, 2016; Kenyon and Blair, 2014). Lucas (1991) defined programming in relation to biological function, as a stimulus or insult that happens during a critical or sensitive period, which results in a permanent change in structure or function of an organism. Such stimuli or insults in the maternal environment may include nutritional factors, infections or other physiological stressors (Salter *et al.*, 2009).

With the 'thrifty phenotype' hypothesis Hales and Barker (1992) proposed that exposure to a nutritional deprivation during a critical period of development in prenatal or early postnatal life would affect growth and development, prioritising organs and tissues critical for immediate survival, such as the brain and heart. This would then predispose the individual to developing metabolic disorders later in life due to dysfunction of the tissues that were not prioritised at the time of nutrient restriction, e.g. kidneys and pancreas. This hypothesis has since been extended with the 'predictive adaptive response' hypothesis, proposing that adaptations made by the foetus *in utero* or in the early postnatal life are made to prepare the offspring for the expected future environment. In the event that the postnatal environment is not predicted correctly, then the adaptation will be inappropriate, and may lead to an increased susceptibility to certain diseases (Gluckman and Hanson, 2004).

There is now ample evidence backing the hypothesis that nutritional insults during prenatal and early postnatal life can change the phenotype of the affected individual. The first evidence, which was the basis for the development of the 'thrifty phenotype' hypothesis, came from epidemiological studies, and these have since been supported by experimental animal studies.

2.1.2 Epidemiological evidence

Epidemiological studies by Barker and colleagues provided some of the first evidence to suggest that health in adult life could be programmed by uterine environment and formed the basis of their

'thrifty phenotype' hypothesis. A study of populations in England and Wales revealed a correlation between increased mortality rates from ischaemic heart disease and previous high infant mortality rates in the same areas. They suggested that the geographical distribution pattern of ischaemic heart disease in adults reflected areas where early life nutrition had been poor at their time of birth, which was reflected in the high infant mortality (Barker and Osmond, 1986).

Furthermore, retrospective cohort studies of a population from Hertfordshire, UK, showed that low birth weight was significantly correlated with development of impaired glucose tolerance and type 2 diabetes (Hales *et al.*, 1991). Hypertension (raised blood pressure) as well as raised plasma lipid concentrations (Barker *et al.*, 1993) and increased mortality from cardiovascular disease (Osmond *et al.*, 1993) was also found to be associated with low birth weight. By using low birth weight as an indicator for poor nutrition in foetal life, this data formed the basis of the 'thrifty phenotype' hypothesis. A cohort study from Helsinki, Finland have likewise found low birth weight and thinness at birth to be correlated with development of coronary heart disease and type 2 diabetes (Eriksson *et al.*, 1999, 2002).

Critique of this type of retrospective epidemiological studies include the fact that birth weight is a crude measure of maternal nutritional status during pregnancy. The famine that occurred in Holland during the winter of 1944-1945 (the Dutch hunger winter) therefore, provided a unique opportunity to study the effect of maternal undernutrition in humans more directly. Food supply was blocked for a period of approximately 6 months and so, during this time, women in different stages of pregnancy were exposed to nutritional deprivation. Follow-up studies of people who were *in utero* during the famine revealed an increased prevalence of chronic diseases, but also that health consequences varied depending on when during pregnancy the famine occurred. Individuals exposed to famine during the first trimester had increased risk of developing coronary heart disease and obesity later in life, while those exposed in the second trimester were more likely to develop obstructive airway disease and reduced kidney function. If the famine exposure happened during the final trimester however, people were instead more at risk of developing insulin resistance, type 2 diabetes and hypertension, thus showing that the timing of exposure to prenatal malnutrition is important to the type health outcome (Painter *et al.*, 2005; Roseboom *et al.*, 2001). Birth weight was reduced in those who were exposed in late gestation, but those exposed in early gestation were slightly heavier at birth than individuals not exposed to famine (Roseboom *et al.*, 2001). Thus, while providing support for the conclusions of cohort studies, in showing an effect of foetal malnutrition on health in later life, the studies concerning the Dutch hunger winter also outlined that

caution should be taken when using birth weight as a measure of maternal nutritional status. Undernutrition is however not the only form of prenatal malnourishment that has been shown to have a programming effect on the offspring. Several studies have found an association between both low- and high birth weight and risk of developing type 2 diabetes (Pettitt and Jovanovic, 2001; Wei *et al.*, 2003), revealing a 'U'-shaped risk curve. Two prospective cohort studies (the Generation R study and the Project Viva) examined possible associations between BMI before as well as weight gain during pregnancy and cardio-metabolic health of the offspring. The Generation R cohort study from the Netherlands found that maternal weight gain during early pregnancy led to increased adiposity and cardio-metabolic risk factors in the children (Gaillard *et al.*, 2015). Similar metabolic risks were found to be associated with high maternal weight in the US cohort study, Project Viva (Perng *et al.*, 2014). This study furthermore found that weight gain during pregnancy also lead to increased adiposity and leptin concentrations in the offspring.

The early postnatal period has also been suggested as a critical period for nutritional programming in humans. There are indications from human studies that effects of nutritional deprivation *in utero* become more pronounced when the postnatal nutrition differs from the prenatal nutrition, which is referred to as nutritional mismatch. This is based on studies showing an association of individuals who were small at birth and subsequently gained weight rapidly, with the development of type 2 diabetes (Crowther *et al.*, 1998) and cardiovascular disease (Barker *et al.*, 2005; Eriksson *et al.*, 1999). Other studies on effects of early postnatal nutrition in humans are based on studies of long-term health benefits of breast feeding, which suggest that programming of disease susceptibility occurs during this time (Langley-Evans, 2015). Factors such as timing of complementary feeding have also been investigated, showing a correlation between high energy and protein intake from complementary feeding at 2-12 months of age and a higher BMI in childhood (Pearce *et al.*, 2013).

Together, numerous retrospective and prospective epidemiological studies have indicated that disease risk in adult life is associated with both under- and overnutrition in pre- and early postnatal life. Problems with such studies include that characteristics such as birth weight is a crude proxy of nutritional status *in utero*, and some studies have found no association between maternal nutrition and foetal growth (Langley-Evans and Langley-Evans, 2003; Mathews *et al.*, 1999). Furthermore there is a danger of unadjusted confounding of retrospective epidemiological studies (Langley-Evans, 2015). The development of animal models has therefore proven valuable in confirming the associations between nutrition in foetal and early postnatal life and later programming of metabolic function that have been indicated in human studies.

2.1.3 Evidence from animal models

Following the findings in human epidemiological studies, researchers began using animal models to provide evidence of a causal link between malnutrition during critical periods of plasticity and later disease risk. In addition, studies of experimental animals have provided an important opportunity for gaining an understanding of the mechanisms underlying such an association.

A range of animal models have been developed using both small and large animals. Small animal models include rats, mice and rabbits while large animal models include e.g. sheep and pigs (Chavatte-Palmer *et al.*, 2016).

There are several different approaches to studying the effects of nutritional challenges on foetal development, including global nutrient restriction, restriction of specific nutrients (e.g. protein, iron, calcium and zinc), high-energy and high-fat diets, and cafeteria diets (McMullen and Mostyn, 2009). Both short- and long term effects of undernutrition, overnutrition and mismatching pre- and postnatal diets can be investigated as well as how the timing of the nutritional challenge affects the results (identification of critical periods). Furthermore, effects can be investigated at different levels, from systemic function, to organ- and tissue level, down to cellular levels (Chavatte-Palmer *et al.*, 2016).

Due to implications for human health, much research in the area of foetal programming has focused on development of metabolic and cardiovascular diseases. Studies of maternal protein restriction and global nutrient restriction during gestation have revealed ample evidence of associations between prenatal nutrient restriction and changes in metabolic function related to development of disease. For example, prenatal protein restriction and global nutrient restriction has been shown to induce hypertension and cardiovascular dysfunction in rat models (Brawley *et al.*, 2003; Langley and Jackson, 1994; Torrens *et al.*, 2006, 2009) and sheep models (Cleal *et al.*, 2007; Gopalakrishnan *et al.*, 2004), though Cleal *et al.* (2007) only found this effect when the sheep had been exposed to a mismatching postnatal diet. A study of protein restriction in rats furthermore suggested a relationship between hypertension and deterioration of renal function (Nwagwu *et al.*, 2000). Insulin sensitivity and glucose tolerance are likewise targets of poor foetal nutrition. Both general nutrient restriction and protein restriction *in utero* have resulted in insulin resistance, revealed via glucose tolerance tests in adult animals (Fernandez-Twinn *et al.*, 2005; Gardner *et al.*, 2005; Kongsted *et al.*, 2014) and via changes in expression of insulin-signalling molecules in muscle and adipose tissues (Fernandez-Twinn *et al.*, 2005; Ozanne *et al.*, 2003).

Intrauterine growth restriction has also been shown to have a programming effect on fat

distribution, food preference and intake as well as activity level in later life. Studies have revealed increased visceral adiposity in sheep (De Blasio *et al.*, 2007; Nielsen *et al.*, 2013) and rats (Bellinger *et al.*, 2006; Joss-Moore *et al.*, 2010). This was accompanied by increased appetite for fat (Bellinger *et al.*, 2004; Nielsen *et al.*, 2013), increased feeding activity (De Blasio *et al.*, 2007; Vickers *et al.*, 2000) and a more sedentary behaviour (Bellinger *et al.*, 2006; Vickers *et al.*, 2003). Furthermore, exposure to a mismatching, obesogenic diet in postnatal life was found to exacerbate several of these effects as well as induce obesity (Nielsen *et al.*, 2013; Vickers *et al.*, 2000, 2003). Remarkably similar programming effects of prenatal overnutrition have been found in animal models. Hypertension, cardiovascular dysfunction and altered vascular structure has been found in offspring of rats exposed to high-fat feeding during gestation (Armitage *et al.*, 2005; Khan *et al.*, 2003, 2005) and mice offspring of obese mothers (Samuelsson *et al.*, 2008). Development of insulin resistance and type 2 diabetes has also been found in rat (Taylor *et al.*, 2005) and mouse models (Gniuli *et al.*, 2008) following exposure to a high-fat diet in foetal life. In addition, many studies report that maternal high-energy diets and maternal obesity during gestation led to increased visceral adiposity in the offspring and increased the risk of obesity in adult life (e.g. Chen *et al.*, 2008; Howie *et al.*, 2009; Khanal *et al.*, 2014; Long *et al.*, 2010; Muhlhausler *et al.*, 2006; Samuelsson *et al.*, 2008).

Several of the mentioned studies also included a postnatal obesogenic diet in their experiments. The results of some studies indicated that exposure to a prenatal obesogenic environment provided an adaptive advantage for offspring that was subsequently exposed to a postnatal obesogenic diet (Khan *et al.*, 2005), while others did not find interactions between the pre- and postnatal diets (Howie *et al.*, 2009; Khanal *et al.*, 2014). Conversely, other studies found that adverse programming effects of prenatal overnutrition were exacerbated by postnatal overnutrition (Chen *et al.*, 2008; Gniuli *et al.*, 2008). These differences might be caused by the fact that the studies did not examine the same parameters. Thus there may be an adaptive advantage in relation to cardiovascular health as was found by Khan *et al.* (2005), while risk of obesity is exacerbated when combining pre- and postnatal overnutrition (Chen *et al.*, 2008; Gniuli *et al.*, 2008).

The above outlines a small sample of experimental studies of foetal programming in animal models. They serve to illustrate that such models have indeed provided evidence to support that foetal programming occurs as an effect of nutrition during periods of developmental plasticity, as was suggested in human epidemiological studies.

2.2 Potential mechanisms involved in foetal programming

Strong evidence has been provided by extensive human and animal studies, showing that malnutrition during pre- and early postnatal life has long-term effects on health and risk of developing diseases. Consequently, much research has focused on investigating underlying mechanisms. Induction of changes so early in life that only manifests as disease-associated phenotypes later in life implies that some form of stable alteration must occur (Salter *et al.*, 2009). Though the precise molecular mechanisms underlying the programming of disease by malnutrition *in utero* are still unknown, several potential mechanisms have been proposed, including alterations of organ structure, cell numbers, clonal selection and epigenetics (Koletzko *et al.*, 2012), which will be explained in the following.

2.2.1 Changed structure and growth of organs and tissues

The timing of exposure to a programming stimulus is critical for the outcome. The sensitivity to programming is greatest during periods of rapid growth and maturation (Langley-Evans, 2015). In precocial species such as humans and sheep, windows of developmental plasticity for organs such as the brain and cardiovascular system occur in early gestation, while for kidneys it occurs during mid-gestation. For adipose tissue and muscle, windows of developmental plasticity occur in late gestation (Symonds *et al.*, 2007). In rodents, which are altricial, the 'critical' window for adipose tissue development also occurs in the last part of gestation, but continues during early postnatal life, until weaning (Lukaszewski *et al.*, 2013).

Clonal selection: Development of organs and tissues occur in an ordered pattern of cell proliferation and differentiation. Clonal selection during early proliferation is a potential mechanism whereby variations in nutrient supply might affect the function of the organ (Waterland and Garza, 1999). Initial proliferation in each organ involves division of similar, though not necessarily identical, progenitor cells. During early proliferation, genetic and epigenetic modifications may occur in individual cells, which are passed on to the progeny of that cell, thus creating subpopulations of cells (clones) with slightly different characteristics. If challenged by e.g. nutritional deprivation of structural fatty acids, cell subpopulations with an increased capacity for lipogenesis might have a selective advantage and proliferate more effectively than other cells. Thus the organ might end up consisting largely of these cell lines, thereby programming the organ for increased lipogenic activity (Waterland and Garza, 1999).

Cell number: Growth of tissues and organs during development either happens through increasing cell numbers (hyperplasia) or by increasing cell size (hypertrophy). Thus lack of nutrients during

critical periods with high levels of hyperplasia may result in a permanent reduction in cell numbers of the affected organ or tissue (Waterland and Garza, 1999). For example, protein and iron restriction during gestation has been shown to reduce nephron numbers in rats, associated with later development of hypertension (Langley-Evans *et al.*, 1999; Swali *et al.*, 2011).
Organ structure: Variations in organ structure can be induced during organogenesis, and may result in e.g. changed organisation of different cell types as well as altered innervation and vascularisation within the organ (Waterland and Garza, 1999).

Thus, changes in nutrient status can result in remodelling of tissues and organs. Such remodelling may be facilitated by changes in gene expression, which affect tissue development and function. These changes in gene expression may in turn be mediated by epigenetic regulation (Langley-Evans, 2015).

2.2.2 Epigenetics

A potential mechanism for foetal programming, that has received much attention, is changes in gene expression caused by nutritionally induced changes of epigenetic regulation.
The term 'epigenetics' refers to heritable changes in gene expression, that occur through modifications other than changes in the DNA sequence (Wilkins, 2005). The major known epigenetic processes are DNA methylation, modification of histone organisation and non-coding RNAs (Lillycrop and Burdge, 2012).
Epigenetics play an important role during development, in regulating tissue-specific gene expression. While cells of an individual contain the same DNA, differentiation of cells into different tissues and organs leads to differential expression of genes, thus facilitating the very different structures and functions of organs (Ford and Long, 2012). Changes in epigenetic modifications of gene expression caused by environmental factors such as nutrition is a possible mechanism for foetal programming (Langley-Evans, 2015).

2.2.2.1 Epigenetic processes

DNA methylation: DNA methylation is a mechanism that inhibits expression of genes. When methylation occurs in mammalian cells it takes place on carbon 5 of the pyrimidine ring of a cytosine base positioned next to a guanine base, which is termed a CpG dinucleotide (Chmurzynska, 2010). Most CpG dinucleotides are clustered in so-called CpG islands at the promoter region of genes, and hypermethylation of a CpG island in a promoter region leads to repression of gene expression, by preventing binding of transcription factors, while

hypomethylation causes activation of transcription (Herrera *et al.*, 2011; Lillycrop and Burdge, 2012).

Histone modifications: DNA in the nucleus of eukaryotic cells is arranged as chromatin, which consist of nucleosomes. A nucleosome is 147 bp DNA wrapped around a histone octamer (two copies of each of the four histone proteins H2A, H2B, H3 and H4) (Lillycrop and Burdge, 2012), and this serves to compact and store the DNA string efficiently (Herrera *et al.*, 2011). Each of the histones has an amino-terminal tail, and modifications of these tails can work to either activate or repress gene expression or can be associated with DNA repair. Known modifications include acetylation, methylation, ubiquitination and phosphorylation. Such modifications can work by loosening or tightening the DNA from the histone complex, thus making the DNA more or less accessible for transcription, or by recruiting factors that are part of the transcription mechanism (Munshi *et al.*, 2009).

Non-coding RNAs: A class of small non-coding RNAs, the micro-RNAs (miRNA), have been shown to have a role in epigenetic gene expression. One mechanism is that the miRNAs bind to target mRNAs which leads to degradation of the mRNA or suppression of translation. Furthermore, miRNAs can induce chromatin remodelling, thereby regulating gene expression (Saidi *et al.*, 2017).

2.2.2.2 Evidence that early life nutrition can alter epigenetic regulation

While epigenetic mechanisms are a crucial part of proper cell differentiation and gene transcription control (Saidi *et al.*, 2017) there is growing evidence that environmental factors such as nutritional challenges can induce permanent changes in epigenetic regulation, and subsequent alterations of phenotype. For example differential feeding of female honeybee larvae produce either fertile queen bees or sterile worker bees, which has been shown to be driven by DNA methylation (Kucharski *et al.*, 2008). Likewise, the coat colour of the Agouti mouse reveal that nutritional programming is affected by DNA methylation. In this mouse the overexpression of a single locus (Avy), caused by hypomethylation of the locus, changes the regular black coat colour to yellow (Waterland and Jirtle, 2003). It has been demonstrated that feeding yellow mice a diet supplemented with methyl donors during pregnancy, led to increased methylation of the gene in the offspring. This resulted in offspring being born with an intermediate brown coat colour (Waterland and Jirtle, 2003).

Nutritional challenges, such as undernutrition, overnutrition or specific nutrient restriction, have been found to modify epigenetic regulation in many studies. Such studies have primarily been conducted on rodents, but evidence also exists from sheep, pigs and humans.

Studies of individuals exposed to the Dutch hunger winter and people living in rural Bangladesh

have revealed that *in utero* undernutrition can induce permanent changes in DNA methylation in humans (Finer *et al.*, 2016; Heijmans *et al.*, 2008; Tobi *et al.*, 2009).

Offspring of rats receiving protein restricted diets during gestation had changed expression of genes involved in carbohydrate and lipid metabolism compared to controls (e.g. Bertram *et al.*, 2009; Burdge *et al.*, 2004; Maloney *et al.*, 2003), which may have been facilitated through changes in epigenetic regulation. In line with this, several epigenetic modifications have been found in offspring exposed to protein restriction *in utero*, along with changed gene expression. Protein restriction throughout gestation (9% casein vs. 18% casein in controls) lead to hypomethylation of peroxisome proliferator-activated receptor-α (PPARα) and the glucocorticoid receptor (GcR) as well as increased expression of these genes in the livers of rat offspring (Lillycrop *et al.*, 2005, 2007, 2008). Furthermore, altered histone modifications have been found at the glucose transporter 4 (GLUT-4) and the CCAAT/enhancer-binding protein (C/EBPβ) promoter regions in skeletal muscle of female rat offspring, coinciding with increased expression of the genes, following *in utero* protein restriction (Zheng *et al.*, 2011, 2012). Reducing dietary the protein level with 50% in sows during gestation and lactation lead to increased expression of the myostatin gene in skeletal muscle of piglets at 28 days of age, associated with altered histone modifications and growth retardation (Jia *et al.*, 2016).

It should be noted, that some studies have found, that reduced expression of leptin in mouse adipose tissue (Jousse *et al.*, 2011) and the glucokinase gene in rat liver (Bogdarina *et al.*, 2004) was associated with hypomethylation of the gene promoter regions, which is not consistent with the knowledge that hypomethylation causes activation of transcription (Lillycrop and Burdge, 2012). This serves to illustrate that much is still not known about epigenetic mechanisms.

Studies of models using global nutrient restriction have likewise found epigenetic modifications associated with altered gene expression. Reducing feed intake to 50% of *ad libitum* intake from mid-gestation and onwards, including the lactation period resulted in altered histone modifications leading to repression of GLUT-4 expression in skeletal muscle in rats (Raychaudhuri *et al.*, 2008). Likewise, in a pig study, offspring of mothers fed 75% of requirements had reduced expression of GLUT-4 in muscle, in this case found to be associated with increased DNA methylation of the gene promoter (Wang *et al.*, 2016). Furthermore, a rat model showed that nutrient restriction in gestation followed by early postnatal overnutrition resulted in increased DNA methylation and reduced expression of the peroxisome proliferator–activated receptor-γ coactivator- 1α (PGC-1α) gene in muscle and liver (Xie *et al.*, 2015).

Overnutrition *in utero* has also been shown to induce epigenetic modifications. Prenatal exposure to a high-fat diet caused DNA methylation of the Pomc promoter and decreased expression of Pomc, a gene involved in control of food intake (Marco *et al.*, 2014). The study also found that the increased methylation was not removed by feeding a standard diet from weaning. In addition, histone modifications at the promoter regions of adiponectin (AdipoQ) and leptin were found to be consistent with reduced expression of AdipoQ and increased expression of leptin in mice exposed to a high-fat diet *in utero* (Masuyama and Hiramatsu, 2012).

These examples serve to illustrate that altered gene expression induced by nutritional challenges early in life may be associated with epigenetic changes, which can affect expression. The genes investigated in the studies above are genes that affect e.g. glucose and lipid metabolism, growth and food intake, and altered expression of such genes are known to be involved in development of metabolic diseases. It however, still remains to be elucidated what mechanism elicits such epigenetic changes.

While much is still not known about the mechanisms underlying the foetal programming of adult disease, the proposed mechanisms outlined here points to changes in gene expression as an important link. As outlined in the previous section 2.1, nutritional insults in foetal and early postnatal life are associated with an increased risk of developing metabolic diseases such as type 2 diabetes and cardiovascular diseases. The same types of diseases have also been associated with adipose tissue dysfunction (Hajer *et al.*, 2008), which makes it relevant to investigate adipose tissue as a potential target of foetal programming. Since the critical window of developmental plasticity for adipose tissue occurs in late gestation for species such as humans and sheep, and in late gestation and during the suckling period in rodents it follows that nutritional challenges in these periods may be especially relevant when investigating effects on adipose tissue development and function.

2.3 Adipose tissue

The previous sections have outlined that nutritional challenges during periods of developmental plasticity is associated with an increased risk of metabolic and cardiovascular disorders. This may be linked to altered development and functional capacity of organs and tissues. The purpose of the following section is to provide insight into normal adipose tissue development and function, as well as known effects of adipose tissue dysfunction.

Adipose tissue is comprised mainly of adipocytes (fat cells), and to a lesser extend other cell types, as well as connective tissue, vascular and nerve tissue (Louveau *et al.*, 2016).
Adipose tissue has great plasticity, and retains its capacity to expand throughout life. Growth of adipose tissue happens via two processes: hyperplasia (increase of cell number) and hypertrophy (increase of cell size). Hyperplasia, which is also referred to as adipogenesis, mainly, though not exclusively, occurs during foetal and early postnatal life. At later stages, the main function of hyperplasia is to renew adipose tissue, rather than to facilitate tissue growth. Hyperthrophy begins in foetal life, and after birth it becomes the main growth mechanism for adipose tissue (Bonnet *et al.*, 2010).
There can be distinguished between two different types of adipose tissue: brown and white. The main function of brown adipose tissue is thermogenesis, and is mainly found in newborns, disappearing shortly after birth, as well as in hibernating mammals (Chen *et al.*, 2014). In the following the term 'adipose tissue' will refer to white adipose tissue.

2.3.1 Adipose tissue development

The embryonic origin of adipocyte progenitors is only partially known, but appear to vary depending on both anatomical location of the adipose tissues (Chau *et al.*, 2014) and the adipocyte type (white or brown) (Chen *et al.*, 2014). Generally it is described that adipocytes derive from multipotent mesenchymal stem cells (MSC). MSCs can differentiate into several cell types including, adipocytes (adipose tissue), osteoblasts (bone tissue), chondrocytes (cartilage) and myocytes (muscle tissue) (Lowe *et al.*, 2011; Otto and Lane, 2005).

The adipogenic differentiation of MSCs can be divided into two major steps that each involve complex, coordinated processes of gene expression, collectively termed adipogenesis:
1. Commitment (stem cell → preadipocyte). In this step the multipotent MSCs commit towards the adipogenic lineage and lose the ability to differentiate into other lineages. Several factors are involved regulating the initiation of this commitment, including bone morphogenetic proteins

(BMPs), which are members of the transforming growth factor β (TGF-β) superfamily and which promote differentiation (Zamani and Brown, 2011). Wnt signalling on the other hand inhibits differentiation (Otto and Lane, 2005). Once committed to the adipogenic lineage the cells are termed preadipocytes.

2. Full differentiation (preadipocyte → adipocyte). In this step the preadipocytes develop into terminally differentiated, mature adipocytes. Adipocyte differentiation is initiated by a combined effect of the transcription factor families peroxisome proliferator-activated receptor (PPAR) and CCAAT/enhancer binding protein (C/EBP) (Chen *et al.*, 2014).

Activation of C/EBPβ and C/EBPδ in preadipocytes initiates mitotic clonal expansion as well as transcription of C/EBPα and PPARγ. C/EBPα and PPARγ induce expression of many genes that are expressed by terminally differentiated adipocytes, and are involved in e.g. lipolysis, lipogenesis and insulin sensitivity (Lowe *et al.*, 2011). Levels of PPARγ and C/EBPα is maintained in the differentiated adipocyte, as they exert positive feedback on each other (Sethi and Vidal-Puig, 2007), thus maintaining adipocyte specific gene expression.

As in the commitment step, the terminal adipocyte differentiation is also regulated by many factors. For example, tumor necrosis factor α (TNFα) and Wnt interact to block differentiation (Cawthorn *et al.*, 2007), and preadipocyte factor 1 (Pref-1) inhibits differentiation by regulating the expression of C/EBPβ and δ (Wang and Sul, 2009). Stimulation of adipogenesis can be initiated by hormonal stimulation with e.g. insulin, glucocorticoids, insulin-like growth factor 1 (IGF1) and growth hormone (Sethi and Vidal-Puig, 2007). The factors mentioned above are only a few of those controlling the steps of adipogenesis, and more are still being discovered.

Mature adipose tissue contains both preadipocytes and MSCs, thus enabling the tissue to maintain and renew itself. These precursor cells have been characterised based on their expression of different cluster of differentiation (CD) markers including CD34 and CD44 (Louveau *et al.*, 2016), and can also be recognised through the expression of Pref-1, which is present in preadipocytes, but disappears in mature adipocytes (Wang and Sul, 2009).

2.3.2 Adipose tissue function

Fat accumulation is the most efficient way of storing energy in mammals, and an important role of adipose tissue is thus to store fat in the form of neutral triglycerides during times of surplus energy as well as to release fatty acids (FA) when energy balance is negative (Klaus, 2004) and provide insulation and mechanical support (Sethi and Vidal-Puig, 2007).

However, adipose tissue is not simply a passive storage site for excess energy. The discovery that

leptin is secreted from adipose tissue (Zhang *et al.*, 1994) initiated a further search for other adipose-derived secreted factors, and since then, numerous of such factors have been identified. This has revealed adipose tissue to be a highly complex endocrine organ, with many secreted hormones, cytokines and other peptides, collectively termed adipokines, that are actively involved in the control of physiological processes with both endocrine, paracrine and autocrine effects. These effects include regulation of metabolism, inflammatory response, angiogenesis (formation of blood vessels), appetite and reproductive function (Desai and Ross, 2011; Sethi and Vidal-Puig, 2007).

The adipokines that have been most extensively studied are leptin and adiponectin.
Leptin is mainly produced by adipocytes, and the level of leptin that is expressed and secreted correlate with total body fat mass. It primarily acts by inhibiting appetite in the hypothalamus, thereby reducing feed intake and by increasing energy expenditure (Krug and Ehrhart-Bornstein, 2005), more specifically lipolysis in adipocytes and muscle cells and lipid oxidation in the liver (Hajer *et al.*, 2008). Leptin receptors (LEPR) are expressed in the hypothalamus and in many peripheral tissues, including kidneys, liver, spleen, lung and reproductive tissues as well as in adipocytes themselves (Harris, 2014). As reviewed by Harris (2014) the expression of leptin receptors by adipocytes creates the opportunity for leptin to act directly on the cells to affect their metabolism, but evidence suggest that such direct effects only make a small contribution to inhibition of lipid accumulation and promotion of lipid mobilisation. Leptin-mediated control of adipose metabolism happens, to a greater extend, via the sympathetic nervous system (Harris, 2014).
Adiponectin promotes insulin sensitivity and has anti-inflammatory effects and, as opposed to leptin, adiponectin levels decrease with increasing fat mass. It is predominantly produced by adipocytes and its biological activity is mediated through receptors, which are primarily found in liver and muscle tissue. The insulin sensitising effect of adiponectin happens through several mechanisms, including increased FA oxidation in liver and muscle, enhanced glucose uptake in muscle and decreased gluconeogenesis in the liver (Fantuzzi, 2014; Hajer *et al.*, 2008).

2.3.2.1 Adipose tissue metabolism

Metabolic processes within the adipocytes include deposition of excess energy through the activity of lipogenic enzymes (lipogenesis) and mobilization of fat during energy shortage (lipolysis) to be released to other tissues where they can be oxidised (Sethi and Vidal-Puig, 2007).

Lipogenesis: Deposition of lipids in adipocytes happens either through uptake of fatty acids from plasma or through de novo synthesis of fatty acids. Subsequent esterification of the fatty acids with glycerol creates triglycerides (TG) which can be stored in the adipocyte (Bonnet *et al.*, 2010). PPARγ activates the lipogenic transcription factor sterol regulatory element binding protein (SREBP1), which in turn induces the expression of the enzymes lipoprotein lipase (LPL), an extracellular lipase, and fatty acid synthase (FAS), an intracellular lipogenic enzyme (Desai and Ross, 2011). When lipids are transported in plasma it is generally as part of large lipoprotein-complexes. Uptake of lipids from plasma into adipocytes is facilitated by LPL, which hydrolyses the TGs in chylomicrons and very low-density lipoproteins (VLDLs).
De novo synthesis is catalysed by FAS (Desai and Ross, 2011). Fatty acids are synthesised mainly from acetate and lactate in ruminants and from glucose in non-ruminants (Drackley, 2000), taken up via glucose transporters such as GLUT-4 (insulin-dependent) and GLUT-1 (insulin-independent).

Lipolysis: In lipolysis TGs are broken down to free fatty acids (FFA) and glycerol, and this primarily takes place at two sites: the adipose tissue and the liver. The relative contribution of each of these tissues depends on the species. In ruminants and pigs the adipose tissue is the major site, while in humans it is the liver (Chen *et al.*, 2014).
Lipolysis is mediated through the activities of lipases, by removing fatty acids one by one from TGs by hydrolysis, first yielding diacylglycerol (DG), then monoacylglycerol (MG) and lastly glycerol and a total of three FFAs. Adipose triglyceride lipase (ATGL) predominately removes the first fatty acid from TGs, yielding a FFA and a DG molecule. Hormone sensitive lipase (HSL) hydrolyses both TGs, DGs and MGs, with highest hydrolytic activity towards DGs; and monoacylglocerol lipase catalyses the hydrolysis of MGs (Jaworski *et al.*, 2007).
The process of lipolysis is complex and tightly controlled. The lipid droplets in adipocytes are coated with proteins of the perilipin family. The most abundant protein on the surface of adipocyte lipid droplets is perilipin (PLIN) and it serves as a barrier, limiting the access of cytosolic lipases to the lipid droplet during basal metabolic conditions, thereby limiting lipolysis and promoting TG storage. During fasting states, plasma concentration of catecholamines (adrenalin and noradrenalin) increases and these in turn bind to β-adrenergic receptors (ADRβ) located in the cell membrane. This leads to the phosphorylation of HSL and PLIN, which allows HSL to gain access to the lipid droplet, and consequently an increase in lipolysis (Brasaemle, 2007). Increased levels of glucocorticoids during fasting also increase liposysis, while insulin inhibits lipolysis by causing dephosphorylation of HSL and down-regulation of ATGL (Jaworski *et al.*, 2007).

ATGL is activated by comparative gene identification-58 (CGI-58) (Lass *et al.*, 2006) and appears to play a greater role than HSL in basal lipolysis. It associates with CGI-58 which binds to PLIN, thus providing access for ATGL to the lipid droplet (Brasaemle, 2007).

2.3.2.2 Inflammatory response

Among the factors secreted by adipocytes and adipose tissue macrophages are pro-inflammatory cytokines such as TNFα and interleukin-6 (IL6). Adipocytes also secrete the pro-inflammatory chemokine, monocyte chemotactic protein-1 (MCP-1) which attract macrophages. These secreted factors all exert an insulin-desensitising effect (Guerre-Millo, 2004; Trayhurn *et al.*, 2011) and TNFα furthermore affects adipocytes to increase lipolysis and decrease lipogenesis (Sethi and Hotamisligil, 1999) and both TNFα and IL6 inhibit adiponectin expression (Guerre-Millo, 2004). Free fatty acids can activate the pattern recognition receptor toll-like receptor 4 (TLR-4), resulting in induction of pro-inflammatory pathways in adipocytes and macrophages, leading to increased expression of inflammatory genes. Activation of TLR-4 can also lead to phosphorylation, and thereby inhibition, of insulin receptor substrate (IRS), an important molecule involved in intracellular insulin signalling (Toubal *et al.*, 2013).

2.3.3 Different adipose tissue depots

Adipose tissue appears in several different anatomical sites with distinct metabolic features (Sethi and Vidal-Puig, 2007). The main depots are found under the skin (subcutaneous depots), and surrounding the inner organs (visceral depots), including perirenal (surrounding the kidneys), mesenteric (surrounding the intestines) and epicardial (located around the heart) depots. Furthermore there are intra- and intermuscular adipose tissue depots located within and between muscles respectively (Bjørndal *et al.*, 2011; Komolka *et al.*, 2014).

Visceral adipose tissue (VAT) is more metabolically active than subcutaneous adipose tissue (SAT) and has higher lipolytic activity, meaning that VAT contributes more to plasma FFA levels than SAT (Bjørndal *et al.*, 2011). A higher level of ADRβ in VAT contribute to the increased sensitivity to catecholamine-induced lipolysis, and furthermore VAT is less sensitive to the anti-lipolytic effect of insulin, and to ADRα_2-dependant inhibition of lipolysis (Ibrahim, 2010). Additionally, VAT has a higher basal and insulin-stimulated glucose uptake than SAT, and a greater glucose metabolism (Komolka *et al.*, 2014).

A further difference between VAT and SAT is that VAT contains more inflammatory and immune cells than SAT, and thus has a larger capacity for synthesising inflammatory mediators such as

TNFα, IL6 and MCP-1 (Ibrahim, 2010). While SAT drains into the systemic circulation abdominal VAT drains into the portal system, leading FFAs, adipokines and other secreted endocrine factors from the VAT directly to the liver (Bjørndal *et al.*, 2011).
SAT has a greater lipid storage capacity than VAT, and has greater avidity for FFA uptake. It therefore acts as a buffer during dietary intake of lipid, thereby protecting other tissues from lipotoxic effects (Bjørndal *et al.*, 2011). This also means that once the capacity for lipid storage has been reached in SAT, fat will begin to accumulate in other, less suited tissues (Ibrahim, 2010).

2.3.4 Dysfunctional adipose tissue and metabolic health

Two theories linking adipose tissue to the metabolic dysfunction observed in foetal programming studies, are those of limited adipose tissue expansion capacity and changed adipokine production (Sethi and Vidal-Puig, 2007; Virtue and Vidal-Puig, 2010).

According to the adipose tissue expandability hypothesis, there is a preset limit to the expansive capacity of adipose tissue in an individual. When this limit is reached, the adipose tissue is unable to store more lipids, and additional lipids will instead be directed to other organs and tissues. Since organs such as muscle, heart, liver and pancreas are not capable of storing large amounts of lipids, this may have lipotoxic effects, including insulin resistance and apoptosis (Virtue and Vidal-Puig, 2010). Foetal programming may affect adipose tissue expandability, as demonstrated by (Nielsen *et al.*, 2016). This study found that nutrient restriction in late gestation altered the morphology of subcutaneous adipose tissue in adolescent and adult sheep, with increased collagen infiltration and an increased amount of small adipocytes. It is suggested that these results indicate reduced expandability of the subcutaneous adipose tissue, leading to greater fat deposition in visceral adipose tissues and non-adipose tissues (Nielsen *et al.*, 2016). Since visceral obesity in particular has been linked to development of insulin resistance, increased risk of developing certain cancers and impaired glucose and lipid metabolism (Shuster *et al.*, 2012), reduced expansive capacity of subcutaneous adipose tissue cannot be considered beneficial.

Adipokines play an important endocrine role in regulating whole body metabolism, and dysfunctional adipose tissue is associated with altered adipokine production (Hajer et al., 2008). Increased secretion of leptin, TNFα and IL6 as well as decreased expression of adiponectin which causes increased release of FFAs from adipose tissue and is e.g. associated with enlarged (hypertrophic) adipocytes as seen in obesity, have a variety of adverse effects on other tissues (Harwood Jr., 2012). These tissues include the liver, skeletal muscle, pancreas, vasculature and the

hypothalamus. For example, it induces insulin resistance in liver and muscle tissue, which leads to decreased glucose uptake in muscle, and increased glucose production and release from the liver. Additional effects in the liver include decreased lipid oxidation and development of fatty liver. Chronically elevated FFA levels inhibit insulin secretion from the pancreatic β-cells. Effects on the hypothalamus causes increased food intake and reduced energy expenditure (Harwood Jr., 2012). Furthermore, inability of adipocytes to take up glucose, as shown in a model of GLUT-4-knockout mice, cause insulin resistance and glucose intolerance in other tissues (Herman and Kahn, 2006), indicating that sensing the plasma glucose level via uptake in adipocytes plays a role in adapting whole body glucose homeostasis (Sethi and Vidal-Puig, 2007).

To sum up, adipose tissue both functions as a storage for excess energy and as an endocrine organ. Adipocyte development and function rely on complex processes, that include many steps and regulatory factors. Changes in these processes may increase the risk of developing metabolic and cardiovascular disorders. Fat accumulation in visceral adipose tissues rather than subcutaneous adipose tissue, altered adipokine production and increased lipolytic activity in adipocytes are factors that may increase the risk of systemic insulin resistance, type 2 diabetes and cardiovascular disorders.

2.4 Adipose tissue as a target for foetal programming

As outlined in the previous sections, adipose tissue dysfunction may increase the risk of developing metabolic and cardiovascular disorders. Alterations in adipose tissue development and function caused by nutritional challenges during critical windows of developmental plasticity may thus be involved in developing metabolic disorders later in life.

This section will therefore focus on studies investigating effects of pre- and early postnatal malnutrition, both in the form of nutrient restriction and excess nutrition, on adipose tissue development and function and associated adaptations of whole body metabolism.

Studies of late gestation malnutrition have been chosen in the case of sheep models, as this represents the period of developmental plasticity of adipose tissue in this species (Symonds *et al.*, 2007). In the case of rodent studies, most experimental designs include malnutrition throughout gestation and in some cases lactation. Additionally, studies of long-term effects have been prioritised when available, since metabolic and cardiovascular disorders often do not occur until adult age.

Nielsen *et al.*, (2013) found in their model of late gestation undernutrition (last 6 weeks of gestation) that offspring subjected to nutrient restriction in foetal life had reduced deposition of subcutaneous adipose tissue and, if subjected to an early postnatal (3 days - 6 months of age) obesogenic diet, were more disposed to develop visceral obesity. This was found to be associated with a changed morphology of the subcutaneous adipose tissue, with an increased occurrence of small adipocytes (diameter <40 µm) and collagen infiltration at 6 months of age (Nielsen *et al.*, 2016). The model was later expanded to include not only late gestation undernutrition, but also overnutrition, combined with early postnatal overnutrition (Khanal *et al.*, 2014). This model revealed that the decreased expandability of subcutaneous adipose tissue observed in the previous study was seen as an effect of both prenatal under- and overnutrition in 6 month old offspring. When combined with exposure to a postnatal obesogenic diet this predisposed for development of visceral adiposity. The postnatal obesogenic diet however, besides causing increased fat deposition in all of the adipose tissues, led to greater deposition of perirenal fat relative to the mesenteric and subcutaneous areas (Khanal *et al.*, 2014). This was accompanied by reduced kidney weight, something that was also observed in the previous study though it was found to disappear in adulthood following dietary correction (Khanal *et al.*, 2016; Nielsen *et al.*, 2013). Returning to the sheep at 2-2½ years of age, after 1.5-2 years of dietary correction, the previously observed reduced expandability of subcutaneous fat (Khanal *et al.*, 2014; Nielsen *et al.*, 2013) was no longer evident

(Khanal *et al.*, 2016; Nielsen *et al.*, 2013). However, the deviating morphology with increased occurrence of small adipocytes and collagen infiltration in prenatally undernourished sheep was still observable at 2 years of age (Nielsen *et al.*, 2016). Alterations in expression of genes related to metabolism found in three different adipose tissues (perirenal, mesenteric and subcutaneous) of adolescent lambs subjected to dietary challenges did not persist into adulthood, but a changed fatty acid composition in perirenal adipose tissue was apparent both in adolescent and adult animals subjected to prenatal nutrient restriction (Nielsen *et al.*, 2016). Furthermore, one of the studies found that, in contrast to the findings in adolescent lambs, those animals that had been exposed to early life obesity lacked any expansion of perirenal adipose tissue from adolescence into adulthood. This led to an altered distribution of visceral fat, with more fat deposited in the mesenteric adipose tissue depot compared to control animals (Khanal *et al.*, 2016). This was in stark contrast to the increased deposition in perirenal adipose tissue seen at six months of age (Khanal *et al.*, 2014).

A predisposition for development of visceral adiposity has also been found in studies of rats. Results from a model of intrauterine growth restriction, induced by uteroplacental insufficiency, showed that the amount of visceral (retroperitoneal) relative to subcutaneous adipose tissue was increased in adolescent male rats, but not in females. This was accompanied by increased expression of the pro-adipogenic transcription factor PPARγ mRNA and protein in male visceral adipose tissue and decreased expression of GLUT-4 mRNA in subcutaneouse adipose tissue in both males and females (Joss-Moore *et al.*, 2010). The increase in PPARγ occurred prior to the increase in adiposity, which made the authors suggest that dysregulated expression of this gene predisposes for visceral obesity. Contrary to that finding, another study found that reduced protein intake during gestation decreased the expression of PPARγ in abdominal adipose tissue of 34 day old rat offspring. Additionally, plasma concentrations of triglycerides and non-esterified fatty acids (NEFA) were increased (Burdge *et al.*, 2004), which might be related to the decreased expression of PPARγ, as it apart from inducing maturation of pre-adipocytes, also induces transcription of genes involved in lipogenic pathways (Kersten *et al.*, 2000).

A study of male rats subjected to low protein nutrition *in utero* followed by catch-up growth during lactation found that they had higher epidydimal fat mass than controls at three weeks of age. They also showed hypertrophic growth of the adipose tissue both at three weeks and at three months of age (Berends *et al.*, 2013). In another study of adult rats, male offspring of mothers that were protein restricted throughout gestation and lactation had increased visceral (mesenteric, omental and retroperitoneal combined) fat mass and a higher ratio of visceral adipose tissue to body weight

(Guan *et al.*, 2005). They found that this coincided with increased mRNA expression of a range of genes involved in adipogenesis and angiogenesis as well as lipogenesis. This suggested that the increased growth of visceral adipose tissue was mediated through hyperplasic growth.
Gnanalingham *et al.* (2005) found no effect of gestational nutrient restriction of sheep on adiposity, but reported that nutrient restriction during late gestation (day 110-147) decreased glucocorticoid action and abundance of uncoupling protein-2 (UCP-2) mRNA in perirenal adipose tissue in offspring at one month of age. Specifically, expression of the glucocorticoid receptor (GcR), 11β-hydroxysteroid dehydrogenase (11βHSD)-1 (a glucocorticoid activating enzyme) and UCP-2 decreased, while expression of 11βHSD2 (a glucocorticoid inhibiting enzyme) increased in perirenal adipose tissue. Since glucocorticoids promote maturation of preadipocytes (Peckett *et al.*, 2011), the altered expression of GcR, 11βHSD1 and 11βHSD2 might lead to reduced adipogenesis.
Nielsen *et al.* (2016) found no effect of late gestation undernutrition on UCP-2 expression in perirenal adipose tissue, but found a decreased expression of the gene in subcutaneous adipose tissue in six month old lambs. In two year old adult sheep, however, this was no longer evident. Meanwhile, a greater abundance of UCP-2 was found in perirenal adipose tissue in another study of late gestation undernutrition when examining offspring lambs at 30 days of age (Mostyn *et al.*, 2003) and at one year of age (Sharkey *et al.*, 2009). UCP-2 is a mitochondrial protein that has been linked to obesity and decreased insulin signaling in adipose tissue (De Souza *et al.*, 2007). Therefore, increased expression in foetally undernourished individuals might be expected to indicate adverse effects. However, despite the fact that all of the studies investigated nutrient restriction in late gestation they showed varying results concerning the expression of UCP-2. The results of Nielsen *et al.* (2016) showed that gene expression in the three investigated adipose tissue depots (perirenal, mesenteric and subcutaneous) changed with ageing, as none of the altered gene expression patterns found in adolescent lambs subjected to dietary challenges persisted into adulthood, and others appeared in adult animals. Thus, the differences between the mentioned studies might partly be explained by sampling taking place at different ages. Variations in the exact composition of the feed provided in the different studies is another possible source of differences in outcomes.
Prenatal nutrient restriction has been shown to alter insulin signalling in visceral adipose tissue depots as well as glucose tolerance and insulin sensitivity. Gardner *et al.*, (2005) found no difference in glucose or insulin response to a glucose challenge in 1 year old sheep that had been nutrient restricted during early gestation nor in their expression of insulin signalling molecules in

perirenal adipose tissue. When nutrient restriction had occurred during late gestation (day 110-147) however, areas under the curve for glucose and insulin following a glucose challenge were greater than in control animals, indicating glucose intolerance and insulin resistance. Furthermore, the expression of the insulin dependent glucose transporter GLUT-4 in perirenal adipose tissue was reduced in late gestation undernourished offspring at 1 year of age. Additionally, in these sheep both absolute and visceral adipose tissue mass was increased, which was not the case in sheep subjected to nutrient restriction in early gestation.

A rat model of prenatal protein restriction combined with postnatal catch-up growth resulted in larger adipocytes and impaired expression of the insulin-signalling proteins IRS-1 and PI3K p110β in epididymal adipose tissue both at 3 weeks and 3 months of age (Berends *et al.*, 2013). The mRNA expression of the genes was however not affected, suggesting that the reduced expression of the proteins is caused by posttranscriptional mechanisms, such as by the actions of miRNAs.

Undernutrition in late gestation was found to be associated with altered expression of inflammatory genes in perirenal adipose tissue of sheep offspring (Sharkey *et al.*, 2009). Late gestation nutrient restriction caused decreased expression of CD68 at birth, while at 1 year of age expression of both CD68 and TLR-4 was increased compared to controls. In addition, expression of the chemokine receptor-2 (CCR2), whose main ligand is MCP-1, was significantly increased in this group. Furthermore, intrauterine growth restricted male rats had increased TNFα mRNA and protein abundance in subcutaneous adipose tissue, but not in visceral adipose tissue (Riddle *et al.*, 2014).

Increased visceral adiposity, as seen in studies of maternal nutrient restriction, have also been found in studies of maternal overnutrition: In a sheep study of the effect of maternal obesity throughout the entire gestation, an effect was observed on adiposity as well as glucose and insulin regulation in mature offspring at 19 months of age (Long *et al.*, 2010). At this age, the offspring of obese mothers had reduced glucose clearance and insulin sensitivity. Following a 12 week period of *ad libitum* feeding, the offspring of obese mothers increased body weight and percentage of body fat more than controls. Furthermore, the differences in glucose and insulin regulation were exacerbated.

In adult offspring of obese mice, increased abdominal adiposity was associated with altered mRNA expression and increased size of adipocytes (Samuelsson *et al.*, 2008). Expression of ADRβ2, ADRβ3 and 11βHSD1 was decreased, while PPARγ was increased at 3 months of age. Such changes in gene expression might make the adipocytes less responsive to catecholamines, and thereby reducing lipolytic activity (Peckett *et al.*, 2011) while increasing lipogenic and adipogenic activity leading to expansion of the tissue.

Chen *et al.*, (2008) included both prenatal and postnatal overnutrition in their rat model and investigated effects at weaning (20 days of age). They found that both challenges separately induced delayed glucose uptake following a glucose tolerance test. Offspring that were exposed to both pre- and postnatal overnutrition had the slowest glucose uptake, showing that the postnatal diet exacerbated the effect of the prenatal diet. Additionally, plasma insulin and triglyceride concentrations were increased by prenatal overnutrition, and both pre- and postnatal overnutrition caused decreased expression of GLUT-4 in muscle tissue and increased plasma leptin. Gene expression of TNFα and lipolytic genes ATGL and CPT-1 was tested in retroperitoneal adipose tissue. While prenatal nutrition did not have an effect on gene expression, postnatal overnutrition increased the expression of all three genes.

An extensive study was conducted by Borengasser *et al.*, (2013) of effects of maternal obesity on expression of genes regulating lipogenesis, insulin signaling, glucose transport and adipogenesis in retroperitoneal adipose tissue of 21 day old rat offspring. It revealed several alterations in gene expression, including increased expression of SREBP-1c, FAS, GLUT-4, PPARγ, C/EBPα and C/EBPβ. Additionally they found that maternal obesity decreased methylation of key adipogenic transcription factors C/EBPβ, PPARγ and zinc finger protein 423 (Zfp423). The results suggested that adipocyte commitment and differentiation is regulated by maternal obesity via epigenetic mechanisms (Borengasser *et al.*, 2013).

Decreased methylation of the promoter region of Zfp423 was also found in a recent study of offspring of obese mice (Liang *et al.*, 2016). The reduced methylation of Zfp423 in epididymal adipose tissue, as well as in progenitor cells extracted from the tissue at weaning was accompanied by increased expression of the gene, along with increased expression of adipogenic markers PPARγ and aP2. At this age, there were significantly more adipocytes in the tissue compared to the control group, indicating that maternal obesity enhanced adipogenic differentiation of progenitor cells at weaning. At three months of age, however, offspring exposed to maternal obesity and subsequently an obesogenic diet had reduced ratio of progenitor cells in the epididymal adipose tissue, and reduced expansive capacity. Adipocytes were larger and showed increased expression of inflammatory markers MCP-1, TNFα and IL6. Additionally this group had increased plasma concentrations of insulin and triglycerides, as well as a slower rate of glucose clearance following a glucose tolerance test. The authors suggested that the early increase in adipogenic activity led to fewer progenitor cells being available later in life, thus preventing further expansion of the tissue in a situation of excess energy. This led to inflammation of the tissue as well as metabolic dysfunction

(Liang *et al.*, 2016).
Another mouse model of maternal gestational obesity, focusing on effects on inflammation, showed increased mRNA expression of TNFα as well as the chemokines Ccl2 and Ccl7. In addition, they found reduced expression of the microRNA miR-706, known to regulate translation of inflammatory proteins (Alfaradhi *et al.*, 2016)
A study of *in utero* overnutrition in mice found increased expression of leptin and decreased expression of adiponectin in mesenteric adipose tissue at 2, 12 and 24 weeks to be associated with histone modifications at the promoters of the same genes (Masuyama and Hiramatsu, 2012). Furthermore, these changes persisted through several generations of normally fed offspring (Masuyama *et al.*, 2015), indicating that *in utero* overnutrition can lead to stable alterations of gene expression. As mentioned previously, in section 2.3.4, increased expression of leptin and inflammatory cytokines as well as decreased adiponectin may have negative effects on other tissues.

Maternal undernutrition during early-mid gestation (~day 28-80 of gestation), during the period of maximal placental growth has also been shown in some studies to influence adiposity of the offspring: Near-term foetuses of nutrient restricted mothers had more adipose tissue than controls (Bispham *et al.*, 2003, 2005) and adolescent lambs had increased perirenal adipocyte size at 6 months (Daniel *et al.*, 2007) as well as more backfat at 4 months and visceral fat at 9 months (Ford *et al.*, 2007) compared to control groups. In addition to increased adiposity, Ford *et al.*, (2007) also found that the adolescent offspring subjected to early gestation undernutrition had increased glucose concentrations and decreased insulin response following a glucose challenge test.
One possible explanation for adipose tissue being targeted by a nutritional insult that occurred outside of the period of adipose tissue formation and growth could be that altered placental growth and vascularisation due to malnutrition might reduce nutrient transfer capacity from the mother to the foetus for the remaining duration of gestation (Wu *et al.*, 2006). This could then create a situation of nutrient restriction in late gestation, during the window of developmental plasticity of adipose tissue.

Together these studies support the hypothesis that adipose tissue is a target of foetal programming. As reviewed in earlier sections increased visceral adiposity and altered developmental and metabolic function have been linked to adverse effects in other tissues, such as liver and pancreas and to development of metabolic disorders.
Many studies have found that both maternal under- and overnutrition lead to increased adiposity,

especially in the visceral area. However, the studies disagree on whether this is caused mainly by hypertrophic or hyperplasic growth, based on investigations of morphology and gene expression. Additionally, in some, but not all, of the studies effects were not evident unless the animals were subsequently challenged with an obesogenic diet. Such differences cause difficulties in drawing conclusions about effects of foetal programming of adipose tissue. There are several variations between studies, that may contribute to such differences. It is notable that most studies investigate only one adipose tissue depot, which is often the perirenal adipose tissue, particularly in sheep studies, and epidydimal or retroperitoneal in rodent studies. It is relevant to choose a visceral depot due to visceral adipose tissue being a known risk factor for development of metabolic disorders (Shuster *et al.*, 2012). However, studies that do investigate effects of foetal programming on more than one adipose tissue depot indicate that effects can vary drastically between different depots, also within the visceral area (e.g. Joss-Moore *et al.*, 2010; Khanal *et al.*, 2014, 2016, Nielsen *et al.*, 2013, 2016). This may contribute to the differences in results reported by different studies, and more knowledge is therefore needed on how different adipose tissue depots react to the same challenge.

Furthermore, variations in which genes are investigated and the nutritional treatments may also be contributing factors to the varying results. Additionally, many studies, especially those using rodent models, only include males, while some of the studies that include both genders do not include gender as a variable. There is, however, ample evidence that males and females react differently to foetal programming (Woods, 2004), and this may therefore also be a contributing factor to differences in results of different studies.

Finally, it needs to be considered that many of the studies examine offspring at different ages. Often, metabolic disease does not appear until adult age, but many studies do not continue beyond weaning or young adulthood. As the studies that examine animals at several ages have revealed that alterations occur with ageing (e.g. Nielsen *et al.*, 2013, 2016; Sharkey *et al.*, 2009), more studies of adult animals might provide a better indication of long-term effects of foetal programming.

The experimental study performed in relation to this thesis, which will be described in the following, is meant to add to the knowledge of long-term effects of foetal programming in adult (2½ years of age) animals and will also consider possible gender differences. Through investigation of gene expression in both prenatally under- and overnourished animals, combined with postnatal overnutrition, further insight will be gained as to whether these widely different nutritional challenges lead to the same or different developmental and metabolic adaptations in adipose tissue.

3 Materials and methods

The experimental work included in this Master's thesis is a part of a larger project. The laboratory analyses have been performed on adipose tissues derived from an experiment designed using the Copenhagen sheep model (Nielsen *et al.*, 2013). The experiment was conducted at the Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark, at the experimental facilities on the farm Rosenlund, Lynge, Denmark. All experimental procedures were approved by the National Committee on Animal Experimentation, Denmark. A detailed description of the experimental design, animals as well as feeding, care and management of the experimental animals used in this study can be found in Khanal *et al.*, (2014, 2016). The following paragraph provides a summary of the experiment (Khanal *et al.*, 2014, 2016).

3.1 Experimental animals and study design

The experiment was made as a 3x2 factorial design combining three prenatal and two postnatal nutrition exposures, and 36 twin-pregnant Texel sheep and their offspring were included. During the last six weeks of gestation (term = 147 days) the sheep were assigned to one of three diets: either NORM (N=9; fulfilling 100% of recommended daily allowances for digestible energy and protein), HIGH (N=13; 150% of recommended daily digestible energy and 110% of recommended daily protein), or LOW (N=14; 50% of recommended daily digestible energy and protein).
After lambing each pair of twin lambs were assigned to separate diets from the age of three days until six months of age. The allocation of the twin lambs was based firstly on gender and secondly on birth weight in order to achieve as even a distribution of sex and birth weight as possible between the groups. The two diets given to the lambs were a conventional diet (CONV; N=35; 16 males, 19 females) and a diet with a high content of carbohydrate and fat (HCHF; N=35; 18 males, 17 females). The CONV diet consisted of hay supplemented with a milk replacer during the first eight weeks, and the daily feeding allowances were adjusted weekly in order to achieve a constant, moderate weight gain of approximately 225 g/day. Lambs fed the HCHF diet had *ad libitum* access to a mixture of 50% milk replacer and 50% dairy cream (until a daily maximum of 2.5 l/day) as well as rolled maize (until a daily maximum of 1 kg/day).
At six months of age, around puberty, 26 lambs were slaughtered, while the remaining animals were kept until adulthood. At this point eight additional lambs (4 males, 4 females) at the same age (six months) from a commercial sheep farm were included in the experiment as external controls (EC). This yielded seven different groups of animals: NORM-CONV (N=6; 4 females, 2 males), NORM-HCHF (N=4; 2 females, 2 males), HIGH-CONV (N=6; 4 females, 2 males), HIGH-HCHF (N=6; 3

females, 3 males), LOW-CONV (N=8; 4 females, 4 males), LOW-HCHF (N=7; 3 females, 4 males) and EC (N=7; 4 females, 3 males). From six months until 2½ years of age all animals received the same diet of high-quality grass hay *ad libitum*, supplemented with rolled barley during the first months. At 2½ years of age the remaining 44 sheep were slaughtered. After slaughtering, samples of tissue were collected, including subcutaneous, mesenteric, perirenal and epicardial adipose tissues. Tissue samples intended to be used for studying gene expression were fixated in RNA*later* (RNA*later*® Solution, Ambion, The RNA Company, USA) immediately upon sampling and were stored at -80°C until analysis, while samples to be used for histological evaluations were transferred to 4% paraformaldehyde (PFA) solution for 24 hours followed by 2% PFA for one week until paraffin embedding.

RNA extraction, cDNA synthesis and quantitative real-time PCR was performed on the four collected adipose tissues, as described in the following. The writer personally performed all of the experimental procedures on the epicardial and the perirenal adipose tissues.

3.2 RNA-extraction

Total mRNA was extracted from the adipose tissue samples by homogenizing approximately 150 mg tissue (TissueLyser II, QIAGEN, Hilden, Germany) in 1000 µL TRIzol® reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). Phase separation was then performed on the resultant supernatant by the use of 120 µL chloroform, resulting in an upper aqueous phase, which was subsequently mixed with 500 µL isopropanol to facilitate precipitation of RNA. Further RNA extraction was performed using the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA). This was in accordance with the manufacturer's protocol with the exception that the amount of tissue used was larger, because of previous experience with low yield of RNA from adipose tissue, and furthermore only 50 µl nuclease-free water was used to elude the RNA instead of 100 µl. Details of the protocol followed can be seen in Appendix A. The concentration of isolated RNA was determined by measuring the absorbance on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Products, Wilmington, USA) (Appendix A).
Integrity of RNA was measured from samples of mesenteric and subcutaneous adipose tissue on an Agilent 2100 Bioanalyzer with an Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany) (the writer was not involved in this analysis).

3.3 cDNA-synthesis

The isolated RNA was used for synthesizing cDNA via reverse transcription. To create a total reaction volume of 25 µL, 16.3 µL RNA sample was added to 8.7 µL master mix.
The master mix used for the reverse transcription reaction consisted of 5 µl M-MLV 5 x Reaction Buffer (Promega), 1.3 µl dNTP Mix (Promega), 0.2 µl Random Primers (Promega), 0.4 µl Oligo(dT)15 Primer (Promega), 0.8 µl RNasin Ribonuclease Inhibitor (Promega), and 1 µl M-MLV reverse transcriptase (Promega). A protocol can be seen in Appendix B. All cDNA samples were then stored at -20°C until further analysis.

3.4 Quantitative Real-time PCR (qPCR)

The mRNA expression levels of target genes in the different adipose tissues were determined by qPCR, using LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany), LightCycler 480 Multiwell Plate 384, white (Roche Diagnostics GmbH), and LightCycler 480 instrument (Roche Diagnostics GmbH). The cDNA was pooled to make standard curves and calibrator. A six-point standard curve was made for each gene using serial dilutions of cDNA (1:4, 1:8, 1:16, 1:32, 1:64, 1:128) to determine the efficiency ($-10^{-1/(\text{slope of standard curve})}$) of each primer set (see Appendix C). Primer sets with efficiencies between 1.8 and 2.2 (which equals an increase between 80% and 120% of target nucleic acid in each amplification cycle) were used for analysis (Table 1).
Each qPCR reaction well had a total reaction volume of 10 µl, which consisted of 2 µl ten-times diluted cDNA, 5 µl 2 x SYBR Green I master mix (Roche Diagnostics GmbH), 1 µl 10 µM forward primer (TAG Copenhagen, Copenhagen, Denmark), 1 µl 10 µM reverse primer (TAG Copenhagen), and 1 µl nuclease-free water. Samples and negative controls (no cDNA template) were run in triplicates (see Appendix D). The amplification cycle program included denaturation (95°C for 10 sec), annealing (60°C for 10 sec) and elongation (72°C for 20 sec), which was repeated 45 times in each qPCR reaction. Melting curves of the PCR products were checked by LightCycler 480 instrument ver. 1.5 software (Roche Diagnostics GmbH) to ensure that a single product was obtained. Data were analyzed using the advanced relative quantification method provided by the LightCycler 480 instrument ver. 1.5 software (Appendix D).
The mRNA expression levels were determined for target genes involved in stimulation of lipogenesis (FAS) and lipolysis (FABP4, LPL, HSL, PPARα, ATGL, PLIN-1, CGI-58, ADRβ1), as well as inhibition of lipolysis (ADRα1). Expression of genes involved in glucose transport (GLUT-4 (insulin stimulated), GLUT-1 (basal)), glucose metabolism (FBPase) and insulin signaling (IRS1)

were also examined. Additionally, genes stimulating (TGF-β1, C/EBPβ, PPARγ, PGC-1α) and inhibiting (Wnt5α, Pref-1) adipogenic differentiation, and cell surface markers of adipose stem cells and preadipocytes (CD34, CD44) were analysed, as well as expression of adipokine genes (AdipoQ, Leptin), inflammatory (TNFα, MCP-1, TLR-4, CD68, IL6) and angiogenic (VEGF, VEGF-A) genes. Finally, mRNA expression levels were determined for cell surface receptors (GcR, IGF1R, LEPR) and genes involved in energy homeostasis (FTO) and controlling mitochondrial-derived reactive oxygen species (UCP-2).

β-actin (ACTB) was used as a reference gene to normalize the expression levels of target genes. The primer sequences used in this study derive from ovine or bovine cDNA sequences and have all been published previously. Primer sequences, their efficiencies and sources of previous publication are listed in Table 1.

Table 1. Primer sequences used for qPCR (F: Forward primer, R: Reverse primer) and their efficiencies and sources

Gene	Primer sequences	Efficiency	Source of primer sequence
ACTB	F: 5'-ACC CAG ATC ATG TTC GAG ACC TT-3' R: 5'-TCA CCG GAG TCC ATC ACG AT-3'	1.94	(Safayi *et al.*, 2010)
AdipoQ	F: 5'-ATC AAA CTC TGG AAC CTC CTA TCT AC-3' R: 5'-TTG CAT TGC AGG CTC AAG-3'	1.90	(Muhlhausler *et al.*, 2007)
ADRα1	F: 5'-ATC CAC ACC ATC TCC CTC AG-3' R: 5'-TCG TCT CTA AGC CCT ACC TCT G-3'	2.34	(Chan *et al.*, 2009)
ADRβ1	F: 5'-CGC TCA CCA ACC TCT TCA TC-3' R: 5'-CAC ACA GGG TCT CAA TGC TG-3'	1.94	(Chan *et al.*, 2009)
ATGL	F: 5'-CAC CAG CAT CCA GTT CAA CCT-3' R: 5'-CTG TAG CCC TGT TTG CAC ATC T-3'	2.02	(Ji *et al.*, 2012)
C/EBPβ	F: 5'-GAC AAG CAC AGC GAC GAG T-3' R: 5'-GTG CTG CGT CTC CAG GTG-3'	3.346 *	(González-Calvo *et al.*, 2015)
CD34	F: 5'-TGA CCT GAG AGA GAT GGG CA-3' R: 5'-CGA GGT GAC CAG TGC AAT CA-3'	2.00	(Ceccarelli *et al.*, 2015)
CD44	F: 5'-GAC CAT GGG GCA AAC ACA AC-3' R: 5'-TCT GCC CAC ACC TTC TCC TA-3'	2.03	(Ceccarelli *et al.*, 2015)
CD68	F: 5'-GTC CTG CTA CCA CCA CCA GT-3' R: 5'-GCT GGG AAC CAT TAC TCC AA-3'	1.92	(Khanal *et al.*, n.d.)
CGI-58	F: 5'-CAG TGA CGG AAT ACA TCT ACC ACT G-3' R: 5'-GCC AAT TCG CTG GAG CAT-3'	1.99	(Ahn *et al.*, 2014)
FABP4	F: 5'-CAT CTT GCT GAA AGC TGC AC-3' R: 5'-AGC CAC TTT CCT GGT AGC AA-3'	2.16	(Hosseini *et al.*, 2012)
FAS	F: 5'-CCC AGC TCA ACG AAA CCA-3' R: 5'-GAC GAG GTC AAC ACC CTT CC-3'	2.08	(Qiao *et al.*, 2007)
FBPase	F: 5'-CGG GAG ATC AAG TGA AGA AGC T-3' R: 5'-CAG GTT CGA CTA TGA TGG CAT GT-3'	2.02	(van Harten *et al.*, 2013)
FTO	F: 5'-ACA CAT GGC TTC CCT ACC TG-3' R: 5'-GAG GAT GCG AGA GAC TGG AG-3'	2.10	(Sébert *et al.*, 2010)
GAPDH	F: 5'-GTC GGA GTG AAC GGA TTT GG-3' R: 5'-AAC GAT GTC CAC TTT GCC AGT A-3'	2.18	(Safayi *et al.*, 2010)
GcR	F: 5'-ACT GCC CCA AGT GAA AAC AGA-3' R: 5'-ATG AAC AGA AAT GGC AGA CAT TTT ATT-3'	2.17	(Chan *et al.*, 2009)
GLUT-1	F: 5'-GCA GGA GAT GAA GGA GGA GAG C-3' R: 5'-GCA GCA CCA CGG AAA TGA G-3'	2.07	(Chan *et al.*, 2009)
GLUT-4	F: 5'-AGT ATG TGG CGG ATG CTA TGG G-3' R: 5'-CGG CGG AAG ACG GCT GAG-3'	1.94	(Chan *et al.*, 2009)

HSL	F: 5'-CTT TCG CAC CAG CCA CAA C-3' R: 5'-CTC GTC GCC CTC AAA GAA GA-3'	2.11	(Xu *et al.*, 2015)
IGF1R	F: 5'-GCC TTT TAC TCT GTA CCG AAT CG-3' R: 5'-GCG CTG CAG CCA AGC T-3'	1.96	(Wallace *et al.*, 2014)
IL6	F: 5'-TCA TCC TGA GAA GCC TTG AGA-3' R: 5'-TTT CTG ACC AGA GGA GGG AAT-3'	1.92	(Wang *et al.*, 2012)
IRS1	F: 5'-CAA GAC CAT CAG CTT CGT GA-3' R: 5'-GTC CAC CTG CAT CCA GAA CT-3'	1.84	(Hosseini *et al.*, 2012)
LEPR	F: 5'-TTT CCT GGA TGC TGT CAC CC-3' R: 5'-CGG TTT CCC TAC TCC TTC CG-3'	2.01	Accession no. NM_001009763.1
Leptin	F: 5'-GCT CCA CCC TCT CCT GAG TTT-3' R: 5'-ACT GGC GAG GAT CTG TTG GT-3'	1.94	(Wallace *et al.*, 2014)
LPL	F: 5'-CGA GTA TGC AGA AGC TCC AAG TC-3' R: 5'-CCT GGT GAA CGT GTG TAA AAC ATC-3'	1.84	(Wallace *et al.*, 2014)
MCP-1	F: 5'-GCT GTG ATT TTC AAG ACC ATC CT-3' R: 5'-GGC GTC CTG GAC CCA TTT-3'	1.95	(Khanal *et al.*, n.d.)
PLIN-1	F: 5'-AGG GTG TCA CTG ACA ACG TGG-3' R: 5'-GTT GTC GAT GTC CCG GAA TT-3'	1.94	(Sanz *et al.*, 2015)
PGC-1α	F: 5'-CCG AGA ATT CAT GGA GCA AT-3' R: 5'-GAT TGT GTG TGG GCC TTC TT-3'	1.86	(Struewing *et al.*, 2007)
PPARα	F: 5'-CGT GTG AAC ATG ACC TAG AAG-3' R: 5'-ACG AAG GGC GGA TTG TTG-3'	1.87	(Bispham *et al.*, 2005)
PPARγ	F: 5'-ACG GGA AAG ACG ACA GAC AAA TC-3' R: 5'-CAC GGA GCG AAA CTG ACA CC-3'	2.17	(Chan *et al.*, 2009)
Pref-1	F: 5'-CCA TGG GCA TCT CTT CCT CA-3' R: 5'-GCC GGC CTC CTT GGT GAA-3'	2.02	(Fahrenkrug *et al.*, 1999)
TGF-β1	F: 5'-AAG CGG AAG GGC ATC GA-3' R: 5'-CGA GCC GAA GTT TGG ACA AA-3'	2.26	(Haçariz *et al.*, 2009)
TLR-4	F: 5'-CTG AAT CTC TAC AAA ATC CC-3' R: 5'-CTT AAT TTC GCA TCT GGA TA-3'	2.03	(Khanal *et al.*, n.d.)
TNFα	F: 5'-CCA CCA ACC ATC ACC AAG GA-3' R: 5'-ACG TTG CGA AGT ATT CCG GT-3'	2.12	(Johnsen, 2014)
UCP-2	F: 5'-ATG ACA GAC GAC CTC CCT TG-3' R: 5'-GGG CAT GAA CCC TTT GTA GA-3'	1.90	(Yiallourides *et al.*, 2009)
VEGF	F: 5'-GGG CTG CTG TAA TGA CGA AAG-3' R: 5'-TGA GGT TTG ATC CGC ATA ATC TG-3'	1.81	(Safayi *et al.*, 2010)
VEGF-A	F: 5'-GCC TTG CCT TGC TGC TCT AC-3' R: 5'-GGT TTC TGC CCT CCT TCT GC-3'	1.98	(Khanal *et al.*, n.d.)
Wnt5a	F: 5'-TCT CCT TCG CCC AGG TTG TA-3' R: 5'-GGC TGT GCT CCT ATG ATA TAT ACT TCT G-3'	2.11	(Kiewisz *et al.*, 2011)

***** Efficiency of C/EBPβ was read as 2, which is why this primer was used. The results achieved with this primer could not be interpreted, due to insufficient quality.

3.5 Histology

The PFA-fixed tissue samples embedded in paraffin were used for tissue sectioning. Paraffin blocks with epicardial adipose tissue samples were cut and stained at the Department of Biomedical Sciences, University of Copenhagen. Sections of 5 µm were cut using a Thermo Fisher rotary microtome (Microm HM355S, Thermo Fisher Scientific, Walldorf, Germany) and mounted on SuperFrost™ glass slides. From each block, two consecutive sections were collected from one site in the block.

The slides were stained using the haematoxylin-eosin staining method. Tissue sections were immersed in Tissue-Clear Xylene Substitute for 10 min to deparaffinise them, and were following rehydrated with a decreasing gradient of ethanol solutions, from 99% to 70%. The sections were

then immersed in hematoxylin dye for 5 min, and thereafter washed under running tap water for 5 min. They were then immersed in eosin dye for 5 min followed by dehydration with an increasing gradient of ethanol solutions, from 70% to 99%, and finally mounted with PERTEX® and a cover glass.
The writer was not involved in the cutting and staining of the samples from the three remaining adipose tissues (subcutaneous, perirenal and mesenteric). These samples were stained using an iron-haematoxylin method, which resulted in a more marked colouring of the cell membranes compared to the haematoxylin-eosin staining method.

Subsequently, slides were scanned on a Zeiss Axio Scan.Z1 automated slide scanner, located at the Core Facility for Integrated Microscopy (CFIM), department of Biomedical Sciences, University of Copenhagen. Slides were scanned using a 5x magnification, as was required for subsequent analysis. Histological analysis was performed by the use of a protocol consisting of three individual apps, designed for the purpose (Visiopharm®, app ID 10113). The 'Membrane app' provided membrane-to-cell ratio, the 'Size app' classified the cells according to size, assigning each cell into one of nine size classes, and the 'Shape app' assigned the cells to one of nine classes based on the circularity of the cell.

3.6 Statistical analysis

All statistical analyses of qPCR data were performed using R Studio (R Core Team, 2017). Separate datasets were created, that either included or excluded the external control group. Only datasets without the external control group were analysed for this thesis.
Due to time constraints statistical analyses of histology data is not included in this thesis.

An initial model was created for each gene, in each adipose tissue including fixed effects of pre- and postnatal diets and gender as well as their interaction. Ewe bodyweight (bw), ewe body condition score (bcs), lamb birth weight, lamb body weight at 6 months and body weight at 2½ year were included as covariates to reduce variation. Additionally, random effects of ewe and lamb were included, as follows:

$$Y_i = \alpha(Prenatal\ diet \times Postnatal\ diet \times Gender_i) + \beta(Ewe\ bw_i) + \gamma(Ewe\ bcs_i) + \delta(Lamb\ birth\ weight_i) + \varepsilon(Lamb\ bw\ at\ 6\ months_i) + \epsilon(bw\ at\ 2½\ years_i) + A(Ewe_i) + B(Lamb_i) + e_i$$

where

Y_i is the relative gene expression

e_i are independent and normally distributed $N(0,\sigma^2)$

A_j are independent and normally distributed $N(0,\sigma_A^2)$

B_i are independent and normally distributed $N(0,\sigma_B^2)$

Due to missing adipose tissue samples *i* and *j* are different for the four tissues:
Epicardial: $i = 37$; $j = 29$. Perirenal: $i = 36$; $j = 28$. Mesenteric and subcutaneous: $i = 31$; $j = 25$.

The initial model was fitted as a mixed linear model in RStudio (the 'lme'-function) (Pinheiro *et al.*, 2017). Model assumptions of normally distributed observations and variance homogeneity were tested for each initial model. Homogeneity of variance was validated using residual plots (raw residuals plotted against predicted values). Normality of residuals was tested by means of a Shapiro-Wilks test (P-value > 0.05 indicates normal distribution) and verified by visual validation of a quantile-quantile plot. Data were log-transformed when necessary to achieve a normal distribution of residuals.

It was tested, whether it was possible to remove parameters from the model by stepwise comparison of the Akaike's Information Criterion (AIC) value for full and reduced models. The model with the smallest AIC value was selected as the final model, indicating the model that best described the data (overview of final models can be seen in Appendix E). An ANOVA analysis was then performed on the final model to reveal whether the parameters remaining in the model had a significant effect on expression of the gene.
Results were expressed as least square means ± standard error of mean (LSmeans ± SEM) using REML estimation. Pairwise comparison of LSmeans for individual treatments was performed using Tukey Honestly Significant Difference (HSD) test (Lenth, 2016). Log-transformed data were back-transformed before reporting. The level of significance was 5% ($P < 0.05$). Examples of R-script are given in Appendix F.

Due to time constraints, statistical analyses were only performed on 23 of the 37 examined genes (AdipoQ, ATGL, CD34, CD44, CGI-58, FABP4, FAS, FBPase, GcR, GLUT-1, GLUT-4, HSL, IGF1R, IL6, Leptin, LPL, MCP-1, PLIN-1, PPARγ, TGF-β1, TLR-4, VEGF and VEGF-A). For mesenteric adipose tissue data were not available for CD44, meaning that 22 genes were analysed for this tissue.

4 Results

In the following presentation of results, no significant effect was detected, unless specifically stated, of the nutritional history in late fetal or early postnatal life, gender, or their interactions for any of the analyzed genes. Note that P-values provided in the text relate to the ANOVA (Appendix E), while significant differences in the figures indicate significant differences between individual treatment groups.

4.1 Perirenal adipose tissue

Effects of prenatal nutrition: The mRNA expression was affected by prenatal nutrition for ten different genes, out of which expression was affected differently in males and females for five genes (Figure 1 Figure 2). The most prevalent pattern was that of increased expression in the LOW animals compared to NORM and HIGH. This occurred in genes related to factors involved in lipid metabolism in both sexes (CGI-58, P=0.0199; FABP4, P= 0.0064 (Figure 1a-b)) and in males only (HSL, P=0.0230 (Figure 2a)), glucose transport in both sexes (GLUT-1, P=0.0025 (Figure 1c)), adipose tissue development in both sexes (VEGF-A, P=0.0084 (Figure 1d)) and in males only (CD44, P=0.0197; TGF-β1, P=0.0001 (Figure 2c-d)) and in the glucagon receptor in males only (GcR, P=0.0139 (Figure 2b)).

The expression of IGF1R (P=0.0156; Figure 1e), which is involved in adipose tissue development, and of insulin dependent glucose transporter, GLUT-4 (males only, P=0.0204 (Figure 2e)) revealed a different pattern, with LOW animals showing higher expression than HIGH animals, but lower expression than NORM animals. In the five genes with varying expression in males and females, the females generally had lower expression levels than the males, and showed less variation between the dietary treatment groups. However a small decrease in expression was evident in LOW females compared to HIGH and NORM females (Figure 2).

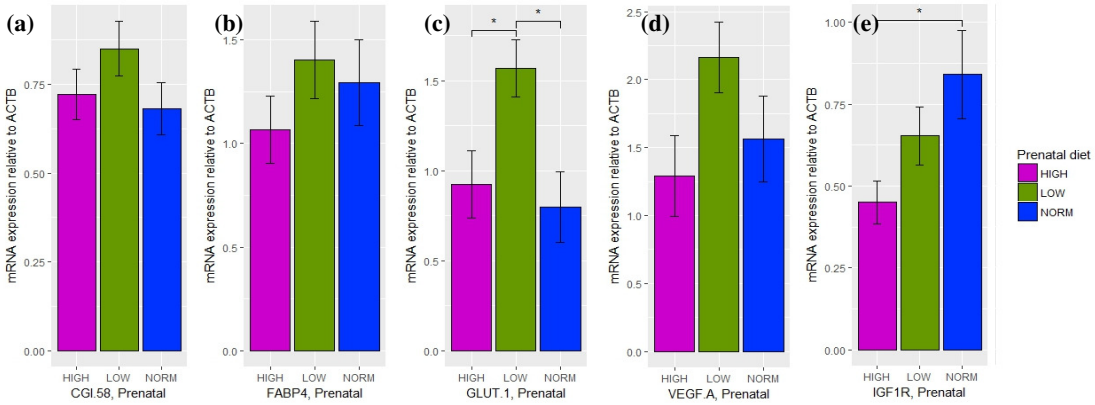


Figure 1. Influence of prenatal nutrition on gene expression in **perirenal adipose tissue**. (a) CGI-58, (b) FABP4, (c) GLUT-1, (d) VEGF-A, (e) IGF1R. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001). Number of animals in the treatment groups: HIGH (N=12); LOW (N=14); NORM (N=10)

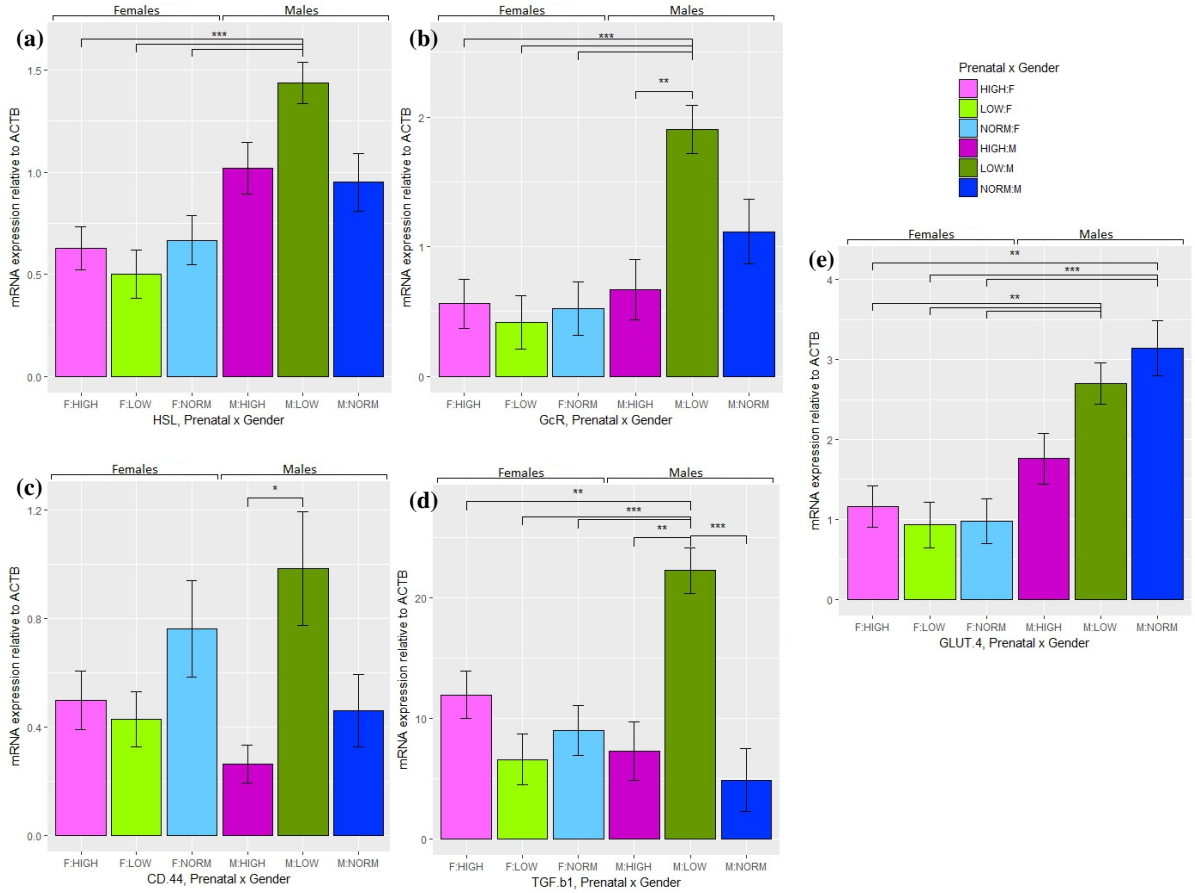


Figure 2. Influence of prenatal nutrition on gene expression in **perirenal adipose tissue** in females (F) and males (M). (a) HSL, (b) GcR, (c) CD44, (d) TGF-β1, (e) GLUT-4. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001). Number of animals in the treatment groups: HIGH,F (N=7); LOW,F (N=6); NORM,F (N=6); HIGH,M (N=5); LOW,M (N=8); NORM,M (N=4)

Effects of postnatal diet: The postnatal obesogenic HCHF diet affected expression of four genes involved in lipid metabolism (PLIN-1, P=0.0109; FABP4, P=0.0256), glucose transport (GLUT-4,

P= 0.0007) and adipose tissue development (VEGF, P=0.0298).

For both males and females, expression of PLIN-1 was increased in animals subjected to the HCHF diet compared to the CONV diet (Figure 3a). The same pattern was seen in females for FABP4, GLUT-4 and VEGF, while expression of these genes were reduced in males when they had been given the HCHF diet (Figure 3b-d).

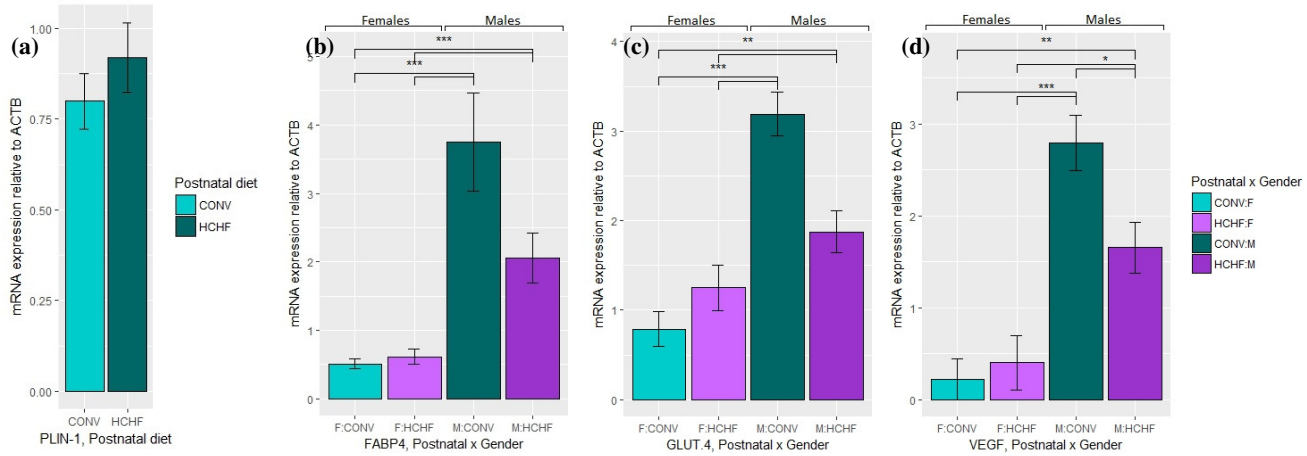


Figure 3. Influence of postnatal diet on gene expression in **perirenal adipose tissue** including significant differences between females (F) and males (M). (a) PLIN-1, (b) FABP4, (c) GLUT-4, (d) VEGF. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001). Number of animals in the treatment groups: CONV (N=20); HCHF (N=16); CONV,F (N=12); CONV,M (N=8); HCHF,F (N=7); HCHF,M (N=9).

Interactions between pre- and postnatal nutrition: For four genes (HSL, VEGF, IL6 and MCP-1) effects of prenatal nutrition varied depending on which postnatal diet the animals subsequently became exposed to, and for further two genes, (FAS and CD34) the pre- and postnatal nutrition interaction differed between males and females. Patterns of changes were not consistent in all genes and significant differences were only observed between certain individual groups, but some general trends could nevertheless be observed.

For four of the six genes, animals exposed to prenatal LOW nutrition responded differently to the postnatal HCHF diet than those exposed to NORM or HIGH nutrition.

For lipolytic gene HSL (P=0.0188 (Figure 4a)), when animals had been exposed to prenatal LOW nutrition, mRNA expression was decreased if they subsequently became exposed to the mismatching HCHF rather than CONV diet, whereas those exposed to prenatal HIGH and NORM nutrition had increased expression if they had received the postnatal HCHF rather than CONV diet in early postnatal life. For angiogenic marker VEGF (P=0.0184 (Figure 4b)), the observed pattern was completely opposite, with the HCHF diet causing increased expression in LOW sheep, and

decreased expression in HIGH and NORM sheep.

Interestingly, for the two genes FAS (lipogenesis, P= 0.0344 (Figure 5a)) and CD34 (stem cell marker, P=0.0478 (Figure 5b)), which were affected differently depending on gender, the reactions to a postnatal HCHF diet was completely opposite in males and females. The females showed the same pattern as was evident in HSL, while males showed the opposite pattern, similar to the one seen in VEGF. It should, however, be noted that conclusions based on the three-way interactions must be made with caution, due to the small group sizes ($2 \leq N \leq 4$).

The two genes involved in inflammation, IL6 (P=0.0456 (Figure 4c)) and MCP-1 (P=0.0051 (Figure 4d)), did not reveal the same type of long-term responses to the interaction of pre- and postnatal nutrition as the other genes. The most striking effect of expression of these genes were seen in NORM sheep, which had decreased expression when having been subjected to the postnatal HCHF diet, equivalent to the reaction seen in VEGF and FAS and CD34 in males.

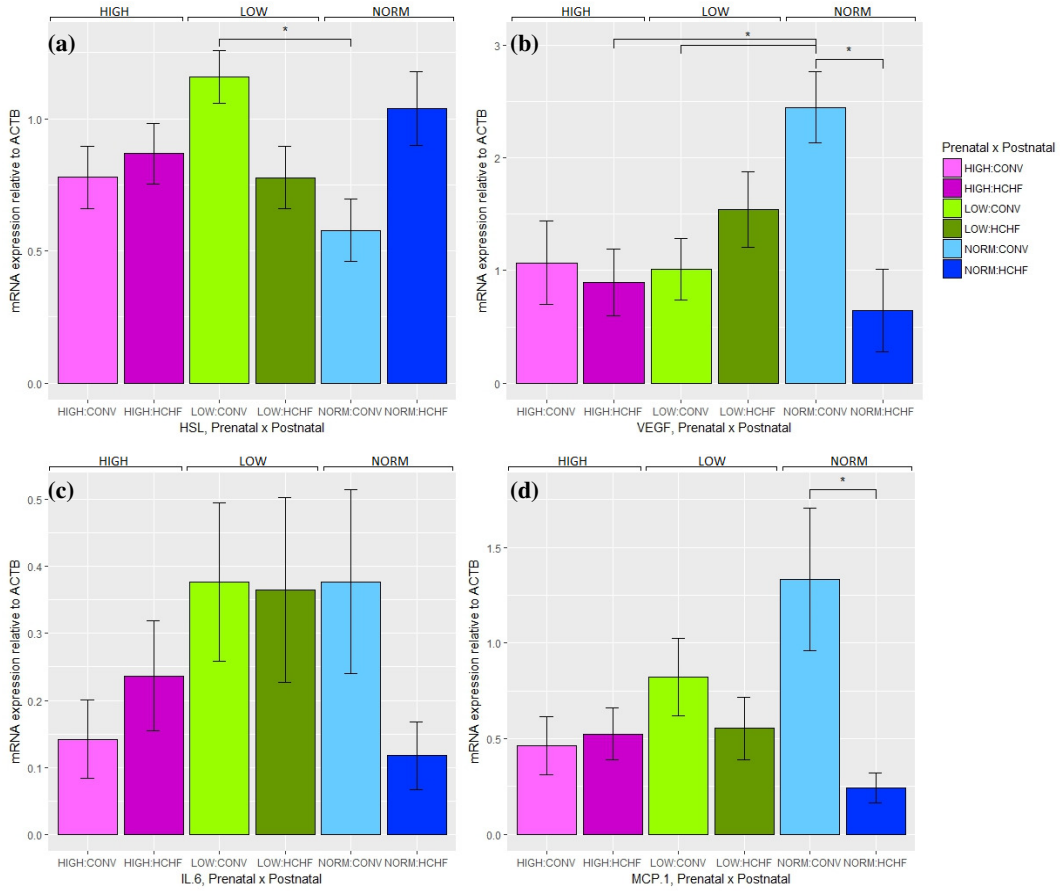


Figure 4. Influence of interaction between prenatal and postnatal nutrition on gene expression in **perirenal adipose tissue**. (a) HSL, (b) VEGF, (c) IL6, (d) MCP-1. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Number of animals in the treatment groups: HIGH,CONV (N=6); HIGH,HCHF (N=6); LOW,CONV (N=8); LOW,HCHF (N=6); NORM,CONV (N=6); NORM,HCHF (N=4)

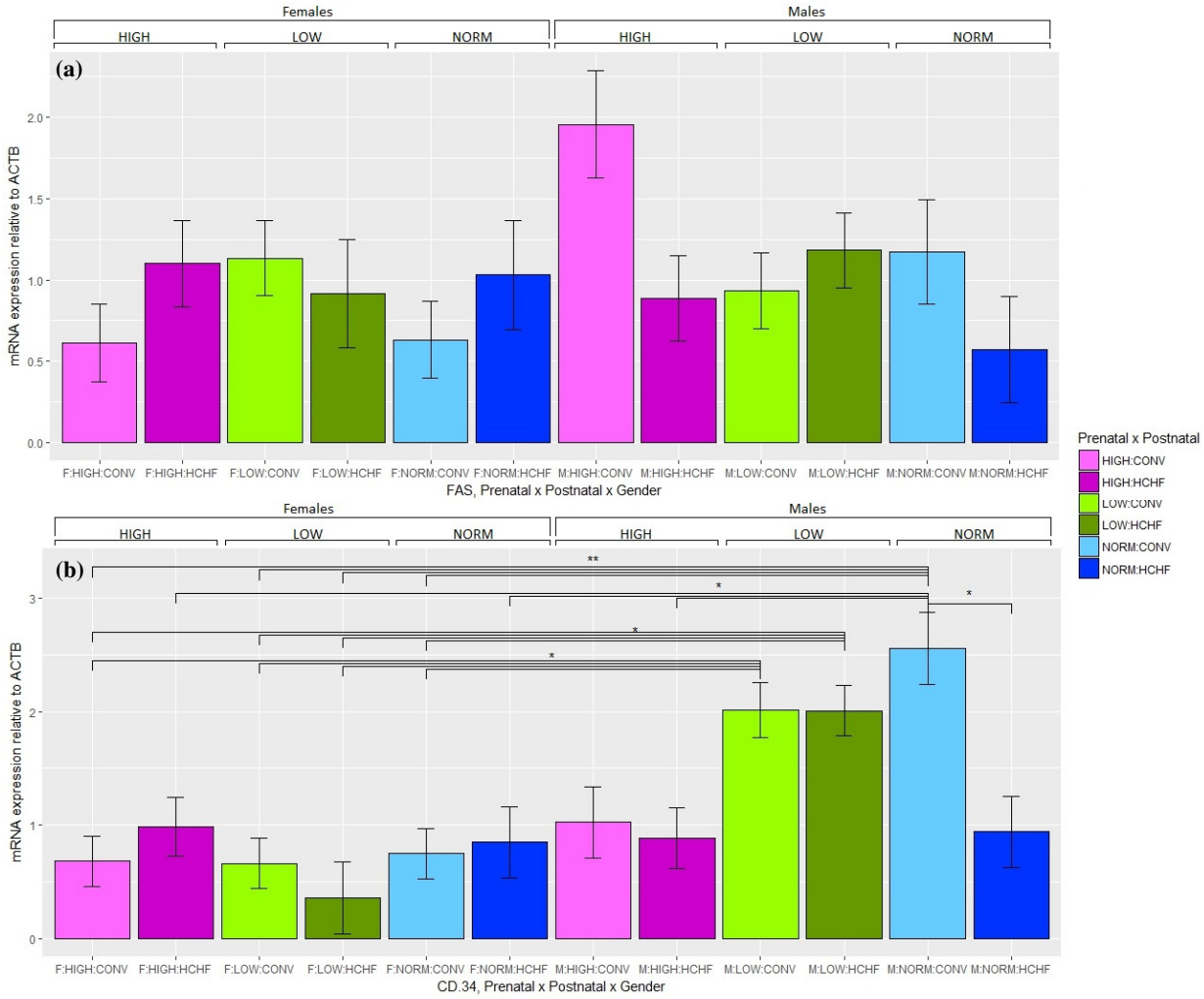


Figure 5. Influence of interaction between prenatal and postnatal nutrition on gene expression in **perirenal adipose tissue** in females (F) and males (M). (a) FAS, (b) CD34.
Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001).
Number of animals in the treatment groups: HIGH,CONV,F (N=4); HIGH,HCHF,F (N=3); LOW,CONV,F (N=4); LOW,HCHF,F (N=2); NORM,CONV,F (N=4); NORM,HCHF,F (N=2); HIGH,CONV,M (N=2); HIGH,HCHF,M (N=3); LOW,CONV,M (N=4); LOW,HCHF,M (N=4); NORM,CONV,M (N=2); NORM,HCHF,M (N=2)

Effects of gender: For the adipokine gene AdipoQ (P=0.0002), genes involved in lipid metabolism (ATGL, P=0,0064; CGI-58, P<0.0001; PLIN-1, P<0.0001), genes for glucose transport and metabolism (GLUT-1, P=0.0003; FBPase, P=0.0001) and adipose tissue development (IGF1R, P=0.0001; PPARγ, P<0.0001; VEGF-A, P<0.0001) (Figure 6b-j, respectively) and FABP4 (lipid metabolism), GLUT-4 (glucose transport) and VEGF (adipose tissue development) (Figure 3b-d, respectively) males had higher expression than females. Only the Leptin gene (P=0.0031) showed the opposite pattern, with lower expression in males compared to females (Figure 6a).

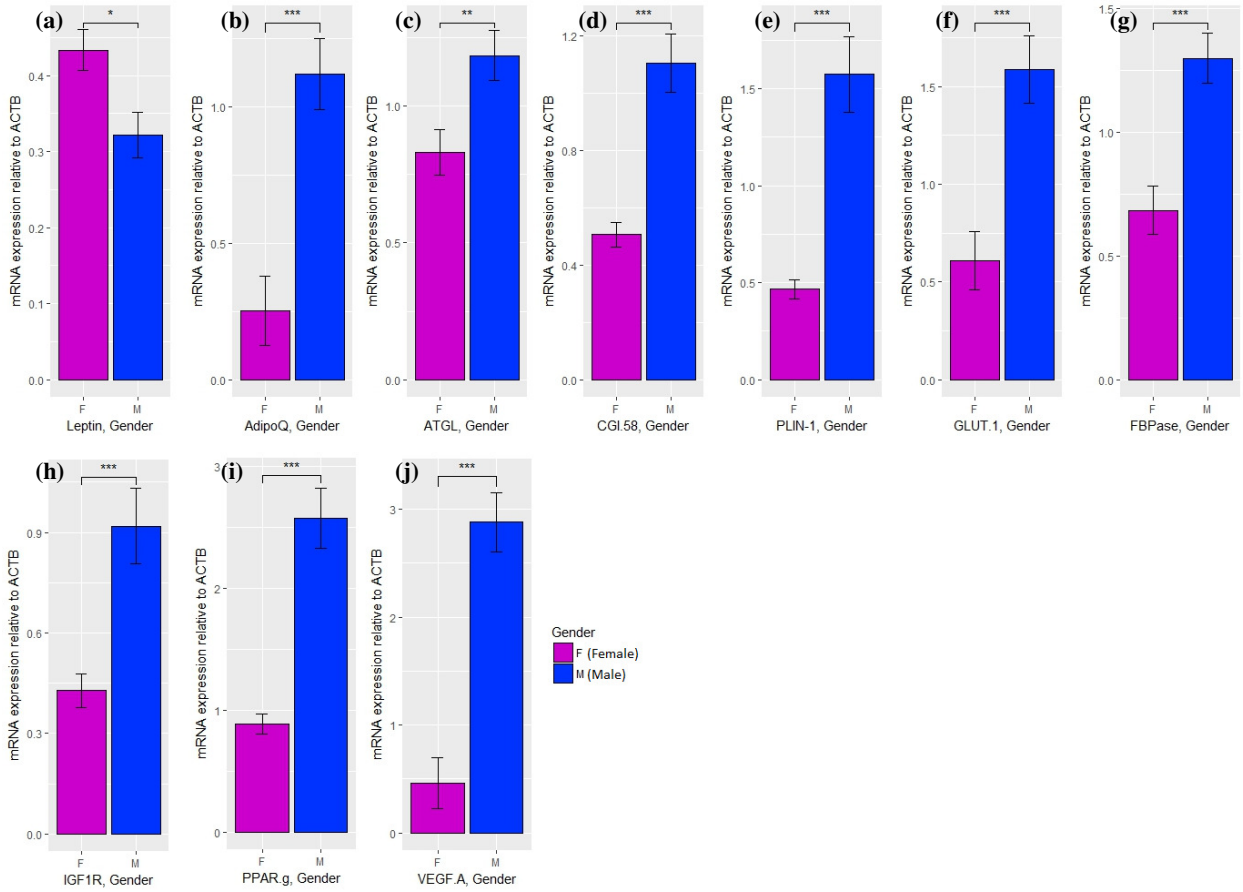


Figure 6. Influence of gender on gene expression in **perirenal adipose tissue**. (a) Leptin, (b) AdipoQ, (c) ATGL, (d) CGI-58, (e) PLIN-1, (f) GLUT-1, (g) FBPase, (h) IGF1R, (i) PPARγ, (j) VEGF-A. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Number of animals in the treatment groups: Females (F) (N=19); Males (M) (N=17)

4.2 Epicardial adipose tissue

Effects of prenatal nutrition: The insulin-dependent glucose transporter, GLUT-4, was the only gene that was similarly affected by prenatal nutrition in males and females (P=0.0163; Figure 7). Expression of this gene was reduced in LOW compared to NORM and HIGH animals.

A similar reduction in LOW animals was observed for genes involved in adipocyte development, namely IGF1R (P=0.0417), TGF-β1(P=0.0144) and VEGF (P=0.0295), but in males only. Surprisingly, females had completely opposite responses to males, and expressions of these three genes were increased in LOW compared to NORM and HIGH females (Figure 8a-c).

For the GcR (P=0.0168) expression, gene expression was low in all females and in LOW and HIGH males compared to NORM males (Figure 8d).

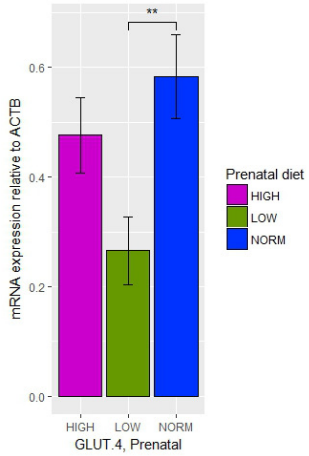


Figure 7. Influence of prenatal nutrition on gene expression in **epicardial adipose tissue**. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001).
Number of animals in the treatment groups: HIGH (N=12); LOW (N=15); NORM (N=10)

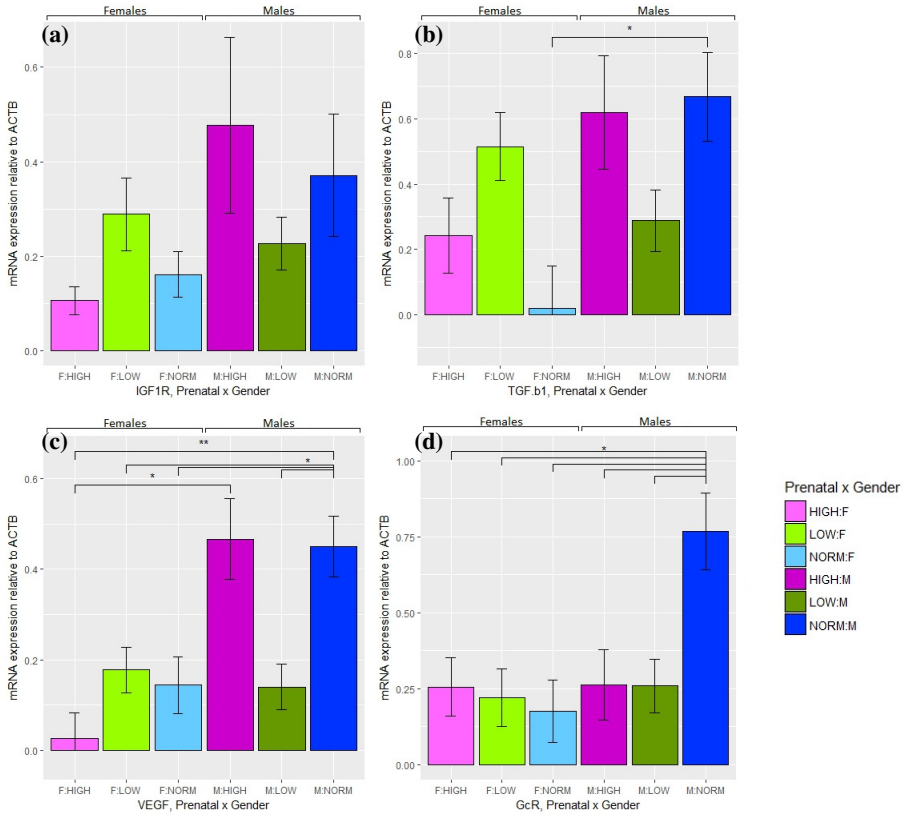


Figure 8. Influence of prenatal nutrition on gene expression in **epicardial adipose tissue** in females (F) and males (M). (a) IGF1R, (b) TGF-β1, (c) VEGF, (d) GcR. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001). Number of animals in the treatment groups: HIGH,F (N=7); LOW,F (N=7); NORM,F (N=6); HIGH,M (N=5); LOW,M (N=8); NORM,M (N=4)

Effects of postnatal diet: Sheep that had been subjected to the obesogenic HCHF diet in early postnatal life showed increased expression of genes related to lipid metabolism (FAS, P=0.0054; HSL, P=0.0021; PLIN-1, P=0.0454), and adipose tissue development (PPARγ, P=0.0222) and AdipoQ (P=0.0304) compared to CONV sheep (Figure 9a-e). The same trend was seen in females for the basal glucose transporter, GLUT-1 (Figure 9f).

However, the opposite was true for GLUT-1 and of the stem cell marker CD34 (P=0.0112 and P=0.0364, respectively, for the interaction between postnatal diet and gender), where expression levels were reduced to lower levels, but in males only, exposed to the postnatal HCHF compared to CONV diet (Figure 9f-g).

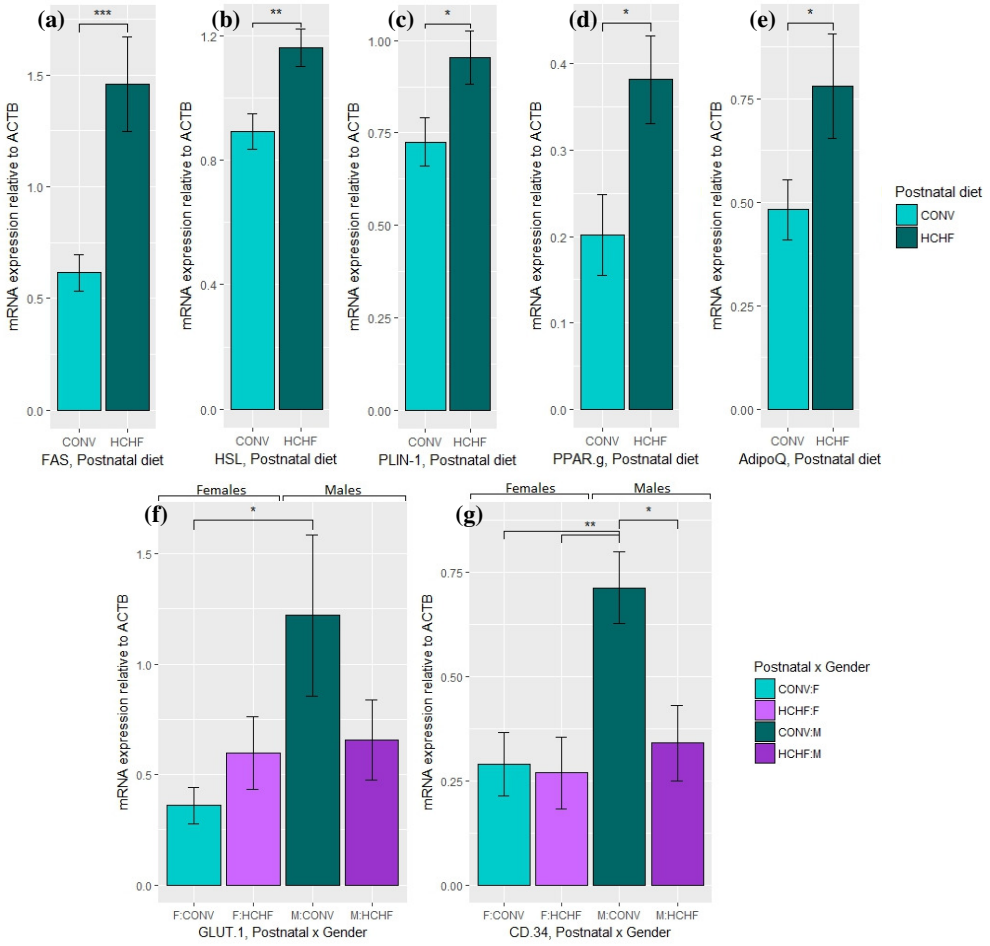


Figure 9. Influence of postnatal diet on gene expression in **epicardial adipose tissue** including significant differences between females (F) and males (M). (a) FAS, (b) HSL, (c) PLIN-1, (d) PPARγ, (e) AdipoQ, (f) GLUT-1, (g) CD34. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001). Number of animals in the treatment groups: CONV (N=20); HCHF (N=17); CONV,F (N=12); CONV,M (N=8); HCHF,F (N=8); HCHF,M (N=9)

Interactions between pre- and postnatal diet: Pre- and postnatal nutrition interactions were seen for two genes involved in basal glucose transport (GLUT-1) and inflammation (TLR-4), and for three genes involved in lipid metabolism (CGI-58, ATGL and FABP4) which were also differentially expressed depending on the gender. Patterns of changes were not always consistent and significant differences were only observed between certain individual groups, but a few general trends could nevertheless be observed.

When LOW sheep were exposed in early postnatal life to the mismatching HCHF diet, expression levels were depressed in adulthood for all genes (GLUT-1, P=0.0419; TLR-4, P=0.0245; ATGL, P= 0.0072; CGI-58, P=0.0121), except for CGI-58 in males (unaffected) and FABP4 (P=0.0044) which

was unaffected in females and increased in males.

Sheep exposed to HIGH prenatal nutrition had the opposite reaction to the HCHF diet compared to those exposed to LOW nutrition for GLUT-1 and TLR-4 (Figure 10). This was also seen in ATGL and FABP4 in males, as well as CGI-58 in females (Figure 11). The three-way interactions should however be interpreted with caution due to the small group sizes.

Gene expression levels were generally higher in LOW-CONV sheep compared to NORM-CONV sheep (Figure 10Figure 11), with the only exception being FABP4 in males (Figure 11c).

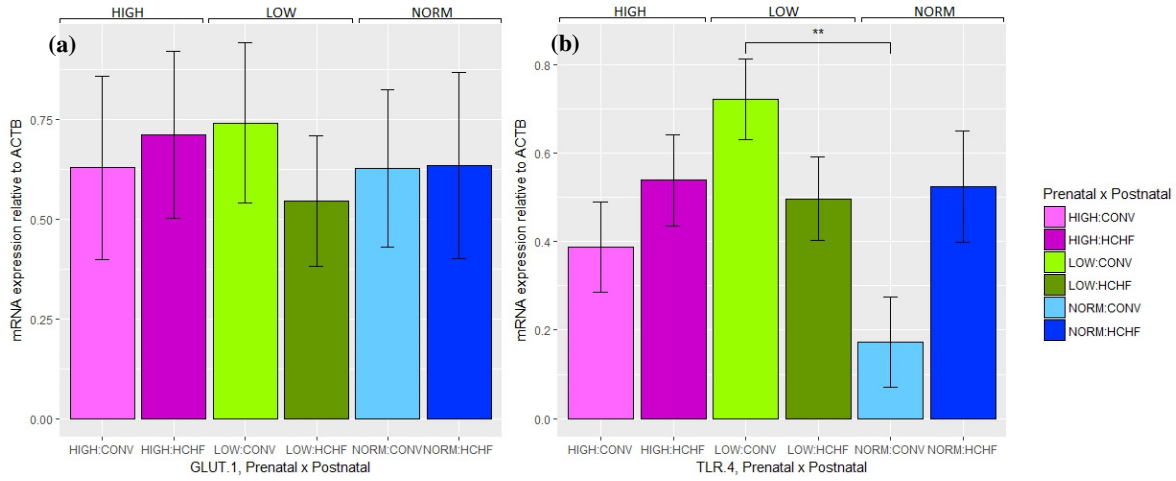


Figure 10. Influence of interaction between prenatal and postnatal nutrition on gene expression in **epicardial adipose tissue**. (a) GLUT-1, (b) TLR-4.

Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Number of animals in the treatment groups: HIGH,CONV (N=6); HIGH,HCHF (N=6); LOW,CONV (N=8); LOW,HCHF (N=7); NORM,CONV (N=6); NORM,HCHF (N=4)

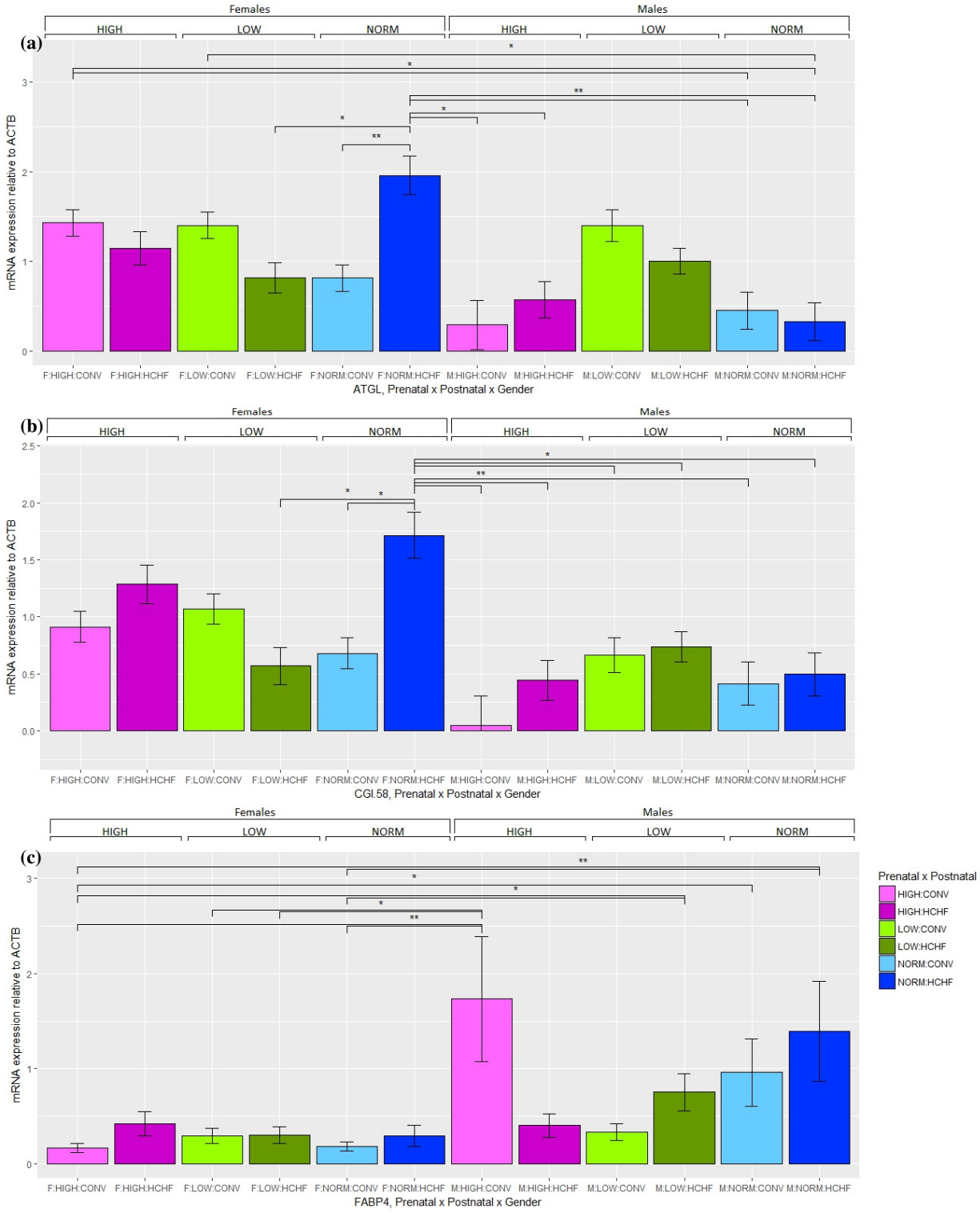


Figure 11. Influence of interaction between prenatal and postnatal nutrition on gene expression in **epicardial adipose tissue** in females (F) and males (M). (a) ATGL, (b) CGI-58, (c) FABP4.
Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).
Number of animals in the treatment groups: HIGH,CONV,F (N=4); HIGH,HCHF,F (N=3); LOW,CONV,F (N=4); LOW,HCHF,F (N=3); NORM,CONV,F (N=4); NORM,HCHF,F (N=2); HIGH,CONV,M (N=2); HIGH,HCHF,M (N=3); LOW,CONV,M (N=4); LOW,HCHF,M (N=4); NORM,CONV,M (N=2); NORM,HCHF,M (N=2)

Effects of gender: For a few genes, there were distinct differences between gene expression levels in males and females (Figure 12). Two, genes related to adipose tissue development, PPARγ (P=0.0470) and VEGF-A (P=0.0053), had higher expression levels in males than females, as was also observed in perirenal adipose tissue, while the opposite was observed for LPL, a gene involved in lipid metabolism (P=0.0001).

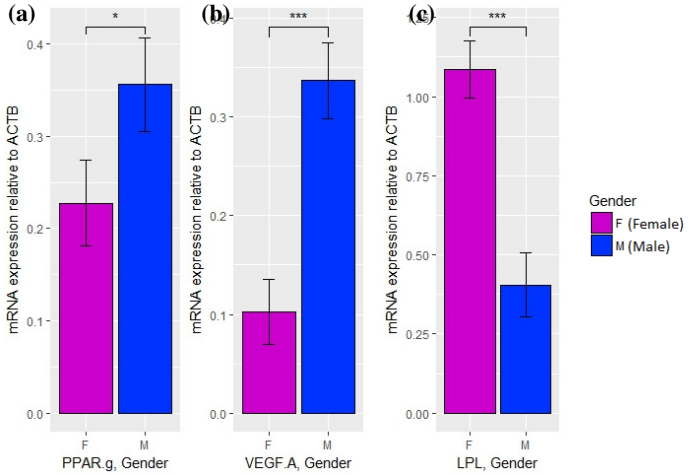


Figure 12. Influence of gender on gene expression in **epicardial adipose tissue**. (a) PPARγ, (b) VEGF-A, (c) LPL.
Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001).
Number of animals in the treatment groups: Females (F; N=20); Males (M; N=17)

4.3 Subcutaneous adipose tissue

Effects of prenatal nutrition: In all genes affected by the prenatal nutrition, the effect was differently manifested in males and females.

In males, expression was decreased in the LOW and HIGH groups compared to the NORM group for lipid metabolism genes FAS (P=0.0346) and HSL (P=0.0291), for genes involved in adipocyte development GcR (P=0.0414), CD34 (P=0.0193) and IGF1R (P=0.0004) as well as inflammatory marker TLR-4 (P=0.0306) (Figure 13a-b;d-g). The only exception to this pattern was lipolytic gene ATGL (P=0.0189 (Figure 13c)) which, in males, was increased in the HIGH and LOW groups compared to NORM. It is however, important to note when interpreting results, that the NORM male group has the smallest group size (N=3). The expression levels were similar between the LOW and HIGH groups for males, except for GcR and CD34 (Figure 13d-e), where expression was

lower in the HIGH group compared to the LOW group.

In females, the pattern of LOW and HIGH groups having decreased expression compared to the NORM group was seen in a few genes, namely ATGL, CD34 and TLR-4 (Figure 13c,e,g). The opposite pattern of increased expression in HIGH and LOW compared to NORM was found only in HSL (Figure 13b). The LOW group had the lowest expression level in GcR, CD34 and TLR-4 in females (Figure 13d,e,g).

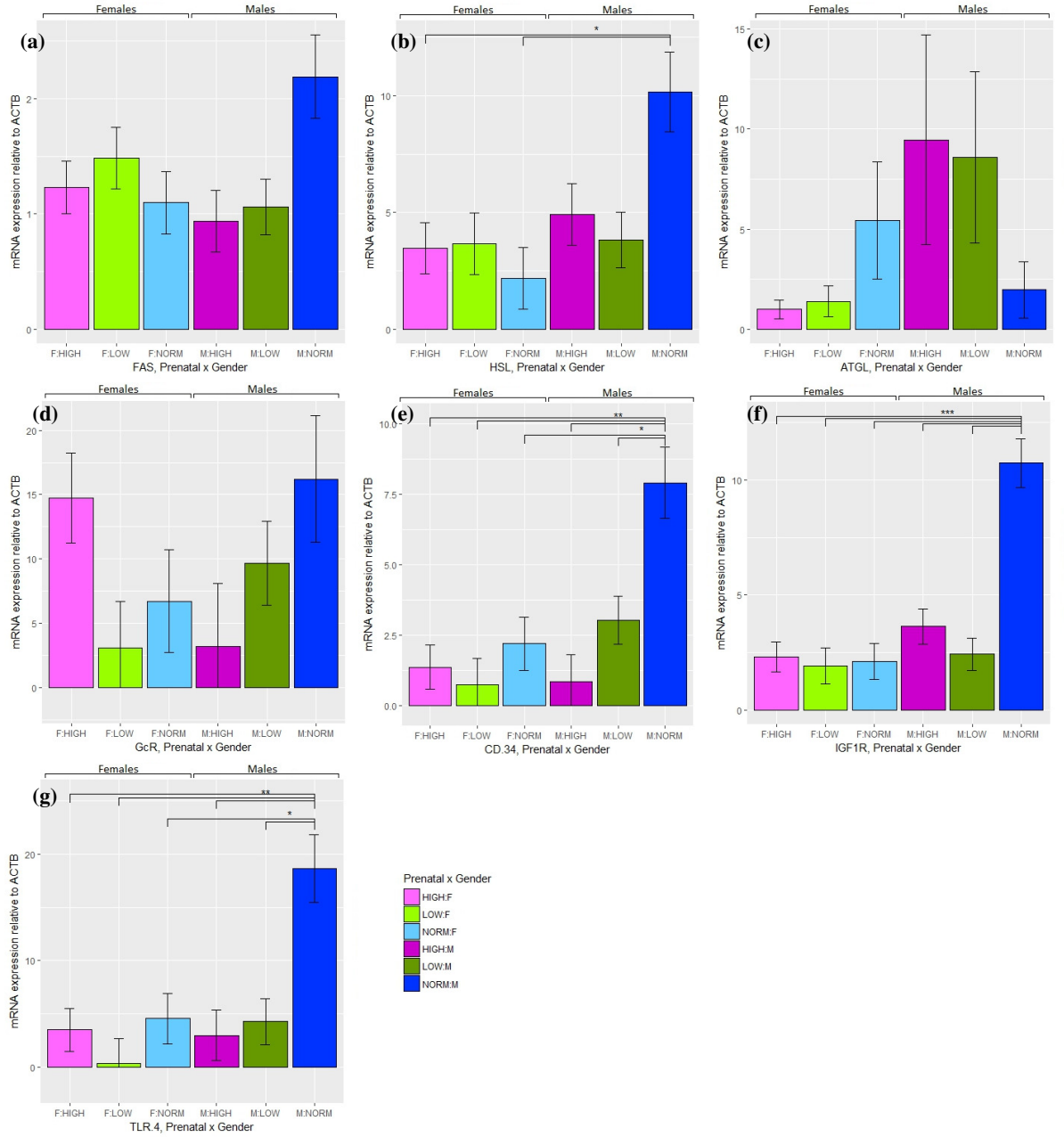


Figure 13. Influence of prenatal nutrition on gene expression in **subcutaneous adipose tissue** in females (F) and males (M). (a) FAS, (b) HSL, (c) ATGL, (d) GcR, (e) CD34, (f) IGF1R, (g) TLR-4. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Number of animals in the treatment groups: HIGH,F (N=7); LOW,F (N=5); NORM,F (N=5); HIGH,M (N=5); LOW,M (N=6); NORM,M (N=3)

Effects of postnatal diet: Only two genes were affected by postnatal diet in the subcutaneous adipose tissue, namely PPARγ, involved in adipose tissue development (P=0.0040) and FBPase, involved in glucose metabolism, in which males and females were affected differently (P=0.0013). Sheep that had been subjected to the obesogenic HCHF diet in early postnatal life showed increased expression of PPARγ compared to CONV sheep, an effect that was also seen in epicardial adipose tissue. The same trend was evident in females for FBPase, while in males, expression of FBPase was reduced in those exposed to the postnatal HCHF diet.

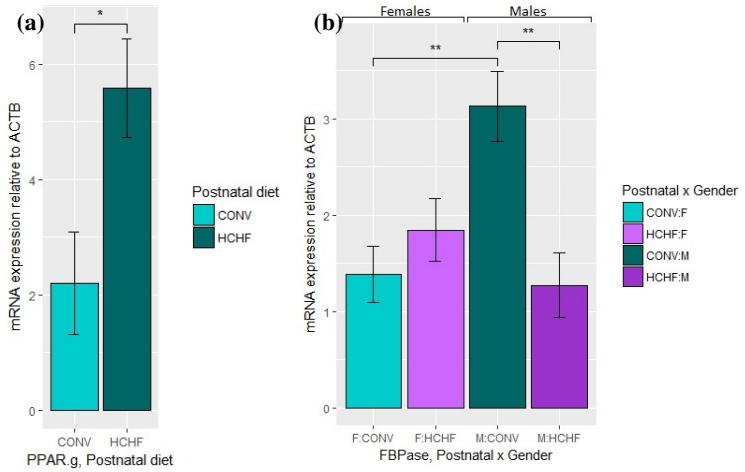


Figure 14. Influence of postnatal diet on gene expression in **subcutaneous adipose tissue** including significant differences between females (F) and males (M). (a) PPARγ, (b) FBPase. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001).
Number of animals in the treatment groups: CONV (N=16); HCHF (N=15); CONV,F (N=10); CONV,M (N=6); HCHF,F (N=7); HCHF,M (N=8)

Interactions between pre- and postnatal nutrition: Sheep subjected to the prenatal LOW nutrition generally tended to have reduced mRNA expression when they had been given the postnatal HCHF diet. This was the case for leptin (P=0.0425, Figure 15b), the glucose metabolism related genes FBPase (P=0.0379, Figure 15c) and GLUT-1 (P=0.0086, Figure 16c), the lipid metabolism related genes PLIN-1 (P=0.0494, Figure 15e) and HSL (P=0.0358, Figure 15f), the inflammatory genes IL6 (P<0.0001, Figure 16d) and MCP-1 (P<0.0001, Figure 16e) though only in males and genes involved in adipose tissue development, IGF1R (P=0.0125, Figure 15g), VEGF (P=0.0047, Figure 15h), CD44 (P<0.0001, Figure 16a) in males and TGF-β1 (P=0.0061, Figure 16b) in females. Only for CGI-58 (P=0.0161, Figure 15d) and LPL (P=0.0207, Figure 16f) in males did the HCHF diet cause increased expression in LOW sheep. For the remaining genes (AdipoQ (P=0.0268, Figure 15a) and VEGF-A (P=0.0033, Figure 15i) in both genders, TGF-β1 in males, and CD44, IL6, MCP-

1 and LPL in females) the postnatal nutrition did not affect gene expression in LOW animals.
In animals subjected to HIGH nutrition prenatally, the subsequent exposure to an obesogenic HCHF diet in early postnatal life had the long-term effect of increasing mRNA expression for most of the affected genes, namely AdipoQ, leptin, CGI-58, PLIN-1, HSL, IGF1R, VEGF and VEGF-A (Figure 15a-b, d-i) as well as CD44, TGF-β1, LPL (Figure 16a,b,f). The only exceptions were a decrease in expression of FBPase (Figure 15c) and in females for GLUT-1, as well as unaffected expression levels in males for GLUT-1, IL6 and MCP-1 (Figure 16c-e).
In all genes with two-way interactions, mRNA expression was decreased in all other groups compared to the NORM-CONV group (Figure 15). For the genes where expression varied for males and females, the same picture was seen in both genders for CD44, IL6 and MCP-1 (Figure 16a,d,e). For the genes with a significant three-way interaction (Figure 16), the NORM-CONV-Male group especially, revealed this response. It is, however, crucial to note that due to missing samples, the NORM-CONV-Male group in subcutaneous adipose tissue consisted of only one animal, making it unadvisable to draw conclusions on the nutritional treatments based on this group. A new statistical analysis ought to be performed for the subcutaneous adipose tissue, excluding this specimen, as it may be the sole cause of some of the significant effects found in this statistical analysis.

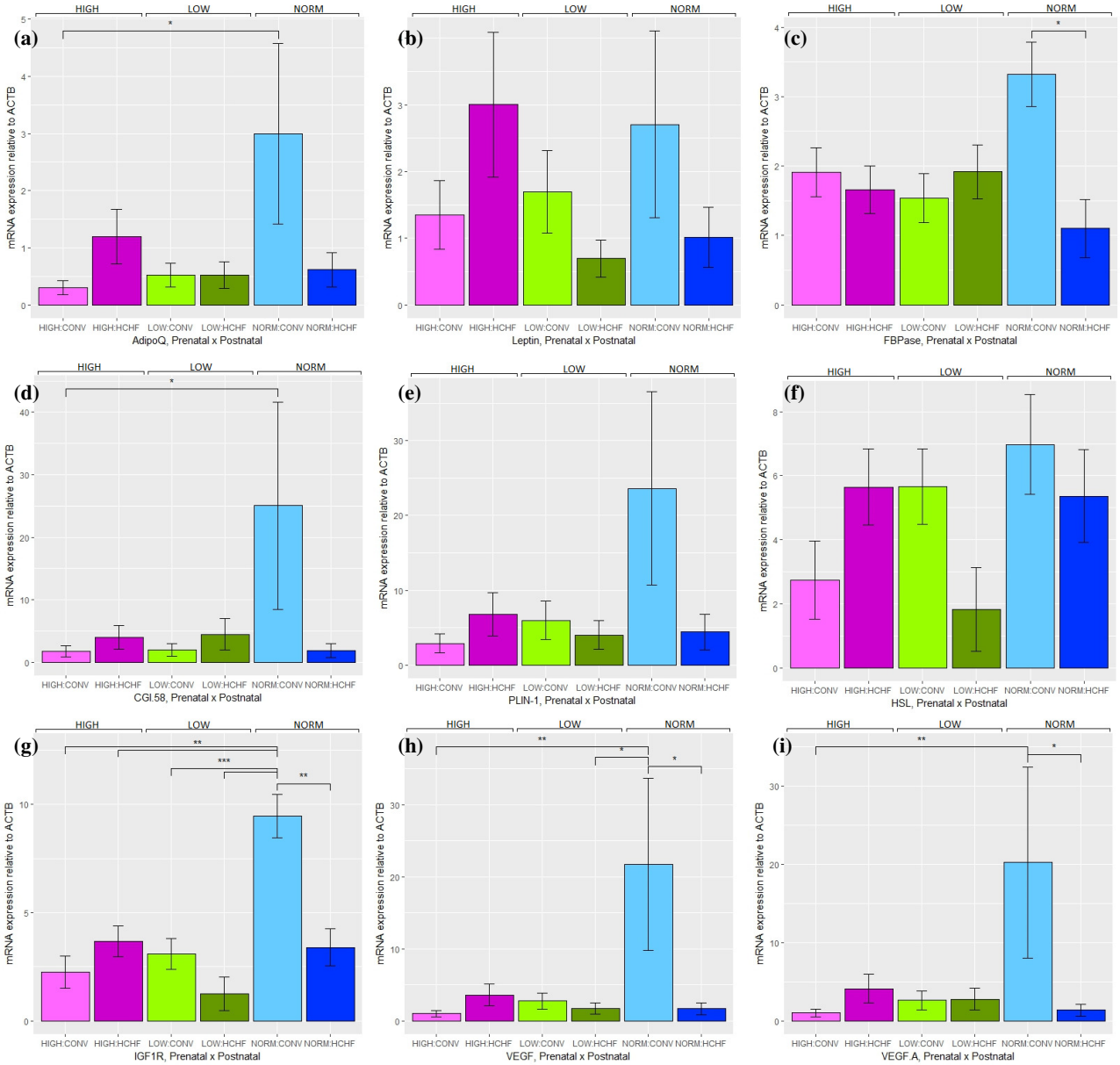


Figure 15. Influence of interaction between prenatal and postnatal nutrition on gene expression in **subcutaneous adipose tissue**. (a) AdipoQ, (b) Leptin, (c) FBPase, (d) CGI-58, (e) PLIN-1, (f) HSL, (g) IGF1R, (h) VEGF, (i) VEGF-A. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Number of animals in the treatment groups: HIGH,CONV (N=6); HIGH,HCHF (N=6); LOW,CONV (N=6); LOW,HCHF (N=5); NORM,CONV (N=4); NORM,HCHF (N=4)

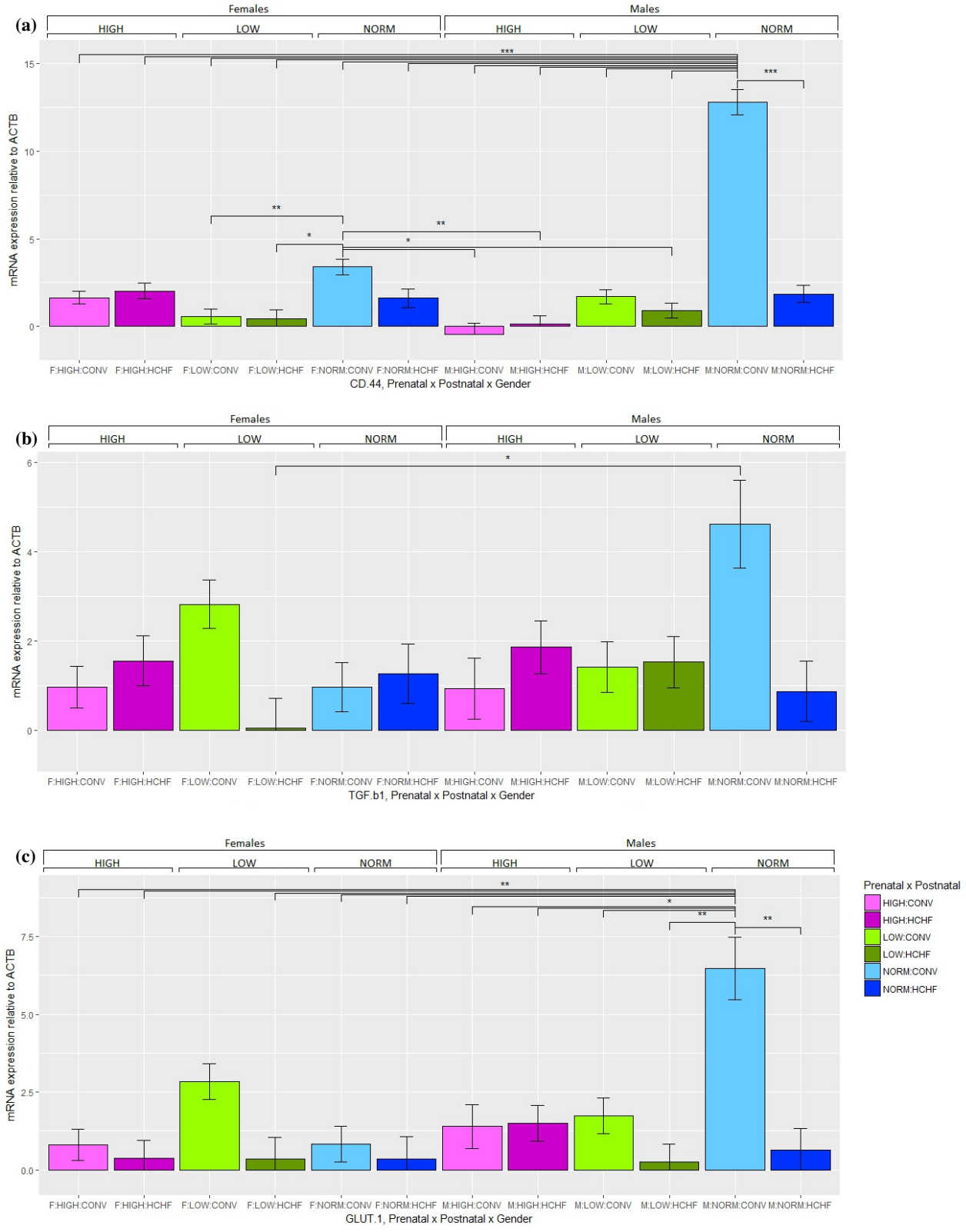


Figure 16. Influence of interaction between prenatal and postnatal nutrition on gene expression in **subcutaneous adipose tissue** in females (F) and males (M). (a) CD44, (b) TGF-β1, (c) GLUT-1, (d) IL6, (e) MCP-1, (f) LPL. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001). Number of animals in the treatment groups: HIGH,CONV,F (N=4); HIGH,HCHF,F (N=3); LOW,CONV,F (N=3); LOW,HCHF,F (N=2); NORM,CONV,F (N=3); NORM,HCHF,F (N=2); HIGH,CONV,M (N=2); HIGH,HCHF,M (N=3); LOW,CONV,M (N=3); LOW,HCHF,M (N=3); NORM,CONV,M (N=1); NORM,HCHF,M (N=2)

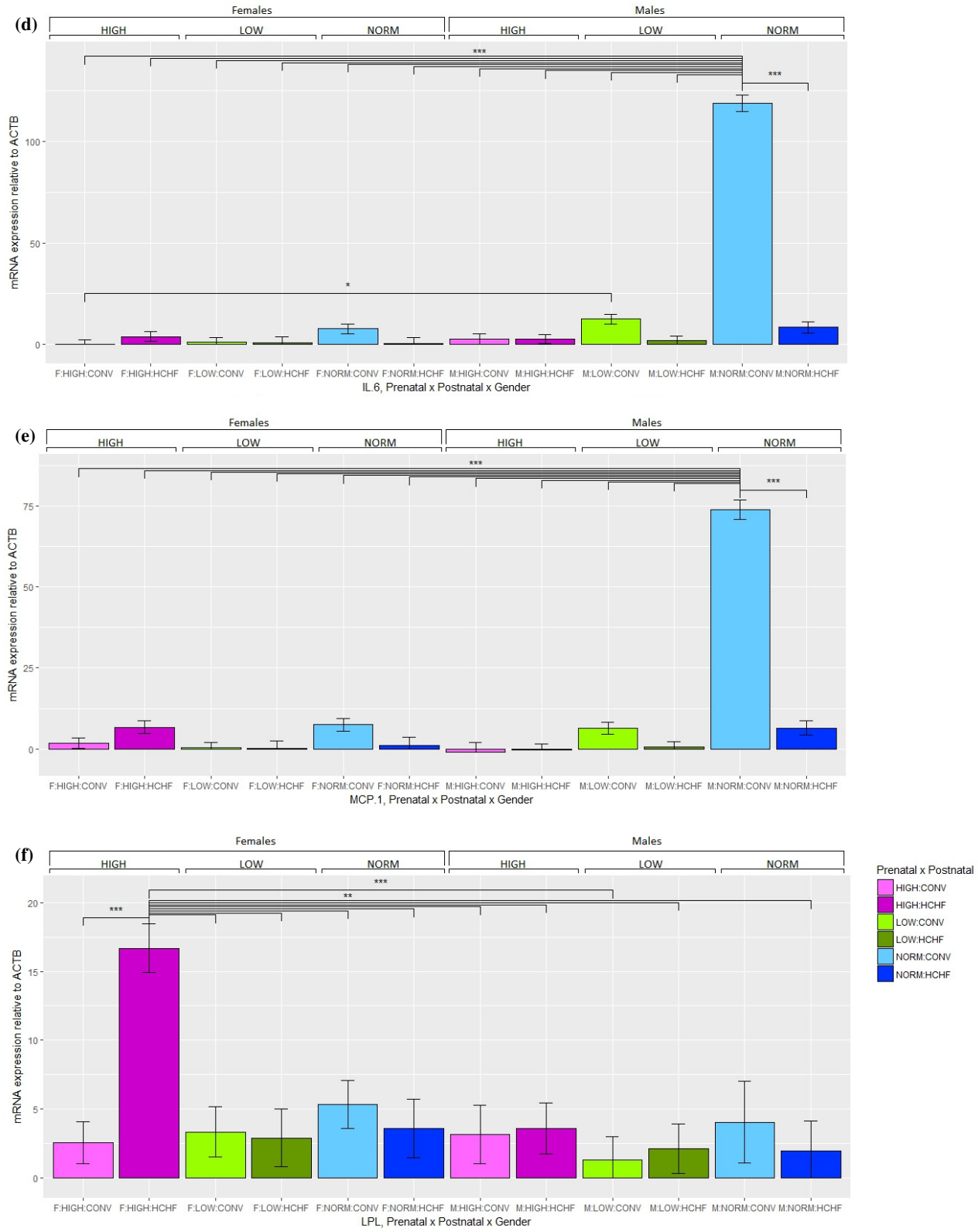


Figure 16 (continued)

Effects of gender: For six genes, there were distinct differences between gene expression levels in males and females. The genes in question were related to lipid metabolism (CGI-58, P=0.0083; FABP4, P=0.0224; PLIN-1, P=0.0006), adipose tissue development (VEGF, P=0.0001; VEGF-A, P=0.0006) and AdipoQ (P=0.0011), and in every case males had higher expression than females (Figure 17), as observed in all other adipose tissues.

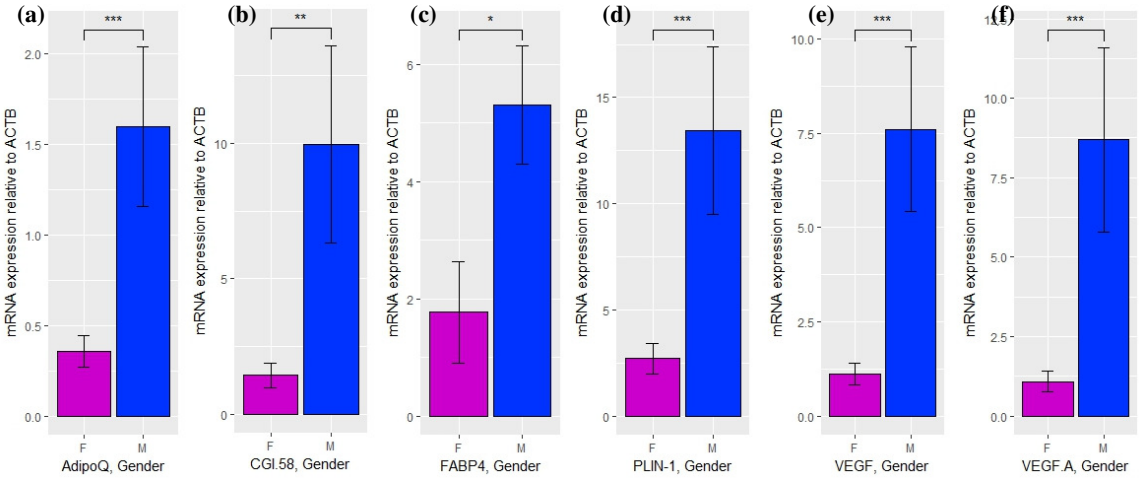


Figure 17. Influence of gender on gene expression in **subcutaneous adipose tissue**. (a) AdipoQ, (b) CGI-58, (c) FABP4, (d) PLIN-1, (e) VEGF, (f) VEGF-A. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001). Number of animals in the treatment groups: Females (F; N=17); Males (M; N=14)

4.4 Mesenteric adipose tissue

Out of the four investigated tissue depots, the mesenteric adipose tissue was the depot where fewest genes were affected by pre- and/or postnatal nutrition as well as gender.

Effects of prenatal nutrition: Long term effects of prenatal nutrition was only evident for one gene in the mesenteric adipose tissue, namely the stem cell marker CD34 (P=0.0255), and the prenatal nutrition had differential impacts in males compared to females. In males, the LOW treatment caused increased expression of the gene compared to HIGH and NORM, while it had the opposite effect in females, where the LOW group had decreased expression compared to the HIGH and NORM groups (Figure 18). Note that the statistical model needs to be adjusted, so that the occurrence of a negative estimates of the expression level, as seen in e.g. Figure 18 cannot occur.

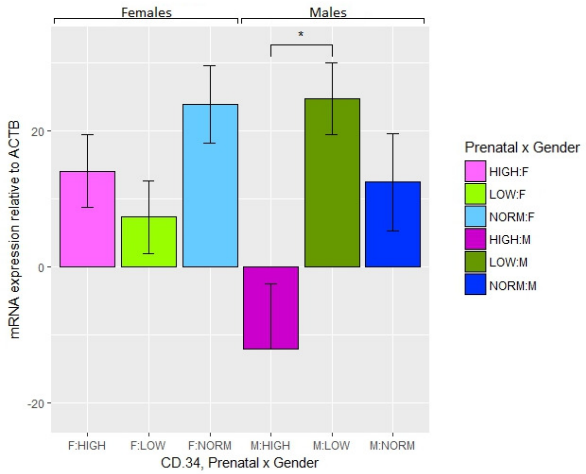


Figure 18. Influence of prenatal nutrition on gene expression in **mesenteric adipose tissue** in females (F) and males (M). Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001).
Number of animals in the treatment groups: HIGH,F (N=6); LOW,F (N=6); NORM,F (N=6); HIGH,M (N=2); LOW,M (N=7); NORM,M (N=4)

Interactions between pre- and postnatal nutrition: For three genes involved in adipose tissue development (CD34, P=0.0209; PPARγ, P=0.0348) and lipid metabolism (FABP4, P=0.0294), the long-term effects of the prenatal nutrition depended on the type of diet the sheep subsequently had been exposed to in early postnatal life.

A trend observed for all three genes was that, LOW sheep that were exposed in early postnatal life to the mismatching HCHF diet had depressed mRNA expression compared to CONV. Variations in gene expression were also found among HIGH and NORM sheep exposed to different postnatal diets, but there was no consistent pattern of increase or decrease as in LOW sheep (Figure 19).

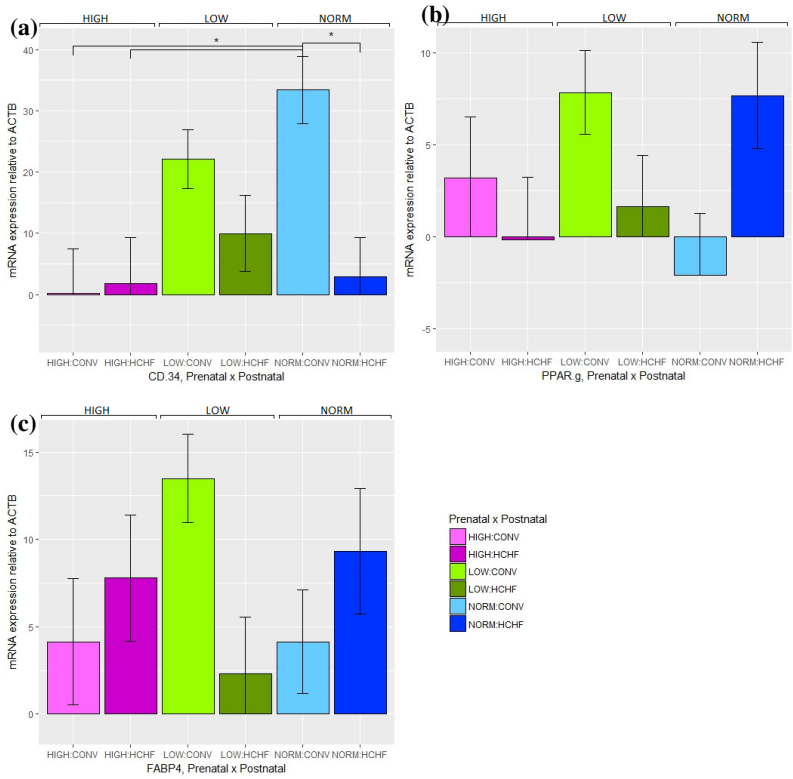


Figure 19. Influence of interaction between prenatal and postnatal nutrition on gene expression in **mesenteric adipose tissue**. (a) CD34, (b) PPARγ, (c) FABP4. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001). Number of animals in the treatment groups: HIGH,CONV (N=4); HIGH,HCHF (N=4); LOW,CONV (N=8); LOW,HCHF (N=5); NORM,CONV (N=6); NORM,HCHF (N=4)

Effects of gender: For two genes involved in glucose metabolism (FBPase; P=0.0035) and adipose tissue development (IGF1R; P=0.0129), expression levels differed between males and females. In both genes expression was higher in males than in females (Figure 20), as seen in the other tissues.

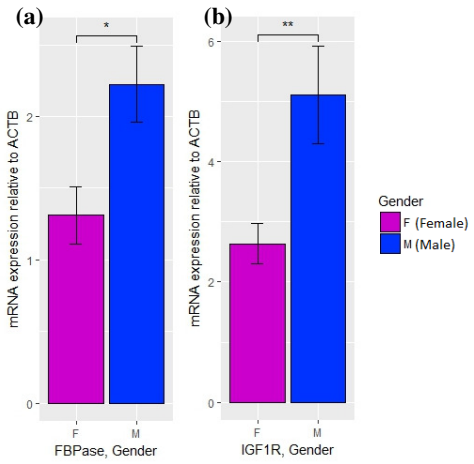


Figure 20. Influence of gender on gene expression in **mesenteric adipose tissue**. (a) FBPase, (b) IGF1R. Values are expressed as LSmeans ± SEM. Asterisks (*) denote significant differences between individual treatment groups as revealed by pairwise comparison (* P < 0.05, ** P < 0.01, *** P < 0.001). Number of animals in the treatment groups: Females (F; N=18); Males (M; N=13)

4.5 Histology

In the following, examples of the results procured with the Visiopharm® app are given. As stated in the 'Materials and Methods'-section, statistical analysis of these data are not included in this thesis and will therefore not be a part of the following discussion.

The Membrane App

Figure 21 shows an example of membranes and cells detected by the Visiopharm® 'Membrane' app. The app registers the total area, the total area of cells, the area of grey shading, the area of excluded area and the area of membrane.

Based on this, it calculates percentage of cell area, percentage of membrane area and percentage of removed cell area.

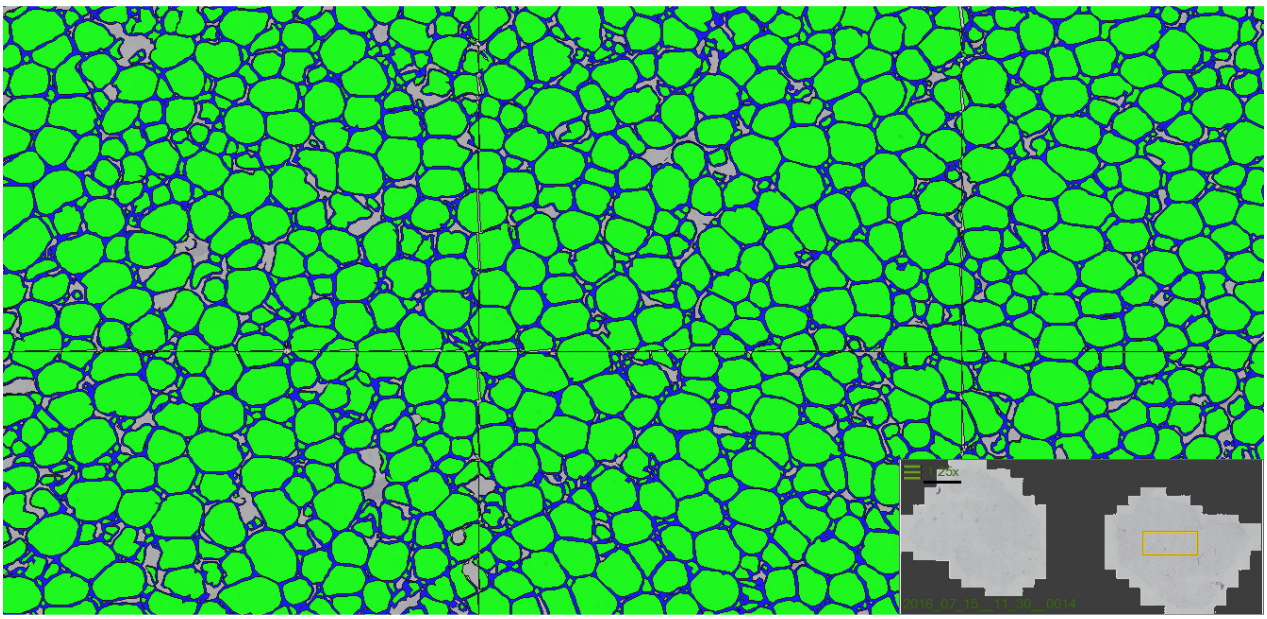


Figure 21. Example of image analysis using the 'Membrane app' by Visiopharm®. The image analysed in this figure is from subcutaneous adipose tissue. The app has detected membranes of cells (blue) and cell areas (green) while cell areas larger than 36000 μm^2 are excluded. (shown in 1.25x magnification)

The Size App

Figure 22 shows an example of how cells are classified according to size in the Visiopharm® 'Size' app. The app divides the cells into one of nine categories based on their size:

'Cells under 200' (Cells with an area larger than 60 μm^2 and smaller than 200 μm^2),

'Cells under 400' (Cells with an area larger than 200 μm^2 and smaller than 400 μm^2),

'Cells under 800' (Cells with an area larger than 400 μm^2 and smaller than 800 μm^2),

'Cells under 1600' (Cells with an area larger than 800 μm^2 and smaller than 1600 μm^2),

'Cells under 3200' (Cells with an area larger than 1600 μm^2 and smaller than 3200 μm^2),

'Cells under 6400' (Cells with an area larger than 3200 μm^2 and smaller than 6400 μm^2),

'Cells under 12800' (Cells with an area larger than 6400 μm^2 and smaller than 12800 μm^2),

'Cells under 25600' (Cells with an area larger than 12800 μm^2 and smaller than 25600 μm^2),

'Cells under 36000' (Cells with an area larger than 25600 μm^2 and smaller than 36000 μm^2),

Based on this, it calculates the percentage of cells in each of the categories.

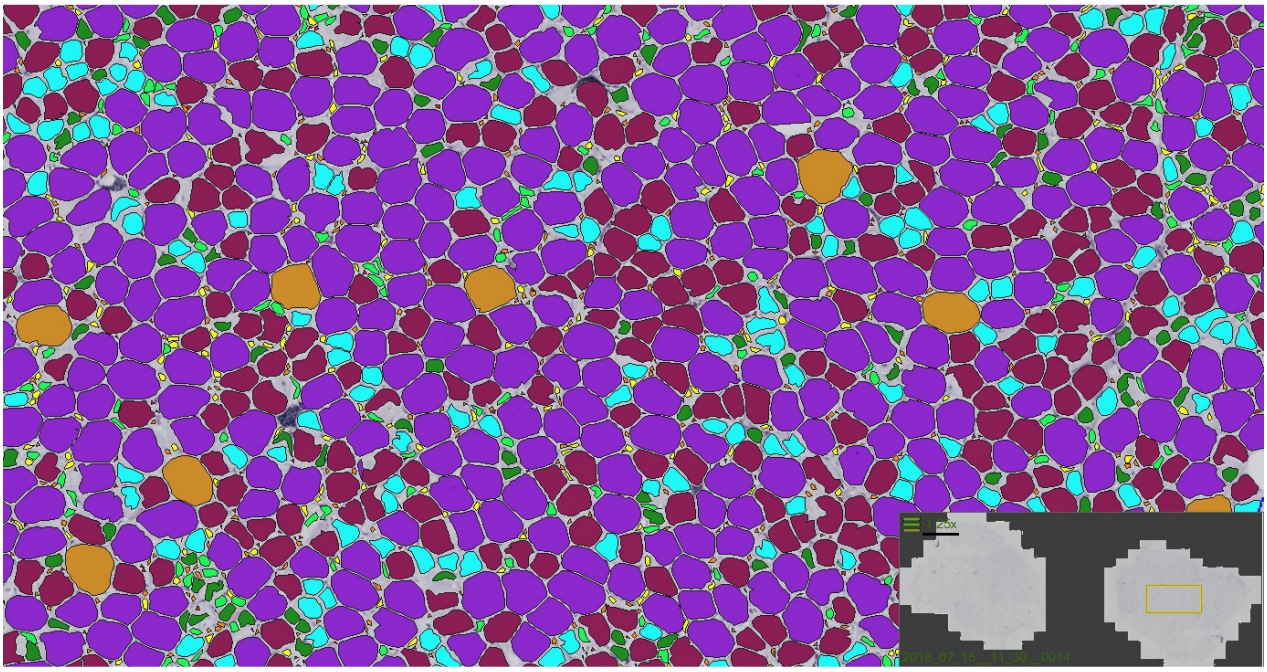


Figure 22. Example of image analysis using the 'Size app' by Visiopharm®. The image analysed in this figure is from subcutaneous adipose tissue. (shown in 1.25x magnification)

The app has detected the size of the cells and classified according to size as indicated below:

- Cells with an area larger than 60 μm^2 and smaller than 200 μm^2
- Cells with an area larger than 200 μm^2 and smaller than 400 μm^2
- Cells with an area larger than 400 μm^2 and smaller than 800 μm^2
- Cells with an area larger than 800 μm^2 and smaller than 1600 μm^2
- Cells with an area larger than 1600 μm^2 and smaller than 3200 μm^2
- Cells with an area larger than 3200 μm^2 and smaller than 6400 μm^2
- Cells with an area larger than 6400 μm^2 and smaller than 12800 μm^2
- Cells with an area larger than 12800 μm^2 and smaller than 25600 μm^2
- Cells with an area larger than 25600 μm^2 and smaller than 36000 μm^2

The Shape App

Figure 23 shows an example of how cells are classified according to size in the Visiopharm® 'Shape' app. The app divides the cells into one of nine categories based on their shape, based on the circularity of the cells (with form factor 0 being a line and form factor 1 being a perfect circle):

'Cells under 0.2' (Cells with a form factor between 0 and 0.2)

'Cells under 0.3' (Cells with a form factor between 0.2 and 0.3)

'Cells under 0.4' (Cells with a form factor between 0.3 and 0.4)

'Cells under 0.5' (Cells with a form factor between 0.4 and 0.5)

'Cells under 0.6' (Cells with a form factor between 0.5 and 0.6)

'Cells under 0.7' (Cells with a form factor between 0.6 and 0.7)

'Cells under 0.8' (Cells with a form factor between 0.7 and 0.8)

'Cells under 0.9' (Cells with a form factor between 0.8 and 0.9)

'Cells under 1.0' (Cells with a form factor between 0.9 and 1.0)

Based on this, it calculates the percentage of cells in each of the categories.

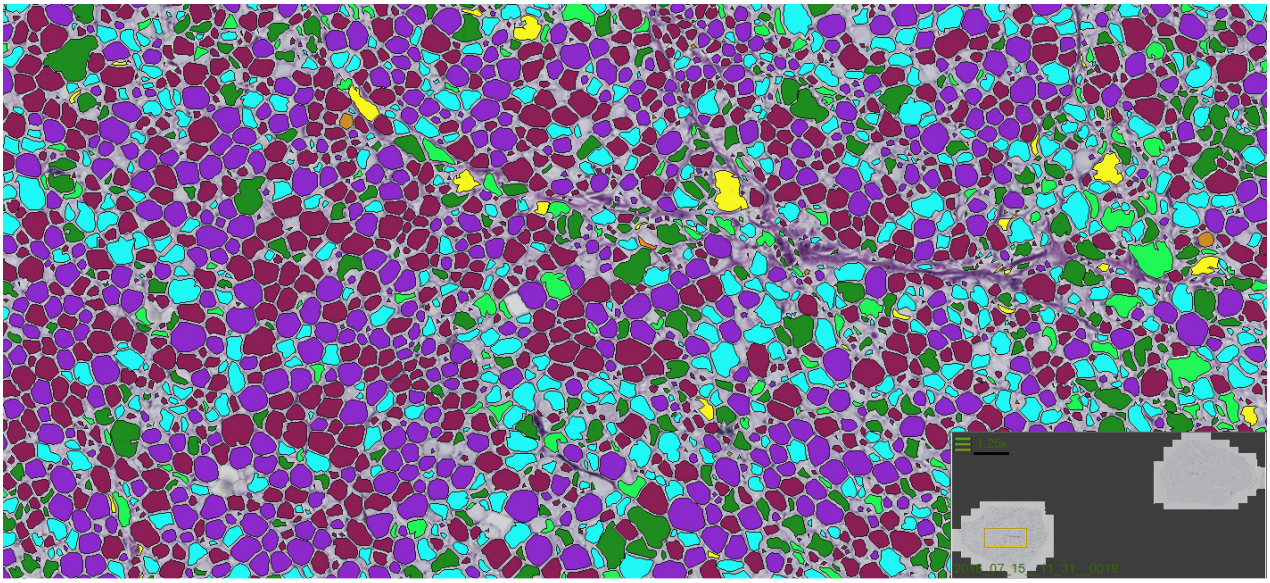
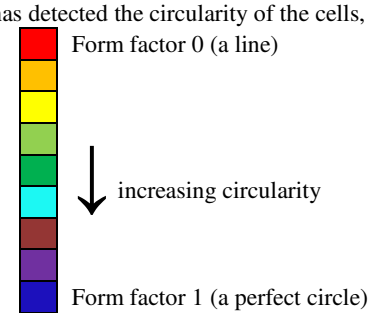


Figure 23. Example of image analysis using the 'Shape app' by Visiopharm®. The image analysed in this figure is from subcutaneous adipose tissue. (shown in 1.25x magnification)
The app has detected the circularity of the cells, and has classified the cells according to circularity as indicated below:



5 Discussion

The objectives of the current study were to investigate if late gestation under- and overnutrition affect adipose tissue in a similar, but depot-specific manner, leading to altered morphology and expression of genes involved in adipocyte development (hyperplasic and hypertrophic growth), metabolism and inflammation in adult sheep. Additionally it was an objective was to investigate if late gestation over- or undernutrition affect the ability to cope with development of obesity in early postnatal life.

Table 2. Summary of effects of pre- and postnatal nutrition on metabolism and adipocyte development/proliferation.
Arrows signify whether expression is higher (↑), lower (↓) or similar (↔) compared to the NORM group in the case of prenatal nutrition and compared to the CONV group in the case of postnatal diet and prenatal-postnatal interaction.
Genes have been divided into one of seven groups for simplicity, even though some genes might be considered for several groups: lipolysis (ATGL, CGI-58, FABP4, HSL, leptin, PLIN-1), lipogenesis (LPL, FAS), glucose metabolism (FBPase, GLUT-1), insulin signaling (AdipoQ, GLUT-4), cell differentiation/proliferation (CD34, CD44, GcR, IGF1R, PPARγ, TGF-β1), angiogenesis (VEGF, VEGF-A) and inflammation (IL6, MCP-1, TLR-4).
When more than one gene from a category showed a significant effect of the same treatment, only general patterns have been reported. Three-way interactions have not been included.
Differences between males and females are shown by blue arrows for males and red arrows for females. A smaller arrow in females is made to indicate that the nutritional challenge impacts gene expression less in females compared to the males.

			Lipolysis	Lipogenesis	Glucose metabolism	Insulin signaling	Cell differentiation /proliferation	Angiogenesis	Inflammation
Perirenal	Prenatal	HIGH	↔		↔	↔↓	↔	↔	
		LOW	↑		↑	↔↓	↓↑	↑	
	Postnatal	HCHF	↔↓			↑↓		↑↓	
	PrenatalxPostnatal (reaction to HCHF)	HIGH	↔					↔	(inconsistent)
		LOW	↓					↑	
		NORM	↑					↓	
Epicardial	Prenatal	HIGH				↔	↔	↓↔	
		LOW				↓	↑↓	↑↓	
	Postnatal	HCHF	↑	↑	↑↓	↑	↔↓		
	PrenatalxPostnatal (reaction to HCHF)	HIGH			↔				(all ↑ compared to NORM-CONV)
		LOW			↓				
		NORM			↔				
Subcutaneous	Prenatal	HIGH	↔↓	↔↓			↔↓		↔↓
		LOW	↔↓	↔↓			↔↓		↔↓
	Postnatal	HCHF			↑↓		↑		
	PrenatalxPostnatal (reaction to HCHF)	HIGH	(all ↓ comp. to NORM-CONV; HIGH-HCHF closer to NORM-CONV)		(all ↓ comp. to NORM-CONV)	(all ↓ comp. to NORM-CONV; HIGH-HCHF closer to NORM-CONV)	(all ↓ comp. to NORM-CONV; HIGH-HCHF closer to NORM-CONV)	(all ↓ comp. to NORM-CONV; HIGH-HCHF closer to NORM-CONV)	
		LOW							
		NORM							
Mesenteric	Prenatal	HIGH					↓		
		LOW					↓↑		
	Postnatal	HCHF							
	PrenatalxPostnatal (reaction to HCHF)	HIGH	↑				(inconsistent)		
		LOW	↓						
		NORM	↑						

Main results are summarised in Table 2, and the discussion will be based on the trends that are indicated in the table. Three-way interactions have not been included in the table summary, as the significant effects of these appeared to be caused by one very small group (N=1-2) standing out. Especially in the case of subcutaneous adipose tissue, the three-way interactions should not be interpreted until a new statistical analysis has been performed without the group which had only one animal of a specific sex.

Overall, the results of this study revealed that gene expression in the four examined adipose tissues are targeted very differently by the foetal and early postnatal nutritional treatments. In the following discussion the four adipose tissues will therefore be discussed separately. The subcutaneous and perirenal adipose tissues were targeted mainly by prenatal malnutrition. Meanwhile, the epicardial adipose tissue was targeted to a greater extent than the other tissues by the early postnatal obesogenic diet, while the mesenteric adipose tissue was largely unaffected in adult animals, of the nutrition received in late foetal and early postnatal life. Furthermore, sexual dimorphism was evident, as males and females showed different responses to the same nutritional challenges, and long-term programming outcomes of early nutrition in gene expression were greater in males than in females. Studies on the same animals used in this experiment, but at six months of age (i.e. by the end of the period with postnatal nutrition intervention), revealed that both prenatal under- and overnutrition altered intrinsic cellularity of the adipose tissue depots (Khanal *et al.*, n.d.), i.e. the cell number in the non-obese state, and also altered the way whereby subcutaneous, perirenal and mesenteric adipose tissues expanded when the sheep were fed an obesogenic diet (Khanal *et al.*, 2014, 2016), relying to a much larger extent on hypertrophic rather than hyperplasic growth compared to NORM sheep. In the following, it will be discussed if the differences in gene expression found in the current study can help to explain these previously observed differences in fat deposition and expandability seen in the adipose tissue depots.

5.1 Males were more sensitive to foetal programming than females

For many genes, significant differences were found between expression levels in males and females, with males having higher expression in all but two cases (Leptin in perirenal adipose tissue and LPL in epicardial adipose tissue), although females had more extensive body fat deposition than males. Interestingly, more than half of the statistically significant effects of pre- and/or postnatal nutrition included an interaction with gender. This was evident in all four tissues and, in most cases, a larger impact of the nutritional treatments was evident in males compared to females. In fact, long-term implications of foetal nutrition on adipose gene expression were in many cases

only observed in males.

In the case of long-term effects of a postnatal obesogenic diet, the variations seen in males and females respectively revealed the same picture, regardless of which adipose tissue it was found in. It was manifested as decreased mRNA expression in males exposed to the HCHF diet, while females had the opposite reaction, though the HCHF diet had less effect in females than in males.

Differences in how mRNA expression in males and females were affected by the prenatal nutrition did not show a uniform pattern, but females generally showed less variation in expression across nutritional treatment groups. Such trends indicate that males are more affected by nutritional foetal programming than females. The same has been found in other studies of foetal programing, e.g. by Joss-Moore *et al.* (2010), who found altered adipose tissue distribution and gene expression in intrauterine growth restricted males, but not in females. A review of animal models further supports that females may be better protected from long-term effects of *in utero* malnutrition with regards to renal disease and hypertension (Woods, 2004).

Interestingly, the gender-specific differences in reaction to foetal programming revealed in the current study were not evident in adolescent sheep (Khanal *et al.*, n.d.). The appearance of such age-related gender variations thus points to the importance of assessing long-term impacts of nutritional foetal programming in both genders.

The underlying reason for the gender differences is unknown. Considering that the nutrition intervention in this experiment was applied during late gestation, it is tempting to speculate that it could be related to epigenetic modifications targeting the sex chromosomes. Females bearing two X-chromosomes could perhaps be better protected against epigenetic alterations targeting an X chromosome compared to males, which bear only one X chromosome. However, it has not been possible to find any studies addressing this issue.

The results of this study provides a rather consistent indication that males may be more susceptible to long-term effects of pre- and early postnatal malnutrition. However, intervention strategies aimed at preventing subsequent development of metabolic diseases should not be targeted at men only, since many effects of malnutrition were found irrespective of gender.

5.2 Different adipose tissue depots are not affected in the same way by late gestation and/or early postnatal malnutrition

The data presented in this study revealed that the four investigated adipose tissues responded very differently to late gestation and early postnatal nutritional insults. This emphasises the importance of considering the (patho)physiological role of individual adipose tissues and sampling site, when programming effects on adipose tissues are to be evaluated.

5.2.1 Prenatal over- and undernutrition and postnatal overnutrition depress gene expression in subcutaneous adipose tissue. Prenatally overnourished sheep may be better protected against adverse effects of postnatal overnutrition

Khanal and colleagues found, as previously mentioned, that late gestation under- as well as overnutrition reduced intrinsic cellularity of the subcutaneous adipose tissue in non-obese lambs at six months of age (Khanal *et al.*, n.d.) and this co-existed with reduced deposition of fat in the subcutaneous depot (Khanal *et al.*, 2014). When exposed to a high-fat diet, the subcutaneous adipocytes grew to a similar size independent of prenatal nutrition, but in the HIGH and LOW lambs, fat deposition was not associates with hyperplasic growth to the same extent as observed in the NORM lambs (Khanal *et al.*, n.d.), leading to less deposition of subcutaneous adipose tissue in HIGH and LOW when they became obese. When HCHF sheep subsequently had been fed the same low fat diet as the CONV lambs for 2 years, the deposition of fat in the subcutaneous depot became similar irrespective of the nutrition history in early postnatal life (Khanal *et al.*, 2016).

The data presented in this thesis indicate that changes in expandability of subcutaneous adipocytes are associated with changes also in gene expression in response to both prenatal under- or overnutrition and, to some extent, postnatal overnutrition. Genes related to lipid- and glucose metabolism, adipose tissue development, insulin signalling and inflammation were reduced in prenatally malnourished animals (over- and undernutrition) compared to those that had been fed adequately during late fetal life.

The reduced expression of genes involved in adipogenesis and angiogenesis in the HIGH and LOW groups (especially evident in males) is consistent with might indicate a reduced ability to undergo hyperplasic growth, as was observed in the six month old lambs. These differences in gene expression, did however not lead to differences in fat mass in identically fed adult animals (Khanal *et al.*, 2016). Together this might indicate that the subcutaneous fat mass expansion that occurred in the animals from six months of age until 2½ years of age may have relied more on hypertrophic growth than hyperplasic growth, although this needs to be confirmed by histological evaluations.

Hypertrophic adipocytes are associated with decreased expression of AdipoQ and increased expression of inflammatory markers (Harwood Jr., 2012). The fact that expression levels of AdipoQ were indeed decreased in the HIGH and LOW groups in this study might thus support the notion that the subcutaneous adipose tissue relies more on hypertrophic growth in HIGH and LOW compared to NORM. On the other hand, there was no indication of any increase in mRNA expression for inflammatory markers in the HIGH or LOW groups, which might indicate that excessive hypertrophy, at least, has not occurred, consistent with the observations in lambs, which indicated that the capacity of subcutaneous adipocytes to undergo hypertrophy is limited compared to the perirenal and mesenteric adipocytes.

For the genes where an interaction between pre- and postnatal nutrition was evident, gene expression was generally reduced in all groups compared to the NORM-CONV group. Here it was, however, notable that those that had received the HIGH-HCHF nutrition tended to show less reduction in gene expression than the other groups compared to NORM-CONV. The HIGH-CONV group on the other hand, appeared to mirror the mismatch seen in LOW-HCHF, by having lower expression than HIGH-HCHF. These results somewhat surprisingly appear to indicate that exposure to prenatal overnutrition may, contrary to the expected, increase one's ability to cope with postnatal overnutrition.

Overall the data presented in this thesis suggest that there are long-term programming effects of both prenatal over- and undernutrition and to some extend also early postnatal overnutrition on gene expression relating to the development and function of subcutaneous adipose tissue, especially in males. It was hypothesised that both late gestation under- and overnutrition would reduce the ability of subcutaneous adipose tissue to expand. Reduced expression of genes involved in adipocyte differentiation and proliferation is in line with this hypothesis, though overall fat mass in identically fed adut sheep was not affected by the early life nutrition history (Khanal *et al.*, 2016).

5.2.2 Late gestation undernutrition changes gene expression in perirenal adipose tissue

Khanal *et al.* (2014) found that exposure to an early postnatal HCHF diet increased total fat mass independently of prenatal nutrition. However, those that had received the HIGH and LOW nutrition in late gestation deposited fat in the perirenal adipose tissue to a much greater extent than the NORM animals. Histological evaluations furthermore revealed that this growth was caused by extreme hypertrophic growth, while hyperplasic growth was decreased compared to NORM (Khanal *et al.*, n.d.). After two years of dietary correction the nutritional groups attained similar body weights and fat deposition with the notable exception of perirenal adipose tissue, which had

failed to expand in the HIGH and LOW animals from adolescence into adulthood (Khanal *et al.*, 2016). From these phenotypic results it might be expected that the HIGH and LOW, or possibly the HIGH-HCHF and LOW-HCHF groups would stand out compared to the other groups with regards to gene expression. The investigation of gene expression in the lambs at six months of age only revealed effects the postnatal obesogenic diet in perirenal adipose tissue (Khanal *et al.*, n.d.). They found that postnatal overnutrition decreased expression of genes related to metabolism and adipocyte development, while upregulating expression of inflammatory markers. This fitted the phenotypic response of hypertrophic growth, though not distinguishing between the NORM and the HIGH and LOW groups. In the adult animals investigated in this study, there were few significant differences in gene expression caused by the HCHF diet, and no distinct pattern was evident. Rather, the main finding concerning gene expression in perirenal adipose tissue was that long-term effects were primarily caused by late gestation undernutrition. For most of the affected genes (involved in lipid metabolism, basal glucose transport and adipose tissue development and vascularisation) this led to up-regulation of expression compared to normally fed sheep, though in some cases only in males. These alterations in gene expression indicates that prenatal undernutrition programs the tissue to be able to grow via hyperplasia and to metabolise glucose and lipids. A link could be drawn to the increased fat deposition seen in response to the HCHF diet in the young animals. However, it does not coincide with the fact that the growth was found to occur via hypertrophy rather than hyperplasia nor with the reduced expansion of the tissue from adolescence to adulthood.

When a different reaction to the prenatal diet occurred in females, it was mostly for markers of adipose tissue development, and though they were consistently less affected by the prenatal nutrition than males, they did tend to have reduced expression when they had been exposed to undernutrition in late foetal life. Effects of the prenatal overnutrition were much less pronounced than those of undernutrition, but it can be noted that the markers of adipose tissue development showed a slight tendency to have reduced expression compared to those fed normally.

There is thus not a clear connection linking the observed morphology and fat deposition patterns to the variations in gene expression found in this study. The observation that is most in line with the phenotypic changes observed in the LOW and HIGH animals as a reaction to the HCHF diet is that of a slightly reduced expression of genes related to adipose tissue development and angiogenesis in HIGH animals and in to some extent in LOW females. This might indicate that the ability to grow via hyperplasia is reduced, thus making them more reliant on (unhealthy) hypertrophic growth.

However the most consistent change was the before mentioned up-regulation of gene expression in LOW animals. It might be speculated if this is a sign of compensatory change in gene expression occurring in the LOW animals as a response to the extreme hypertrophic growth seen in the HCHF groups at six months of age. Nonetheless, it is not manifested in the phenotypic outcome in the 2½ year old animals, where the expansive capacity of the tissue appeared to have collapsed since adolescence (Khanal *et al.*, 2016). It will be interesting to see if the ongoing evaluation of the histological data from these sheep can provide further clues as to the cause of the collapse of the perirenal adipose tissue.

An interesting observation is that the alteration in gene expression seen in the HIGH and LOW groups compared to NORM were opposite (up-regulation in LOW and down-regulation on HIGH), while they had the same phenotypic response to the HCHF diet, both at six months of age (extreme hypertrophic growth) and at 2½ years of age (collapse). This might indicate that different mechanisms cause the same phenotypic outcome in the two prenatally malnourished groups.

5.2.3 Mesenteric adipose tissue is not programmed by late gestation malnutrition

Visceral adipose tissue depots, with emphasis on the mesenteric adipose tissue are outlined as risk factors in development of metabolic diseases (Bjørndal *et al.*, 2011).

In the six month old lambs it was found that intrinsic cellularity was decreased in mesenteric, just as in subcutaneous adipose tissue, by late gestation under- and overnutrition. When they were subjected to the postnatal HCHF diet, this tissue exhibited increased hypertrophic growth, though not as extreme as in the perirenal adipose tissue (Khanal *et al.*, n.d.).

After two years of dietary correction the animals that had received the HCHF diet still had slightly increased deposition in the mesenteric adipose tissue compared to the CONV. Due to the lack of expansion of the perirenal adipose tissue in HIGH and LOW groups that had received the HCHF diet, the distribution of visceral adipose tissue was altered in these groups, with an increased mesenteric:perirenal adipose tissue ratio (Khanal *et al.*, 2016).

Interestingly, though the tissue revealed phenotypic adaptations following the nutritional insults, very few effects were found in gene expression in the adult animals. The study of gene expression in the adolescent lambs did not reveal any effects of the prenatal nutrition, with the exception of increased expression of an inflammatory marker in the LOW-HCHF group (Khanal *et al.*, n.d.). This indicates that mesenteric adipose tissue may not be sensitive to nutritional programming during the last six weeks of gestation. The alterations seen in the tissue more likely represents carry-over effects from the other adipose tissues, that showed a greater response to the nutritional insults.

5.2.4 Early postnatal overnutrition targets epicardial adipose tissue

The tissue that was most sensitive to long-term impacts of the postnatal obesogenic HCHF diet was the epicardial adipose tissue. The increased expression of genes relating to lipid metabolism in HCHF-fed animals suggested that the early postnatal obesogenic environment has primed the epicardial adipose tissue for processing lipids to a greater extent than in normally fed animals. This, along with increased expression of adipogenic marker PPARγ may put the HCHF-fed animals at increased risk of fat accumulation in the epicardial adipose tissue, in the event of nutritional excess. People that have been overweight in childhood might thus be at increased risk of developing coronary artery disease and cardiac abnormalities, as well as metabolic syndrome, which are associated with increased amounts of epicardial adipose tissue (Iacobellis, 2015).

Both animal and human studies have shown that males had a higher incidence of hypertension and vascular dysfunction compared to females (Grigore *et al.*, 2008). In relation to this, it is interesting to note that the HCHF diet caused decreased expression of a stem cell marker in males only. Fewer stem cells in the tissue means that it might rely more on hypertrophic growth in the event of excess nutrition, potentially leading to an increased inflammatory response (Harwood Jr., 2012). Inflammatory cytokines secreted from epicardial adipose tissue has been found to be associated with cardiovascular disease (Iacobellis, 2015). Gender differences were also observed in genes that were affected by late gestation undernutrition. This will be discussed further below.

5.2.5 Developmental capacity of the visceral adipose tissues are affected differently by prenatal undernutrition

A curious observation is that, when prenatal undernutrition caused changed expression of markers of adipocyte development, the change in expression was opposite in perirenal and epicardial adipose tissues. In perirenal adipose tissue the expression adipogenic markers was increased in adult males, but decreased in females that had been subjected to late gestation undernutrition. In epicardial adipose tissue on the other hand, expression was decreased in males and increased in females exposed to prenatal undernutrition. To the author's knowledge, such a pattern has never been reported previously.

As mentioned before, reduced ability of the tissue to expand via hyperplasia will mean that expansion will have to rely more on hypertrophic growth in the event of energy excess, increasing the risk of inflammation and hypoxia (Virtue and Vidal-Puig, 2010). Therefore, it might be speculated that prenatal undernutrition cause more adverse effects in epicardial adipose tissue in males, while it instead preferentially targets the perirenal adipose tissue in females. In relation to

this, it is noteworthy that males haves been found to have higher prevalence of hypertension and vascular dysfunction compared to females as a consequence of foetal undernutrition (Grigore *et al.*, 2008).

One of the few long-term effects of foetal programming that was found in mesenteric adipose tissue was for the stem cell marker CD34. The expression of this gene was, as in perirenal adipose tissue, up-regulated in prenatally undernourished males, and down-regulated in prenatally undernourished females, consistent with the preference for hypertrophic growth when they were fed an obesogenic diet.

In the case of the insulin dependent glucose transporter, GLUT-4, males showed reduced expression both in prenatally undernourished and especially in prenatally overnourished sheep. This suggests that prenatal malnutrition, both over- and undernutrition, causes the perirenal adipose tissue to be less responsive to insulin signalling. This response was furthermore also found in males that had been overnourished in early postnatal life. GLUT-4 expression was also reduced by prenatal undernutrition in epicardial adipose tissue. Further investigations of other genes related to insulin signalling are necessary to gain a clearer picture of whether these two visceral adipose tissues become insulin resistant as a long-term consequence of foetal programming.

5.2.6 Tissue specific differences in programming effects might be related to the time window of development for the different depots

It was hypothesised that late gestation under- and overnutrition interferes with mechanisms controlling hyperplasic and hypertrophic growth and metabolism in adipocytes in a depot specific manner. This was indeed found to be the case, as is evident from the discussion above. It is tempting to speculate that such differences in response might be related to the timing of the nutritional manipulation (last six weeks of gestation. The major part of adipocyte differentiation and adipogenesis occurs during late gestation in precocial species like the sheep and humans (Symonds *et al.*, 2007). However, there are variations in the exact time window of growth of the distinct adipose tissue depots. In a foetal sheep study it was found that perirenal-abdominal as well as pericardial adipose tissues were detectable from day 70 of gestation, while subcutaneous adipose tissue appeared 2-3 weeks later (Alexander, 1978).

The pericardial adipose tissue has been shown to be especially susceptible to foetal programming in early-mid gestation (Symonds *et al.*, 2016), indicating that adipose tissue depots around the heart may initiate their development earlier in gestation than the perirenal-abdominal and the subcutaneous adipose tissue depots. In line with this, fewer gene expression changes were found to

be caused by the late gestation under- and overnutrition in the epicardial adipose tissue compared to the subcutaneous and perirenal depots in the current study. The same may be the case for the mesenteric adipose tissue, which was practically unaffected by the pre- and early postnatal nutritional insults. However, the exact timing of initial development of this tissue is not known, as it is not distinguished from other abdominal depots in the foetal sheep.

5.3 Methodological considerations

In relation to the experimental work completed for this thesis there are some challenges and potential sources of error that should be taken into consideration.

The design of this study intended to allow for differentiation between treatment groups while also separating gender effects. However, initial group sizes were reduced due to culling of animals that developed pneumonia or diarrhoea during the experimental period, thus resulting in smaller group sizes than anticipated. This means that interpretation of three-way interactions might be considered inadvisable. It might be considered to leave out this parameter in further statistical analyses of the data.

The prioritisation of which genes should be included in the statistical analysis was made based on the quality of the qPCR results. All samples were run in triplicates to allow for the calculation of standard deviation of the Crossing Point value, while still having the option of removing an outlier result. However in some cases it was not possible to reduce the standard deviation below 0.4 for a sample. Therefore, the genes with no, or as few as possible, samples with a standard deviation above 0.4 were prioritised to be included in the statistical analysis.

For a few of the genes, namely ADRβ1, CD68, PPARα and especially C/EBPβ it might be advisable to perform additional qPCR analyses, perhaps with alternative primers, due to these genes having many samples with a large standard deviation in all four adipose tissues. An alternative primer will indeed be necessary to get results for C/EBPβ, as the efficiency and slope of the standard curve for this primer was read wrong, resulting in this primer being accepted by mistake. Additionally, it was found that the primer sequence used had been copied incorrectly from the paper it had previously been published in, which might explain the poor results obtained with that primer. The remaining genes that were not included in the statistical analysis all had good qPCR results in at least one of the four adipose tissues. This suggests that the primers were not the cause of the varying standard deviations or undetectable crossing points. It might instead be caused by either technical error during the qPCR analysis or alternatively that expression of the gene was so low in the tissue in question that it was not detectable.

It was intended to include the glucagon receptor and insulin receptor subunit β (INSRβ) in the analysis, but though several primers were tested, no working primers were found. Both of these receptors have been reported as present in adipose tissue (Kershaw and Flier, 2004) and as INSRβ has been investigated in previous studies of the Copenhagen sheep model (Nielsen *et al.*, 2016) it should be possible to find a primer for this gene that works in adipose tissues of sheep.
Glucagon is known to stimulate lipolysis in adipose tissue in birds (Scanes, 2009), and has also been suggested to promote lipolytic activity in adipose tissue in other species, though much less is known about how the hormone affects this tissue compared to hepatic tissue, which is the main target of glucagon (Ali and Drucker, 2009). Expression of glucagon receptor mRNA has been found in adipose tissue in rats (Hansen *et al.*, 1995; Svoboda *et al.*, 1994) and in mice (Burcelin *et al.*, 1995), though in low amounts compared to expression in the liver. Studies investigating expression of the glucagon receptor in ruminant adipose tissue are, however, lacking. The inability to find a working primer for the glucagon receptor in adipose tissue in the current study could indicate that the receptor is not markedly expressed in adipose tissue in sheep. In line with this, different studies have failed to find lipolytic effects of glucagon in adipose tissue in ruminants (Brockman, 1978; Hippen *et al.*, 1999; She *et al.*, 1999).

6 Conclusions

The results of this study suggested that gene expression of adipose tissue is susceptible to long-term programming effects of late gestation and, to less extent, early postnatal malnutrition. Furthermore, the results suggested that long-lasting programming outcomes target males to a larger extent than females, which is curious considering that females have a larger fat mass than males.

The findings of this study showed that expression of genes related to adipocyte differentiation and proliferation as well as lipid- and glucose metabolism and inflammation was reduced in subcutaneous adipose tissue of animals that had been either under- or overnourished in late gestation, compared to those that had been adequately nourished. These data therefore support the hypothesis that late gestation under- and overnutrition reduces the ability of subcutaneous adipose tissue to expand in adult animals. However, a limited expansive capacity of the subcutaneous adipose tissue was only observed in adolescent lambs when they had been exposed to a high-fat diet in early postnatal life. When provided with an adequate diet from six months of age until 2½ years of age however, fat deposition in the subcutaneous adipose tissue depot was similar between treatment groups. Thus the ability of the subcutaneous adipose tissue to expand may only be compromised in a situation of dietary excess.

The investigation of gene expression in the adult sheep furthermore revealed that the four studied adipose tissues were programmed differently by late gestation under- and overnutrition as well as postnatal overnutrition. Subcutaneous adipose tissue was targeted by both prenatal over- and undernutrition, and to some extend also postnatal overnutrition, which caused decreased expression of genes that promote hyperplasic growth and metabolic functions. Expression of the same types of genes in perirenal adipose tissue was targeted mainly by prenatal undernutrition, which led to increased expression compared to normally fed animals. This was especially evident in males. Prenatal overnutrition had less impact on gene expression in this tissue, but generally showed the opposite reaction than that caused by undernutrition. The similar phenotypic alterations seen in prenatally under- and overnourished animals when subjected to a postnatal obesogenic diet might therefore be caused by different mechanisms. Gene expression in mesenteric adipose tissue showed very little programming. Any phenotypic outcomes related to the early life nutrition history in this tissue might therefore be caused by carry-over effects from the other adipose tissues. This warrants further studies, since mesenteric adiposity is believed to be particularly linked to development of dietary induced pancreas and metabolic disorders. Prenatal effects on adipogenic genes in the epicardial tissue were gender specific and reacted opposite than what was seen in the perirenal

adipose tissue. Decreased ability of epicardial adipose tissue to grow via hyperplasia in males might be related to the higher incidence of cardiovascular disease seen in men.

Long-term effects of the postnatal HCHF diet were mostly evident in epicardial adipose tissue and were mostly independent of prenatal diet. The NORM animals did not appear to be protected any more than the other groups. The altered gene expression in the epicardial adipose tissue indicated that it was primed to metabolise lipids to a greater extent than in conventionally fed animals, potentially increasing the risk of fat accumulation in the tissue. This is a feature associated with an increased risk of developing coronary artery disease and cardiac abnormalities, as well as metabolic syndrome.

7 Perspectives

The results presented in this thesis have added to the evidence that prenatal malnutrition causes long-term alterations in adipose tissue development and function, seen in adult sheep. This may indicate a causal link between prenatal malnutrition and later development of metabolic and cardiovascular diseases, although validity of findings from animal studies needs to be confirmed in humans.

In order to get a further understanding of the mechanisms involved in alterations in gene expression, further studies might include investigations of whether epigenetic changes in the promoter regions of the affected genes can help to explain the observed changes in mRNA expression. Furthermore, while the current study used mRNA expression as a measure for gene expression, it would be relevant to investigate if protein abundance matched the mRNA expression. Berends *et al.* (2013) found that protein expression of insulin-signalling proteins was impaired, while mRNA expression was not. Final gene expression might thus be affected post-transcription e.g. by microRNAs. Clinically relevant outcomes of foetal programming have largely been found in ageing individuals. While the current study goes far in relation to examining long-term effects of foetal programming including gender differences, an extended study where the animals were kept for an even longer period would make it possible to draw more direct connections between health of ageing individuals and e.g. gene expression in adipose tissue and other organs.

The results obtained in this study may also have relevance in relation to livestock production, since content and distribution of fat are of great importance in the assessment of meat quality. Furthermore, in the event that metabolic dysfunction leads to pathological states in the production animals, it might increase veterinary costs for the producer and reduce performance of the animal. Within dairy production, there may be an effect of maternal nutrition during gestation on later milk production of the offspring. Since milk yield is affected by nutrient partitioning, alterations in lipid and glucose metabolism mediated through programming of adipose tissue might contribute to an altered milk yield (Bach, 2012). Dairy cows beyond their first gestation are lactating throughout most of gestation, meaning that the developing foetus competes with the mammary glands for nutrients (Bell and Bauman, 1997). Therefore, calves in current dairy production systems may be exposed to nutrient restriction, especially in the final part of gestation. It can therefore be relevant to examine if this affects productivity.

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Appendix A - Protocol for RNA extraction with notes on how to measure RNA concentration on the NanoDrop ND-1000 Spectrophotometer

RNA-purification with Promega-kit
Homogenizing (the procedure must be performed in the hood!)
Transfer 1 stainless steel bead (5mm) to a 2ml eppendorff tube
Allow to overnight at -20°C
Turn on centrifuge and set it to 4°C
Centrifuge MaXtract tube 2ml in 1min at 12,000xg
Add 1000µl Trizol to the eppendorff tube with 1 stainless steel bead and set the tube on ice (if you have more tubes, prepare them all and put on ice)
Weigh approx. 150mg tissue, note the weight (can be done in advance and kept in -80°C freezer)
Add each tissue sample to a tube with Trizol
Transfer the samples to tissue lyser (Qiagen) on program 3 (300 rpm for 6 min). Remember to tighten the lock!
Set the samples on ice and transport them back to the hood in the PCR lab
Incubate for 5min at room temperature. During the 5min, transfer the liquid to a new 2ml tube
Centrifuge for 10 min at 12,000xg at 4°C
Transfer supernatant to MaXtract tube 2 ml (approx. 1000µl)
Phase separation
Add 120µl of chloroform and shake the tube vigorously for 15 sec
Incubate on ice for 10 min
Add 240µl of water to the water phase and turn the tube x2
Centrifuge for 10 min at 12,000xg at room temperature
Transfer 600µl of the upper colorless aqueous phase to new 2ml tubes
Condensation with isopropanol
Add 500µl isopropanol per 600µl aqueous phase
Incubate for 10min at room temperature OR overnight at -20°C
RNA Clean-up with Promega SV Total RNA isolation system (Z3100)
Make DNase Incubation mix: Per sample: Yellow Core Buffer - 40µl $MnCl_2$ 0.09M - 5µl DNase 1 - 5µl --> mix gently --> on ice
Mix the sample vigorously with a pipette
Transfer 700µl of the sample to Promega column
Centrifuge for 15 seconds at 13,400rpm, discard elute
If there is more of the sample, add it to the column and centrifuge, discard elute
Add 600µl RNA wash solution (RWA)
Centrifuge for 1min at 13,400rpm, discard elute
Add 50µl of DNase-mix in the middle of the membrane
Incubate for 15min at room temperature
Add 200µl of DNase stop solution (DSA)
Centrifuge for 1min at 13,400rpm, discard elute
Add 600µl RNA wash solution (RWA)
Centrifuge for 1min at 13,400rpm, discard elute
Add 250µl RNA wash solution (RWA)
Centrifuge for 2min at 13,400rpm, discard elute and transfer column to new tubes
Add 50µl of RNase-free water
Incubate for 1min
Centrifuge for 1min at 13,400rpm, save elute
Take the 50µl from the tube and pipette them onto the filter
Centrifuge for 1min at 13,400rpm -> discard filter
Measure the nanodrop
Store RNA in -80°C freezer

Measuring on NanoDrop:
- add 1 µl nuclease-free water to the petastal on the NanoDrop -> click 'OK' to initialize instrument -> wipe water off
- choose 'RNA'
- add 1 µl nuclease-free water -> click 'Blank' -> wipe water off
- add 1 µl sample -> write the name of the sample + click 'Measure' -> note results -> wipe off
- (repeat with all samples) -> save results
- add 1 µl nuclease-free water -> click 'Measure' -> wipe off
- Clean up before leaving

Appendix B - Protocol for cDNA synthesis

cDNA mastermix reagents	1x	46x
5 x M-MLV RT Buffer	5.0 µl	230 µl
dNTP 10 mM	1.3 µl	59.8 µl
Random hexamer primer 2 µg/µl	0.2 µl	9.2 µl
Oligo(dt) 0.5 µg/µl	0.4 µl	18.4 µl
RNase Inhibitor	0.8 µl	36.8 µl
MMLV enzyme (reverse transcriptase)	1.0 µl	46 µl
Sum	8.7 µl	
RNA sample	16.3 µl	
Total sum	25.0 µl	

Procedure for making cDNA from RNA:

- Write names of samples on lids of PCR tubes

- Add matermix reagents to a 1.5 ml tube (make x46 for 44 samples) → vortex + centrifuge

- Use electronic pipette to add 8.7 µl mastermix to each PCR tube (1 per sample) → centrifuge

- Vortex + centrifuge RNA sample → Add 16.3 µl RNA sample → vortex + centrifuge

- Run RT-PCR program on G-storm in PCR lab
(25°C for 10 min → 42°C for 60 min → 95°C for 5 min → 4°C → -20°C freezer)
    - Turn on machine
    - Open hood → Place the tubes with samples in the middle → Close hood
    - Press 'Run Program' → choose the folder named 'HOLLY' →
    - Choose the cDNA program ('CDNA.scr') in the folder → press 'Run Selected'
    - Enter the sample volume (25 µl) → press 'OK' to run the program
    - When the program is finished, remove samples and turn off machine

- Store cDNA in -20°C freezer

Appendix C: Protocol for making standard curves (qPCR), incl. quick guide to LightCycler

Bring ice *keep all tubes on ice at all times!

Serial dilution of cDNA:

Make cDNA pool:

- Mix + centrifuge cDNA samples
- 2 µl of each cDNA sample is collected in a 1.5 ml eppendorff tube
- Vortex and centrifuge

Dilute cDNA pool: (There will be enough for testing 8 primers (3 wells with 2 µl per well for each primer))

- Write on lids of six 1.5 ml eppendorff tubes: 1:4 ; 1:8 ; 1:16 ; 1:32 ; 1:64 ; 1:128
- Add 75 µl water to the 1:4-tube
- Add 50 µl water to the other 5 tubes
- Add 25 µl of the cDNA pool (vortexed + centrifuged) to the 1:4-tube --> vortex + centrifuge
- Take 50 µl from the 1:4-tube, and add it to the 1:8-tube --> vortex + centrifuge
- Take 50 µl from the 1:8-tube, and add it to the 1:16-tube --> vortex + centrifuge
- Take 50 µl from the 1:16-tube, and add it to the 1:32-tube --> vortex + centrifuge
- Take 50 µl from the 1:32-tube, and add it to the 1:64-tube --> vortex + centrifuge
- Take 50 µl from the 1:64-tube, and add it to the 1:128-tube --> vortex + centrifuge

---

Mastermix:

Dilute primers 1:10:

- Write name of primer on an eppendorff tube (two tubes per primer - one for "F" and one for "R")
- Vortex and centrifuge the primer
- Add 270 µl water and then 30 µl of the primer to the eppendorff tube --> vortex and centrifuge

Make Mastermix:

- Write name of primer + "M" on an eppendorff tube
- Make 24x Mastermix for each primer ---------->

(Remember to vortex + centrifuge primers before adding them to the Mastemix)

Mastermix	**1x**	**24x**
SYBR Green	5	120
Primer 10 µM F	1	24
Primer 10 µM R	1	24
dH_2O	1	24

Prepare well plate:

- Put well plate on ice + draw lines on it to divide it into sections
- Add 8 µl Mastermix to each well (21 wells for each primer)
- Add 2 µl diluted cDNA to each well (1:4 in three wells, then 1:8 in three wells, then 1:16 in three wells, then 1:32 in three wells, then 1:64 in three wells, then 1:128 in three wells, then water in three wells) - repeat for each primer
- Cover with plastic seal

		1	2	3	4	5	6	7	8	9	10	11	12
Primer 1	a	1:4	1:4	1:4	1:8	1:8	1:8	1:16	1:16	1:16	1:32	1:32	1:32
	b	1:64	1:64	1:64	1:128	1:128	1:128	W	W	W			
Primer 2	c	1:4	1:4	1:4	1:8	1:8	1:8	1:16	1:16	1:16	1:32	1:32	1:32
	d	1:64	1:64	1:64	1:128	1:128	1:128	W	W	W			
Primer 3	e	1:4	1:4	1:4	1:8	1:8	1:8	1:16	1:16	1:16	1:32	1:32	1:32
	f	1:64	1:64	1:64	1:128	1:128	1:128	W	W	W			
Primer 4	g	1:4	1:4	1:4	1:8	1:8	1:8	1:16	1:16	1:16	1:32	1:32	1:32
	h	1:64	1:64	1:64	1:128	1:128	1:128	W	W	W			

<u>Using LightCycler for making standard curves</u>: (Nb. no gloves when using the computer)

- Centrifuge plate + turn on machine
- Login in
- Choose "New Experiment from Template"
- Choose "Run Protocol Anne" --> ⊘
- **Go to "Subset editor"** --> Press ⊕ once for each primer --> Name new subsets after primers --> Choose which rows a primer belongs to (use Ctrl) --> Press "Apply" (for each primer)
- **Go to "Sample editor"** -->

in "*Step 1 - Select Workflow*": select "Rel Quant"
in "*Step 2 - Select Samples*": select a primer
in "*Step 3 - Edit Rel Quant Properties*": select "Standard" and "Reference"
--> fill in the following information:

Color	Repl Of	Sample Name	Combined Sample and Target Type	Concentration	
	A1	1,4	Ref Standard	0,25	
	A1	--II--	(Nb. all are automatically changed to Ref Standard by selecting "Standard Concentration" and "Reference" in "Step 3...")	--II--	
	A1	--II--		--II--	
	A4	1,8		0,125	
	A4	--II--		--II--	
	A4	--II--		--II--	
	A7	1,16		0,0625	
	A7	--II--		--II--	
	A7	--II--		--II--	
	A10	1,32		0,03125	
	A10	--II--		--II--	
	A10	--II--		--II--	
	B1	1,64		0,015625	
	B1	--II--		--II--	
	B1	--II--		--II--	
	B4	1,128		0,0078125	
	B4	--II--		--II--	
	B4	--II--		--II--	
	B7	W	Ref Negative		
	B7	--II--	Ref Negative		
	B7	--II--	Ref Negative		
	B10				
	B10				
	B10				

Nb. letter in "Repl Of" must correspond to the row on the plate (for first primer: A and B, for second primer: C and D ...)

- Save by clicking on the  -sign
- Put plate in machine --> **Go to "Experiment"** --> choose "Start Run" (takes 1½ h)

- **Go to "Analysis"** --> For each primer do the following:

Create new analysis: Tm calling --> choose the primer under "Subset" --> ⊘
--> unclick the empty wells (double click) --> Ctrl + A --> press Calculate.
*All curves should create the same top. If they do not then it is not a good primer.

- Click on  to return to overview.

Create new analysis: Abs Quant/2nd Derivate Max --> choose the primer under "Subset" --> ⊘
--> unclick the empty wells (double click) --> Ctrl + A --> press calculate.
*Efficiency should be 2.000, Slope should be -3.000.
- Look at details about the samples (wells).
*MeanCp should be below 30, STD Cp below 0.4. If it is above 0.4 then check if one of the wells has Cp that deviates from the others - if so try to unclick it (as odd results may be caused by bad pipetting in one well).
- Go back --> mark all of the samples again (Ctrl + A) --> press calculate.

- When all samples have been analyzed, close the window (it will ask if you want to save --> "Yes")
- Remove the well plate from the machine --> throw away
- Turn off machine

Appendix D: Protocol for qPCR, incl. quick guide to LightCycler

1. Make calibrator (cDNA pool diluted 1:10)

2. Dilute cDNA-samples (1:4)

3. Make Mastermix

	1x	141x
SYBR Green	5	705
Primer 10 µM F	1	141
Primer 10 µM R	1	141
dH_2O	1	141

Remember:
Always vortex and centrifuge
Only touch the edge of the tubes with cDNA to avoid heating the samples

4. Prepare 384 well plate (illustration below)

- Put well plate on ice + draw lines on it to divide it into sections
- Add 8 µl Mastermix to each well
- Add 2 µl calibrator to each of the first 3 wells, then 2 µl cDNA sample to 3 wells per sample, then 2 µl water to each of the last 3 wells
- Cover with plastic seal

Epicardial adipose tissue (44 samples):

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	calibrator			1755			1759			1761			1763			1764			1766			1767		
B	1768			1769			1770			1772			1774			1775			1777			1778		
C	1782			1783			1784			1785			1787			1788			1789			1792		
D	1796			1799			1802			1804			1807			1810			1816			1818		
E	1821			1822			1823			1826			1827			1828			12966			13204		
F	13366			13436			13489			14083			14104			water								
G	calibrator			1755			1759			1761			1763			1764			1766			1767		
H	1768			1769			1770			1772			1774			1775			1777			1778		
I	1782			1783			1784			1785			1787			1788			1789			1792		
J	1796			1799			1802			1804			1807			1810			1816			1818		
K	1821			1822			1823			1826			1827			1828			12966			13204		
L	13366			13436			13489			14083			14104			water								
M																								
N																								
O																								
P																								

Perirenal adipose tissue (43 samples)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	calibrator			1755			1759			1761			1763			1764			1766			1767		
B	1768			1769			1770			1772			1774			1777			1778			1782		
C	1783			1784			1785			1787			1788			1789			1792			1796		
D	1799			1802			1804			1807			1810			1816			1818			1821		
E	1822			1823			1826			1827			1828			12966			13204			13366		
F	13436			13489			14083			14104			water											
G	calibrator			1755			1759			1761			1763			1764			1766			1767		
H	1768			1769			1770			1772			1774			1777			1778			1782		
I	1783			1784			1785			1787			1788			1789			1792			1796		
J	1799			1802			1804			1807			1810			1816			1818			1821		
K	1822			1823			1826			1827			1828			12966			13204			13366		
L	13436			13489			14083			14104			water											
M																								
N																								
O																								
P																								

<u>Using LightCycler for advanced relative quantification analysis</u>: (Nb. no gloves when using the computer)

- Centrifuge plate --> back on ice
- Change the blocks in the machine (to the ones that fits the 384 well plate)
- Login → choose "New experiment from template" → choose "384 Run Protocol" → ⓥ
- **Go to "Subset editor"** → Press ⊕ once for each primer → Name new subsets after primers → Choose which rows a primer belongs to (use Ctrl) → Press "Apply" (for each primer)
- **Go to "Sample editor"** → in "*Step 1 - Select Workflow*": select "Rel Quant"

Fill in information in 'Repl Of', 'Sample Name' and 'Combined Sample and Target Type'
In 'Combined Sample and Target Type' the calibrator = 'Target PosCalibrator', while samples = 'Target Unknown'
(NB when analyzing ACTB (reference gene) the information must be changed from "Target .." to "Ref ..")

- Put plate in machine --> Go to "Experiment" --> choose "Start Run" (takes ≈ 1 h)

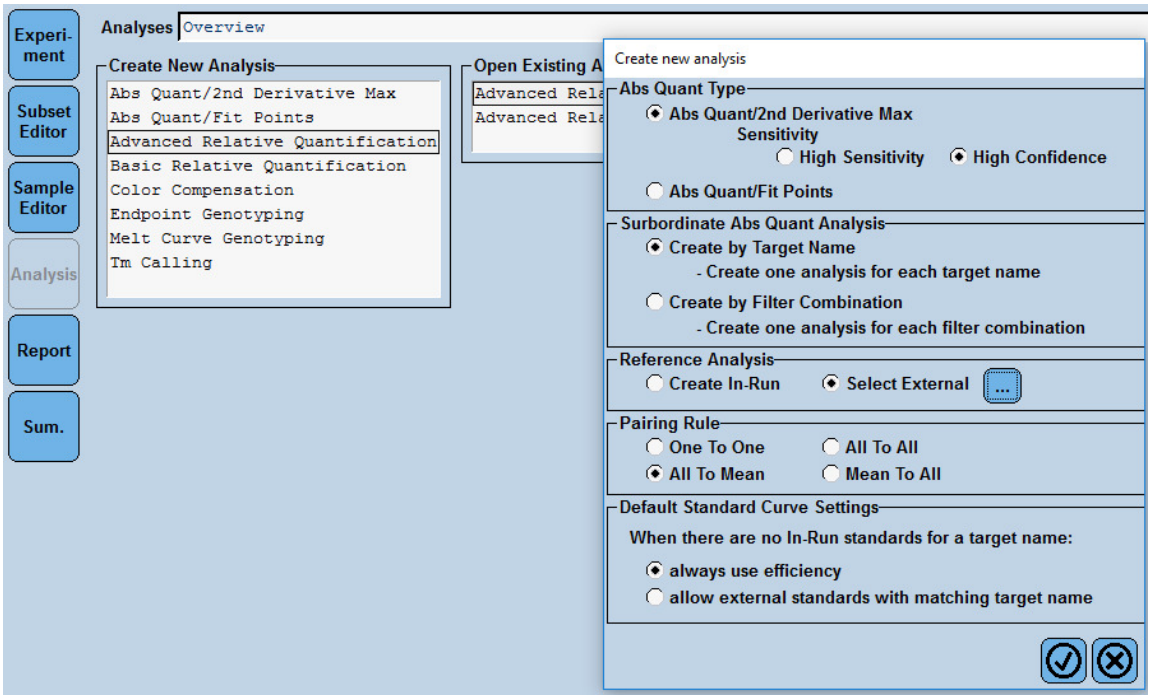
When the run is complete close the window (it will ask if you want to save --> "Yes")

- Remove plate --> Turn off machine --> Change the two blocks back to the standard ones (fits 96 well plates)

How to perform an Advanced Relative Quantification analysis (Adapted from 'LightCycler® 480 Instrument Operator's Manual, Software Version 1.5', 4.3.5 Performing an Advanced Relative Quantification Experiment. Roche Diagnostics GmbH, Mannheim, Germany):

- **Go to "Sample editor"** --> Change the efficiency for each primer to the efficiency of the standard curve

- **Go to "Analysis"** --> For each primer do the following:
Create new analysis: Advanced Relative Quantification --> choose the primer under "Subset" --> ⊘
In the following dialog box 'Create new analysis' the following boxes should be chosen: --> ⊘



The *Relative Quantification Analysis* screen opens.
Go to 'Target Name' --> double-click on the target gene to get to the **Abs Quant/2nd Derivate Max** analysis window --> choose "Replicate of" (above list of samples) --> (unclick the empty wells) --> Ctrl + A --> press calculate.
*For each sample, check that STD Cp is 0.40 or less.
*If it is above 0.40, one well must be unclicked (the one with Cp furthest from the others)
Return by clicking 'Back to Rel Quant'
Do the same for the reference gene
Go back to results --> press calculate

Appendix E: Overview of final models and results of ANOVA analysis

Perirenal adipose tissue. Final model (model with lowest AIC value; only fixed effects shown) and results of ANOVA analysis (significant P-values).

Gene	Final model	Ewed:Lambd:Sex	Ewed:Lambd	Ewed:Sex	Lambd:Sex	Ewed	Lambd	Sex
AdipoQ	AdipoQ ~ Lambd + Sex + Birthw + Lambbw2.5 + Lambd:Sex				NS		NS	0.0002
ATGL	ATGL ~ Ewed + Lambd + Sex + Ewed:Lambd + Ewed:Sex		NS	NS		NS	NS	0.0064
CD-34	CD.34 ~ Ewed + Lambd + Sex + Birthw + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	0.0478	NS	0.0062	NS	0.0432	NS	<0.0001
CD-44	log(CD.44) ~ Ewed + Lambd + Sex + Ewebw + Birthw + Ewed:Lambd + Ewed:Sex		NS	0.0197		0.0197	NS	NS
CGI-58	log(CGI.58) ~ Ewed + Lambd + Sex + Lambbw6 + Ewed:Sex + Lambd:Sex			NS	NS	0.0199	NS	<0.0001
FABP4	log(FABP4) ~ Ewed + Lambd + Sex + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex		NS	NS	0,0256	0,0064	NS	<0.0001
FAS	FAS ~ Ewed + Lambd + Sex + Ewebw + Ewebcs + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	0.0344	NS	NS	NS	NS	NS	NS
FBPase	FBPase ~ Lambd + Sex + Lambd:Sex				NS		NS	0.0001
GcR	GcR ~ Ewed + Lambd + Sex + Birthw + Ewed:Sex + Lambd:Sex			0.0139	NS	0.0100	NS	<0.0001
GLUT-1	GLUT.1 ~ Ewed + Lambd + Sex + Ewebcs + Birthw + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	0.0025	NS	0.0003
GLUT-4	GLUT.4 ~ Ewed + Lambd + Sex + Birthw + Ewed:Sex + Lambd:Sex			0.0204	0.0007	NS	NS	<0.0001
HSL	HSL ~ Ewed + Lambd + Sex + Ewebcs + Ewed:Lambd + Ewed:Sex		0.0188	0.0230		0.0257	NS	<0.0001
IGF1R	log(IGF1R) ~ Ewed + Lambd + Sex + Ewebcs + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	0.0156	NS	0.0001
IL-6	log(IL.6) ~ Ewed + Lambd + Sex + Ewebw + Lambbw2.5 + Ewed:Lambd + Ewed:Sex		0.0456	NS		0.0329	NS	NS
Leptin	Leptin ~ Ewed + Lambd + Sex + Birthw + Lambbw6 + Lambbw2.5 + Ewed:Lambd		NS			NS	NS	0.0031
LPL	LPL ~ Ewed + Lambd + Sex + Lambbw6 + Ewed:Lambd + Ewed:Sex		NS	NS		NS	NS	NS
MCP-1	log(MCP.1) ~ Ewed + Lambd + Sex + Ewebw + Birthw + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	0.0051	NS	NS	NS	0.0308	NS
PLIN-1	log(Perilipin.1) ~ Ewed + Lambd + Sex + Ewebw + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	0.0109	<0.0001
PPAR-γ	log(PPAR.g) ~ Lambd + Sex + Lambbw2.5 + Lambd:Sex				NS		NS	<0.0001
TGF-β1	TGF.b1 ~ Ewed + Sex + Birthw + Ewed:Sex			0.0001		0.0095		0.0237
TLR4	TLR.4 ~ Ewed + Lambd + Sex + Ewebw + Birthw + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	NS
VEGF	VEGF ~ Ewed + Lambd + Sex + Ewebw + Ewebcs + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	0.0184	NS	0.0298	0.0464	NS	<0.0001
VEGF-A	VEGF.A ~ Ewed + Sex + Ewebw + Birthw + Lambbw2.5 + Ewed:Sex			NS		0.0084		<0.0001

Epicardial adipose tissue. Final model (model with lowest AIC value; only fixed effects shown) and results of ANOVA analysis (significant P-values).

Gene	Final model	Ewed:Lambd:Sex	Ewed:Lambd	Ewed:Sex	Lambd:Sex	Ewed	Lambd	Sex
AdipoQ	log(AdipoQ) ~ Lambd + Ewebcs						0.0304	
ATGL	ATGL ~ Ewed + Lambd + Sex + Ewebw + Birthw + Lambbw6 + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	0.0072	0.0154	0.0076	NS	NS	NS	NS
CD-34	CD.34 ~ Lambd + Sex + Lambbw6 + Lambbw2.5 + Lambd:Sex				0.0364		NS	0.0011
CD-44	log(CD.44) ~ Sex + Ewebw + Lambbw2.5							NS
CGI-58	CGI.58 ~ Ewed + Lambd + Sex + Ewebw + Birthw + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	0.0121	NS	NS	NS	NS	0.0326	0.0020
FABP4	log(FABP4) ~ Ewed + Lambd + Sex + Ewebcs + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	0.0044	NS	NS	NS	NS	0.0327	<0.0001
FAS	log(FAS) ~ Lambd + Ewebw + Ewebcs + Birthw + Lambbw6						0.0054	
FBPase	FBPase ~ Ewed + Lambd + Ewebw + Ewed:Lambd		NS			NS	NS	
GcR	GcR ~ Ewed + Sex + Lambbw6 + Ewed:Sex			0.0168		NS		0.0168
GLUT-1	log(GLUT.1) ~ Ewed + Lambd + Sex + Ewebw + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	0.0419	NS	0.0112	NS	NS	NS
GLUT-4	GLUT.4 ~ Ewed + Sex + Ewebw					0.0163		NS
HSL	HSL ~ Lambd + Sex + Birthw + Lambd:Sex				NS		0.0021	NS
IGF1R	log(IGF1R) ~ Ewed + Sex + Ewebw + Lambbw2.5 + Ewed:Sex			0.0417		NS		NS
IL-6	log(IL.6) ~ Sex + Birthw + Lambbw6							NS
Leptin	Leptin ~ Lambd + Sex + Birthw + Lambbw6 + Lambbw2.5 + Lambd:Sex				NS		NS	NS
LPL	LPL ~ Ewed + Lambd + Sex + Birthw + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	0.0001
MCP-1	log(MCP.1) ~ Ewed + Lambd + Sex + Ewebw + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	NS
PLIN-1	Perilipin.1 ~ Ewed + Lambd + Sex + Birthw + Ewed:Lambd		NS			NS	0.0454	NS
PPAR-γ	PPAR.g ~ Ewed + Lambd + Sex + Ewebcs + Ewed:Lambd		NS			NS	0.0222	0.0470
TGF-β1	TGF.b1 ~ Ewed + Lambd + Sex + Ewebw + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	0.0144	NS	NS	NS	NS
TLR4	TLR.4 ~ Ewed + Lambd + Birthw + Ewed:Lambd		0.0245			NS	NS	
VEGF	VEGF ~ Ewed + Lambd + Sex + Ewebw + Ewebcs + Birthw + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	0.0295	NS	NS	NS	0.0107
VEGF-A	VEGF.A ~ Ewed + Lambd + Sex + Ewebw + Ewebcs + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	0.0053

Subcutaneous adipose tissue. Final model (model with lowest AIC value; only fixed effects shown) and results of ANOVA analysis (significant P-values).

Gene	Final model	Ewed:Lambd:Sex	Ewed:Lambd	Ewed:Sex	Lambd:Sex	Ewed	Lambd	Sex
AdipoQ	log(AdipoQ) ~ Ewed + Lambd + Sex + Ewed:Lambd + Ewed:Sex + Lambd:Sex		0.0268	NS	NS	NS	NS	0.0011
ATGL	log(ATGL) ~ Ewed + Sex + Ewebw + Lambbw6 + Ewed:Sex			0.0189		NS		NS
CD-34	CD.34 ~ Ewed + Lambd + Sex + Ewebw + Ewed:Lambd + Ewed:Sex		NS	0.0193		0.0173	NS	0.0245
CD-44	CD.44 ~ Ewed + Lambd + Sex + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	<0.0001	<0.0001	<0.0001	0.0005	<0.0001	0.0010	0.0005
CGI-58	log(CGI.58) ~ Ewed + Lambd + Sex + Ewebcs + Lambbw6 + Ewed:Lambd + Lambd:Sex		0.0161		NS	NS	NS	0.0083
FABP4	FABP4 ~ Ewed + Lambd + Sex + Ewebw + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	0.0224
FAS	FAS ~ Ewed + Lambd + Sex + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	0.0346	NS	NS	NS	NS
FBPase	FBPase ~ Ewed + Lambd + Sex + Ewebcs + Lambbw6 + Ewed:Lambd + Lambd:Sex		0.0379		0.0013	NS	NS	NS
GcR	GcR ~ Ewed + Lambd + Sex + Ewebcs + Lambbw2.5 + Ewed:Lambd + Ewed:Sex		NS	0.0414		NS	NS	NS
GLUT-1	GLUT.1 ~ Ewed + Lambd + Sex + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	0.0086	NS	0.0123	NS	NS	0.0037	NS
GLUT-4	log(GLUT.4) ~ Ewed + Lambd + Sex + Lambbw6 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	NS
HSL	HSL ~ Ewed + Lambd + Sex + Ewed:Lambd + Ewed:Sex		0.0358	0.0291		NS	NS	0.0221
IGF1R	IGF1R ~ Ewed + Lambd + Sex + Ewebw + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	0.0125	0.0004	NS	0.0191	NS	0.0009
IL-6	IL.6 ~ Ewed + Lambd + Sex + Ewebcs + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Leptin	log(Leptin) ~ Ewed + Lambd + Sex + Ewebw + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	0.0425	NS	NS	NS	NS	NS
LPL	LPL ~ Ewed + Lambd + Sex + Lambbw6 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	0.0207	0.0052	NS	0.0425	0.0118	NS	0.0009
MCP-1	MCP.1 ~ Ewed + Lambd + Sex + Ewebw + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	<0.0001
PLIN-1	log(Perilipin.1) ~ Ewed + Lambd + Sex + Ewed:Lambd + Lambd:Sex		0.0494		NS	NS	NS	0.0006
PPAR-γ	PPAR.g ~ Ewed + Lambd + Sex + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	0.0040	NS
TGF-β1	TGF.b1 ~ Ewed + Lambd + Sex + Birthw + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	0.0061	NS	NS	NS	NS	NS	NS
TLR4	TLR.4 ~ Ewed + Lambd + Sex + Ewebw + Ewed:Lambd + Ewed:Sex		NS	0.0306		0.0282	NS	0.0375
VEGF	log(VEGF) ~ Ewed + Lambd + Sex + Ewed:Lambd + Ewed:Sex + Lambd:Sex		0.0047	NS	NS	NS	NS	0.0001
VEGF-A	log(VEGF.A) ~ Ewed + Lambd + Sex + Ewebcs + Lambbw6 + Ewed:Lambd + Lambd:Sex		0.0033		NS	NS	NS	0.0006

Mesenteric adipose tissue. Final model (model with lowest AIC value; only fixed effects shown) and results of ANOVA analysis (significant P-values).

Gene	Final model	Ewed:Lambd:Sex	Ewed:Lambd	Ewed:Sex	Lambd:Sex	Ewed	Lambd	Sex
AdipoQ	log(AdipoQ) ~ Birthw							
ATGL	ATGL ~ Ewed + Lambd + Sex + Ewebw + Lambbw6 + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	NS
CD-34	CD.34 ~ Ewed + Lambd + Sex + Ewebw + Ewebcs + Birthw + Lambbw6 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	0.0209	0.0255	NS	0.0386	NS	0.0222
~~CD-44~~	~~(CD.44)~~	~~Not available~~	——	——	——	——	——	——
CGI-58	CGI.58 ~ Ewed + Lambd + Sex + Ewebw + Ewebcs + Lambbw6 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	NS
FABP4	FABP4 ~ Ewed + Lambd + Ewebw + Ewebcs + Ewed:Lambd		0.0294			NS	NS	
FAS	log(FAS) ~ Ewed + Lambd + Sex + Ewebw + Ewebcs + Birthw + Lambbw6 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	NS
FBPase	FBPase ~ Ewed + Lambd + Sex + Ewebcs + Birthw + Ewed:Lambd + Ewed:Sex		NS	NS		NS	NS	0.0035
GcR	log(GcR) ~ Ewed + Sex + Ewebw + Ewed:Sex			NS		NS		NS
GLUT-1	log(GLUT.1) ~ Sex + Birthw							NS
GLUT-4	log(GLUT.4) ~ Ewed + Lambd + Ewebcs + Birthw + Lambbw2.5 + Ewed:Lambd		NS			NS	NS	
HSL	HSL ~ Lambd + Sex + Lambd:Sex				NS		NS	NS
IGF1R	log(IGF1R) ~ Ewed + Lambd + Sex + Birthw + Lambbw2.5 + Ewed:Lambd		NS			NS	NS	0.0129
IL-6	IL.6 ~ Ewed + Lambd + Sex + Ewebw + Birthw + Lambbw6 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	NS
Leptin	Leptin ~ Sex							NS
LPL	LPL ~ Ewed + Lambd + Sex + Ewebw + Lambbw6 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	NS
MCP-1	MCP.1 ~ Ewed + Lambd + Sex + Ewebw + Birthw + Lambbw6 + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	NS
PLIN-1	log(Perilipin.1) ~ Sex + Birthw + Lambbw2.5							NS
PPAR-γ	PPAR.g ~ Ewed + Lambd + Sex + Ewebw + Ewebcs + Lambbw6 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	0.0348	NS	NS	NS	NS	NS
TGF-β1	log(TGF.b1) ~ Ewed + Lambd + Sex + Ewebw + Lambbw2.5 + Ewed:Lambd + Ewed:Sex + Lambd:Sex + Ewed:Lambd:Sex	NS	NS	NS	NS	NS	NS	NS
TLR4	log(TLR.4) ~ Ewed + Lambd + Sex + Ewebcs + Ewed:Lambd		NS			NS	NS	NS
VEGF	log(VEGF) ~ Ewed + Lambd + Sex + Ewebcs + Lambbw2.5 + Ewed:Lambd + Ewed:Sex		NS	NS		NS	NS	NS
VEGF-A	VEGF.A ~ Lambd						NS	

Appendix F: Examples of R-scripts (using CGI-58 from perirenal adipose tissue as an example)

```
> setwd("C:/Users/Lise/Desktop/Dataanalyse/Perirenal/no_control/")
> Expression_with_control <- read.table("All data Perirenal.txt", header=T, sep="\t")
> Expression_no_control <- Expression_with_control[1:36,]
> Expression_no_control$Lamb <- factor(Expression_no_control$Lamb)
> Expression_no_control$Ewe <- factor(Expression_no_control$Ewe)
> library(nlme)
> library(MASS)
> library(estimability)
> library(lsmeans)
> library(ggplot2)
> library(ggsignif)
```

Initial model:

```
> CGI.58_lme_no_control <- lme(CGI.58 ~ Ewed*Lambd*Sex + Ewebw + Ewebcs + Birthw + Lambbw6 +
Lambbw2.5, random=~1|Lamb/Ewe, na.action = na.omit, data=Expression_no_control, method="ML")
```

Model validation: Residual plot

```
> plot(fitted(CGI.58_lme_no_control),residuals( CGI.58_lme_no_control), ylim = c(-0.25,0.25))
> abline(h=c(-1.96*sd(residuals(CGI.58_lme_no_control)), 0,1.96*sd(residuals(
CGI.58_lme_no_control))), lty=2)
```

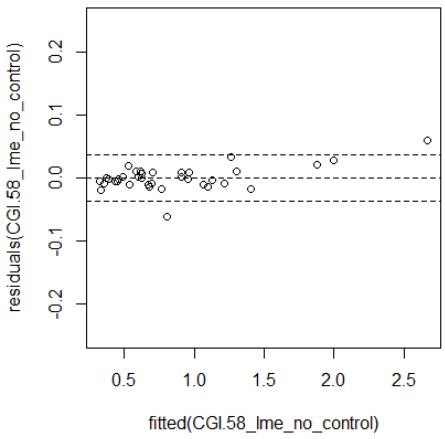
Model validation: Shapiro-Wilks test

```
> shapiro.test(residuals(CGI.58_lme_no_control))

        Shapiro-Wilk normality test

data:  residuals(CGI.58_lme_no_control)
W = 0.90157, p-value = 0.003769
```

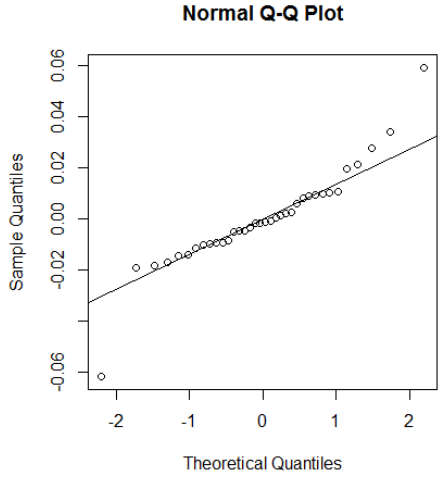
Model validation: Quantile-quantile plot

```
> qqnorm(residuals(CGI.58_lme_no_control));qqline(residuals( CGI.58_lme_no_control))
```

Residual plot:



Quantile-quantile plot



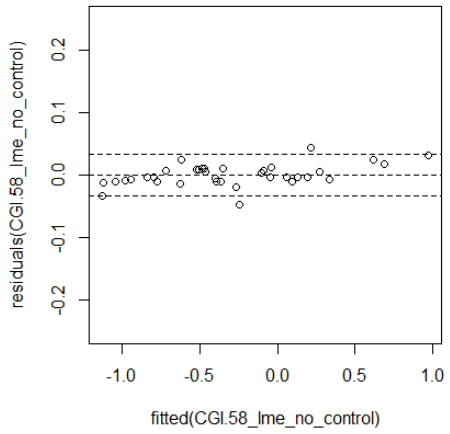
*Shapiro-Wilks normality test and plots indicate that data must be log-transformed

Log-transformed model:

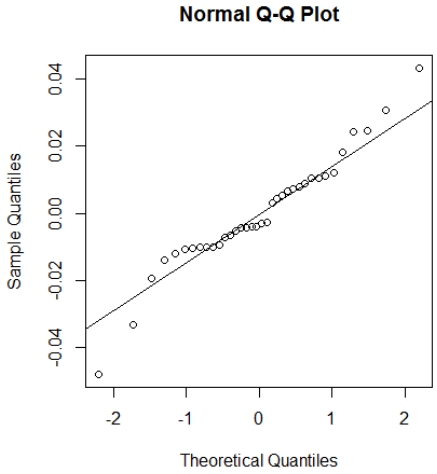
```
> CGI.58_lme_no_control <- lme(log(CGI.58) ~ Ewed*Lambd*Sex + Ewebw + Ewebcs + Birthw +
Lambbw6 + Lambbw2.5, random=~1|Lamb/Ewe, na.action = na.omit, data=Expression_no_control,
method="ML")

> shapiro.test(residuals(CGI.58_lme_no_control))

        Shapiro-Wilk normality test

data:  residuals(CGI.58_lme_no_control)
W = 0.95582, p-value = 0.1594
```

(the residual plot and quantile-quantile plot are extracted with the same formula as written above)

Residual plot



Quantile-quantile plot



Stepwise comparison of AIC values

```
> CGI.58_T<- stepAIC(CGI.58_lme_no_control,direction="both")
```

[output not shown. Model with smallest AIC value is extracted with the command below]

```
> summary(CGI.58_T)
[entire output not shown]
Fixed effects: log(CGI.58) ~ Ewed + Lambd + Sex + Lambbw6 + Ewed:Sex + Lambd:Sex
```

Final model:

```
> CGI.58_Final_REML <- lme(log(CGI.58) ~ Ewed + Lambd + Sex + Lambbw6 + Ewed:Sex + Lambd:Sex,
random=~1|Lamb/Ewe, na.action = na.omit, data=Expression_no_control, method="REML")
```

ANOVA performed to find significant effects:

```
> anova(CGI.58_Final_REML)
            numDF denDF  F-value p-value
(Intercept)     1    27 29.94056  <.0001
Ewed            2    27  4.54658  0.0199   ← Significant p-value
Lambd           1    27  1.06212  0.3119
Sex             1    27 43.40121  <.0001   ← Significant p-value
Lambbw6         1    27  2.69759  0.1121
Ewed:Sex        2    27  2.65990  0.0882
Lambd:Sex       1    27  2.48501  0.1266
```

Pairwise comparison of the significant parameters:

- Pariwise comparisons of the prenatal dietary treatments (Ewed):

```
> lsmeans(CGI.58_Final_REML, pairwise~Ewed)
$lsmeans
 Ewed     lsmean         SE df   lower.CL    upper.CL
 HIGH -0.3262608 0.09717605 27 -0.5256496 -0.12687203
 LOW  -0.1621696 0.09000081 27 -0.3468360  0.02249685
 NORM -0.3826262 0.10693491 27 -0.6020386 -0.16321394

Results are averaged over the levels of: Lambd, Sex
Results are given on the log (not the response) scale.
Confidence level used: 0.95

$contrasts
 contrast       estimate        SE df t.ratio p.value
 HIGH - LOW  -0.16409126 0.1326813 27  -1.237  0.4424
 HIGH - NORM  0.05636543 0.1433914 27   0.393  0.9186
 LOW - NORM   0.22045668 0.1390481 27   1.585  0.2690

Results are averaged over the levels of: Lambd, Sex
Results are given on the log (not the response) scale.
P value adjustment: tukey method for comparing a family of 3 estimates
```

- Pariwise comparisons of the genders (Sex; F=female, M=male):

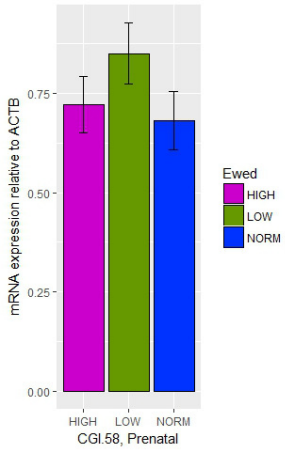
```
> lsmeans(CGI.58_Final_REML, pairwise~Sex)
$lsmeans
 Sex      lsmean         SE df    lower.CL   upper.CL
 F   -0.68022759 0.08555918 27 -0.85578051 -0.5046747
 M    0.09952317 0.09254985 27 -0.09037343  0.2894198

Results are averaged over the levels of: Ewed, Lambd
Results are given on the log (not the response) scale.
Confidence level used: 0.95

$contrasts
 contrast   estimate        SE df t.ratio p.value
 F - M    -0.7797508 0.1367087 27  -5.704  <.0001

Results are averaged over the levels of: Ewed, Lambd
Results are given on the log (not the response) scale.
```

Graph production

(the command type = "response" back-transforms the log-transformed data. NB when data have not been log-transformed the ggplot command must include 'y=lsmean' instead of 'y=response', as the column name changes depending on whether data has been transformed or not)

Graph of expression levels in the prenatal nutrition groups:

```
> d <- summary(lsmeans(CGI.58_Final_REML, pairwise~Ewed)$lsmeans, type = "response")
> ggplot(d, aes(x=Ewed, y=response, fill=Ewed)) +
+   scale_fill_manual(values=c("#CC00CC","#669900","#0033FF")) +
+   geom_bar(stat="identity", color="black", position=position_dodge())+
+   geom_errorbar(aes(ymin=response-SE, ymax=response+SE), width=.2) +
+   ylab("mRNA expression relative to ACTB") + xlab("CGI.58, Prenatal")
```



Graph of expression levels in females and males:

```
> d <- summary(lsmeans(CGI.58_Final_REML, pairwise~Sex)$lsmeans, type = "response")
> ggplot(d, aes(x=Sex, y=response, fill=Sex)) +
+   scale_fill_manual(values=c("#CC00CC","#0033FF")) +
+   geom_bar(stat="identity", color="black", position=position_dodge())+
+   geom_errorbar(aes(ymin=response-SE, ymax=response+SE), width=.2) +
+   ylab("mRNA expression relative to ACTB") + xlab("CGI.58, Gender")+
+   geom_signif(comparisons = list(c("M", "F")), annotations = "***")
```