FACULTY OF HEALTH AND MEDICAL SCIENCES
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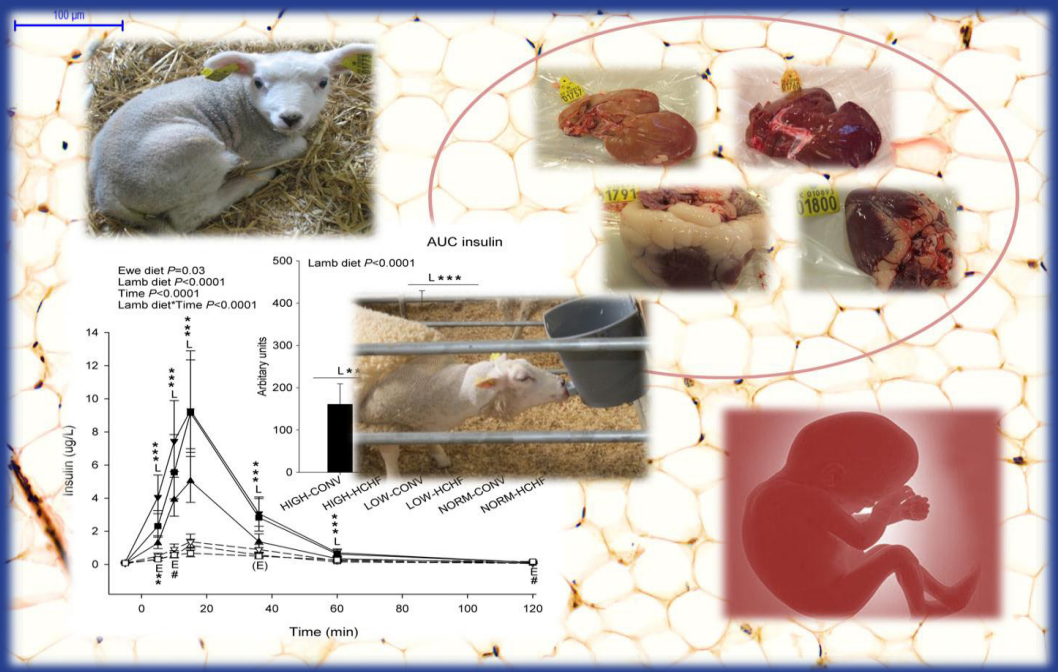
Impacts of late gestation malnutrition and an early postnatal high-fat diet on metabolic, endocrine and adipose tissue function and development in sheep

PhD thesis Prabhat Khanal



Impacts of late gestation malnutrition and an early postnatal high-fat diet on metabolic, endocrine and adipose tissue function and development in sheep

PhD thesis 2014

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Front cover:

Background, van Giessen stained adipocyte; Upper left corner; about a week old lamb; Bottom left corner; insulin responses during propionate fasted tolerance test; Centre; lamb suckling cream-milk mix; Upper right corner (encircled); liver and heart in postnatally high-fat fed (left side) or normally-fed lambs (right side); Bottom right corner, human foetus found at the internet (http://ayurveda.iloveindia.com/shows-pictures.php?%20img=foetal-development.jpg).

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Preface

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List of publications

Paper I: Khanal, P., Husted, S.V., Axel, A.M.D., Johnsen, L., Pedersen, K.L., Moretensen, M.S., Kongsted, A.H. & Nielsen, M.O. 2014. Late gestation over- and undernutrition predispose for visceral adiposity in response to a post-natal obesogenic diet, but with differential impacts on glucose–insulin adaptations during fasting in lambs. *Acta Physiol* **210,** 110–126.

Paper II: Khanal P., Axel, A.M.D, Kongsted, A.H., Husted, S.V., Johnsen, L., Pandey, D., Pedersen, K.L, Birtwistle, M., Markussen, B., Kadarmideen, H.N., & Nielsen, M.O. Maternal under- and overnutrition have differential impacts when combined with a postnatal obesogenic diet on glucose-lactate-insulin adaptations during metabolic challenges in adolescent sheep. *Acta Physiol* (passed first review).

Paper III: Khanal, P., Pedersen, K.L., Pandey, D., Safayi, S., Hou, L., Birtwistle, M., Symonds, M., Kadarmideen, H.N., & Nielsen, M.O. Differential impacts of late gestational under- and overnutrition on adipose tissue development and function and abdominal adiposity risk upon exposure to a postnatal high-fat diet in adolescent sheep. Manuscript.

Paper IV: Khanal, P., Axel, A.M.D., Johnsen, L., Hansen, P., Kongsted, A.H., Lyckegaard, N.B., & Nielsen, M.O. Long-term consequences of late gestation malnutrition and early postnatal high-fat diet on growth characteristics and metabolic adaptations to fasting in adult sheep. Manuscript.

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Abbreviations

11β-HSD2	11β-Hydroxysteroid Dehydrogenase type 2
ACCCA	Acetyl Co-enzyme A Carboxylase
AOC	Area Over the Curve
APOC3	Apolipoprotein C3
APOE	Apolipoprotein E
APOEA1	Apolipoprotein A1
ATM	Adipose Tissue Macrophages
AUC	Area Under Curve
BCS	Body Condition Score
BOHB	β-Hydroxy Butyrate
BUN	Blood Urea Nitrogen
C/EBP-β	CCAATT Enhancer Binding Protein-β
CD 68	Cluster of Differentiation 68
CFP	Centre for Fetal Programming
CNI	Cell Number Index
CONV	Conventional, Moderate
CSF2	Colony Stimulating Factor 2
DOHaD	Developmental Origins of Health and Disease
EC	External Controls
FABP4	Fatty Acid Binding Protein 4
FAS	Fatty Acid Synthase
FGF7	Fibroblast Growth Factor 7
FGR2IIB	Fibroblast Growth Factor Receptor 2IIB ligands
FOaD	Foetal Origins of Adult Disease
GAPDH	Glyceraldehyde 3 Phosphate Dehydrogenase
GGT	γ-Glutaryl Transferase
GLUT2	Glucose Transporter 2
GLUT4	Glucose Transporter 4
GTT	Glucose Tolerance Test
HCHF	High-Carbohydrate-High-Fat
HDL	High-Density Lipoprotein
HIGH	Overnutrition, 150% of energy and 110% of protein requirements

HSL	Hormone Sensitive Lipase
HSL	Hormone Sensitive Lipase
IDF	International Diabetes Foundation
IGF-1	Insulin-like Growth Factor-1
IGFBP-2	Insulin-like Growth Factor Binding Protein-2
IL6	Interleukin 6
INSRβ	Insulin Receptor β subunit
ITT	Insulin Tolerance Test
IUGR	Intrauterine Growth Restriction
IVGTT	Intravenous Glucose Tolerance Test
LDLr	Low Density Lipoprotein receptor
LGA	Large for Gestational Age
LOW	Undernutrition, 50% of energy and protein requirements
LPL	Lipoprotein Lipase
LSmeans	Least Square of Means
MCP-1	Monocyte Chemoattractant Protein-1
MGLL	Monoacylglycerides Lipase
mRNA	messenger Ribonuclecic Acid
NAFLD	Non-Alcoholic Fatty Liver Disease
NORM	Normal, fulfilling normal requirements of energy and protein
NOS2	Nitric Oxide Synthase 2
PAR	Predictive Adaptive Response
Pdx1	Pancreatic Duodenal Homeobox1
PI3K-p110	Phosphatidylinositol 3 Kinases p110 β catalytic subunit
PLIN1	Perilipin 1
PPARα	Perioxisome Proliferator-Activated Receptor α
PPARγ	Perioxisoe Proliferator-Activated Receptor γ
PPI	Protein-Protein Interactions
PTT	Propionate tolerance test
PUFA	Polyunsaturated Fatty Acids
qPCR	quantitative Polymerase Chain Reaction
SEM	Standard Error of the Mean
SGA	Small for Gestational Age

SREBP1	Sterol Regulatory Binding Protein 1
TG	Triglycerides
TLR4	Toll-Like Receptor 4
TNF-α	Tumour Necrosis Factor-α
UCP-2	Uncoupling Protein-2
VEGF	Vascular Endothelial Growth Factor
VFA	Volatile Fatty Acids
WHO	World Health Organization

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Summary

Aim: In this study the aim was to test the following hypotheses that i) late gestation under- and overnutrition have similar adverse impacts on fat deposition patterns and visceral adiposity risk but through differential impacts on metabolic and endocrine plasticity and functional development of different adipose tissues; ii) individuals exposed to late gestation under- or overnutrition have different susceptibilities towards intake of a high-carbohydrate-high-fat diet in early postnatal life, and a history of prenatal overnutrition provides a superior ability to cope with an early postnatal high-fat diet; iii) dietary (and body fat) correction later in life can to a great extent reverse adverse outcomes of an early postnatal high-fat diet.

Methodology: In a 3×2 factorial design, 36 twin-pregnant ewes were fed a NORM (fulfilling 100% of daily requirements for energy and protein), LOW (50% of NORM) or HIGH diet (150% of energy and 110% of protein requirements) during the last six weeks of gestation (term=147 days). Postnatally, the twin-lambs were subjected to a high-carbohydrate-high-fat (HCHF) or a moderate conventional diet (CONV) from three days to six months of age (just after puberty). At six months of age, intravenous tolerance tests using glucose (GTT), insulin (ITT) and propionate (PTT; gluconeogenetic precursor) were conducted and animals were exposed to 2-days of fasting and blood samples were collected at different time points during these tests. Sub-groups of animals were then slaughtered, and different organs and tissues were weighed and sampled. Morphological characteristics and gene expression for markers of lipid metabolism, angiogenesis and inflammation were studied in four different adipose depots (subcutaneous, mesenteric, perirenal, and epicardial). From six months to 2½ years of age, the remaining animals (including additional acquired undisturbed age-matched external controls from the same flock delivering the pregnant sheep) were fed a moderate, low-fat diet, and at 2½ years of age, metabolic and endocrine adaptability to fasting were again

examined, whereafter all animals were sacrificed and different organs and tissues were weighed and sampled.

Results: Adolescent sheep that had been exposed to either prenatal LOW- or HIGH nutrition had reduced subcutaneous fat deposition when fed a high-fat diet, and higher ratios of mesenteric and perirenal fat relative to subcutaneous fat compared to NORM. The HIGH and LOW lambs had lower expression of fatty acid binding protein-4 (FABP4), hormone sensitive lipase (HSL) and vascular endothelial growth factor A (VEGFA) as compared to NORM lambs in subcutaneous adipose tissue, and lower expression of FABP4, GLUT4 and PPAR-γ genes in epicardial adipose tissue. Moreover, both HIGH and LOW lambs had reduced non-obese cellularity in subcutaneous and mesenteric fat and reduced obesity-induced hyperplasia in subcutaneous, mesenteric and perirenal fat and had marked expandability of perirenal adipocytes in response to an early postnatal HCHF diet. The LOW fetal nutrition induced higher expression of monocyte chemoattractant protein 1 (MCP-1) and interleukin-6 (IL-6) genes in perirenal adipose tissue compared to HIGH and NORM lambs. Prenatal malnutrition differentially impacted adaptations of particularly plasma lactate followed by glucose, cholesterol and insulin. This was most clearly expressed during PTT in fasted lambs and much less convincingly during ITT and GTT. In fasted lambs, propionate challenge induced more dramatic increases in lactate than glucose, and HIGH lambs became more hyperglycaemic, hyperlactataemic and secreted less insulin compared to the hypercholesterolaemic LOW lambs. Propionate-induced insulin secretion was virtually abolished in fasted HCHF lambs, but upregulated in fasted compared to fed CONV lambs. HCHF lambs had the greatest insulin secretory responses during GTT. Irrespective of the prenatal diet, high-fat fed lambs developed characteristics resembling what is observed in humans suffering from the metabolic syndrome with higher plasma glucose, cholesterol, non-esterified fatty acids, triglyceride, and lactate combined with abdominal obesity. In all

adipose depots studied, the postnatal HCHF diet increased adipocyte cross-sectional area and expression of inflammatory markers and decreased the expression of genes associated with lipid metabolism.

Even after dietary correction for 2 years, LOW-HCHF sheep had the highest plasma levels of creatinine, cholesterol and highest fasting induced urea-nitrogen among all groups, however, the prenatal impacts on metabolic and endocrine plasticity disappeared in the NORM and HIGH sheep. LOW and NORM, but not HIGH, sheep previously fed the HCHF diet tended to have higher mesenteric fat deposition than the CONV fed. Most of the changes in metabolic function resembling the metabolic syndrome (higher plasma glucose, cholesterol, non-esterified fatty acids, triglyceride) due to the early postnatal high-fat diet also disappeared upon dietary correction in adulthood except that the previously HCHF-fed sheep had increased subcutaneous to perirenal and mesenteric to perirenal fat deposition ratios as adults. These long-term impacts of early life nutrition were found among both males and females.

Conclusion: Findings from this study highlight that adipose tissue, liver and pancreas are key targets of adverse foetal programming. Both prenatal under- and overnutrition predisposed for early development of visceral adiposity, apparently by interfering with lipid metabolism (subcutaneous fat), non-obese cellularity (subcutaneous and mesenteric fat) and obesity-induced hyperplasia (subcutaneous, mesenteric and perirenal fat), which was associated with increased obesity-induced hypertrophy in perirenal fat. Prenatally undernourished animals had lower tolerance towards the postnatal high fat diet as they had increased obesity-induced hypertrophy and reduced obesity-induced hyperplasia in mesenteric fat and such a diet in early life had long-term adverse consequences related to cholesterol and nitrogen (urea and creatinine) metabolism. Prenatally overnourished animals were not protected from the hyperglycaemic effect of a high-fat diet in early life as such, but they did appear to have

superior ability compared to the other groups to recover from the adverse consequences when they underwent dietary correction and body fat loss. Prenatal over- but not undernutrition also interfered with pancreatic insulin secretory plasticity by non-glucose-dependent pathways during early life. This shows that late gestation under- and overnutrition can predispose for early abdominal adiposity and associated metabolic disorders, but the underlying mechanisms are not identical. The potentials for recovery later in life upon dietary correction appeared to be more favourable in individuals exposed to late gestation overnutrition compared to undernutrition. Although, exposure to a high-fat diet in early postnatal life had long-term implications for fat deposition patterns, most of the metabolic features of the early postnatal high-fat diet disappeared upon dietary correction to a moderate low-fat diet in adult life except in prenatally undernourished individuals. This suggests that differential nutritional strategies are needed targeting individuals born at the different ends of the birth weight spectrum. Recommendation for moderate weight gain and prevention of development of adiposity during early life is crucial particularly in individuals exposed to prenatal undernutrition.

Sammendrag (Dansk)

Formål: Dette studie havde til formål at teste følgende hypoteser: i) under- og overernæring i sen drægtighed har samme negative indvirkning på fedtdeponeringsmønster og visceral fedme risiko, men gennem forskellig indvirkning på metabolisk og endokrin plasticitet og funktionel udvikling i forskellige fedtvæv; ii) individer udsat for under- eller overernæring i sen drægtighed har forskellig følsomhed overfor indtagelse af en høj-kulhydrat-høj-fedt diæt i det tidlige postnatale liv, og en fortid med prenatal overernæring forbedrer evnen til at håndtere en tidlig postnatal høj-fedt diæt; iii) diæt (og kropsfedt) korrektion senere i livet kan i stor udstrækning omvende de negative effekter af en tidlig postnatal høj-fedt diæt.

Metode: I et 3×2 faktorielt design, blev 36 tvillinge-drægtige får fodret med en NORM (svarende til 100% af det daglige energi- og proteinbehov), LOW (50% af NORM) eller HIGH (150% og 100% af det daglige energi- hhv. proteinbehov) diæt i de sidste seks uger af drægtigheden (drægtighedslængde=147 dage). Postnatalt blev tvillingelammene fodret op på hver deres diæt, enten en høj-kulhydrat-høj-fedt (HCHF) eller en moderat konventionel (CONV) diæt fra de var tre dage til seks måneder gamle (lige efter puberteten). I en alder af seks måneder, blev der udført intravenøse tolerance tests med glukose (GTT), insulin (ITT) og propionat (PPT; glukoneogenetisk substrat). Dyrene gennemførte desuden en faste på to dage, og blodprøver blev indsamlet på forskellige tidspunkter under disse tests. En undergruppe af dyrene blev herefter aflivet, og forskellige organer og væv blev indsamlet og vejet. Morfologiske karakteristika og genekspression af markører for fedtmetabolisme, angiogenese og inflammation blev studeret i fire forskellige fedtdepoter (subkutant, mesenterisk, perirenalt og epikardial). Fra en alder af seks måneder til 2½ år blev de resterende dyr samt en yderligere gruppe af eksterne kontroldyr (med samme alder og indkøbt fra den samme besætning, der leverede de drægtige får) fodret en moderat lav-fedt

diæt. Ved 2½ års alderen blev der gennemført studier over metaboliske og endokrine tilpasning til faste, hvorefter alle dyrene blev slagtet og forskellige organer og væv blev vejet og prøver blev udtaget.

Resultater: Lam, der havde været udsat for enten prenatal LOW eller HIGH ernæring, havde en reduceret subkutan fedtdeponering, når de blev fodret en høj-fedt diæt samt en højere andel af mesenterisk og perirenalt fedt i forhold til NORM dyrene. HIGH og LOW lammene havde lavere ekspression af fatty acid binding protein-4 (FABP4), hormone sensitive lipase (HSL) og vascular endothelial growth factor A (VEGFA), sammenlignet med NORM lammene, i det subkutane fedtvæv og lavere ekspression af FABP4, GLUT4 og PPAR-γ i det epikardielle fedtvæv. Ydermere havde både HIGH og LOW lammene reduceret cellularitet i subkutant og mesenterisk fedt i ikke-overvægtig tilstand og reduceret fedme-induceret hyperplasi i subkutant, mesenterisk og perirenalt fedt, og de havde samtidig en enorm ekspansionsevne af perirenale fedtceller som respons til den postnatale HCHF diæt. Føtal LOW ernæring fremkaldte en højere ekspression af monocyte chemoattractant protein 1 (MCP-1) og interleukin-6 (IL-6) i perirenalt fedtvæv sammenlignet med HIGH og NORM lam. Prenatal fejlernæring påvirkede tilpasningsevnen forskelligt af især plasma laktat fulgt af glukose, kolesterol og insulin. Dette var tydeligst ved PTT hos de fastede lam og mindre tydeligt under ITT og GTT. Hos de fastede lam fremkaldte propionat mere dramatiske stigninger for laktat end for glukose og HIGH lam blev mere hyperglykæmiske, hyperlaktatæmiske og udskilte mindre insulin sammenlignet med de hyperkolesterolæmiske LOW lam. Det propionat-inducerede insulin sekretions respons blev stort set elimineret hos de fastede HCHF lam, mens det hos de CONV lam blev opreguleret under faste i forhold til i fodret tilstand. HCHF lam havde den største insulinsekretions respons under GTT. Uafhængig af den prenatale diæt, udviklede de HCHF fodrede lam karakteristika, der ligner dem der er observeret hos mennesker som lider af det metaboliske syndrom, dvs. højere

plasma niveauer af glukose, kolesterol, ikke-esterificerede frie fedtsyrer, triglycerider og laktat kombineret med abdominal fedme. I alle de studerede fedtvæv inducerede den postnatale HCHF diæt forøget adipocyt tværsnits areal og øget ekspression af inflammationsmarkører og nedsat ekspressionen af gener associeret med fedtmetabolisme.

Selv efter en diæt korrektion gennem to år havde LOW-HCHF fårene fortsat de højeste plasma niveauer af kreatinin, kolesterol og det højeste faste-inducerede urea-nitrogen blandt alle grupper. Men de prenatale påvirkninger på metabolisk og endokrin plasticitet var ikke længere synlige hos NORM og HIGH får. LOW og NORM får, men ikke HIGH, der tidligere havde modtaget en HCHF diæt, havde en tendens til øget mesenterisk fedtdeponering i forhold til dem der havde modtaget CONV diæten. De fleste af de forandringer, der associerer til det metaboliske syndrom (øget plasma glukose, kolesterol, ikke-esterificerede frie fedtsyrer, triglycerider) forårsaget af den tidlige postnatale høj-fedt diæt, forsvandt også efter en diæt korrektion hos de voksne får bortset fra, at de tidligere HCHF-fodrede får fortsat havde en øget subkutan til perirenal og mesenterisk til perirenal fedtdeponerings ratio som voksne. Disse langsigtede påvirkninger af tidlig ernæring blev fundet hos både hanner og hunner.

Konklusion: Resultaterne fra dette studie viser at fedtvæv, lever og bugspytkirtel er centrale målorganer for føtal programmering. Både prenatal under- og overernæring disponerer for tidlig udvikling af visceral fedme, umiddelbart ved at interagere med fedtmetabolismen (subkutant fedt), intrinsic cellularitet (subkutant og mesenterisk fedt) og fedme-induceret hyperplasi (subkutant, mesenterisk og perirenalt fedt), hvilket prædisponerede for øget fedmeinduceret hypertrofi i perirenalt fedtvæv. Prenatalt underernærede dyr havde lavere tolerance overfor en postnatal høj-fedt diæt, eftersom de udviste øget fedme-induceret hypertrofi og reduceret fedme-induceret hyperplasi i mesenterisk fedt, og det var forbundet med langsigtede negative konsekvenser relateret til kolesterol og nitrogen (urea og kreatinin)

metabolisme. Prenatalt overernæret dyr var ikke bedre beskyttet mod den hyperglykæmiske effekt af en høj-fedt diæt i det tidlige liv som sådan, men de så ud til at have en bedre evne, sammenlignet med de andre grupper, til at komme sig oven på de negative konsekvenser efter langvarig diætetisk korrektion og vægt (fedt-) tab. Eksponering for prenatal over- men ikke underernæring interfererede med plasticiteten i pankreas' insulinsekretion via glukose-uafhængige pathways hos lam. Dette viser at under- og overernæring i sen drægtighed kan disponere for tidlig abdominal fedme og associerede metaboliske lidelser, men de underlæggende mekanismer er ikke identiske. Udsigten til genopretning senere i livet via diætetisk korrektion viste sig dog at være mere favorabel hos individer der har været udsat for overernæring i sen drægtighed sammenlignet med underernæring. Selvom eksponering for en høj-fedt diæt i det tidlige postnatale liv havde livslange implikationer for fedtaflejringsmønstre, forsvandt de fleste af de metaboliske effekter induceret af den tidlige postnatale høj-fedt diæt ved diæt korrektion til en moderat lav-fedt diæt i det voksne liv, dog ikke hos individer der havde været prenatalt underernæret. Det tyder på at der er et behov for at udvikle differentierede ernæringsstrategier målrettet individer født i hver sin ende af fødselsvægt spektret. Anbefaling om moderat vægtøgning og forebyggelse af fedmeudvikling i det tidlige livs lader til at være vigtig, især for individer udsat for prenatal underernæring.

सारांश (नेपाली)

उदेश्य:

यस अध्ययनको उदेश्य निम्न लिखित परिकल्पनाहरु (Hypotheses) लाई परिक्षण गर्नु थियो: क) अन्तिम गर्भावस्था (Late Gestation; अन्तिम ६ हप्ता) मा खुवाइएको न्युन तथा अधिक मातृपोषणले सन्ततीहरुको शरीरमा बोसो जम्मा हुने प्रकृया र visceral मोटोपनको खतरामा समान किसिमको नकारात्मक असर पुर्याउछन् तर metabolic तथा endocrine तरलता (Plasticity) र बिभिन्न बोसो तन्तुहरुको कार्यगत बिकासमा भिन्न प्रभाव रहन्छ। ख) अन्तिम गर्भाबस्थामा न्युन तथा अधिक मातृपोषणबाट गुज्रिएका जनावरहरुमा उनीहरुको जन्मपश्चात जीवनको प्रारम्भिक चरणमा खुवाइएको उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त (HCHF) आहारले फरक प्रकारको सम्बेदनशीलता सृजना गर्छ र अन्तिम गर्भावस्थामा अधिक मातृपोषण प्राप्त गरेका जनावरहरुमा जीवनको प्रारम्भिक चरणमा खुवाइएको उक्त उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त आहारलाई सहन गर्ने क्षमता बढी हुन्छ। ग) जीवनको पछिल्लो चरणहरुमा गरिने आहार सुधारले जीवनको प्रारम्भिक चरणमा दिइएको उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त खानाको नकारात्मक प्रभावहरुलाई धेरै हदसम्म उल्ट्याउन सक्छ।

कार्यपद्धति:

यो अध्ययन ३×२ फ्याक्टोरियल डिजाइन (Factorial Design) मा आधारित थियो। ३६ वटा जुम्ल्याहा-गर्भवती भेडीहरुलाई गर्भावस्थाको अन्तिम ६ हप्ता (जम्मा गर्भअबधी = १४७ दिन) को अबधीमा सामान्य (दैनिक आबश्यक पर्ने शक्ती तथा प्रोटिनको मात्रा शतप्रतिशत पुरा गरिएको), न्युन (सामान्य दैनिक पोषणको ५०% शक्ती तथा प्रोटिन) तथा अधिक पोषण (सामान्य दैनिक पोषणको १५०% शक्ती तथा ११०% प्रोटिन) उपलब्ध गराइयो। उक्त भेडीहरुबाट पाठापाठीहरु जन्मिएपश्चात ३ दिन देखी ६ महिना सम्म जुम्ल्याहा पाठापाठीहरुमध्य एकलाई मध्यम (Conventional or moderate) तथा अर्कोलाई उच्च-

कार्बोहाइड्रेट-उच्च-बोसोयुक्त खाना खुवाइयो। पाठापाठीहरुको ६ महिनाको उमेरमा अन्तशिरा (Intravenous) गुल्कोज (glucose), इन्सुलिन (Insulin), प्रोपायोनेट (Propionate) सहनशिलता परिक्षणहरु (Tolerance tests) गरियो साथै उपबास सहनशिलता परिक्षण (Fasting tolerance test) समेत गरियो। उक्त परिक्षणहरुमा बिभिन्न समयमा रगतका नमुनाहरु संकलन गरियो। त्यसपछी बिभिन्न उप-समुहका पाठापाठीहरुलाई बध गरी विभिन्न अंग तथा तन्तुहरुलाइ तौल गरी नमुना संकलन गरियो। बिभिन्न बोसो तन्तुहरुमा (Subcutanenous, Mesenteric, Perirenal and Epicardial), कोषका बाह्य बिशेशताहरु (Morphological characteristics), lipid metabolism, angiogenesis तथा inflammation को अवस्थाबारे अध्ययन गरियो। बध नगरिएका बाँकी पाठापाठीहरुलाई ६ महिना देखी अढाइ बर्षसम्म मध्यम (न्युन बोसोयुक्त) खाना खुवाइ, अढाइबर्षको उमेरमा उपबास सहनशिलता परिक्षण गरी सम्पूर्ण पाठापाठीहरुलाई बध गरी बिभिन्न अंग तथा तन्तुहरुलाई तौल गरी नमुना संकलन गरियो।

परिणामहरु:

अन्तिम गर्भावस्थामा न्युन अथवा अधिक पोषण खुवाइएका भेडीहरुबाट जन्मिएका पाठापाठीहरुलाई उनीहरुको जीवनको प्रारम्भिक चरणमा (जन्मेको ३ दिन देखि ६ महिनासम्म) उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त आहार खुवाउदा subcutaneous बोसोको मात्रा घट्नुको साथै उक्त बोसो तन्तुको अनुपातमा mesenteric र perirenal बोसो तन्तुको मात्रा गर्भाबस्थामा सामान्य पोषण खुवाइएका पाठापाठीहरुको तुलनामा उच्च पाइयो साथै subcutaneous बोसो तन्तुमा FABP4, HSL र VEGFA जीनहरु र epicardial बोसो तन्तुमा FABP4, GLUT4 र PPAR-γ जीनहरुको अभिब्यक्ती कम देखियो। यसका साथै अन्तिम गर्भावस्थामा न्युन तथा अधिक मातृपोषण खुवाइएका सन्ततीहरुमा मोटोपन नभएको अवस्थामा subcutaneous र mesenteric बोसोमा कोषाबस्था (Cellularity) घटेको, मोटोपन भएको अवस्था subcutaneous, mesenteric र perirenal बोसोमा कोषसंख्या

घटेको अनि perirenal कोषको साइज (पार-अनुभागीय क्षेत्रफल) अधिकतम बढेको पाइयो। गर्भावस्थामा न्युन मातृपोषण दिइएका सन्ततीहरुमा perirenal बोसो तन्तुमा MCP-1 र IL6 जीनहरुको अभिब्यक्ती अधिक देखियो।

गर्भावस्थाको न्युन अथवा अधिक पोषणले सबैभन्दा बढी प्लाज्मा lactate को अनुकुलता (adaptibility) लाई भिन्न किसिमले असर गरेको पाइयो त्यसपछी ग्लुकोज (Glucose), कोलेस्टेरोल (Cholesterol) र इन्सुलिन (Insulin) को मात्रामा असर पुगेको थियो। यस किसिमका असरहरु बिशेष गरी propionate सहलशिलता परिक्षणमा बढी देखिएको थियो भने ग्लुकोज र इन्सुलिन सहनशिलता परिक्षणहरुमा यी असरहरु कम देखिए। गर्भावस्थामा अधिक पोषण खुवाइएका सन्ततीहरुमा उपबास पछी गरिएको propionate सहनशिलता परिक्षणमा ग्लुकोजको साथै प्लाज्मा lactate को मात्रा बढी देखियो भने इन्सुलिनको मात्रा कम देखिएको थियो। अर्कोतर्फ, न्युन पोषण खुवाइएका सन्ततीहरुमा प्लाज्मा कोलेस्टेरोलको मात्रा बढी पाइयो। उपबास पछिको propionate सहनशिलता परिक्षणमा जीवनको प्रारम्भिक चरणमा उच्च बोसोयुक्त खाना खुवाइएको पाठापाठीहरुमा इन्सुलिनको उत्पादन मध्यम आहार खुवाइएका पाठापाठीहरुको तुलनामा असाध्यै न्युन थियो। उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त आहार खुवाइएका पाठापाठीहरुमा ग्लुकोज सहनशिलता परिक्षणमा इन्सुलिन उत्पादन सबैभन्दा धेरै देखिएको थियो। गर्भाबस्थामा न्युन वा अधिक पोषणको बाबजुद, जन्मपश्चात प्रारम्भिक चरणमा उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त आहार खुवाइएका पाठापाठीहरुमा abdominal मोटोपनसंगसंगै असामान्य metabolic लक्षणहरु जस्तै उच्च ग्लुकोज, कोलेस्टेरोल, NEFA, TG आदी देखियो। त्यसै गरी जन्मपश्चातको उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त आहारले अध्ययन गरिएका सबै प्रकारका बोसो तन्तुहरुमा पार-अनुभागीय कोष क्षेत्रफल र inflammatory सुचकहरुको अभिब्यक्ती बढेको पाइयो भने लिपिड metabolism सँग सम्बन्धित जीनहरुको अभिब्यक्ती घटेको देखियो।

६ महिनादेखी अढाइ बर्षसम्म आहार सुधार गर्दा पनि गर्भाबस्थामा न्युन मातृपोषण तथा जन्मपछी उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त आहार खुवाइएका प्रौढ भेडाभेडीहरुमा उपबास पश्चात प्लाज्मा कोलेस्टेरोल, creatinine र blood urea nitrogen को मात्रा अन्य समुहहरुमा भन्दा बढी देखियो। तथापि सामान्य र अधिक पोषण खुवाइएका भेडीहरुका प्रौढ सन्ततीहरुको metabolic तरलतामा भने कुनै त्यस्तो प्रभाब देखिएन। गर्भावस्थामा अधिक पोषण खुवाइएका प्रौढ भेडाभेडीहरु भन्दा न्युन तथा सामान्य पोषण खुवाइएका र उनीहरुको प्रारम्भिक जीवनमा उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त आहार खुवाइएका भेडाभेडीहरुमा मध्यम आहार खुवाइएका भेडाभेडीहरुको तुलनामा mesenteric बोसोको मात्रा केही बढी देखियो। त्यसैगरी जीवनको प्रारम्भिक चरणमा खुवाइएको उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त आहारका नकारात्मक metabolic प्रभाबहरु (उच्च प्लाज्मा ग्लुकोज, कोलेस्टेरोल, NEFA, TG) प्रौढ अवस्थामा आहार सुधार मार्फत लोप भएको पाइयो। तर प्रारम्भिक चरणमा उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त आहार खुवाइएका पाठापाठीहरुमा प्रौढ अवस्थामा आहार सुधार पश्चात पनि subcutaneous र perirenal तथा mesenteric र perirenal बोसोको अनुपातहरु बढी देखियो। जीवनको अघिल्लो चरणको पोषण तथा आहारका दिर्घकालीन प्रभाबहरु कुनै लिङ्ग बिशेषसँग सम्वन्धित थिएनन्।

निष्कर्ष:

यस अध्ययनको परिणामहरुले बोसो तन्तु, कलेजो र pancreas, foetal programming ले प्रभाव पार्ने प्रमुख अंगहरु भएको प्रकाश पार्छ। अन्तिम गर्भावस्थामा खुवाइने न्युन तथा अधिक मातृपोषण दुबैले लिपिड metabolism, मोटोपन नभएको अबस्थाको बोसो तन्तु cellularity (subcutaneous, mesenteric) र मोटोपनले गर्दा हुने कोष संख्या बृदि (subcutaneous, mesenteric, perirenal) र मोटोपन हुँदाको perirenal कोष आकार (पार-अनुभागीय कोष क्षेत्रफल) मा उच्च बृद्दि गरी जन्मश्चात सन्ततीहरुमा visceral मोटोपनको सुरुवाती बिकासलाई समान प्रभाब पार्छन।

अन्तिम गर्भावस्थामा न्युन पोषण दिइएको भेडीहरुबाट जन्मेका भेडाभेडीहरुमा जन्मपश्चातको प्रारम्भिक चरणहरुमा उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त आहार खुवाउदा ति जनावरहरुमा दिर्घकालिनरुपमा उच्च कोलेस्टेरोल र blood urea nitrogen मात्रा देखियो। गर्भावस्थामा अधिक पोषण खुवाइएका जनावरहरु जीवनको प्रारम्भिक अवस्थाको उच्च बोसोयुक्त आहारको hyperglycaemic असरबाट बच्न सकेनन् तर तिनीहरुमा पछी आहार सुधार गर्दा सकारात्मक सुधार हुने र मोटोपन घटाउने क्षमता अन्य समुहका जनावरहरुमा भन्दा उत्कृष्ट पाइयो। गर्भावस्थामा अधिक पोषण खुवाइएका जनवारहरुमा non-glucose dependent pathways सँग सम्वन्धित pancreatic इन्सुलिनको उत्पादनको तरलतामा नकारात्मक असर देखियो। यसले के देखाउन्छ भने गर्भावस्थामा खुवाइएको न्युन तथा अधिक पोषणले सुरुवाती abdominal मोटोपन र यससँग सम्बन्धित metabolic प्रभाबलाई बढाउछ तर यसमा अन्तर्निहित कारणहरु फरक-फरक हुनसक्छन्। जीवनको पछिल्ला चरणहरुमा गरिने आहार सुधारबाट सुरुवाती जीवनमा देखिएका नकारात्मक प्रभावहरुबाट मुक्त हुने क्षमता अन्तिम गर्भावस्थामा न्युन पोषण खुवाइएका जनवारहरुको तुलनामा अधिक पोषण दिइएका जनावरहरुमा राम्रो देखिन्छ। जीवनको प्रारम्भिक अवस्थामा दिइएको उच्च-कार्बोहाइड्रेट-उच्च-बोसोयुक्त आहारले दिर्घकालिनरुपमा शरिरमा बोसो तन्तु बितरण हुने ढाँचालाई प्रभाब पारेपनी गर्भावस्थामा न्युन पोषण खुवाइएका जनवारहरुमा बाहेक अन्य समुहहरु आहार सुधार पश्चात उक्त अधिक बोसोयुक्त आहारको नकारात्मक metabolic प्रभाबहरुबाट हुन सक्छन्। यसर्थ गर्भावस्थामा न्युन तथा उच्च पोषण उपलब्ध जनवारहरुमा जन्मपश्चात फरक-फरक पोषण रणनितीको आबश्यक पर्छ र गर्वबस्थामा न्युन पोषण पाएका जनावरहरुमा (वा व्यक्तिहरुमा) मध्यम तौल कायम गर्ने र जीवनको सुरुवाती समयदेखि नै मोटोपनबाट टाढै रहने रणनिती आबश्यक देखिन्छ।

GENERAL INTRODUCTION

The World Health Organization (WHO) defines obesity as "a condition of abnormal or excessive fat accumulation in adipose tissue, to the extent that health may be impaired" (WHO 2000) and abdominal obesity, a marker of "dysfunctional adipose tissue" has been emphasized to be the central indicator of metabolic syndrome (Despres & Lemieux 2006). The adverse effects of this global obesity epidemic, associated with a range of other disorders, are increasingly manifested around the world and have important implications for the quality of human life. Developed countries are adversely impacted from the obesity epidemic and a recent study from the 2009-2010 National Health and Nutrition Examination Survey in US shows that 35.5% of adult men and 35.8% of adult women were obese (Flegal Km 2012). In Europe, the prevalence of obesity was found to be 4.0 – 28.3% in men and 6.2 – 36.5% in women with considerable geographic variation (Berghofer *et al.* 2008). Previously, obesity and associated disorders were used to be considered only problems of the developed world with undernutrition and food security problems being the major issues in developing countries. Now, prevalence of obesity and the metabolic syndrome is rising rapidly in the developing world as well affecting people of all age groups associated with improvements in their economic situation (Misra & Khurana 2008). It has been reported that in 2005, 33% of the world's adult population were overweight or obese and it is further predicted that if the recent trend continues, 57.8% of the total world's population (3.3 billion people) could be either overweight or obese by 2030 (Kelly *et al.* 2008). Thus, presently obesity is considered a common disorder worldwide and a serious public health issue both in the developed as well as in the developing world (Prentice 2006).

The International Diabetes Federation (IDF), aiming to establish a diagnostic tool which could be used in clinical practice world-wide, defines the metabolic syndrome as central obesity plus any two of the following: raised triglycerides, reduced high-density lipoprotein

(HDL) cholesterol, raised fasting plasma glucose and raised blood pressure (Alberti *et al.* 2006). Understanding the underlying mechanisms behind the development of obesity and associated disorders plays a crucial role to limit and even to prevent a further increase of this epidemic. The development of obesity and metabolic disorders is generally related to sedentary and unhealthy life styles such as overnutrition and lack of physical activity. Now the obvious question arises why some individuals appear to be more susceptible to increased adiposity than others. Previous epidemiological and animal studies have shown that obesity and associated metabolic disorders are not only related to genotype and/or environmental factors but also influenced by the circumstances taking place during foetal and early postnatal life (Taylor & Poston, 2007, Dyer & Rosenfeld 2011). Therefore, a better understanding of how the early life nutrition can program for adiposity and health outcomes later in life is essential to design proper nutritional interventions to contribute to reverse the obesity epidemic (Fall 2011). Since early life stage is increasingly recognized as being a critical factor influencing future health and disease risks, the current study aimed to evaluate the programming impacts of prenatal malnutrition, both in the form of over- and undernutrition, in combination with an early postnatal high-carbohydrate-high-fat (HCHF) diet on growth, fat deposition and organ development, metabolic and endocrine plasticity, and adipose tissue development and function.

Hypotheses

The present PhD thesis rests on the hypothesis that both maternal over- and undernutrition during the last six weeks of gestation in sheep causes similar foetal metabolic programming and the effects in the offspring will be manifested at a young age.

- Late gestation under- and overnutrition programme adipose tissue development in a similar manner to predispose for development of visceral adiposity later in life.

- Late gestation under- and overnutrition induce similar impacts on metabolic and endocrine adaptability in response to fasting and glucose (GTT), insulin (ITT) and propionate tolerance tests (PTT).
- Late gestational over- and undernutrition leads to a similar but depot-specific alternation in expression of genes involved in lipid metabolism (adipo-lipogenesis) and inflammatory responses and modify the expandability of adipocytes at different adipose depots resulting in increased visceral adiposity.
- An early postnatal obesogenic high-fat diet would further exacerbate the impacts of prenatal nutrition and individuals with a foetal history of overnutrition will have a superior ability to cope with a high-fat diet in early postnatal life.
- Dietary correction in individuals exposed to an obesogenic high-fat diet in early postnatal life can reverse the postnatal impacts of such an obesogenic diet but not the adverse impacts in adult life of having been exposed to malnutrition during late foetal development.

Objectives

The major objectives of this PhD project was thus to test these hypotheses by conducting studies in the Copenhagen sheep model, to investigate whether:

- Both late gestation under- and overnutrition impact development and function of different adipose tissues in a way that predisposes for development of visceral adiposity later in life.
- Late gestation under- and overnutrition have differential implications for the metabolic and endocrine adaptability including hepatic glucostatic function, as evidenced during intravenous tolerance tests (TT) simulating nutrient surplus situations, by acute provision of, glucose (GTT) or insulin (ITT), and nutrient scarcity

situations, by fasting coupled with acute provision of propionate (gluconeogenetic precursor; PTT) in the fasted and fed states.

- Late gestation over- and undernutrition lead to similar but depot-specific alterations in expression of genes involved in lipid metabolism (adipo-lipogenesis) and inflammatory responses, and modify the expandability of adipocytes in subcutaneous adipose tissue thereby resulting in increased visceral adiposity risk.
- An early postnatal HCHF diet further exacerbates the impacts of prenatal nutrition and whether individuals with a foetal history of overnutrition have a superior ability to cope with a high-fat diet in early postnatal life.
- Dietary correction in individuals exposed to an obesogenic high-fat diet can reverse the adverse outcomes in adulthood of such a postnatal impact early in postnatal life, but not the adverse impacts of having been exposed to malnutrition in late foetal life.

Outline

The outline of the PhD thesis is given below.

Chapter 1 consists of a review of the background literature on different foetal programming hypotheses, impacts of prenatal malnutrition on growth and development, metabolic and endocrine responses and adipose tissue development and function.

Chapter 2 presents and discusses the suitability of the Copenhagen sheep model used in the experiment and presents the experimental procedures with respect to feeding treatments, assessment of growth and development of different tissues and organs in the body and evaluation of metabolic and endocrine function and functional development of four different adipose tissues (subcutaneous, mesenteric, perirenal and epicardial).

Chapter 3 describes the effects of late gestation over- or undernutrition combined with a high-fat diet after birth on growth, fat deposition patterns and fasting adaptation in lambs **(Paper I)**.

Chapter 4 reports that maternal under- and overnutrition would have differential impacts on metabolic and insulin-axis function in adolescent sheep when combined with an early postnatal high-carbohydrate-high-fat diet **(Paper II)**.

Chapter 5 presents the effects of late gestational malnutrition and an early postnatal high-fat diet on adipose tissue development and function in adolescent sheep **(Paper III)**.

Chapter 6 evaluates whether changes in metabolic function and fat deposition pattern due to prenatal malnutrition and early postnatal HCHF diet could be reversed by dietary correction later in life **(Paper IV)**.

Chapter 7 is an overall discussion and conclusion of the results presented in this PhD thesis

Chapter 8 describes future perspectives on programming impacts of maternal nutrition on metabolic and endocrine function.

Chapter I: INTRODUCTION

Foetal Programming

Exposure to nutritional insults during critical periods of early life development can induce long-term or permanent changes in the structure and function of an organism (Godfrey & Barker 2000). The term 'foetal metabolic programming' was defined some 20 years ago to account for such changes in the structure and function of an organism in response to nutritional insults during critical periods of foetal development (Lucas 1991, Godfrey & Barker 2000). Intrauterine undernutrition has been increasingly recognized as a risk factor for postnatal development of metabolic disorders like obesity, type-2 diabetes etc., since the "thrifty phenotype hypothesis" was first brought forward in 1992 (Hales & Barker 1992). Subsequent studies have confirmed that foetal undernutrition can lead to permanent and irreversible changes in body composition, metabolism and endocrine function, thus increasing the risks of metabolic disorders later in life particularly upon exposure to a mismatching diet in postnatal life (Ravelli *et al.* 1999, Godfrey & Barker 2000, Jones & Ozanne 2009).

In recent years, attention has also been directed towards the potential programming effects of maternal overnutrition, since mounting evidence suggests that foetal overnutrition somewhat surprisingly may have similar adverse health implications later in life as foetal undernutrition (Muhlhausler *et al.* 2006, Grattan 2008, Ford & Long 2011). Previous studies have thus revealed a 'U' – shaped curve relating birth weight to the risk of adult obesity (Curhan *et al.* 1996, Godfrey & Barker, 2000, Dyer & Rosenfeld, 2011). This suggests that widely different nutritional insults (over- versus undernutrition) during gestation, resulting in individuals being born at the extremes of the birth weight spectrum, may result in similar adverse outcomes on health and disease later in life. However, it is not known, whether these widely

different nutritional insults in foetal life affect the development of organs and body functions through similar underlying physiological and molecular biological mechanisms.

The foetal programming hypothesis based on epidemiological studies

Using official data from Norway, it was earlier shown that poverty during childhood followed by prosperity later in life was adversely correlated with risks of coronary heart disease suggesting that early life nutrition may be a critical determinant for disease risks later in life although no explanation was provided at that time (Forsdahl 1977). In 1989, Barker and colleagues tested the hypothesis that impaired growth and development during prenatal and early postnatal life would predispose for ischemic heart disease later in life (Barker *et al.* 1989). In an epidemiological study based on 5654 men born in 1911-30, they reported that men with the lowest weights at birth and at one year of age had the highest deaths from ischaemic heart disease later in life (Barker *et al.* 1989). Later, in a study based on men born during 1920-30 it was demonstrated that reduced growth in early life is strongly associated with impaired glucose tolerance, hypertension and non-insulin dependent diabetes and elevated plasma 32-33 split proinsulin concentrations (Hales *et al.* 1991). Barker *et al.* (1992) subsequently analysed the occurrence of syndrome X in men aged 64 years in Heartfordshire, UK, and performed glucose tolerance tests on a sample of men and women aged 50 years in Preston, UK. Those studies revealed that the prevalence of syndrome X was strongly associated to birth weight and it fell progressively in both men and women with increasing birth weights, and this adverse association between low birth weight and later development of the metabolic syndrome encouraged the authors to suggest that the metabolic syndrome should be renamed “the small-baby syndrome”. The risk of developing syndrome X among the 64-year-old men with birth weights of 2.95 kg or less was more than ten times higher compared to that of men with birth weights of 4.31 kg or higher. Similarly, the percentage of

men and women aged 50 years with the syndrome fell progressively from 10 among those with birth weights of 2.5 kg or less to 1 among those with birth of more than 3.41 kg. These previous studies generated the Forsdahl-Barker hypothesis regarding early nutritional environment and later risks of diseases, recognizing Forsdahl as the original source of the idea and Barker as the developer of the concept (Vangen *et al.* 2005). In 1992, Hales and Barker proposed the "thrifty phenotype hypothesis" suggesting that poor nutrition in foetal and early postnatal life leads to impaired development and function of pancreatic β-cells and increases susceptibility towards development of type 2 diabetes later in life (Hales and Barker 1992). Later on it has been reported that nutrient deprivation at different stages of gestation may lead to specific outcomes such as poor embryonic growth, foetal wasting and lower birth weight and size, and such metabolic adaptations to undernutrition are linked to defects in endocrine systems such as insulin and the growth hormone- insulin like growth factor-1 (IGF-1) axis (Barker *et al.* 1993). In 1995, Barker proposed the "foetal origins hypothesis" stating that disproportionate foetal growth due to foetal undernutrition in middle to late gestation leads to programming of coronary heart diseases later in life (Barker 1995).

Maternal malnutrition during the specific periods of foetal life may affect specific physiological systems in offspring, since as shown in **Figure 1** different organs have their own pattern of organogenesis and maturation and are associated with distinct developmental stages during foetal life (Symonds *et al.* 2009). Retrospective data from the Dutch Hunger Famine (1944-45) have revealed that exposure to famine during early gestation influences the cardiovascular system and increasing the risk of coronary heart disease in adult life (Roseboom *et al.* 2000) whereas prenatal exposure to famine during late gestation is associated with decreased glucose tolerance and increased insulin concentration thus

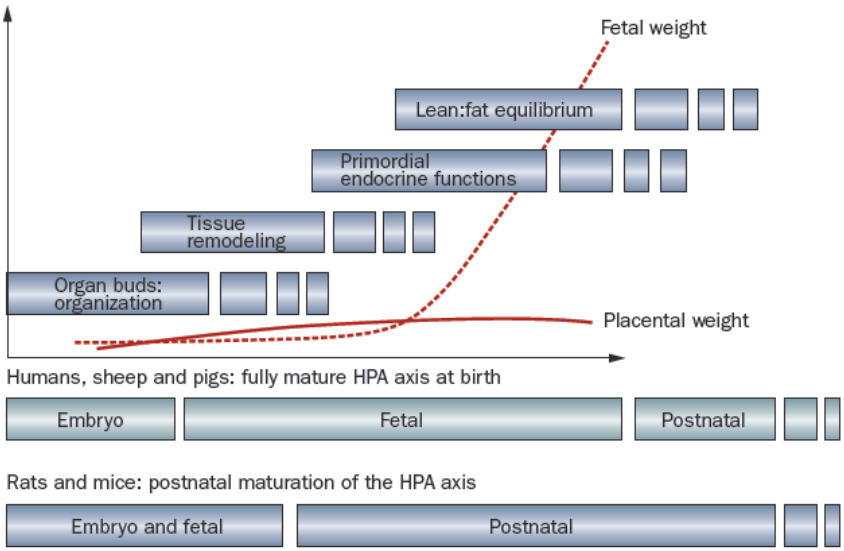

Figure 1: Critical windows for foetal organogenesis and changes in placental and body weight between conception and early postnatal life, in a range of species; HPA axis, hypothalamic–pituitary–adrenal axis [adapted from (Symonds *et al.* 2009)].

influencing the glucose-insulin homeostasis and increasing type 2 diabetes risk (Ravelli *et al.* 1998). The concept of "foetal origins of adult diseases" (FOAD) describing the association between size at birth and subsequent risks of developing cardiovascular disease and type 2 diabetes later in life and later the term "foetal origins of adult disease" of adult disease has been replaced by "developmental origins of health and disease" (DOHaD) suggesting that risks of adult disease are not only influenced by events during foetal life but also during periods even prior to conception and after birth (Gluckman & Hanson 2006). Hence, the DOHaD concept expanded the focus to take into account that adult disease risk may be affected by an interaction of pre- and postnatal environmental factors. It was hypothesized that adaptations in a foetus in response to adverse maternal environments could have short term advantages to improve immediate survival, while increasing the risk of later disease, if the postnatal life (nutritional) environment did not match the foetal prediction of

environment. In this context, the "Predictive Adaptive Responses" (PAR) was suggested meaning that environmental factors during early life can induce adaptive responses due to developmental plasticity, and thereby modify the phenotype to match the predicted environmental exposure later in life (Gluckman *et al.* 2005).

Historically, investigations on developmental programming have primarily focused on the maternal undernutrition and specific nutrient deficiencies. However, a few previous human epidemiological studies have also demonstrated that individuals who are exposed to elevated nutrient supply during early periods of life are at a higher risk of developing obesity and associated disorders later in life. For example, an American cohort study on 854 participants showed that prenatal obesity may double the risk of adult obesity later in life (Whitaker *et al.* 1997). Moreover it has been shown that infants of mothers with diabetes during pregnancy are predisposed to develop overweight and obesity during childhood (Plagemann *et al.* 1997).

Maternal malnutrition and postnatal metabolism – evidence from animal studies

Maternal undernutrition affects postnatal growth in offspring and may lead to alterations in development of body functions. Nutrient deprivation in pregnant rats (30% of *ad libitum* intake throughout pregnancy) led to reduced early postnatal growth and elevated blood pressure in adulthood showing that maternal undernutrition during pregnancy may alter cardiovascular homeostasis in offspring (Woodall *et al.* 1996). In a sheep study, prenatal undernutrition during late gestation, but not during early gestation, altered glucose-insulin homeostasis in adult offspring by increasing the area under the curve (AUC) for glucose and insulin during intravenous glucose tolerance tests (IVGTT) and this was associated with reduced adipose GLUT4 protein expression (glucose transporter 4) and increased adipose tissue mass (Gardner *et al.* 2005). Recently, we have developed the 'Copenhagen sheep

model' to facilitate studies of long-term programming effects of pre- and early postnatal nutrition. In this model we have shown that undernutrition (50% of estimated energy and protein requirements) of twin pregnant ewes during late gestation (last six weeks of gestation) predisposed for visceral obesity in the offspring by altering fat distribution patterns and reducing subcutaneous fat deposition (Nielsen *et al.* 2013). Studies using different animal models have shown that poor intrauterine nutrition can induce changes in beta cell function and insulin action as reviewed earlier (Jones & Ozanne, 2009). Maternal energy restriction during late gestation in sheep (Kongsted *et al.* 2014) or throughout gestation in guinea pigs (Kind *et al.* 2003) have been shown to reduce insulin sensitivity in adolescent offspring with potentially also decreased glucose tolerance and increased fasting insulin levels, and these effects remained evident in adulthood (Kongsted *et al.* 2014). From another sheep study, it was found that pancreatic insulin secretion and hence β-cell function in young adolescent offspring, may also be adversely affected by prenatal exposure to undernutrition, and that can be associated with a compensatory upregulation of insulin sensitivity with no overall change in glucose tolerance (Husted *et al.* 2007). Reduced pancreatic insulin secretory plasticity has been revealed in adult female sheep with a history of undernutrition during late foetal life (Husted *et al.* 2008). It has further been shown in sheep that insulin secretion in young lambs was predictive of the insulin secretion in young adults, suggesting that events in early life may permanently determine the capacity of glucose-stimulated insulin secretion (Gatford *et al.* 2004).

Along with poor maternal nutrition, evidence is accumulating to suggest that maternal malnutrition also in the form of overnutrition can have long-tern adverse impacts on the offspring even when the offspring are fed a normal diet during postnatal life (Muhlhausler *et al.* 2006, Grattan 2008, Ford & Long 2011). Therefore, maternal overnutrition also appears to be a crucial factor contributing to foetal programming of aetiology of obesity and associated

disorders later in life. And recent studies have also tried to evaluate the programming outcomes of maternal overnutrition during critical periods of foetal development and subsequent risks in offspring later in life, but as yet, information is scarce regarding the nature of the underlying mechanisms linking prenatal overnutrition to increased disease risk later in life.

Impacts of postnatal nutrition on manifestation of prenatal programming

It has been suggested that alterations of normal foetal growth, metabolism and endocrine regulatory systems are foetal adaptations taking place in order to prepare the foetus to a given postnatal (nutritional) environment. If the postnatal nutritional environment does not correspond to what the foetus was prepared for, various disorders can develop, such as cardiovascular dysfunctions and altered renal functions as shown in adult sheep (Cleal *et al.* 2007). Similar observations have also reported from human studies. For example, a high prevalence of diabetes was reported among young adult Ethiopian immigrants, who had lived in Israel for up to 4 years with different dietary habits than in Ethiopia (Cohen *et al.* 1988). Moreover, in pigs it has been shown that a maternal atherogenic diet is protective against early atherosclerosis development in offspring upon exposure to an atherogenic diet postnatally (Norman & LeVeen 2001). Similarly, rodent offspring of high-fat fed mothers which were raised under normal diet developed endothelial dysfunction whereas such impacts were not observed in the offspring fed similar high-fat diet as during foetal life (Khan *et al.* 2004). Furthermore, a sedentary behaviour induced by prenatal undernutrition in rat offspring was further exacerbated by postnatal hypercaloric diet (Vickers *et al.* 2003). Thus, postnatal nutritional environment plays an important role in determining how foetal adaptations due to parental nutrition express after birth later in life. This PhD thesis emphasizes on interactions of parental over- and undernutrition with an early postnatal

obesogenic high-fat diet on the metabolic and endocrine plasticity, and adipose tissue deposition, distribution, development and function.

Mechanism of foetal programming

The mechanisms underlying the phenomenon foetal programming are still incompletely understood. In this section, some of the important mechanisms known or suggested to be implicated in foetal programming will be illustrated. Since, the scope of the PhD thesis was to investigate the impacts of malnutrition on various metabolic and endocrine adaptabilities and adipose tissue development and function, the main focus will be on prenatal programming of some of the major organs in this context, such as the endocrine pancreas, liver and adipose tissues.

Placental function and overexposure of glucocorticoids

Modification of maternal environmental factors may lead to changes in placental function which lead to adaptive changes in the foetus with implications for subsequent foetal growth and development **(Figure 2)** (Seckl & Holmes 2007), which as mentioned have been thought to improve chances of survival in the extrauterine environment. Glucocorticoids are hormones which are important for foetal growth, tissue development and organ maturation. It has been shown that increased foetal exposure to maternal glucocorticoids may lead to low birth weights and subsequent disorders later in life, and in rats a decreased activity of the fetoplacental enzyme 11 β-hydroxysteroid dehydrogenase that acts as a placental barrier to maternal glucocorticoids has thus been associated with low birthweight (Edwards *et al.* 1993). The enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) acts as a protective barrier to glucocorticoids as it inactivates glucocorticoids to physiologically inert 11-keto forms ensuring that high maternal glucocorticoids levels are largely excluded from the foetus (Brown *et al.* 1996).

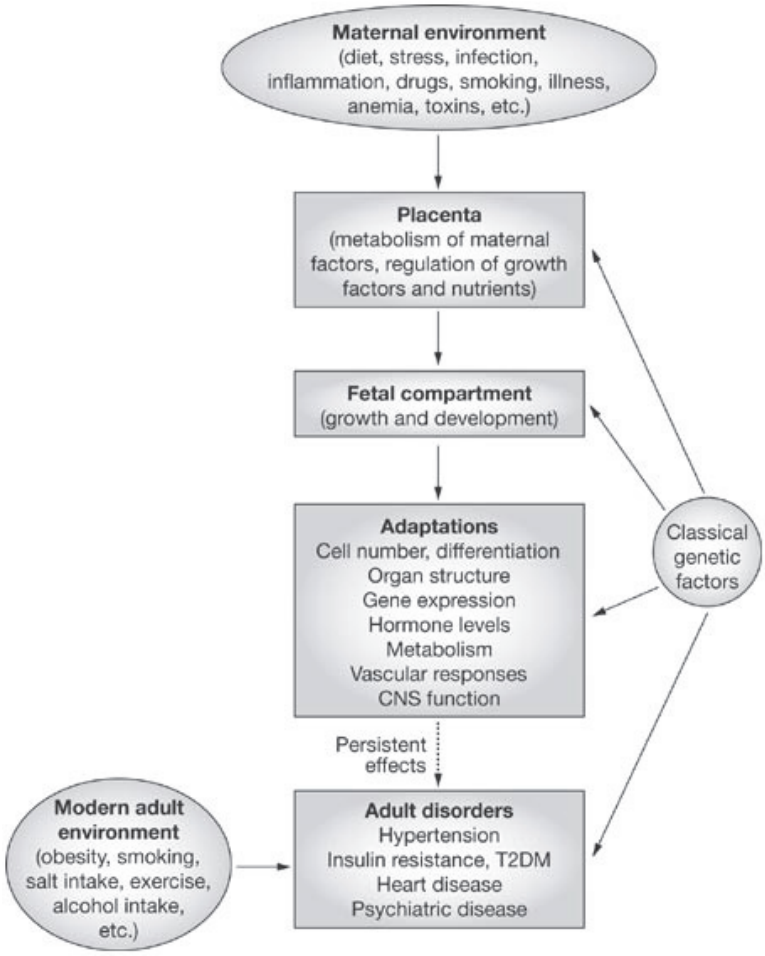

Figure 2: The concept of developmental programming [adapted from (Seckl & Holmes 2007)].

Different intergenerational factors or exposures or environments associated with one generation can relate to the health and disease of the next generation (Emanuel 1986). Such programming impacts of low birth weight and later risks of cardiovascular diseases may not merely limited to the first generation offspring, rather they may be inherited across a numbers of generations as reviewed earlier (Drake & Walker 2004).

Foetal growth is primarily determined by nutrient supply which is dependent upon placental transport functions. Foetal growth restriction can therefore be associated with limitations in nutrient and oxygen transfer and accelerated foetal growth on the other hand can be a consequence of elevated nutrient supply leading to development of LGA (large for gestational age) individuals. Birth weight itself is, however, considered a poor marker for intrauterine development, since e.g. intrauterine catch-up growth can occur to mask alterations in early gestation development, and long-term programming outcomes can occur without necessarily affecting size at birth (Godfrey 2002). It has previously been suggested that altered placental phenotypes would provide better predictive information regarding abnormal maternal nutrition and risks of health and disease later in life (Sibley *et al.* 2005). In human, it has been shown that maternal obesity increased placental weight and modulated fatty acid transport (lower expression of placental fatty acid binding proteins) without affecting foetal growth (Dube *et al.* 2012). Thus, altered maternal nutrition, exposure of maternal hormones levels, placental nutrient transfer and utero-placental blood flow may be important in determining the intrauterine environment, and hence foetal adaptations coupled to development of adult diseases (Jansson & Powell 2007).

Programming of the endocrine pancreas

It has now been established that foetal programming in prenatally growth restricted individuals can lead alternation in pancreatic β-cell function and insulin action in target tissues (Jones & Ozanne 2009) and the long-term impacts of maternal malnutrition on endocrine pancreas are illustrated in **Figure 3**. Defects in the pancreatic β-cell population and insulin-sensitive tissues function due to foetal malnutrition could be one of the underlying mechanisms explaining altered foetal growth and subsequent risks of the metabolic syndrome later in life (Remacle *et al.* 2007). It has previously been reported in an IUGR rat model that

uteroplacental insufficiency leads to impaired function and development of the β-cell which might be due to oxidative stress and mitochondrial dysfunction, and epigenetic modifications of key β-cell regulators (Simmons 2007a). Maternal malnutrition can induce epigenetic modifications of key genes responsible

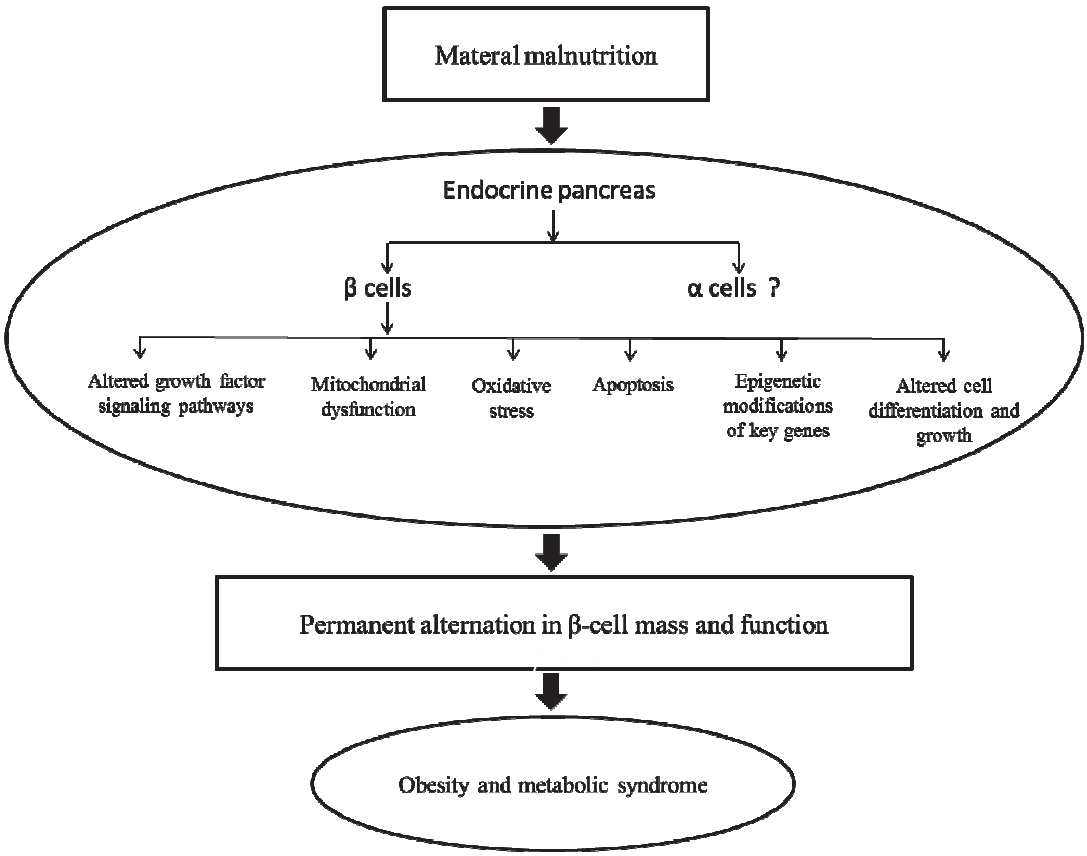

Figure 3: Maternal malnutrition and impacts on endocrine pancreas

for β-cell development, thus contributing to later development of type 2 diabetes in adult life as reviewed earlier (Simmons 2007b). For example, expression of Pdx1 (pancreatic and duodenal homeobox 1) transcription factor, a critical regulator of β-cell development and function, was permanently reduced in the intrauterine growth restricted (IUGR) rodent β-cell and underwent epigenetic modification throughout development (Park *et al.* 2008). Intrauterine programming of the endocrine pancreas has been studied also in precocious

species like sheep which are closer to humans in terms of timing of islet development and remodelling during foetal development. Decreased mRNA expression of IGF-1, fibroblast growth factor 7 (FGF7), fibroblast growth factor receptor 2IIB ligands (FGR2IIB), and Pdx-1, and increased expression of insulin-like growth factor binding protein-2 (IGFBP-2) genes have been shown in placental insufficiency-induced IUGR foetal pancreatic tissues in sheep suggesting that growth factor signalling pathways may be targeted in IUGR individuals to reduce β-cell mass and function (Chen *et al.* 2012).

Maternal overnutrition has also been shown to have long-lasting adverse impacts on growth and development of the endocrine pancreas contributing to the metabolic syndrome later in life. In sheep, it has been shown that maternal obesity induced by a high plane of nutrition by mid-gestation led to maternal and foetal hyperglycaemia and hyperinsulinaemia, over-growth of the pancreatic islets and accelerated β-cell development due to hyperplasia whereas such impacts were not evident in pancreatic α-cells (Ford *et al.* 2009). In contrast, when maternal obesity continued throughout pregnancy in the same obese sheep model, a marked reduction in foetal pancreatic weight and accelerated foetal β-cell apoptosis in late gestation and hypoinsulinaemia at birth were observed suggesting that maternal obesity may have differential impacts on foetal pancreatic growth during depending on the timing during early and late foetal development, and this may have differential long-term influences on pancreatic function, and glucose-insulin homeostasis later in life (Zhang *et al.* 2011). Rat pups exposed to nutrient deficit or excess during foetal development are born with fewer β cells and more susceptible to oxidative stress as reviewed earlier (Reusens *et al.* 2011). Thus, adverse programming of development and function of the foetal endocrine pancreas could affect steps in the insulin secretion process **(Figure 4)** as well as insulin signalling thereby affecting the risks of obesity and type 2 diabetes later in life.

Past studies have mainly focused on programming impacts on β-cell and investigations on long-term effects of maternal malnutrition on pancreatic α-cell development and function are few although glucagon is also a very important regulator of glucose homeostasis.

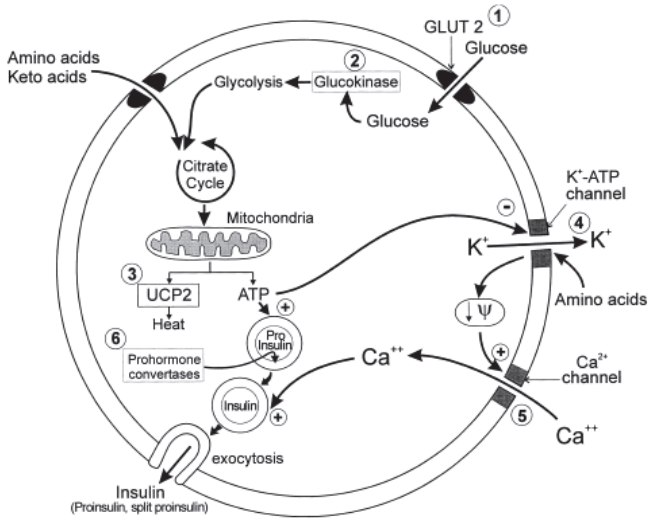

Figure 4: Diagram of stimulus-secretion coupling in the pancreatic β-cell illustrating the processes which may be programmed *in utero*. 1. Abundance of the glucose transporter-GLUT 2. 2. Abundance and activity of glucokinase. 3. Abundance and activity of uncoupling protein (UCP)-2. 4. ATP-dependent K+ channels responsible for the potential difference (ψ) across the cell membrane. 5. Voltage sensitive Ca^{2+} entry and $[Ca^{2+}]_i$. 6. Abundance and activity of prohormone convertases which metabolize pro-insulin to insulin [adapted from (Fowden & Hill 2001)].

Programming of the liver

The liver is an important metabolic and endocrine organ in our body and it has been highlighted that multiple metabolic disorders are associated with liver disease and dysfunction (Marchesini *et al.* 2003). It has been suggested that non-alcoholic fatty liver disease (NAFLD) may be considered as an additional feature of the metabolic syndrome (Marchesini *et al.* 2001), and early life nutrition can affect hepatic function and metabolism, which may have long-lasting effects later in life. For example, aged female offspring born to

nutrient restricted ewes during early gestation had higher hepatic lipid and glycogen content suggesting increased propensity for nutrient storage in the liver (George *et al.* 2012). Moreover, maternal protein restriction throughout pregnancy increased hepatic PPARα in rat offspring along with elevated plasma TG and fatty acids concentrations, indicating impaired lipid homeostasis (Burdge *et al.* 2004). Similarly, hepatic lipid accumulation was increased in obese sheep subjected to suboptimal nutrition during early gestation (Hyatt *et al.* 2011). Moreover, our previous study also demonstrates that the liver can be implicated in altered insulin sensitivity, in prenatally programmed individuals, and that it is associated with altered expression of insulin signally-related genetic markers and altered fatty acid composition in structural lipids (Hou *et al.* 2013).

Maternal overnutrition and high-fat diet can also have long-term adverse impacts on hepatic function and regulation later in life. In nonhuman primates, it has been shown that chronic maternal high-fat diet consumption, independent of maternal obesity or diabetes, led to increase in liver triglycerides (TG) levels, and activation of oxidative stress and gluconeogenic pathways, thus increasing the risks of NAFLD (McCurdy *et al.* 2009). Similarly, long-term feeding of maternal high-fat diet has been shown to increase the risks of hypertension, raised plasma lipids and fatty liver in mice offspring, thus developing the features of the metabolic syndrome in postnatal life (Elahi *et al.* 2009). Although it is clear that both maternal over- and undernutrition can have similar adverse programing impacts on liver function and glucose-lipid regulation, it remains to be established whether such adverse programming outcomes of prenatal nutrition are associated with common mechanisms.

Adipose tissue – a target of foetal programming

Adipose tissue, commonly called ‘fat’ is a type of loose connective tissues comprised of lipid-filled cells (adipocytes) surrounded by a matrix of collagen fibres, blood vessels,

fibroblasts and immune cells and it is considered an important organ with vital metabolic and endocrine properties (Kershaw & Flier 2004). Adipose tissue can be classified into brown adipose tissue (BAT) or white adipose (WAT). As reviewed earlier (Enerbäck 2010), BAT is made up of cells with multilocular lipid droplets, rich in mitochondria and they express uncoupling protein 1 (UCP1) enabling to produce heat by uncoupling mitochondrial respiration from ATP production and it disappears after birth. On the other hand, WAT is the predominant type of adipose tissue developing postnatally in humans and other species and it is characterized with a single lipid inclusion and eccentrically located nucleus and involved in various biological functions. This study focuses on changes in development and function of WAT in response to different nutritional exposures during late foetal and early postnatal life. WAT is distributed throughout the whole organism in different depots without physical connection and coordination between them is still unclear. Adipose tissue basically has two major functions. They play an important role in whole body energy balance through storage of fat in situations with energy surplus, and this energy depot can be mobilized in times of energy scarcity. Adipose tissues further secrete chemicals called adipokines that are involved in energy homeostasis, glucose and lipid metabolism and inflammation (Ahima & Osei 2008, Fantuzzi 2005). It is important to understand the process of adipose development since any disturbances in this development may increase the risk of deposition of fat in non-adipose tissues, also called ectopic fat accumulation, and this is associated with insulin resistance and development of type 2 diabetes (Gastaldelli 2011).

Adipose tissue development

Adipose tissue development is mainly regulated by two processes adipogenesis and lipogenesis. Adipogenesis refers to the differentiation of pre-adipoctyes into mature fat cells. Transcription factors such as sterol regulatory binding protein-1 (SREBP-1) and peroxisome

proliferator-activated receptor γ (PPARγ) play important role in the process of adipose tissue development and PPARγ is involved in the regulation of both adipogenesis and lipogenesis (Kersten 2001). Adipose tissue adipogenesis is also coupled to angiogenesis, and regulatory factors involved in angiogenesis can therefore potentially affect adipose development. Vascular endothelial growth factor (VEGF) is one of the most critical angiogenetic factors, which initiates the formation of immature blood vessels (Hausman & Richardson 2004). The expression of the VEGF gene is influenced by different factors and it has been reported that hypoxia induces transcription of the VEGF gene by binding the hypoxia inducible factor to the *cis* elements in the VEGF promoter (Liekens *et al.* 2001).

Programming of adipose growth, deposition and distribution

It is widely accepted that obesity and associated disorders are traditionally associated with increased intake of energy-rich foods (Putnam *et al.* 2002) or sedentary lifestyles (Sturm 2004). Obesity and lipotoxicity caused by ectopic fat accumulation lead to a progressive defect in pancreatic insulin secretion coupled with a progressive enhancement in insulin resistance and other adverse effects on major organ function which subsequently results in development of type 2 diabetes (Golay & Ybarra 2005, van Herpen & Schrauwen-Hinderling, 2008). In recent years it has been recognized that fat deposition patterns and metabolic and endocrine functions of adipose tissue depend upon the maternal nutrition (Symonds *et al.* 2004) and adipose tissue is now believed to be a major target of foetal programming, which can contribute to predispose for development of obesity and associated disorders later in life (Desai & Ross 2011). For example it has been revealed that humans with low birth weights (average: 2.76 kg) had a higher percentage of body fat and fat mass and a higher trunk to limb fat ratio compared to higher birth weight individuals (average: 4.23 kg) (Kensara et al., 2005).

Regional distribution of fat is an important factor determining the association of obesity to alterations in carbohydrate and lipid metabolism since adipose tissue distributed at different depots may have unique structural and functional properties. Excess accumulation of abdominal fat may be associated to poor expandability of subcutaneous fat, which will reduce the ability of subcutaneous adipose tissue to act as a buffer, i.e. 'sink', in situations with excessive availability of energy and nutrients (Lemieux 2004) and in such a situation the risk of nutrient overflow and hence excess accumulation of abdominal fat and also ectopic fat is increased (Miranda *et al.* 2005). Therefore, subcutaneous fat is considered a relatively healthy fat due to a specific function in preventing lipid-overflow to visceral areas and non-adipose tissues related to its expandability, and due to unique intrinsic properties related to e.g. improvement of insulin sensitivity (Tran *et al.* 2008). Foetal adipose tissue in both sheep and humans is mainly deposited during the last part of gestation where the major part of foetal adipogenesis and adipose differentiation takes place (Symonds and Lomax 1992). We have previously developed the Copenhagen sheep model in order to be able to investigate the long-term consequences of malnutrition during third trimester development and shown that maternal undernutrition during late gestation increases the risks of abdominal obesity in offspring possibly due to a reduced expandability of subcutaneous adipose tissue (Nielsen *et al.* 2013). Previously, it has been reported that the growth of perirenal-abdominal adipose tissue commences at about day 70 days of gestation in sheep and rapid increase in fat mass along with parallel increase in lipid content occurs until 110-120 days of gestation and the growth of subcutaneous fat commences 2-3 weeks later than perirenal-abdominal fat depots and a significant regression of subcutaneous fat occurs about day 115, which virtually disappears by full term (Alexander 1978). Another study also confirms that both perirenal and subcutaneous adipose tissue in foetal sheep begins to grow at about day 70 of gestation and a marked increase in proliferation of mitochondria and development of nerves are visible

in the perirenal fat depot during late gestation (Gemmell & Alexander 1978). Thus nutritional insults during pregnancy potentially can have different impacts on development of adipose tissues depending upon timing of their development.

In the past, a few studies have attempted to investigate the molecular biological mechanisms behind the altered structure and function of prenatally programmed adipose tissue. For example, maternal protein restriction throughout gestation and lactation in rats increased the expression of glucose transporter-4 (GLUT-4), fatty acid synthase (FAS) and CCAATT enhancer-binding protein (C/EBP-β) genes (Guan *et al.* 2005), indicating that glucose and lipid metabolism pathways in adipose tissue in part could be the possible targets of maternal malnutrition leading to development of obesity in offspring later in life. Recent studies indicate that maternal overnutrition may also have long-term adverse impacts on adiposity in sheep offspring (Ford & Long 2011). However, it is not known whether prenatal overnutrition results in similar alterations in adipose growth, development and deposition patterns as undernutrition and whether similar physiological mechanisms would be involved. Moreover, it remains to be investigated whether individuals exposed to prenatal over- and undernutrition would respond to an early postnatal nutrition in a similar way with respect to depot-specific fat deposition and distribution coupled to associated disease risks. During review of available literature, it was found that impacts of early life nutrition on mesenteric and renal fat have never been studied specifically in humans as the two visceral compartments are anatomically distinct located and may have specific functional properties.

Programming of inflammatory responses in adipose tissue

Adipose tissue is not only a passive responder to nutrient provision and signals from different hormonal systems, but also acts as an important endocrine organ secreting and expressing different factors (adipokines), which have important endocrine functions (Kershaw & Flier

2004). Changes in adipose tissue mass are associated with altered endocrine and metabolic functions of adipose tissue. Adipose tissue macrophage numbers increase in obesity and are involved in different inflammatory pathways in obese individuals. A macrophage marker (F4/80) in perigonadal, perirenal, mesenteric and subcutaneous adipose tissues of mice was positively correlated with adipocyte size and body mass (Weisberg *et al.* 2003). Adipose tissue macrophages (ATM) are the source of inflammatory adipokines such as TNF-α (tumour necrosis factor) and IL-6 (interleukin) and rodent studies show that ATMs may significantly contribute to systemic insulin resistance (Zeyda & Stulnig 2007).

The implications of foetal programming for inflammatory responses in adipose tissue and how this related to adipose structure and function has only been addressed in a few previous studies, which mainly focused on impacts of prenatal undernutrition on expression of different inflammatory markers in adipose tissue. Such studies have shown that late gestational nutrient restriction in sheep can upregulate the expression profile of inflammatory markers such as cluster of differentiation 68 (CD68) and toll-like receptor (TLR4) (Sharkey *et al.* 2009). However, information is very scarce when it comes to implications of foetal overnutrition. However, both maternal over- and undernutrition can apparently lead to altered adipocyte function development in offspring, which alters lipid metabolism (Desai & Ross 2011) and predisposes for abdominal obesity.

It remains to be investigated, however, whether potentially different underlying mechanisms in adipose programming has implications for the susceptibility towards unhealthy diets in postnatal diet of individuals with a history of foetal over- as compared to undernutrition.

Chapter 2: GENERAL METHODOLOGY

Experimental design and dietary treatments

In this study, we investigated impacts of late gestation over- or undernutrition in combination with an early postnatal obesogenic diet on metabolic and endocrine plasticity and adipose tissue development and function in sheep. This allows us to document to what extent our sheep model can resemble what has been found in other species and to allow us to compare outcomes of late gestation insults with insults induced earlier in gestation. The experiment was a 3×2 factorial design with three prenatal and two postnatal nutrition exposures, as illustrated in **Figure 5 (Paper I)**. Thirty six twin-pregnant multiparous (parities ranging from three to five) cross-bred Texel ewes and their off-spring were used in the experiment. During late gestation (last six weeks of pregnancy; term = 147 days), the ewes were allocated to one of three different dietary regimens: NORM (N = 9) fulfilling daily requirements for energy and protein according to Danish feeding standards; LOW (N = 14) providing only 50% of energy and protein requirements; or HIGH (N = 13) designed to provide 150% of energy and 110% of protein requirements. The ewes were allocated to these treatments six weeks prior to expected parturition, and it was attempted to reach as even a distribution of body weight and body condition score (BCS) across treatment groups as possible at the entry into the experiment. It was ensured that all newborn lambs received colostrum within three hours after birth. The lambs suckled their dam at will until three days after parturition, and then the dam was removed from her offspring. From three days to six months of age, one lamb from each twin pair received a high-carbohydrate–high-fat (HCHF; N=35; 18 males; 17 females; high-carbohydrate high-fat diet consisting of a cream-milk replacer mix in a 1:1 ratio supplemented with rolled maize; **Figure 6A**) and the other lamb a moderate, conventional (CONV; N=35; 16 males, 19 females; conventional diet consisting of milk replacer and hay

until 8 weeks and hay only thereafter and adjusted to achieve moderate and constant growth rates of appr. 225 g day-1) diet. Allocation of the twin lambs to their respective postnatal dietary treatment groups was done right after birth using sex (first priority) and birth weight (second priority) as allocation criteria to achieve as even a sex and birth weight distribution as possible across the two postnatal treatment groups within each prenatal treatment. The experimental design thus gave rise to 6 treatment groups: HIGH-CONV HIGH-HCHF; LOW-CONV; LOW-HCHF; NORM-CONV; NORM-HCHF and details about experimental animals have been shown in **Appendix I**.

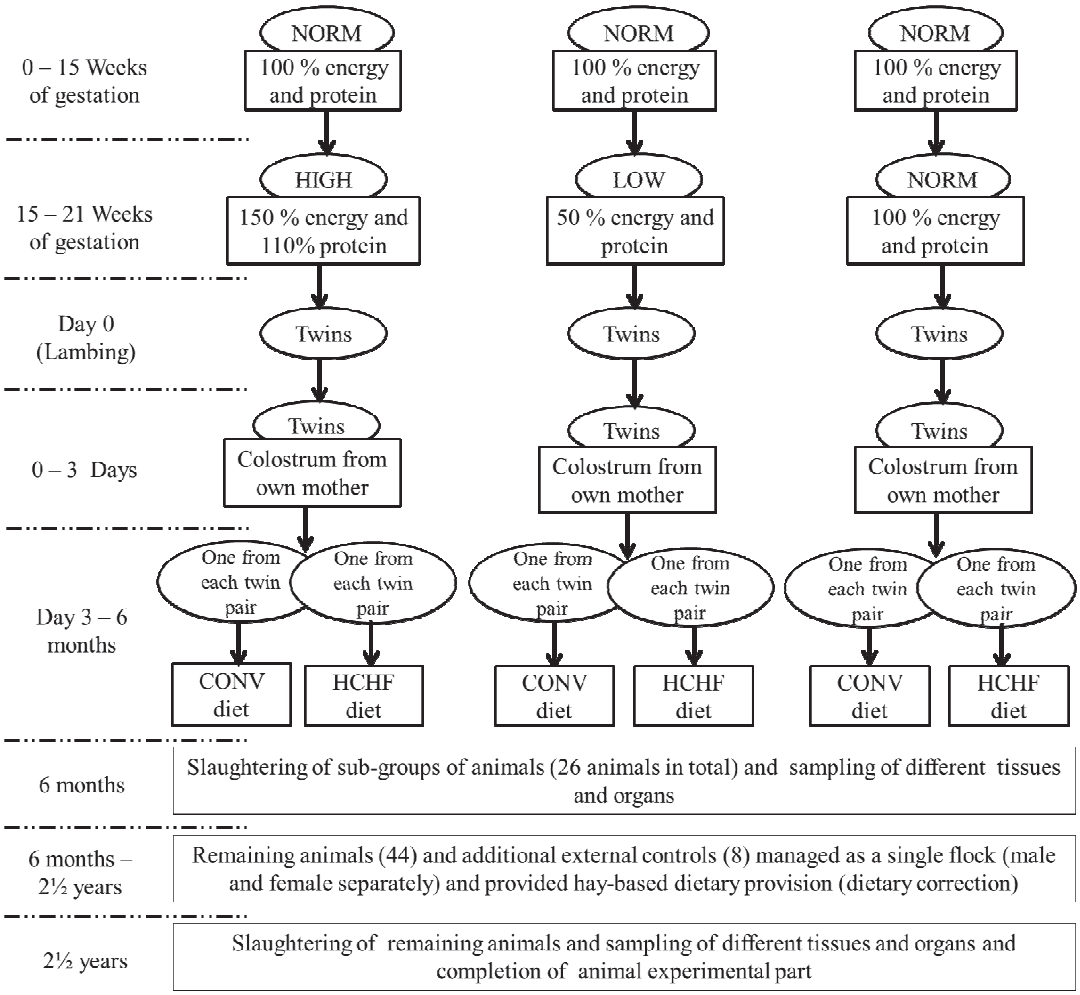

Figure 5: Schematic presentation of the experimental 3×2 factorial design.

In our study, we used sheep as an experimental model for humans, since sheep, as reported earlier (Nielsen *et al.* 2013), has a long gestation period and offspring are born with similar physiological maturity at birth as humans, which is an important issue when the studies focused on impacts of maternal malnutrition during the last trimester on health and disease later in life. The sheep obviously has limitations as an animal model for humans since it is a ruminant animal having a distinctly different digestive system compared to monogastrics with a four-chambered stomach, where significant fermentation of dietary carbohydrates take place. However, it is possible to induce similar phenotypic attributes as in human through specific dietary manipulations in sheep. In our studies, the postnatal HCHF diet consisted of a milk-dairy cream mix supplemented with ground maize. The milk-dairy cream mix was provided in suckling buckets **(Figure 6B)**, to stimulate the so-called oesophageal groove reflex, which in young ruminant animals is induced by suckling, and it causes muscle flaps to close and form a tube connecting the oesophagus with the lower segments of the digestive tract (Comline & Titchen 1951, Orskov & Benzie 1969), whereby a significant part of such a diet can escape forestomach fermentation and be digested in the small intestine as in monogastric animals. We chose to use maize as the starch supplement in the HCHF diet, as starch in maize has a relatively low degradability in the forestomachs and therefore can escape forestomach fermentation to be digested in the small intestine (Orskov 1986) like in monogastric animals. By this dietary manipulation, we were able to feed an obesogenic diet with a high content of starch and fat to the lambs for the first 6-months of postnatal life, which is otherwise difficult in a ruminant animal without disrupting rumen fermentation and compromising health of the animal.

From six months of age, all lambs regardless of their previous pre- and early postnatal dietary treatments, were fed the same diet consisting of artificially dried green hay (dietary correction of the early postnatal HCHF diet) *ad libitum* supplemented by barley and

managed as sex divided flocks and all animals received water *ad libitum.* An additional eight (four males and four females) age-matched lambs were included in this experiment as undisturbed external controls (EC) and raised in the same flocks with the other experimental animals from 6-months until 2½ years of age. This resulted in seven different groups: HIGH-HCHF, HIGH-CONV, LOW-HCHF, LOW-CONV, NORM-HCHF, NORM-CONV, EC **(Paper IV)**.

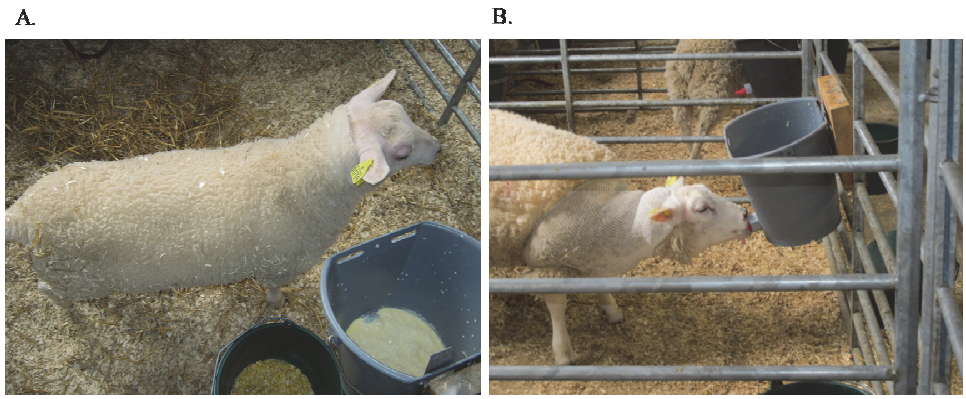

Figure 6: A. HCHF diet: maize and milk-cream mix, B. Suckling of milk-cream mix by HCHF animals through bucket

Recordings and measurements

We anticipated that foetal programming would alter growth trajectory as it has previously been reported that early nutrition can programme later growth trajectory and body composition (Gale *et al.* 2001, Sayer & Cooper 2005). Hence, we recorded body weights, BCS and feed intake in twin-pregnant ewes **(Paper I)**. The lambs were examined and weighed immediately after birth and lamb body weights, body proportions, BCSs and feed intakes were recorded **(Paper I)**. In adult sheep, body proportions and body weights were determined (crown-rump length, girth circumference, height over withers, head width) **(Paper IV)**.

Slaughtering of animals

To investigate age related and tissue-specific prenatal programming outcomes on different organs and tissue systems, we slaughtered sub-groups of animals at six months **(Paper I)** and the remaining animals at 2½ years of age **(Paper IV)**. Twenty six lambs (only one lamb from each twin pair) in total were selected for slaughtering at six months of age and remaining animals were continued in the experiment for later studies as adults. Just before slaughtering, animals were anaesthetized with Propofol (5-6 mg i.v. kg^{-1} body weight; B. Braun, Melsungen Tyskland, Germany) and they were thereafter sacrificed by decapitation. Immediately after decapitation, different organs were excised and weighed to be able to evaluate the impacts of late gestation malnutrition on organ and tissue growth and development and to assess morphological changes and molecular mechanisms at different adipose depots in fatally programmed individuals.

Assessment of metabolic and endocrine plasticity

To evaluate metabolic status, we took blood samples all ewes by jugular venipuncture once on weeks (-7, -5, -3, and -1 week) prior to expected parturition, and on the day after lambing. Additionally, blood samples from lambs were collected the day after birth (day one) by jugular venipuncture. At six months **(Paper I and II)** and 2½ years **(Paper IV)** of age we conducted specific tolerance tests to be able to study metabolic adaptability and plasticity of endocrine regulatory systems to situations resembling nutrient surplus and nutrient scarcity situations and to evaluate hepatic gluconeogenetic function. To facilitate repeated collection of blood samples during the tolerance tests, catheters were inserted into a jugular vein at least one day prior to initiation of tolerance tests **(Figures 7A and 7B)**.

A. B.

Figure 7: A. Chatherization of animals for repeated blood samplings during challenge tests. B. Blood sampling by using home-made indwelling catheter during challenge test.

Fasting tolerance test

Fasting tolerance test is the simplest and easiest nutritional challenge to evaluate changes in glucose and lipid metabolism blood glucose and diagnose alterations in endocrine changes like leptin and IGF-1 axis. Fasting tolerance tests were conducted in lambs at both six months **(Paper I)** and 2½ years of age **(Paper IV)**.

Glucose tolerance test

Oral and intravenous glucose tolerance tests (IVGTT) are normally conducted in humans to evaluate insulin sensitivity and β-cell function (Tura *et al.* 2008), however, oral glucose tolerance test is not possible to conduct in sheep since glucose is fermented by microbes to lactate and then to volatile fatty acids (VFA) in rumen. Previously, glucose tolerance test has successfully conducted in sheep and has been identified a useful tool to assess the insulin secretory response to glucose and the rate of disappearance of injected glucose (Sasaki *et al.* 1984, Kongsted *et al.* 2014). Prior to the intravenous glucose tolerance test, six months old

lambs were fasted overnight to avoid interference in insulin secretory responses dependent of the distance from last feed intakes **(Paper II)**.

Insulin tolerance test

Insulin tolerance test was conducted in six month old lambs to estimate glucose disappearance rate in response to injected insulin and to assess alterations in insulin resistance **(Paper II)**. This tolerance test was conducted in the fed state except for the HCHF lambs, which were not fed the milk-cream mix to avoid very high levels of lipids in the blood, which could potentially interfere with the later analyses of blood samples.

Propionate tolerance tests during fasted and fed states

Propionate is a normal metabolite of ruminant fermentation and plays an important role in the ruminant metabolism as a primary gluconeogenic metabolism precursor and it is also a major microbial fermentation metabolite in the human gut (Hosseini *et al.* 2011). Propionate metabolism in the ruminant occurs nearly exclusively in the liver and propionate has physiological effects on a number of metabolic and endocrine systems and propionate loading tests have successfully been used to evaluate hepatic and endocrine function of ruminants (Grohn *et al.* 1985, Bruss *et al.* 1986). In this study, we conducted propionate tolerance test in six months old lambs to evaluate liver function (gluconeogenic capacity of liver) in relation to both adequate nutrient availability and nutrient scarcity situations **(Paper II)**. Thus, two different propionate tolerance tests were performed, one in the fed state (the milk-cream mix was not fed to the HCHF lambs to avoid interference of high plasma lipid levels in the later analyses) and the other by the end of a 2-day fasting period.

Laboratory analyses of metabolites and hormones

Overview of different metabolites and hormones analysed during different tolerance tests is presented in Table 1. Glucose and insulin **(Appendix II)** levels were measured to examine overall glucose-insulin homeostasis and non-esterified fatty acids (NEFA), TG, cholesterols were determined to investigate changes in lipid metabolism. Blood urea nitrogen (BUN) provides information hepatic and kidneys function as it is the amount of nitrogen in blood that comes from urea produced by liver and filtered by kidneys as a waste product. Creatinine is also considered an indicator of renal function as it by-product of muscle metabolism and cleared by kidneys. β-hydroxy butyrate (BOHB), a ketone body was measured was measured to examine ketosis status and γ-glutaryltransferase (GGT) was determined as an index of liver dysfunction. Lactate, were determined in plasma samples to investigate interplay between liver and extra-hepatic tissues during metabolic adaptions. Analyses for leptin, IGF-1, cortisol, and ghrelin were determined in the fasting samples to investigate overall changes in regulation energy expenditure, gluconeogenesis and appetite during fasting **(Paper I)**.

Morphological and molecular analyses of adipose tissue

Adipose tissue is considered as one of the important targets of abnormal early life nutrition increasing risks of obesity and associated metabolic disorders later in life (Desai & Ross 2011). At six months of age, sub-groups of animals from each treatment group were slaughtered, and tissues samples were randomly sampled from subcutaneous (above the muscularis *longissimus dorsii* at the level of the last rib), mesenteric, perirenal and epicardial adipose tissues to investigate depot-specific morphological and molecular changes of adipose tissue development and lipid metabolism **(Paper III)**. Adipocyte tissue size (area), and proportions of adipocytes, collagen and connective tissues and microvessles, and cell number

Table 1: Plasma metabolites and hormones analysed during the different tolerance test

Tolerance tests	*Fasting*	*Glucose*	*Insulin*	*Propionate (fasted)*	*Propionate (fed)*
Metabolites/Hormones					
Glucose	**X**	**X**	**X**	**X**	**X**
Insulin	**X**	**X**	**X**	**X**	**X**
Lactate	**X**	**X**	**X**	**X**	**X**
TG	**X**	**X**	**X**	**X**	**X**
NEFA	**X**	**X**	**X**	**X**	**X**
Cholesterol	**X**	**X**	**X**	**X**	**X**
BOHB	**X**	**X**	**X**	**X**	**X**
Creatinine	**X**	**X**	**X**	**X**	**X**
BUN	**X**	**X**	**X**	**X**	**X**
GGT	**X**	**X**	**X**	**X**	**X**
Leptin	**X**				
IGF-1	**X**				
Cortisol	**X**				
Ghrelin	**X**				

TG, Triglycerides; NEFA, Non-esterified fatty acids; BOHB, β-hydroxy butyrate; BUN, Blood urea nitrogen; GGT, γ-glutaryl transferase; IGF-1, Insulin like growth factor-1

index (CNI) to investigate cellularity and morphological changes of adipose tissue **(Paper III; Appendices III and IV)**. Prenatal and early postnatal nutrition can affect changes in expression of genes associated with adipose tissue development, lipid metabolism and inflammation; we aimed to investigate changes in expression levels of different adipose markers at different depots. For gene expression studies, pieces of tissues were fixated in RNAlater (RNAlater® Solution, Ambion, The RNA Company, USA) for 24 hours and then all samples were stored at -80ºC pending analyses. RNA extractions (Appendix V), RNA

integrity determination (Appendix VI), cDNA synthesis (VII) were performed by using standard protocols. The mRNA expression levels of target genes related to lipid metabolism, adipose development and inflammation at different adipose tissue depots (subcutaneous, mesenteric, perirenal and epicardial) were determined by using quantitative real time polymerase chain reaction (qPCR) as described previously (Hou *et al*., 2013) **(Paper III; Appendix VIII)**.

Protein-protein interaction (PPI) networks analyses for candidate genes

The thirteen candidate genes studied here are known gene targets/biomarkers of adipose tissue metabolism, development and inflammation. It could be hypothesized that if the pre- and postnatal nutritional insults can modify the expression levels of these markers, it could also potentially affect genes/markers that are closely co-regulated along with the target genes. This would result in corresponding changes in protein/metabolite abundance in different tissues. In order to study the regulatory and interaction networks including our target genes, protein-protein interaction (PPI) networks were built by anchoring target genes and derived known and predicted interactions by running the network analyses STRING (Search Tool for the Retrieval of Interacting Genes/Proteins; http://string.embl.de/ (Jensen *et al.* 2009), which is a database of known and predicted protein interactions. STRING uses one protein per gene. If there is more than one isoform per gene, the longest isoform is selected, unless there is information to suggest that another isoform is better annotated. The interactions include direct (physical) and indirect (functional) associations; these are derived from four sources: Genomic context, High-throughput experiments, Co-expression databases and previous knowledge (text mining).

Accompanying Papers

PAPER I

Khanal, P., Husted, S.V., Axel, A.M.D., Johnsen, L., Pedersen, K.L., Moretensen, M.S., Kongsted, A.H. & Nielsen, M.O. 2014. Late gestation over- and undernutrition predispose for visceral adiposity in response to a post-natal obesogenic diet, but with differential impacts on glucose–insulin adaptations during fasting in lambs. *Acta Physiol* **210,** 110–126.

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Late gestation over- and undernutrition predispose for visceral adiposity in response to a post-natal obesogenic diet, but with differential impacts on glucose–insulin adaptations during fasting in lambs

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Abstract

Aim: To investigate if late gestation under- or overnutrition has similar adverse impacts on visceral adiposity, metabolic and endocrine function in sheep, and if subsequent exposure to a high-fat diet in early post-natal life exaggerates the prenatal programming outcomes later in life.

Methods: Thirty-six twin-pregnant ewes were fed a NORM (fulfilling 100% of daily requirements for energy and protein), LOW (50% of NORM) or HIGH diet (150% of energy and 110% of protein requirements) during the last 6 weeks of gestation (term = 147 days). Post-natally, the twin lambs were subjected to a high-fat or a moderate diet until 6 months of age (around puberty), where metabolic and endocrine adaptability to fasting was examined, and subgroups of animals were killed.

Results: Animals exposed to either prenatal under- or overnutrition had reduced subcutaneous fat deposition when fed a high-fat diet, resulting in higher ratios of mesenteric and peri-renal fat relative to subcutaneous fat compared to controls. This was not related to prenatal influences on plasma glucose or insulin. Irrespective of the prenatal diet, high-fat-fed lambs underwent changes resembling the metabolic syndrome with higher plasma glucose, cholesterol, non-esterified fatty acids, triglyceride and lactate combined with abdominal obesity. Peri-renal fat appeared to be a particular target of a high-fat diet post-natally.

Conclusion: Both prenatal under- and overnutrition predisposed for abdominal adiposity, apparently by reducing the expandability of subcutaneous adipose tissue and induced differential physiological adaptations to fasting. This study does not suggest that exposure to gestational overnutrition will provide a protective effect against development of hyperglycaemia later in life.

Keywords fat deposition, foetal malnutrition, metabolic profiles, obesity, post-natal overnutrition.

It has been known for many years that foetal undernutrition can lead to permanent and irreversible changes in body composition, metabolism and endocrine function, thus increasing the risks of metabolic disorders later in life (Hales & Barker 1992, Ravelli *et al.* 1999, Godfrey & Barker 2000). In recent years, mounting evidence has suggested that maternal overnutrition can have similar long-term adverse effects on the offspring (Muhlhausler *et al.* 2006, Grattan 2008, Ford & Long 2011). Previous studies have revealed a

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'U' – shaped curve relating birth weight to the risk of adult obesity (Curhan *et al.* 1996, Godfrey & Barker 2000, Dyer & Rosenfeld 2011). This suggests that widely different nutritional insults (over- vs. undernutrition) during gestation, resulting in individuals being born at the extremes of the birth weight spectrum, may predispose for similar adverse outcomes on health and disease later in life. However, it is not known, whether these widely different nutritional insults, that is, over- or undernutrition in foetal life are affecting development of organs and body functions through similar physiological mechanisms. It is well recognized that nutrition during early post-natal life may also play an important role in determining the growth trajectory and post-natal obesity risk, and the immediate post-natal period has also been suggested as a period where long-term programming of health and disease can be induced (Patel & Srinivasan 2011). It has been reported in several studies that exposure to overnutrition with an energy dense, high-fat diet during early post-natal life increases the risk of metabolic syndrome later in life, particularly in offspring subjected to prenatal nutrient restriction, which might be due to an altered metabolic capacity to tolerate energy-rich diets or rapid catch-up growth in post-natal life (Guilloteau *et al.* 2009). However, it is not well known, whether such a worse-case scenario of mismatching pre- and post-natal exposures also applies for those individuals, who have been exposed to overnutrition during both foetal and early post-natal life. This question is important to elucidate in order to be able to establish efficient strategies for intervention in the future for individuals exposed to different kinds of foetal malnutrition.

Late gestation is the period, which contributes most to determine the birth weight in humans, and it has previously been reported that the majority of adipose tissue deposition occurs during late gestation in species born precocial, such as humans and sheep (Symonds & Lomax 1992). We have developed the Copenhagen sheep model in order to be able to investigate the long-term consequences of malnutrition in a stage of gestation corresponding to the human third trimester (Nielsen *et al.* 2012), which are not possible to study in rodent or pig models, as these animal species are born altricial. In our sheep model, we have shown that late gestation undernutrition increases the risk of abdominal obesity upon exposure to an energy dense, high-fat diet during early post-natal life, possibly due to a reduced expandability of subcutaneous adipose tissue. Other sheep model studies have demonstrated similar outcomes in response also to early and mid-gestation undernutrition (Ford *et al.* 2007).

Recent studies indicate that maternal overnutrition may also have long-term adverse effects on adiposity in sheep offspring (Ford & Long 2011), and such increased adiposity in offspring during early post-natal life has been associated with higher blood glucose levels (Muhlhausler *et al.* 2006).

In the present study, we aimed to test the hypothesis, using the Copenhagen sheep model, that both late gestation under- and overnutrition programme adipose tissue development to predispose for development of visceral adiposity later in life, based on induction of similar changes in metabolic adaptability to variations in nutrient supply, but despite similarities, individuals with a foetal history of overnutrition will have a superior ability to cope with a high-fat diet in early post-natal life.

Materials and methods

The present experiment was conducted at the experimental facilities on the farm Rosenlund, Lynge, Denmark under the auspices of the Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark. All experimental procedures were approved by the National Committee on Animal Experimentation, Denmark.

Experimental animals and design

The experiment was a 3 × 2 factorial design with three prenatal and two post-natal nutrition exposures, as illustrated in Figure 1. Thirty-six twin-pregnant multiparous (parities ranging from 3 to 5) cross-bred Texel ewes and their offspring were used in the experiment. During late gestation (last 6 weeks of pregnancy; term = 147 days), the ewes were allocated to one of three different dietary regimens: NORM ($N = 9$) fulfilling daily requirements for energy and protein according to Danish feeding standards; LOW ($N = 14$) providing only 50% of energy and protein requirements; or HIGH ($N = 13$) designed to provide 150% of energy and 110% of protein requirements. The ewes were allocated to these treatments 6 weeks prior to expected parturition, and it was attempted to reach as even a distribution of body weight and body condition score (BCS) across treatment groups as possible at the entry into the experiment (Table 1). It was ensured that all newborn lambs received colostrum within 3 h after birth. The lambs suckled their dam at will until 3 days after parturition, and then the dam was removed from her offspring. From 3 days to 6 months of age, one lamb from each twin pair received a high-carbohydrate–high-fat (HCHF) and the other lamb a moderate, conventional (CONV) diet. Allocation of the twin lambs to their respective post-natal dietary treatment groups was made right after birth using gender (first priority) and birth

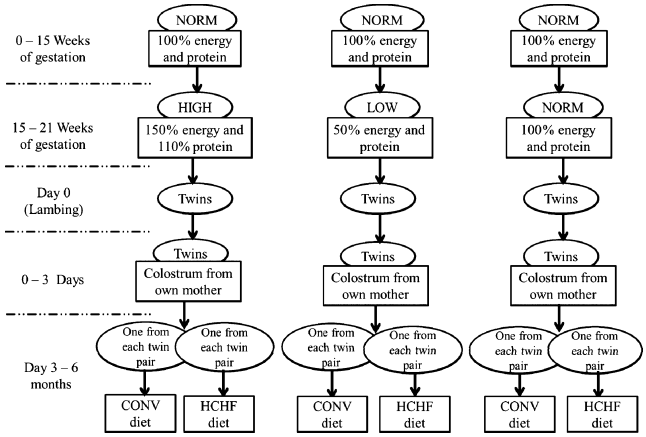

Figure 1 Schematic presentation of the experimental 3 × 2 factorial design. During the last 6 weeks of pregnancy, the pregnant ewes were given either a HIGH (N = 13; 150% of daily requirements for digestible energy and 110% of daily requirements for protein, respectively) or LOW (N = 14; 50% of requirements for energy and protein) or NORM diet (N = 9; 100% of requirements for energy and protein). After birth, one twin lamb was assigned to a HCHF (N = 35; 18 males; 17 females; high-carbohydrate high-fat diet consisting of a cream-milk replacer mix in a 1 : 1 ratio supplemented with rolled maize) or CONV (N = 35; 16 males, 19 females; conventional diet consisting of milk replacer and hay until 8 weeks and hay only thereafter and adjusted to achieve moderate and constant growth rates of approx. 225 g day^{-1}). The twin lambs were assigned to their respective post-natal treatment groups right after birth ensuring as uniform a distribution of gender (first priority) and birth weight (second priority) as possible in the two post-natal feeding groups derived from a given prenatal treatment. The experimental design thus gave rise to six treatment groups: HIGH-CONV (N = 13), five males, eight females; HIGH-HCHF (N = 13), five males, eight females; LOW-CONV (N = 13), six males, seven females; LOW-HCHF (N = 13), seven males, six females; NORM-CONV (N = 9), five males, four females; NORM-HCHF (N = 9), six males, three females.

Table 1 Daily energy and protein intake of twin-pregnant sheep during the last 6 weeks of gestation (term = 147 days), and gender distribution and birth weights of their offspring

	Pre-partum feeding levels (ewe)		
Parameters	HIGH	NORM	LOW
No. of twin-pregnant ewes	13	9	14
Weight of ewes 6 weeks pre-partum, kg	76.3 ± 1.5	77.6 ± 1.9	75.1 ± 1.5
BCS of ewes 6 weeks pre-partum	3.87 ± 0.10	3.69 ± 0.12	3.64 ± 0.10
Weight of ewes just prior to partum, kg	92.5 ± 2.1^{a}	89.7 ± 3.15^{a}	79.4 ± 1.4^{b}
BCS of ewes just prior to partum	4.31 ± 0.06^{a}	3.75 ± 0.06^{b}	2.79 ± 0.12^{c}
DE, MJ day^{-1} (average for the last 6 weeks pre-partum)	34.8 ± 0.6^{a}	22.9 ± 0.8^{b}	11.0 ± 0.6^{c}
DCP intake, g day^{-1} (average for the last 6 weeks pre-partum)	284 ± 4.5^{a}	263 ± 5.7^{b}	125 ± 4.6^{c}
No. of lambs	26	18	28
No. of each gender (M/F)	10/16	11/7	15/13
Birth weight, kg	4.38 ± 0.15^{a}	4.35 ± 0.18^{a}	3.89 ± 0.15^{b}
Range in birth weight, kg	2.98–5.83	3.56–5.05	2.05–5.41

NORM, normal diet fulfilling requirements for energy and protein; HIGH, 150% of normal requirements for energy and 110% of normal requirements for protein; LOW, 50% of normal requirements for energy and protein; BCS, body condition score, scale from 1 to 5 (very lean to very fat); DE, digestible energy; DCP, digestible crude protein; M, male; F, female.
Data are presented as least square means ± SEM. Data presented within rows with different superscript letters are significantly different at $P < 0.05$. The DE intake increased from 6 weeks pre-partum to just prior to partum from 20.8 to 39.4, 17.8 to 24.3 and 8.70 to 11.7 MJ day^{-1} for HIGH, NORM and LOW ewes respectively. The increase in DCP from 6 weeks pre-partum to just prior to partum were 199–311, 204–278 and 99.7–134 g day^{-1} for HIGH, NORM and LOW ewes respectively.

weight (second priority) as allocation criteria to achieve as even a sex and birth weight distribution as possible across the two post-natal treatment groups within each prenatal treatment. In the following sections, HIGH, LOW and NORM will refer to prenatal nutrition exposures, and HCHF and CONV denote post-natal dietary treatments.

Experimental housing and feeding of ewes

All ewes were transferred to the experimental facility 1 week before initiation of the experiment for adaptation to the indoor experimental conditions and were given a subcutaneous bolus injection with vitamins (10 mL ADEsan and 10 mL Becomplex SC; Boehringer Ingelheim, Copenhagen, Denmark). The ewes were housed in individual bar-walled pens (1.5 × 1.5 m) with sawdust as bedding material.

The diet for NORM-fed sheep consisted of artificially dried green hay (DanGrønt Speciality Feeds; DLG, Ølgod, Denmark) and commercial concentrate (Fårefoder F; Nordsjællands Andels Grovvareforening, Helsinge, Denmark) to fulfil 100% of the daily requirements for metabolizable energy (ME) and crude protein (NRC 2007). LOW sheep received 50% of the amount of hay and concentrate amounts fed to NORM-fed sheep on a metabolic body weight (MBW) basis. HIGH sheep were fed the same amount of hay and concentrate on a MBW basis as LOW sheep, but were supplemented with whole barley to provide 150% of ME and 110% of daily protein requirements. The average digestible energy and crude protein intakes during the last 6 weeks of gestation are given in Table 1. The LOW ewes were offered small amounts of barley straw (insignificant contribution to energy and protein intake) to avoid discomfort from sensation of hunger. All ewes were fed twice daily, one-half of the rations being given at each meal, and they had access to fresh drinking water *ad libitum,* and received a vitamin-mineral mix (Får Min; VILOMIX, Mørke, Denmark) to fulfil requirements (NRC 2007). During the first 3 days after parturition, all ewes were fed with hay *ad libitum* and supplemented with 300 g of concentrate and 300 g barley per day (in two daily meals) to ensure sufficient colostrum production for the offspring.

Experimental housing and feeding of lambs

Three days after parturition, the ewes were separated from their lambs, and the pens were subdivided into two smaller pens (1.5 × 0.75 m) with sawdust as bedding material for individual housing and feeding of lambs. When the lambs reached the age of 2 months, they were transferred to larger pens (1.5 × 1.5 m), where they were housed for the remainder of the experimental period.

The chemical composition of feedstuffs provided to lambs is given in Table 2. From 3 days till 8 weeks of age (milk-feeding period), CONV lambs were fed milk replacer (180 g milk powder L^{-1}; Elitemilk Lamb, Vilofarm; DLA Group, Galten, Denmark) from a suckling bucket, and in addition to that they received a good quality hay from the age of 14 days. From 3 to 7 days of age, the lambs were fed four times a day and twice daily thereafter. Daily allowances of milk and hay were adjusted on a weekly basis to achieve daily moderate live weight gains of approx. 225 g day^{-1}.

The HCHF lambs were fed a mixture of 50% of the milk replacer and 50% dairy cream (Osted Ost og Mejeri ApS, Lejre, Denmark) *ad libitum* (until they reached the daily maximum of 2.5 L day^{-1}) until the end of the experimental feeding period (6 month). Rolled maize (Maize flakes; R2 Feed Partner A/S, Hedensted, Denmark) was fed *ad libitum* (until they reached the maximum allowance of 1 kg day^{-1}). The milk replacer-dairy cream mix was bottle-fed four

Table 2 Chemical composition and energy content of experimental feeds

Feeds	DM (%)	Ash (% of DM)	aNDF (% of DM)	ADF (% of DM)	ADL (% of DM)	CP (% of DM)	Cfat (% of DM)	DE (MJ kg DM^{-1})
Sheep diet								
Hay	91.4	5.6	47.7	27	3.1	20.8	4.8	13.7
Barley	89.0	2.3	14	6	1.1	12.5	3.1	17.1
Concentrate	87.7	7.7	25.8	18	2.8	15.3	3.8	12.8
Lamb diet								
Hay	93.1	6.8	50.4	32.3	3.5	19.1	3.7	13.5
Maize	89.5	0.6	4.1	<5	0.9	8.5	1.9	16.3
Milk powder	95.6	7.1	–	–	–	22.5	23.6	19.2
Cream	42.9	0.8	–	–	–	4.3	38.0	30.5

DM, dry matter; aNDF, amylase-treated neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; CP, crude protein; Cfat, crude fat; DE, digestible energy.

times daily during day 3–7 post-partum and twice daily thereafter from a suckling bucket. The digestible energy and protein intake of different lamb groups from 3 days to 6 months of age are provided in Table 3. Both lamb groups were supplemented daily with small amounts of barley straw (negligible contribution to energy and protein intake). Water was available *ad libitum* at all times and vitamin-mineral mix was provided based on requirements (NRC 2007).

All ewes gave birth to two live lambs. Two lambs died within the first 2 weeks after birth: one LOW-HCHF male due to infectious peri-carditis, and one LOW-CONV male for unknown reasons (without showing any prior symptoms of illness). Thus, 72 lambs were included in registrations and samplings from birth until 1 week of age, and 70 lambs were included in registrations thereafter. At 4 weeks of age, one HIGH-CONV and one LOW-CONV lamb developed symptoms of pneumonia and were treated with antibiotics (Curamox^{R} Vet., 150 mg mL^{-1}; Boehringer Ingelheim) for 3 days, whereafter they fully recovered. One HIGH-CONV lamb was successfully treated for diarrhoea (Baycox Sheep vet., Bayer HealthCare, Leverkusen, Germany) at the age of 14 weeks.

For twin-pregnant ewes, feed intake was recorded over a 24-h period twice weekly, and body weights and BCS were recorded once a week. The lambs were examined and weighed immediately after birth. Lamb body weights were recorded weekly until 6 months of age. Body proportions [crown-rump length, girth circumference, height over withers, head width (using slide gauge from eye to eye)] were measured at day 1, and at 3 and 6 months of age. Feed intakes were recorded daily during the first week of life and once weekly over a 24-h period thereafter.

Blood samplings

Blood samples were collected from all ewes at approx. 11:00 AM by jugular venipuncture once on weeks −7, −5, −3 and −1 week prior to expected parturition, and on the day after lambing. Additionally, blood samples from lambs were collected the day after birth (day 1) by jugular venipuncture at approx. 11:00 AM.

At 6 months of age, the lambs were subjected to a 44-h fasting period. They had free access to drinking water during that time. Catheters (INTRAFLON 2, 2.7 mm; Laboratories Pharmaceutiques, VYGON, Écouen, France) were inserted into both jugular veins at least 1 day prior to initiation of the fasting period to facilitate repeated collection of blood samples as described by Husted *et al.* (2007). The first sample was taken prior to removal of the food (zero sample, at approx. 13:00 PM) and thereafter at times 24 (at approx. 13:00 PM) and 44 h (at approx. 09:00 AM) after onset of the fasting period.

All blood samples were collected in EDTA-coated tubes and immediately placed on ice until centrifugation at 1800 ***g*** for 15 min at 4 °C. Plasma was transferred to cryo-vials and stored at −20 °C for later analysis.

Laboratory analyses

Glucose, insulin, non-esterified fatty acids (NEFA), triglycerides (TG), blood urea nitrogen (BUN), creatinine, lactate, β-hydroxy butyrate (BOHB), γ-glutamyltrans-

Table 3 Digestible energy and protein intakes of experimental lambs from 3 days to 6 months of age

Parameters	NORM-CONV	NORM-HCHF	HIGH-CONV	HIGH-HCHF	LOW-CONV	LOW-HCHF
Number of lambs (M/F)	5/4	6/3	5/8	5/8	6/7	7/6
DE intake (MJ day^{-1})						
3 days–8 weeks	4.5 ± 0.5^{b}	8.4 ± 0.5^{a}	4.3 ± 0.4^{b}	8.0 ± 0.4^{a}	4.3 ± 0.4^{b}	7.7 ± 0.4^{a}
8 weeks–6 months	11.9 ± 0.9	12.5 ± 0.9	12.4 ± 0.7	11.5 ± 0.7	11.7 ± 0.7	10.9 ± 0.7
DCP (g day^{-1})						
3 days–8 weeks	42.9 ± 1.6^{a}	18.1 ± 1.6^{b}	41.2 ± 1.3^{a}	17.2 ± 1.3^{b}	40.5 ± 1.3^{a}	17.2 ± 1.3^{b}
8 weeks–6 months	122 ± 8.6^{a}	29.8 ± 8.6^{b}	129 ± 7.1^{a}	27.2 ± 7.1^{b}	120 ± 7.1^{a}	26.7 ± 7.2^{b}

NORM, HIGH, LOW, see legends to Table 1; HCHF, high-carbohydrate high-fat diet; CONV, conventional diet to achieve moderate and constant growth rates of approx. 225 g day^{-1}; M, male; F, female; DE, digestible energy; DCP, digestible crude protein.

Data are presented as least square means ± SEM and within rows data with different superscript letters are significantly different at $P < 0.05$. The experimental lambs were offspring of NORM or HIGH or LOW ewes during the last 6 weeks of gestation. From 3 days to 6 months of age, lambs were fed either the HCHF or the CONV diet. The HCHF diet included rolled maize and dairy cream-milk replacer mix. The CONV diet consisted of hay and milk replacer until 8 weeks of age and hay only thereafter. The HCHF lambs had significantly higher intake of energy and lower intake of protein until 8 weeks as compared to CONV lambs ($P < 0.0001$). From 8 weeks to 6 months, CONV lambs had significantly higher intake of protein compared to HCHF lambs ($P < 0.0001$).

ferase (GGT) and cholesterol levels were determined in plasma samples obtained from ewes and all lambs at the University of Copenhagen, Frederiksberg, Denmark as previously described (Kongsted *et al.* 2013). The intra- and interassay coefficients of variation (CV) were below 5 and 10%, respectively for all assays.

Analyses for leptin, IGF-1, cortisol and ghrelin were performed at The University of Western Australia, Perth, Australia. Plasma leptin was measured in duplicate by a double-antibody radio-immunoassay (Blache *et al.* 2000). Plasma IGF-1 was assayed in duplicate by a double-antibody radio-immunoassay using human recombinant IGF-1 (30-A 188; Fitzgerald Industries, Concord, MA, USA) and antihuman IGF-1 antiserum (AFP4892898; National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Disease, Torrance, CA, USA) following an acid–ethanol extraction and cryo-precipitation (Breier *et al.* 1991). Plasma cortisol levels were determined using a commercial radio-immunoassay kit (Clinical AssaysTM, GammaCoatTM, cortisol 125I RIA Kit; DiaSorin, Stillwater, MN, USA; Beausoleil *et al.* 2008). Plasma ghrelin was measured in duplicate by a modified double-antibody RIA method based on the Linco total Ghrelin RIA kit (GHRT-89HK; Linco, St Charles, MI, USA) as previously described by Miller *et al.* (2009). The samples were processed in a single assay for leptin, IGF-1 and ghrelin where intra-assay CVs were below 7%, whereas plasma cortisol was analysed in two assays with intra- and inter-CVs of below 2 and 5% respectively.

Slaughtering of subgroups of experimental animals at 6 months of age

Twenty-six lambs in total [NORM-CONV: 3 (3M, 0F), NORM-HCHF: 3 (3M, 0F), HIGH-CONV: 5 (2M, 3F), HIGH-HCHF: 5 (2M, 3F), LOW-CONV: 5 (2M, 3F), LOW-HCHF: 5 (3M, 2F)] were selected for slaughtering at 6 months of age. Only one lamb from each twin pair was selected for slaughtering allowing another lamb within each twin pair to continue in the experiment for later studies as adults. The subgroups of lambs were selected within each treatment group so that slaughtered and surviving animals (continuing in the experiment) had as uniform a distribution of body weight and gender as possible, except that we chose to slaughter only males from the NORM-CONV and NORM-HCHF groups due to the smaller group size. This was done to allow a sufficient number of females to continue in the experiment to assess the long-term consequences of early life exposures in both genders, which will be published subsequently. Prior to slaughtering, body weight and body proportions were determined. Just before slaughtering, animals were anaesthetized with propofol (5–6 mg i.m. kg^{-1} body weight; B. Braun, Melsungen, Germany) and they were thereafter killed by decapitation. Immediately after decapitation, different organs were excised and weighed.

Statistical analyses

Statistical evaluation of all data was performed in SAS (v.9.2; SAS Institute, Inc., Cary, NC, USA). Homogeneity of variance was evaluated by visual inspection of residual plots and normality of residuals was tested by means of quantile–quantile plots. Log transformations were applied when it was needed to obtain normal distribution of residuals. The birth weights of lambs were analysed by the PROC GLIMMIX procedure, where ewe was included as *random effect* to account for the twin effect. Different organ and tissue weights were analysed using the PROC GLM procedure to compare effects of pre- and post-natal nutrition and lamb gender. The remaining data, including fasting challenge tests, were analysed as repeated measures by the PROC GLIMMIX procedure. For the repeated measurements, different correlation structures between measurements and inhomogeneous variances were tested, and the structure yielding the best fit was chosen. The model included *fixed effects* of feeding level, gender and time of sampling; individual sheep and lambs within feeding level were included as *random effects*, and samples within sheep and lambs were considered *repeated measurements*. Within the post-natal HCHF group, a few animals (one from the NORM, two from the HIGH and two from the LOW prenatal groups) had poorer growth rates compared to other animals in their groups, which was evident from 4 to 6 weeks of age, and they reached substantially lower body weights at 6 months of age (all <30 kg) and compared to all the rest of the HCHF-fed animals (all >35 kg), and they attained a characteristic skinny appearance not observed in any of the other animals irrespective of post-natal feeding, but despite the skinny appearance, they had extensive abdominal fat deposition as observed at slaughter. In the statistical analyses, we therefore also decided to test if these 'small-skinny-fat' animals, although few in number, showed any indications of being different from the rest of the animals within their group with 'normal' appearances and growth trajectories for the parameters studied, and the selection criteria was based on a body weight below 30 kg. The model included *fixed effects* of phenotype, age of animal or time of sampling and phenotype ('small-skinny-fat' or 'normal'). Individual sheep and lambs were included as *random effects*, and parameters within sheep and lambs were

treated as *repeated measurements*. Differences in least square means (LS means) were compared by Tukey's multiple comparison test, and presented results are expressed as LS means with standard error of mean (LS means ± SEM). The level of significance was set at $P < 0.05$.

Results

Unless it is specifically stated in the following, we did not for the studied parameters detect any significant effects of the prenatal nutrition, the interactions between pre- and post-natal diets, interactions with time (age of the animal during development or the time course of changes in metabolic and endocrine profiles during the fasting exposure), effects of gender or indications of differences between the 'small-skinny-fat' versus 'normal' HCHF lambs.

Feed intake, body weight and BCS of pregnant sheep

At the onset of the experiment (6 weeks pre-partum), all sheep had similar body weights and BCS (Table 1), but during the last 6 weeks of gestation, NORM and HIGH ewes increased their body weights more, and they obtained significantly higher body weights than LOW ewes during the last 2 weeks of gestation. In the last week of gestation, the BCS of the ewes reflected the feeding level, as HIGH ewes attained significantly higher BCS followed by NORM and then LOW ewes ($P < 0.0001$; Table 1).

Metabolic responses in pregnant sheep

The plasma metabolite levels were similar across treatment groups at the onset of the experiment. During the last 6 weeks of gestation, concentrations changed as would be expected based from fed intakes and gestational changes in the ewes, and HIGH ewes compared to NORM and LOW ewes increased plasma concentrations more of glucose (3.70 ± 0.12–4.5 ± 0.10 mM in HIGH; 3.52 ± 0.16–4.15 ± 0.10 mM in NORM, $P = 0.0004$; and 3.70 ± 0.12–3.99 ± 0.12 mM in LOW, $P < 0.0001$) and GGT (48.3 ± 3.1–55.5 ± 3.2 U L^{-1} in HIGH; 50.4 ± 4.2–48.1 ± 3.3 U L^{-1} in NORM, $P = 0.02$; and 46.0 ± 2.9–37.8 ± 2.1 U L^{-1} in LOW, $P = 0.002$). The LOW ewes, however, increased their plasma levels of creatinine (69.3 ± 3.8–95.2 ± 3.6 μmM) and NEFA (0.27 ± 0.07–0.66 ± 0.05 mM) more during late gestation compared to NORM ewes (78.9 ± 4.9–79.0 ± 4.5 μmM for creatinine and 0.34 ± 0.09–0.30 ± 0.07 mM for NEFA; $P = 0.05$ and $P = 0.01$, respectively), and HIGH ewes (82.8 ± 3.9–76.6 ± 3.7 μmM for creatinine and 0.39 ± 0.07–0.21 ± 0.05 mM for NEFA; $P = 0.009$ and $P < 0.0001$ respectively). Overall, the NORM-fed ewes had higher TG (0.44 ± 0.02 mM) and BUN (8.9 ± 0.30 mM) concentrations compared to HIGH (0.29 ± 0.02 mM TG and 7.5 ± 0.25 mM BUN; $P < 0.0001$ and $P = 0.0007$, respectively) and LOW ewes (0.32 ± 0.02 mM TG and 6.7 ± 0.24 mM BUN; $P = 0.0001$ and $P < 0.0001$, respectively), and BUN levels were in turn significantly higher in HIGH than in LOW ewes ($P < 0.05$).

Birth weight and body proportions in lambs

The prenatally undernourished LOW lambs had reduced birth weights compared to lambs born to HIGH ($P = 0.009$) and NORM dams ($P = 0.03$), whereas no significant difference was obtained in lamb birth weights from NORM and HIGH ewes (Table 1). HIGH and NORM lambs had similar growth patterns until 8 weeks of age and grew at a higher rate than LOW lambs (Fig. 2). After 8 weeks of age, the NORM lambs grew faster than both HIGH and LOW lambs resulting in a significant interaction between prenatal diet and animal age ($P = 0.0004$). The CONV lambs had significantly higher body weights than HCHF lambs from 4 to 8 (age of weaning for CONV lambs) weeks of age, but from week 10, HCHF lambs increased their body weight gain faster

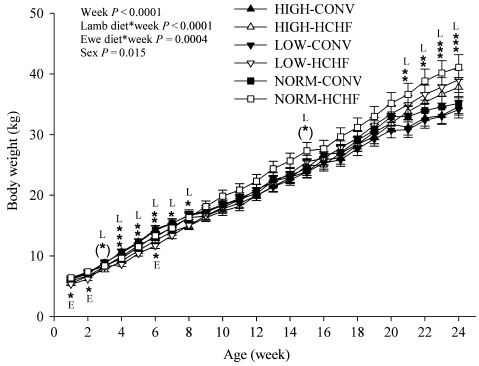

Figure 2 Changes in body weights of lambs from birth to 24 weeks (around puberty) of age. NORM; diet fulfilling daily requirements for energy and protein; HIGH, NORM, LOW, HCHF, CONV: see legends to Figure 1. From 4 to 8 weeks of age, CONV lambs had significantly higher body weights than HCHF lambs, but from week 21 and onwards, HCHF lambs became significantly heavier than CONV lambs. Values are least square (LS) means ± SEM represented by vertical bars. Within week, LS means were significantly different at (*), $P = 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

than the CONV lambs, and from week 21 onwards HCHF lambs became significantly heavier than CONV lambs ($P < 0.0001$ for the interaction between post-natal diet and age; Fig. 2). Within HCHF lambs, a few animals did not grow well from the beginning, and these 'small-skinny-fat' lambs by 24 weeks of age attained significantly lower body weights (>30 kg) compared to the rest of HCHF lambs (>35 kg; $P < 0.001$). Female lambs had lower body weights than male lambs, and this difference amounted to on average approx. 2 kg at 6 months of age ($P = 0.015$). The HCHF lambs in general attained higher girth circumference ($P = 0.005$) and head width ($P = 0.012$) compared to CONV lambs. Overall, male lambs had higher head circumference ($P < 0.0001$), height above wither ($P = 0.017$) and crown-rump length ($P < 0.05$) than female lambs.

Dietary intakes by lambs

There were no effects of gender or prenatal diet on the post-natal intakes of individual feeds over the time course of this experiment ($P > 0.24$ for all parameters and interactions; data not shown). During the milk-feeding period (from day 3 to 8 weeks of age), digestible energy intakes were higher in the HCHF lambs than in the CONV lambs ($P < 0.001$; Table 3), for which feed intakes were adjusted weekly to achieve the same moderate and constant weight gain. However, protein intake was significantly lower in HCHF lambs compared to CONV lambs for the first 8 weeks of life. From 8 weeks (weaning of CONV lambs) to 6 months of age, the average daily intakes of digestible energy were in the same range for all experimental groups; however, protein intakes were markedly lower in HCHF (approx. 26.7–29.8 g day^{-1}) lambs compared to CONV lambs (120–128 g day^{-1}; $P < 0.0001$). The 'small-skinny-fat' HCHF lambs had lower daily average intakes of cream (714 vs. 1153 mL day^{-1}), daily digestible energy (7.01 vs. 11.0 MJ day^{-1}; $P < 0.0001$) and digestible protein (16.1 vs. 25.0 g day^{-1}; $P < 0.0001$) compared with the other HCHF animals.

Metabolic and endocrine adaptations during fasting

Glucose. Plasma glucose levels were similar in CONV lambs irrespective of their prenatal diet ($P = 0.0008$), but in HCHF lambs, HIGH-HCHF lambs had higher blood glucose levels compared to LOW-HCHF lambs prior to fasting at time 0 ($P = 0.0003$) and 44 h after fasting ($P = 0.024$; Fig. 3a). The HCHF lambs had substantially higher baseline plasma glucose levels compared to CONV lambs ($P < 0.0001$), but this difference decreased during fasting and after 44 h of fasting, glucose levels in HCHF lambs had dropped to levels only slightly higher than those observed in CONV lambs ($P < 0.05$).

Lactate. Baseline lactate levels were almost twice as high in HCHF lambs compared to CONV lambs ($P < 0.01$; Fig. 3b). Plasma lactate level decreased as fasting progressed, but more in HCHF-fed animals compared to CONV animals and 44 h after fasting, lactate levels had decreased in HCHF lambs to similar levels as observed in CONV animals, that is, a pattern resembling that observed for glucose. The female lambs had higher lactate level than the male lambs ($P = 0.004$).

Non-esterified fatty acids. The HCHF lambs had more than fourfold basal NEFA levels than CONV lambs ($P < 0.0001$; Fig. 3c). Plasma NEFA steadily increased throughout the fasting period, but much more pronounced in CONV lambs compared to HCHF lambs, and after 44 h of fasting, the NEFA levels were only slightly higher in HCHF compared to CONV lambs.

β-Hydroxy butyrate. The baseline BOHB level was more than twofold higher in CONV compared to HCHF lambs ($P < 0.0001$), but as fasting proceeded, BOHB levels in CONV lambs were more than halved, whereas plasma BOHB levels were increased by more than threefold in HCHF lambs, thus creating the opposite picture after 44 h of fasting ($P < 0.0001$ for the interaction between post-natal diet and time; Fig. 3d).

Blood urea nitrogen. The baseline BUN was similar in CONV and HCHF lambs, but the BUN levels were increased much more in CONV lambs, and they attained higher BUN levels (>3-fold) during fasting than HCHF lambs ($P < 0.0001$; Fig. 3e).

Triglycerides. The HCHF lambs had more than twofold higher plasma TG levels compared to CONV lambs at all time points prior to and during fasting ($P < 0.0001$; Fig. 3f). As fasting proceeded, the TG level in HCHF lambs dropped sharply over the first 24 h and then stabilized at levels, which remained higher than in CONV lambs ($P = 0.04$ for the interaction between post-natal diet and time).

Creatinine. The HCHF lambs (104.43 μmM) had approx. 50% higher basal plasma creatinine level than CONV lambs (70.68 μmM), and this difference was evident at all time points during fasting ($P < 0.0001$; data not shown). Both HCHF and CONV lambs increased creatinine levels until 24 h after fasting followed by a small decrease in HCHF lambs

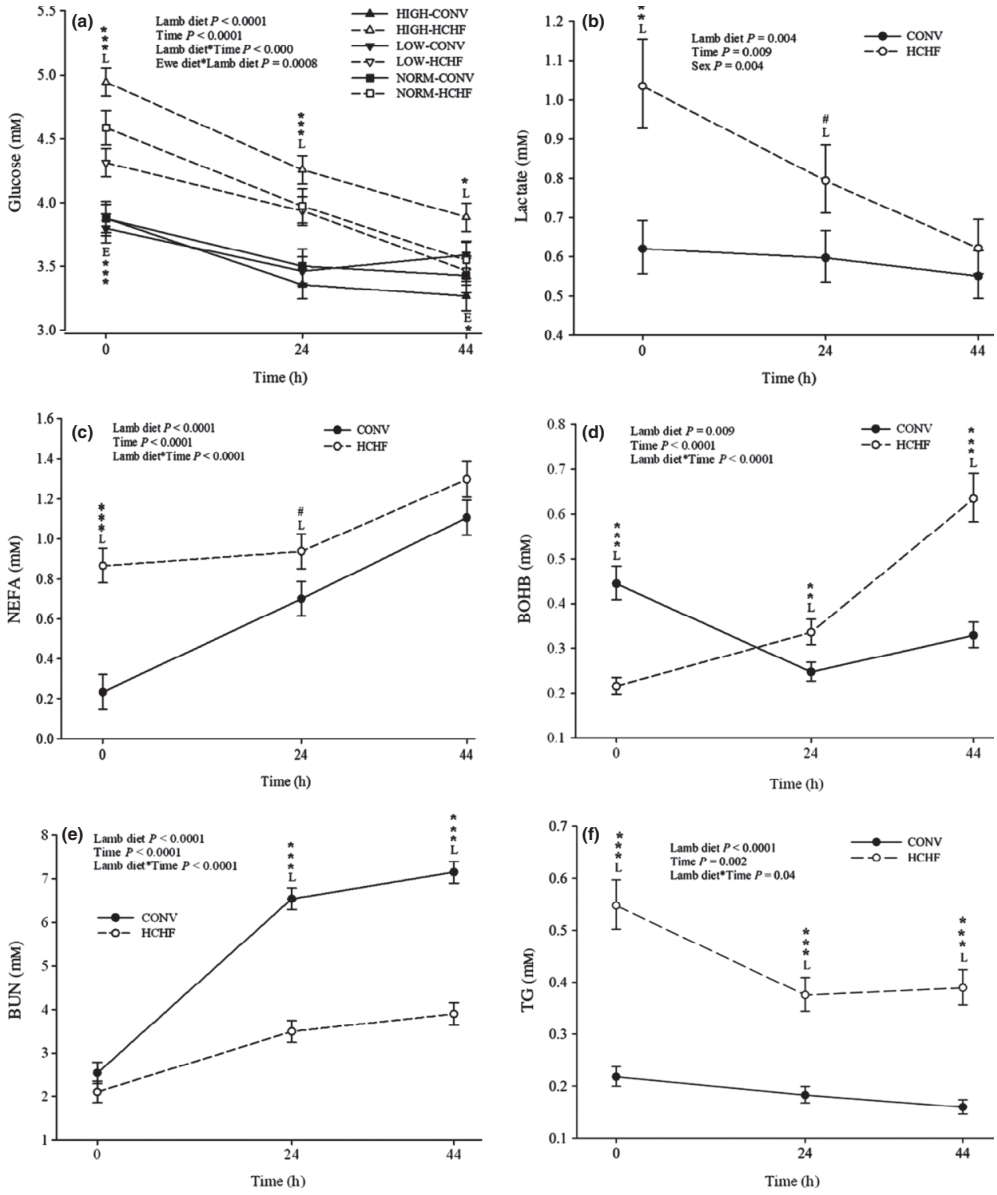

Figure 3 Changes in plasma levels during a 44-h fasting period of (a) glucose (b) lactate (c) non-esterified fatty acids (NEFA) (d) β-hydroxy butyrate (BOHB) (e) blood urea nitrogen (BUN), and (f) creatinine. HIGH, NORM, LOW, HCHF, CONV: see legends to Figure 1. E, ewe diet (prenatal) effect; L, lamb diet (post-natal) effect. Values are least square (LS) means ± SEM represented by vertical bars. Within sampling hour, LS means were significantly different at $*P < 0.05$; $**P < 0.01$; $***P < 0.0001$.

($P = 0.0027$ for the interaction between post-natal diet and time). The small-skinny-fat HCHF lambs had lower creatinine levels compared to the rest of the HCHF lambs (87.9 vs. 115 μmM; $P = 0.004$).

Cholesterol and GGT. Overall, GGT and cholesterol levels were significantly higher in the HCHF (161.70 U L^{-1} and 4.31 mM, respectively) compared to the CONV lambs (61.70 U L^{-1} and 1.60 mM,

respectively; $P < 0.001$; data not shown), but levels were not affected by fasting or the prenatal diet.

Endocrine responses during fasting

Insulin. The baseline insulin concentrations were highest in the unfasted state and higher in the HCHF than CONV lambs ($P = 0.0183$; Fig. 4a). Insulin levels decreased as fasting progressed. Within HIGH and NORM lambs, there were no effects of the post-natal diet, but LOW-CONV lambs maintained higher insulin levels throughout fasting and higher significantly higher than in LOW-HCHF lambs by the end of the 44-h fasting period ($P = 0.014$). CONV lambs in general had less pronounced reductions in insulin levels during fasting and maintained higher insulin levels at time points 24 ($P = 0.0166$) and 44 h ($P = 0.0229$) compared to HCHF lambs. Glucose/insulin ratios were calculated as a measure of insulin sensitivity. There was no effect of the post-natal on this ratio within the HIGH and NORM lambs, but among the LOW lambs, the LOW-CONV-fed lambs (22.60 ± 3.58 mmol μg^{-1}) had lower glucose/insulin ratio ($P = 0.0056$) as compared to the LOW-HCHF-fed lambs (40.59 ± 6.43 mmol μg^{-1}; $P = 0.0466$ for the interaction between the pre- and post-natal nutrition).

Leptin and IGF-1. The HCHF lambs had approximately twofold higher leptin and IGF-1 levels compared to CONV lambs at all time points during the pre-fasted and fasted states ($P < 0.0001$), and plasma leptin and IGF-1 levels decreased during fasting in a

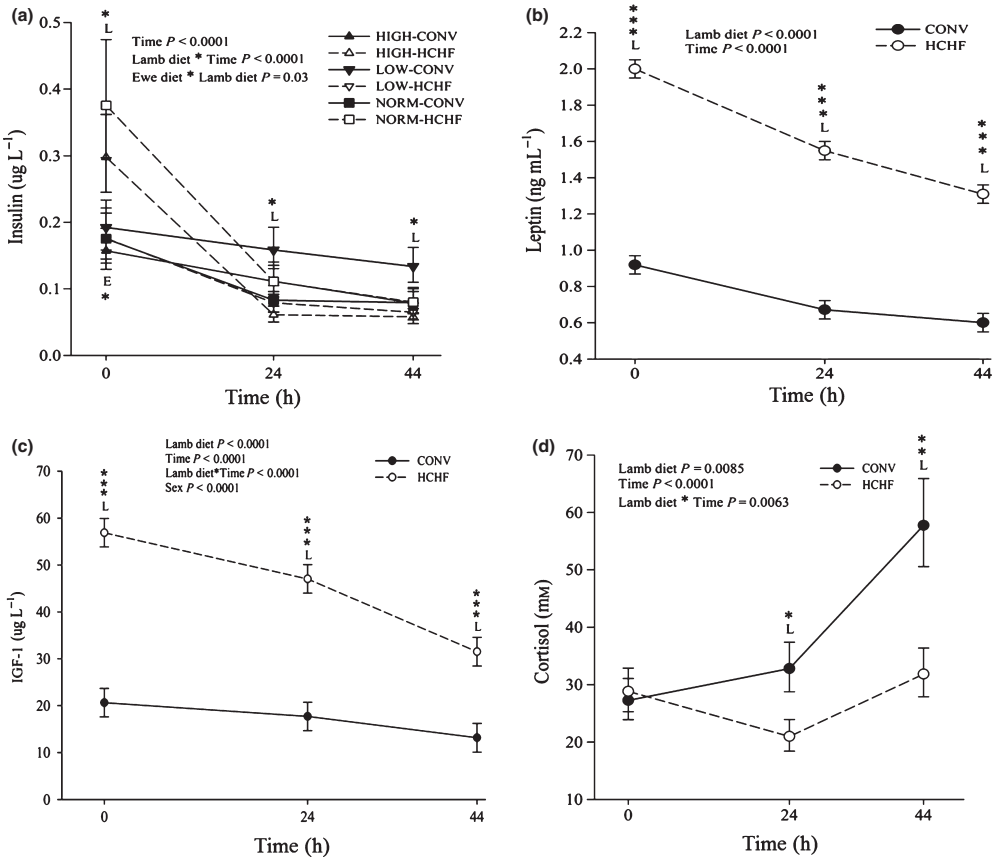

Figure 4 Changes in plasma levels during a 44-h fasting period of (a) insulin, (b) leptin, (c) insulin-like growth factor-1 (IGF-1) and (d) cortisol. HIGH ($N = 26$; ten male, 16 female); NORM ($N = 18$; 11 male, seven female); LOW ($N = 26$; 13 male, 13 female); HCHF ($N = 35$; 18 male, 17 female) and CONV ($N = 35$; 16 male, 19 female): see legends to Figure 1. E, ewe diet (prenatal) effect; L, lamb diet (post-natal) effect. Values are least square (LS) means ± SEM represented by vertical bars. Within sampling hour, LS means were significantly different at $*P < 0.05$; $**P < 0.01$; $***P < 0.0001$.

similar fashion in all treatment groups irrespective of the pre- and post-natal diet (Fig. 4b,c). The 'small-skinny fat' lambs had lower levels of leptin (in the fed state: 1.26 vs. 1.76 ng mL^{-1}; $P = 0.031$) and IGF-1 (in the fed state: 14.3 vs. 49.1 ng mL^{-1}, $P = 0.0071$; 24 h after fasting: 28.3 vs. 56.5 ng mL^{-1}, $P = 0.028$) compared to the rest of the HCHF lambs.

Cortisol. The baseline cortisol levels were similar in HCHF and CONV lambs (Fig. 4d). However, CONV lambs had more pronounced increases in cortisol levels during fasting ($P < 0.05$), whereas plasma cortisol was unaffected by fasting in HCHF animals.

Ghrelin. Plasma ghrelin levels were not affected by neither pre- or post-natal nutrition, and they in general increased during the first 24 h of fasting and normalized again thereafter in all groups ($P < 0.0029$; data not shown).

Fat-deposition patterns and organ weights

Fat deposition. The subcutaneous fat deposition was significantly higher in NORM lambs compared to HIGH lambs both expressed as absolute weights and when expressed relative to body weight ($P = 0.01$ and $P = 0.02$, respectively), whereas there were no significant differences between HIGH and LOW lambs or NORM and LOW, although LOW had numerically lower values for subcutaneous fat weights than HIGH (Table 4). The impact of the prenatal diet could be ascribed solely to the animals that had been exposed to a post-natal obesogenic diet, which resulted in an almost significant interaction of pre- and post-natal diet ($P = 0.0595$). The NORM-HCHF lambs had significantly higher subcutaneous fat deposition than HIGH-HCHF ($P < 0.0001$). There were no significant influences of the prenatal nutrition on deposition of neither mesenteric, peri-renal nor sternal fat.

The HCHF lambs had significantly higher depositions in absolute weights of subcutaneous (approx. 5.5-fold), mesenteric (approximately fivefold), peri-renal (approximately ninefold) and sternal (approx. 4.6-fold) fat than CONV lambs, and this picture was also seen after correction for body weight ($P < 0.0001$, for all fat depots; Table 4). The HCHF female lambs had significantly higher bodyweight-corrected peri-renal fat deposition than the HCHF male lambs ($P = 0.0025$), but a gender effect was not observed in CONV lambs. There were no gender effects for any of the other fat depots, when correcting for differences in body weight.

Fat-deposition pattern. The prenatal diet influenced the preference for fat deposition in the subcutaneous area compared to the abdominal region. The NORM lambs (0.33 ± 0.04) increased subcutaneous fat: mesenteric fat ratios compared to both HIGH (0.22 ± 0.03, $P = 0.018$) and LOW lambs (0.22 ± 0.03, $P = 0.02$; Fig. 5a). Similarly, the NORM lambs (0.34 ± 0.03) had significantly higher ratios of subcutaneous to peri-renal fat as compared to both HIGH (0.23 ± 0.02, $P = 0.009$) and LOW lambs (0.22 ± 0.02, $P = 0.005$; Fig. 5c). The prenatal diet did not have any significant effect on the distribution of fat within the abdomen, that is, between the mesenteric and peri-renal depots (Fig. 5e).

The post-natal diet did not have any significant effect on the relative distribution of fat between the subcutaneous and mesenteric region (Fig. 5b). However, the HCHF diet induced increased deposition of peri-renal fat relative to deposition of fat in both the subcutaneous and mesenteric regions, as reflected by significantly lower ratios of subcutaneous to peri-renal fat in HCHF lambs (0.19 ± 0.02) compared to CONV lambs (0.34 ± 0.02; $P < 0.0001$; Fig. 5d), and reduced mesenteric to peri-renal fat ratio in HCHF lambs (0.74 ± 0.07) compared to CONV lambs (1.39 ± 0.07; $P < 0.0001$; Fig. 5f).

Other organ and tissue weights. The prenatal nutrition exposure had no significant impact on gross or bodyweight-corrected weights of any of the other organs or tissues studied, except for the thyroids. The NORM and LOW lambs had significantly higher gross thyroid weights than HIGH lambs ($P < 0.05$).

The HCHF-fed lambs has significantly higher gross weights of thyroids ($P = 0.0005$), liver ($P < 0.0001$), heart ($P < 0.0001$), *longissimus dorsii* ($P < 0.0003$) and *biceps femoris* ($P = 0.02$) compared to lambs fed the CONV diet. In fact, liver weight was approximately doubled in HCHF compared to CONV lambs, and except for the skeletal muscles *longissimus dorsii* and *biceps femoris*, the post-natal diet effects persisted after correction for body weight. In contrast to the other organs and tissues, kidney weights, both gross and as a proportion of body weight, were found to be significantly reduced in lambs fed the HCHF as compared to CONV diet ($P < 0.002$; Table 4). The male lambs had significantly higher gross kidney and heart weights than female lambs ($P < 0.03$), but these gender effects disappeared upon correction for body weight (Table 4). Female lambs had significantly higher weight of *biceps femoris* compared to male lambs, but only when expressed relative to body weight ($P < 0.05$).

Discussion

We aimed to evaluate whether maternal malnutrition in the form of over- or undernutrition would have

Table 4 Effects of pre- and post-natal nutrition and gender on organ and adipose tissue weights in 6-month-old lambs

Parameters (actual weight, g or % of total body weight)	Prenatal nutrition			Post-natal nutrition		Gender	
	HIGH	NORM	LOW	HCHF	CONV	Male	Female
Body weight	36.5 ± 1.05^{b}	44.8 ± 1.36^{a}	38.5 ± 1.05^{ab}	42.3 ± 0.9^{a}	37.0 ± 0.9^{b}	40.0 ± 0.9	38.8 ± 1.2
Subcutaneous fat, g	140 ± 16^{b}	234 ± 23^{a}	182 ± 16^{ab}	315 ± 15^{a}	57 ± 14^{b}	197 ± 14	174 ± 19
Subcutaneous fat, %	0.36 ± 0.04^{b}	0.52 ± 0.05^{a}	0.45 ± 0.04^{ab}	0.74 ± 0.03^{a}	0.16 ± 0.03^{b}	0.44 ± 0.03	0.46 ± 0.04
Mesenteric fat, g	718 ± 111	845 ± 149	771 ± 112	1279 ± 97^{a}	257 ± 97^{b}	736 ± 102	837 ± 136
Mesenteric fat, %	1.86 ± 0.24	1.89 ± 0.34	1.82 ± 0.24	3.0 ± 0.18^{a}	0.66 ± 0.18^{b}	1.77 ± 0.18	1.92 ± 0.20
Perirenal fat, g	876 ± 95	990 ± 139	1124 ± 95	1793 ± 86^{a}	198 ± 85^{b}	877 ± 80	1109 ± 106
Perirenal fat, %	2.35 ± 0.18	2.37 ± 0.26	2.69 ± 0.18	4.46 ± 0.17^{a}	0.48 ± 0.16^{b}	2.07 ± 0.15^{b}	2.87 ± 0.18^{a}
Sternal fat, g	92 ± 11	95 ± 14	114 ± 10	168 ± 9^{a}	37 ± 9^{b}	108 ± 8	94 ± 10
Sternal fat, %	0.24 ± 0.02	0.22 ± 0.03	0.27 ± 0.02	0.40 ± 0.02^{a}	0.10 ± 0.02^{b}	0.26 ± 0.01	0.24 ± 0.02
Thyroid, g	2.27 ± 0.14^{b}	3.05 ± 0.19^{a}	2.80 ± 0.14^{a}	2.99 ± 0.13^{a}	2.32 ± 0.13^{b}	2.76 ± 0.13	2.58 ± 0.18
Thyroid, %	0.007 ± 0.0004	0.007 ± 0.0005	0.007 ± 0.0004	0.007 ± 0.0003^{a}	0.006 ± 0.0004^{b}	0.007 ± 0.0003	0.007 ± 0.0004
Adrenals, g	2.62 ± 0.20	2.77 ± 0.31	2.44 ± 0.20	2.65 ± 0.19	2.58 ± 0.18	2.39 ± 0.17	2.82 ± 0.23
Adrenals, %	0.007 ± 0.0004	0.007 ± 0.0006	0.006 ± 0.0004	0.007 ± 0.0004	0.007 ± 0.0003	0.006 ± 0.0003	0.007 ± 0.0004
Pancreas, g	30.80 ± 2.5	39.29 ± 3.26	30.39 ± 2.52	35.12 ± 2.30	30.09 ± 2.30	31.78 ± 21.13	35.92 ± 2.85
Pancreas, %	0.09 ± 0.007	0.10 ± 0.011	0.09 ± 0.008	0.10 ± 0.007	0.087 ± 0.007	0.09 ± 0.006	0.10 ± 0.008
Liver, g	485 ± 27	515 ± 36	565 ± 28	703 ± 25^{a}	343 ± 25^{b}	524 ± 20	511 ± 26
Liver, %	1.37 ± 0.06	1.16 ± 0.08	1.39 ± 0.06	1.66 ± 0.05^{a}	0.95 ± 0.05^{b}	1.31 ± 0.06	1.29 ± 0.07
Kidney, g	74.5 ± 2.86	72.1 ± 4.2	81.6 ± 2.93	70 ± 3^{b}	84 ± 3^{a}	81 ± 2^{a}	73 ± 3^{b}
Kidney, %	0.21 ± 0.008	0.18 ± 0.011	0.21 ± 0.01	0.17 ± 0.007^{b}	0.23 ± 0.007^{a}	0.21 ± 0.007	0.19 ± 0.009
Heart, g	164 ± 8	175 ± 12	186 ± 8	202 ± 7^{a}	147 ± 7^{b}	186 ± 7^{a}	163 ± 8^{b}
Heart, %	0.45 ± 0.02	0.44 ± 0.03	0.46 ± 0.02	0.50 ± 0.02^{a}	0.41 ± 0.01^{b}	0.46 ± 0.01	0.45 ± 0.02
Longissimus dorsii, g	464 ± 20	509 ± 22	529 ± 21	555 ± 18^{a}	447 ± 18^{b}	502 ± 19	501 ± 25
Longissimus dorsii, %	1.26 ± 0.05	1.27 ± 0.08	1.34 ± 0.05	1.34 ± 0.05	1.22 ± 0.04	1.25 ± 0.04	1.33 ± 0.05
Biceps femoris, g	301 ± 9	309 ± 13	308 ± 9	304 ± 8^{a}	308 ± 8^{b}	305 ± 11	309 ± 14
Biceps femoris, %	0.78 ± 0.02	0.78 ± 0.03	0.77 ± 0.02	0.77 ± 0.02	0.79 ± 0.02	0.76 ± 0.02^{b}	0.81 ± 0.02^{a}

SD, sheep diet; LD, lamb diet; NORM (N = 6; six male, zero female), normal diet fulfilling requirements for energy and protein; HIGH (N = 10; four male, six female), 150% of requirements for energy and 110% of requirements for protein; LOW (N = 10; five male, five female), 50% of requirements for energy and protein; HCHF (n = 13; eight male, five female), high-carbohydrate high-fat diet; CONV (N = 13; seven male, six female), conventional diet to achieve moderate and constant growth rates of approx. 225 g day^{-1}.
There was a significant interaction of pre- and post-natal nutrition only for adrenal weights after correction for body weight (P = 0.02) without any consistent pattern (Adrenals, % of body weight: HIGH-HCHF, 0.008 ± 0.0005; HIGH-CONV, 0.007 ± 0.0005; NORM-HCHF, 0.007 ± 0.0005; NORM-CONV, 0.006 ± 0.0007; LOW-HCHF, 0.005 ± 0.0005; LOW-CONV, 0.0008 ± 0.0005). Data are presented as least square means ± SEM. Effects of prenatal nutrition, post-natal nutrition or gender were significant $P < 0.05$ if the data within a row and within the respective columns are marked by different superscript letters. The subcutaneous fat represents the fat layer above the *longissimus dorsii* from the right side of the animal.

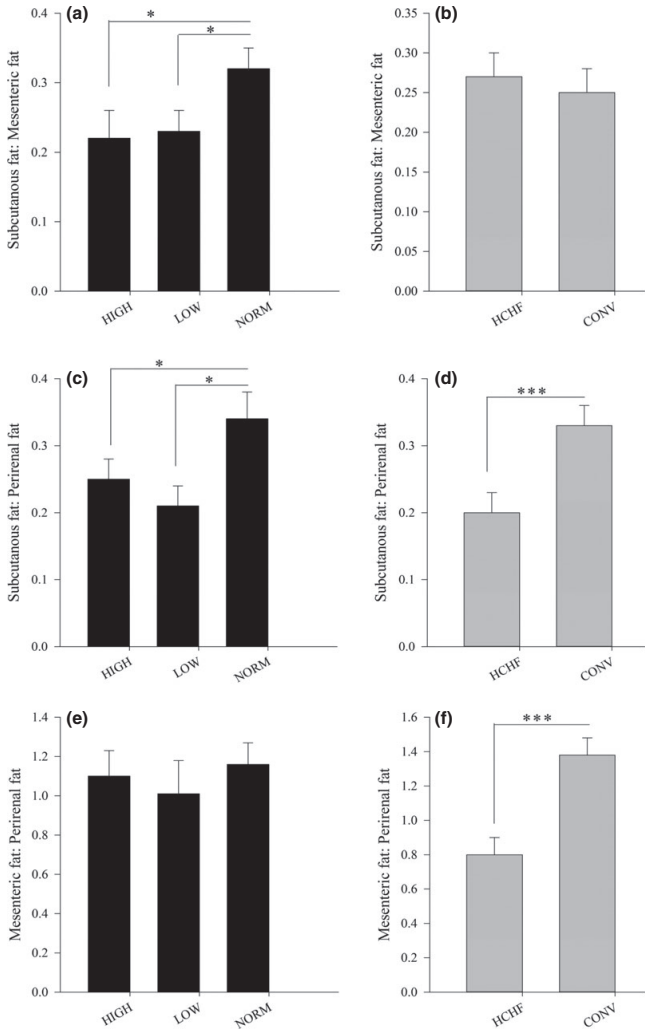

Figure 5 Changes in ratios of subcutaneous to mesenteric (a, b), subcutaneous to peri-renal (c, d) and mesenteric to peri-renal (e, f) fat deposition at post-mortem. HIGH (N = 10; four male, six female); NORM (N = 6; six male, zero female), LOW (N = 10; five male, five female); HCHF (N = 13; eight male, five female) and CONV (N = 13; seven male, six female): see legends to Figure 1. The subcutaneous fat represents fat above the *longissimus dorsii* from the right side of the animal. Values are least square (LS) means ± SEM represented by vertical bars were significantly different at $*P < 0.05$; $***P < 0.0001$.

similar impacts on growth and development, and physiological function in 6-month-old sheep offspring. The major findings of this study were that (i) exposure to widely different types of malnutrition (either over-nutrition or undernutrition) in late gestation can depress expandability of the subcutaneous adipose tissue in post-natal life thereby predisposing for development of visceral adiposity later in life upon exposure to an obesogenic diet, (ii) renal fat may be a possible target of post-natally induced obesity, and this is co-manifested with depressed kidney development, and (iii) metabolic adaptability to variations in nutrient supply during fasting is differentially programmed in response to late gestation exposure to over- as compared to undernutrition.

When evaluating the results from the present study, it should be borne in mind that pre- and post-natal nutrition and gender-specific effects could be evaluated based on all data from all animals from all groups for growth, morphometric measurements, feed intake and metabolic and endocrine responses during fasting, whereas slaughter data were obtained from smaller subgroups of animals, and gender effects could only be evaluated for the LOW and HIGH prenatal groups.

Prenatal under- and overnutrition resulted in similar alterations in regional fat-distribution patterns

Regional distribution of adipose tissue plays a vital role in understanding how obesity is related to distur-

bances in carbohydrate and lipid metabolism (Bouchard *et al.* 1993). In humans, the site of fat accumulation can play a central role regarding the health risks and disorders associated with obesity. Several studies have reported that abdominal fat is a particularly important risk factor for the development of obesity-related complications such as the metabolic syndrome (Wajchenberg 2000). Subcutaneous fat on the other hand has been suggested to be a 'healthy' fat, and increased amounts of subcutaneous fat has been associated with improved insulin sensitivity and decreased risk of developing type 2 diabetes (Misra *et al.* 1997, Snijder *et al.* 2003). The healthy features of subcutaneous fat have further been verified by transplanting subcutaneous fat into the visceral area in mice. In such studies, subcutaneous fat was shown to have unique intrinsic properties, which provided beneficial effects such as improved control of body weight as well as improvements of whole body insulin sensitivity and glucose metabolism (Tran *et al.* 2008). These studies strongly indicate that subcutaneous fat may behave as a 'healthy' fat depot compared to visceral fat, which is not simply due to the anatomical location, but also due to depot-specific intrinsic properties.

In the present study, we found that late gestation overnutrition just as undernutrition (Nielsen *et al.* 2012) interferes with subcutaneous adipose tissue function resulting in decreased ability or preference to accumulate fat in the subcutaneous area. Completely different types of exposures in late gestation may, therefore, lead to the same adverse outcomes later in life and alter fat-distribution patterns in a way, which can increase the risk of abdominal fat deposition and obesity-induced metabolic disorders. This agrees with the previously reported U-shaped relationship between birth weight and obesity risk later in life (Curhan *et al.* 1996, Godfrey & Barker 2000, Dyer & Rosenfeld 2011). Abdominal obesity is considered a marker of adipose dysfunction and the most prevalent indicator for development of metabolic disorders (Despres & Lemieux 2006). It has been shown in overweight and obese women that changes in fat deposition in legs were negatively correlated with risk factors of coronary heart disease, whereas truncal fat deposition was positively correlated with these risk factors, suggesting that relocation from central to peripheral fat deposition is important to reduce the coronary heart disease risk (Okura *et al.* 2004). Excess accumulation of abdominal fat has been related to poor expandability of subcutaneous fat, as this can reduce the ability of subcutaneous adipose tissue to act as a buffer, that is, 'sink', in situations with excessive availability of energy and nutrients (Lemieux 2004), and thereby the risk of nutrient overflow leading to increased abdominal and ectopic fat deposition (Miranda *et al.* 2005) is increased. Further investigations are needed to uncover the underlying mechanisms explaining why prenatal malnutrition both in the form of over- and undernutrition leads to a lowered fat-deposition capacity in the subcutaneous area, and to determine whether it is simply due to alterations in the subcutaneous adipose tissue, or whether modifications of metabolic and endocrine functions of other fat depots are involved as well.

Renal fat as a possible target of post-natally induced obesity, and co-manifestation with depression of kidney development

In our study, the post-natal HCHF diet enormously increased peri-renal fat deposition (by a factor of 9) regardless of the prenatal nutrition. The early post-natal high-fat diet appeared to have the peri-renal area as a primary target, as fat deposition in this area was favoured relative to deposition in the other fat depots studied.

Abdominal fat accumulation is proposed to be a major determinant for the increased risk of chronic kidney, cardiovascular and metabolic diseases associated with obesity-induced metabolic syndrome (Chen *et al.* 2004). Previous studies have suggested that NEFA and adipokines released by visceral adipose tissue play a central role in the development of organ dysfunctions and metabolic disorders in obese individuals (Kahn *et al.* 2006). When lipid droplets are accumulated in non-adipose tissues (ectopic fat deposition), then it may lead to cellular dysfunctions or even cell death due to lipotoxicity (Unger & Orci 2001). To what extent peri-renal fat contributes to these adverse impacts of abdominal adipose tissue in humans on development of metabolic disorders and organ dysfunction remains to be established.

We have both in this study, where male and female lambs were studied, and in a previous sheep study including only male lambs (Nielsen *et al.* 2012), found that kidney weights can be substantially reduced by exposure to an early post-natal HCHF diet. This could simply be a consequence of limitations in amino acids supply and hence inability to sustain normal development of the organ, as dietary protein content of the HCHF diet was quite low and not fulfilling normal requirements for protein (see Table 3). However, for none of the other organs or tissues did we observe any indication of a similar depressive effect of the HCHF diet on their development and gross weights in the 6-month-old lambs. This was even true for the skeletal muscles, *biceps femoris* and *longissimus dorsii*, and it proves to show that there are strong compensatory mechanisms enabling the body to

improve the efficiency of amino acid utilization for protein synthesis and deposition, when energy supply is abundant, presumably through depression of their entry into catabolic pathways. In support of this, we observed much lower plasma levels of BUN in HCHF-fed as compared to CONV-fed lambs, indicating lower rates of amino acid oxidation particularly during fasting. We therefore raise the question, whether the reduced kidney development in HCHF lambs could be related to the excessive peri-renal fat deposition? In diabetic patients, it has in fact been shown that peri-renal fat expansion irrespective of general adiposity can be an independent risk factor for kidney dysfunction, although the mechanism is still not clear, but both physical compression or adipose secretion of locally acting substances have been suggested as possible mechanisms (Lamacchia *et al.* 2011). In our HCHF lambs, we also observed much higher levels of plasma creatinine (approx. 50%), which is considered a rough index of glomerular filtration rate and hence kidney function (Perrone *et al.* 1992). Plasma creatinine levels can be influenced by other body characteristics as well, and it is known to correlate with total gross muscle mass (Perrone *et al.* 1992). However, there were no indications suggesting that muscle mass should have been decreased in the HCHF compared to the CONV lambs. The scientific information on peri-renal fat is remarkably scarce, and it has not been possible to find information on the specific metabolic and physiological functions of peri-renal fat relative to other visceral fats, including such aspects as vascular perfusion and drainage and thus potential routes for direct impacts of peri-renal fat on other organ systems in the body. Future studies appear to be needed to elucidate the potential role of peri-renal fat for kidney development.

Some of the HCHF-fed lambs stood out from the rest of the lambs fed this diet. They did not grow well and attained a smaller body size and skinny appearance at 6 months of age (around puberty). These 'small-skinny-fat' animals appeared to have lower plasma levels of creatinine, which corresponds with a lower muscle mass, and lower plasma IGF-1 levels, corresponding to their lower dietary energy and protein intake. But they also had lower plasma leptin levels irrespective of the fact that they had similar quantitative accumulation of fat as the other HCHF lambs and mainly in the peri-renal area. A 'lean obese' phenotype has been reported in humans, such as the Pima Indians, who have a high proportion of their body mass located in the abdominal area, and they are known to be more prone to development of peripheral insulin insensitivity and earlier in life (Abbott & Foley 1987). Future studies are required to reveal if our 'small-skinny-fat' sheep potentially could represent an animal model for the human 'lean obese' phenotype, and the potential role of peri-renal obesity and altered leptin regulation in the development of such a phenotype.

Adaptation of glucose–insulin homoeostasis during fasting is differentially programmed by late gestation over- vs. undernutrition

With our post-natal HCHF feeding, we were able to produce obese lambs with distinct metabolic and endocrine profiles compared to CONV lambs. In humans, there is a strong relationship between a high-fat diet and development of pancreatic β-cell failure, insulin resistance and type 2 diabetes (Cerf 2007). The HCHF lambs in our studies became hyperglycaemic, hypercholesterolaemic and hyperlipidaemic with very high NEFA levels, and prolonged exposure of such high levels of free fatty acids has been shown to have cytostatic (reduced glucose stimulated insulin secretion) and proapoptotic effects (caspase mediated) on human pancreatic β-cells *in vitro* (Lupi *et al.* 2002). In the present study, the lambs fed a high-fat diet in early post-natal life generally showed signs of reduced glucose tolerance with higher glucose and insulin (except in LOW-HCHF lambs) levels in the fed state, and also higher glucose levels during fasting, although the animals were able to suppress glucose levels during fasting as efficiently as were the CONV lambs. The HIGH lambs with a history of foetal overnutrition attained higher blood glucose levels upon exposure to the HCHF diet compared to LOW lambs, whereas NORM lambs had intermediate glucose levels, and these differences between HIGH and LOW lambs persisted throughout the fasting period. This does not lend support to our hypothesis that individuals with a foetal history of overnutrition will have a superior ability to cope with a high-fat diet in early post-natal life. The data presented here do not allow us to distinguish exactly where in the glucose–insulin axis, the foetally induced alterations occurred. We are conducting more detailed investigations as part of this study to address these issues. However, the outcomes from the current study may point to differential programming of the mechanisms involved in glucose–insulin homoeostasis by prenatal undernutrition as compared to overnutrition, where HIGH become hyperglycaemic with an apparently lowered peripheral insulin sensitivity, whereas LOW lambs appeared to be more resistant to the hyperglycaemic effect of the HCHF diet, even though they had the lowest insulin levels of the three groups of HCHF-fed lambs.

We did not find any indications that prenatal nutrition exposures would influence adaptability of lipid metabolism, that is, NEFA, TG, BOHB and choles-

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terol levels or regulatory factors, that is, IGF-1 and leptin in the young lambs, as these parameters were exclusively affected by the post-natal diet.

In conclusion, prenatal overnutrition just as undernutrition predisposed for abdominal adiposity, apparently by reducing the expandability of subcutaneous adipose tissue. When prenatal effects were observed, they were in general additive to post-natal nutrition effects. Renal fat appeared to be a special target of post-natal overnutrition, and future studies are needed to reveal whether the comanifestation of excessive fat deposition in the peri-renal area with reduced kidney development and function reflects a causal relationship. Exposure to gestational overnutrition did not provide a protective effect against development of hyperglycaemia later in life. Although prenatal over- and undernutrition resulted in similar changes in adipose deposition patterns, they may have differential impacts on underlying regulatory systems such as glucose–insulin homoeostasis.

Conflict of interest

The authors declare that they have no conflict of interest.

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PAPER II

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Maternal under- and overnutrition have differential impacts when combined with a postnatal obesogenic diet on glucose-lactate-insulin adaptations during metabolic challenges in adolescent sheep

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Running head: Effects of maternal malnutrition on metabolic and glucose-insulin regulation

Abstract

Aim: Determine whether late gestation under- (LG-UN) and overnutrition (LG-ON) programme metabolic plasticity in postnatal life in a similar way, and whether metabolic responses to an obesogenic diet in early postnatal life depend on the foetal nutrition history.

Methods: In a 3×2 factorial design, twin-pregnant ewes were for the last six weeks of gestation (term=147 days) assigned to HIGH (150% and 110% of energy and protein requirements, respectively), NORM (100% of requirements) or LOW (50% of requirements) diets. The twin offspring were raised on high-carbohydrate-high-fat (HCHF) or conventional (CONV) diets from 3-days to 6-months of age (around puberty). Then intravenous glucose (GTT), insulin (ITT) and propionate (gluconeogenetic precursor; PTT) tolerance tests were conducted to evaluate (hepatic) metabolic plasticity.

Results: Prenatal malnutrition differentially impacted adaptations of particularly plasma lactate followed by glucose, cholesterol and insulin. This was most clearly expressed during PTT in fasted lambs and much less convincingly during ITT and GTT. In fasted lambs, propionate induced more dramatic increases in lactate than glucose, and HIGH lambs became more hyperglycaemic, hyperlactataemic and secreted less insulin compared to the hypercholesterolaemic LOW lambs. Propionate-induced insulin secretion was virtually abolished in fasted HCHF lambs, but upregulated in fasted compared to fed CONV lambs. HCHF lambs had the greatest insulin secretory responses during GTT.

Conclusion: Prenatal nutrition differentially programmed hepatic C-metabolism in glucose-lactate synthetic pathways and cholesterol homeostasis. LG-ON predisposed for hyperglycaemia and hyperlactataemia, whereas LG-UN predisposed for hypercholesterolaemia upon exposure to an obesogenic diet. LG-ON (not LG-UN) interfered with pancreatic plasticity by non-glucose-dependent pathways.

Key words: *cholesterol, gluconeogenesis, insulin secretion, lactate, propionate tolerance test*

Introduction

Previous human clinical and experimental animal studies have shown that maternal undernutrition alone or in combination with a mismatching postnatal obesogenic diet can programme metabolic plasticity in the offspring in postnatal life, and this is associated with increased risk of development of a range of metabolic disorders such as type 2 diabetes, obesity and cardiovascular disease later in life (Cleal *et al.* 2007, Langley-Evans 2001, Taylor & Poston 2007, Torrens *et al.* 2009). It has been reported that an increased numbers of babies are now born with a high birth weight and large for gestational (LGA) in the developed world like North America and Europe (Ananth & Wen 2002, Surkan *et al.* 2004). It appears that being born LGA and/or by mothers with gestational diabetes increases the risk of obesity, diabetes and associated metabolic disorders later in life (Boney *et al.* 2005, Dabelea 2007, Long *et al.* 2010), just as it is the case for progenies of undernourished mothers that are born small for gestational age (SGA). Hitherto, only a few studies have attempted to assess whether the similar adverse postnatal consequences of exposure to overnutrition and undernutrition in foetal life can be tracked back to similar (molecular) biological alterations and phenotypes. Previous studies in rodents (Fiorotto *et al.* 1995) and sheep (Ford & Long 2011) have indicated that both maternal overnutrition and undernutrition may result in similar impacts on body composition and adaptations of metabolic functions and organ systems. We have also shown that both late-gestation over- (LG-ON) and undernutrition (LG-UN) can alter fat distribution patterns later in life with higher priority on fat distribution in the visceral area. However, our studies indicated that the similar changes in fat deposition patterns induced by the widely different prenatal nutrition exposures could very well arise from different underlying metabolic adaptations (Khanal *et al.* 2014). In order to be able to develop targeted recommendations and/or interventions in the future, we need to know whether individuals exposed to overnutrition during gestation are programmed to have improved

ability to cope with a high plane of nutrition after birth, or if they, like SGA offspring, have an impaired ability to handle a postnatal environment with excess nutrients.

In this study we aimed to test the following hypotheses: i) Late gestational over- or undernutrition lead to differential impacts on metabolic and insulin regulation in different tissue and organ systems, ii) Alterations in metabolic function induced by different prenatal malnutrition exposures will be further exacerbated when combined with an obesogenic diet in early postnatal life, iii) Individuals with a history of prenatal overnutrition have superior ability to cope with an early postnatal obesogenic diet.

We used the Copenhagen sheep model to assess the long-term consequences of different maternal nutrition exposures in a stage of gestation corresponding to the human third trimester (Nielsen *et al.* 2013), which is not possible in rodent models. In lambs exposed to undernutrition or overnutrition during late foetal life and raised on a moderate or obesogenic diet in early postnatal life, we tested the adaptation of plasma metabolites and insulin during glucose and insulin tolerance tests, as it is well-established that LG-UN programmes glucose-insulin axis function in humans (Ravelli *et al.* 1998), sheep (Gardner *et al.* 2005, Husted *et al.* 2007, Kongsted *et al.* 2014) and rodents (Fagundes *et al.* 2007, Latorraca *et al.* 1998). We suspected that glucose and insulin tolerance tests would reveal the efficiency of metabolic adaptability to an acute nutrient surplus situation, but not necessarily the efficiency of adaptation to nutrient scarcity situations, which the undernourished foetus would have been required to adapt to. We therefore also wished to test the efficiency of metabolic plasticity in response to nutrient scarcity. This was done by exposing the lambs to tolerance tests with the gluconeogenetic precursor propionate, in both fed and fasted conditions.

Materials and methods

Experimental set-up

The Copenhagen sheep model (Nielsen *et al.* 2013) was used in this experiment, and details about experimental animals, experimental design, feeding, care and management of the experimental animals used in this study have been described previously (Khanal *et al.* 2014). In short, the experiment was a 3×2 factorial design where twin-pregnant Texel ewes were assigned to one of three feeding treatments during the last six weeks of gestation (term=147 days): NORM (N=9; fulfilling 100% of required digestible energy and protein), HIGH (N=13; 150% of digestible energy and 110% of protein provided to NORM), LOW (N=14; 50% of digestible energy and protein provided to NORM). After lambing, the twin lambs were assigned to either a conventional (CONV; N=35; 16 male, 19 female) or an obesogenic high-carbohydrate-high-fat (HCHF; N=35; 18 male, 17 female) diet from three days until six months of age (around puberty). The CONV diet consisted of milk replacer and hay until eight weeks of age and green hay only from eight weeks to six months of age. The daily allowances of milk and hay were adjusted weekly to ensure that a constant and moderate daily live weight gain (appr. 225 g day^{-1}) was achieved. The HCHF lambs were fed a mixture of 50% milk replacer and 50% dairy cream (*ad libitum* until a daily maximum of 2.5 L was reached) and rolled maize (*ad libitum* until a maximum allowance of 1 kg day^{-1} was reached). All animals had *ad libitum* access to water and were provided vitamin and mineral supplements according to their requirements throughout the experiment.

Catheterization and blood samplings

Permanent indwelling catheter tubes were placed in jugular veins using a percutaneous Intraflon 2 catheter (2.7 mm, Laboratories Pharmaceutiques VYGON, France) as introducer. Catheterizations were performed at least one day prior to the first tolerance test to facilitate repeated collection of blood samples. The catheter tubing was cut into lengths of 60 cm. Prior

to insertion, they were placed in a hibitane (HiBiSCRUB®, 40 mg ml^{-1}, Molnlycke Healthcare Limited, Manchester, UK) – ethanol solution (70% ethanol) for disinfection. The animal was shaved on the neck and the insertion site was disinfected with iodine, whereafter 1 ml local anaesthetic (lidocain, 10 mg ml^{-1}, DAK) was applied under the skin. After a few minutes, the Intraflon catheter was inserted percutaneously, the needle was removed and the permanent catheter tube inserted through the Intraflon catheter, whereafter the Intraflon catheter was also removed. The catheter tube was introduced appr. 15 cm into the jugular vein, but adjusted according to the size of the animal. Finally a close-fitting rubber band was pulled over the catheter tube and positioned at the exit point from the skin, and the catheter was fixed by suturing the rubber band to the skin. Blood samples were collected at different time points (according to the specific tolerance tests) in EDTA coated tubes which were subsequently stored on ice until centrifugation (1800×g_{av} at 4 ^{O}C for 15 min). After centrifugation, plasma samples were transferred to labelled cryo tubes (Th. Geyer Danmark Aps, Roskilde, Denmark) and stored at -20 ^{O}C until later analysis.

Glucose tolerance test in overnight fasted lambs

Prior to the intravenous glucose tolerance test, lambs were fasted overnight and only allowed access to water. Two baseline samples (-15 and -5 min) were taken in the morning at app. 08.00 prior to glucose injection. Then, each lamb received an intravenous bolus injection of 0.45 $g/kg^{0.75}$ glucose (0.5 g ml^{-1} in sterile water, SAD, Copenhagen, Denmark) at time 0 min into the jugular vein, whereafter blood samples were collected at times: +2.5, +10, +20, +30, +60 and +120 minutes after injection.

Insulin tolerance test in fed lambs

Prior to the insulin tolerance test, lambs were fed their normal afternoon ration, except for the HCHF lambs, which were not fed the milk-cream mix to avoid very high levels of lipids in the blood, which could potentially interfere with the later analyses of blood samples. The

baseline samples were taken in EDTA coated tubes prior to the insulin injection (-15 and -5 min) in the afternoon at app. 13.00 and then lambs received an insulin bolus injection of 0.15 U kg^{-1} body weight (1000 U ml^{-1}, Recombinant human insulin, Eli Lilly, Lyngby, Denmark) into the jugular vein. Then blood samples were collected at times: +2.5, +5, +10, +20, and +60 minutes after the insulin injection.

Propionate tolerance tests during fasted and fed states

Two different propionate tolerance tests were performed, one in the fed state and the other at the end of a 2-day fasting period. The tests were conducted in the morning at app. 08.00. During the propionate fasted tolerance test, lambs were subjected to a 44-hour fasting period and then the tolerance test was performed. Lambs were fed their normal morning meal during the propionate fed tolerance test, except for the HCHF lambs, which were not fed the milk-cream mix to avoid interference of high plasma lipid levels in the later analyses. One basal blood sample was collected (-5 min) in the morning at appr. 08:00, and then a bolus injection of sodium propionate (2.5 M in sterile water, Silkeborg Pharmacy Denmark, 2.5 ml $kg^{-0.75}$; 6.25 mmol sodium propionate $kg^{-0.75}$) was given into the jugular vein at time 0, whereafter blood samples were collected at times +5, +10, +15, +36, +60 and +120 minutes after the injection.

Laboratory analyses

Glucose, non-esterified fatty acids (NEFA), triglycerides (TG), blood urea nitrogen (BUN), creatinine, lactate, β-hydroxy butyrate (BOHB), γ-glutamyltransferase (GGT) and total cholesterol levels were determined in all plasma samples by spectrophotometric methods using an ADVIA 1800 chemistry analyzer (Diamond Diagnostics, USA). Lactate, NEFA and BOHB were determined using RANDOX kits (RANDOX Laboratories, Ardmore, UK). Analyses for the rest of the parameters were performed using specific reagents from Siemens Healthcare Diagnostics (Benedict Ave/Tarrytown, NY USA) as described previously (Khanal

et al. 2014). Insulin concentrations were determined by a commercial ELISA kit (Mercodia Ovine Insulin ELISA Kit, Uppsala Sweden; intra- and interassay coefficients of variation <10%). Samples with insulin concentrations below the lower detection level (<0.05 μg L^{-1}) were assigned a value of 0.03 μg L^{-1} to allow their inclusion in the statistical analysis.

Statistical analyses

Test of significance of pre- and postnatal diet, time and sex within each tolerance test

Statistical evaluation of data was performed in the SAS software (v.9.2; SAS Institute, USA). Homogeneity of variance was evaluated by visual inspection of residuals plots and normality of residuals was tested by means of quantile-quantile plots. The data were analysed as repeated measures by the GLIMMIX procedure. Prior to analysis, suitable transformation of data was performed when needed to obtain normal distribution of residuals. Different correlation structures between measurements and heterogeneous variances were tested and the structure yielding the best fit was chosen. The model included *fixed effects* of pre- and postnatal feeding level, and the time of blood sampling, *random effects* of individual sheep and lambs within each feeding treatment, and *repeated measurements* of samples within lambs. Within the postnatal HCHF group, a few animals had poorer growth rates compared to other animals in their group, which became evident from four to six weeks of age, and they reached substantially lower body weights at six months of age (all <30 kg) compared to the rest of the HCHF fed animals (all >35 kg). Despite the skinny appearance, they turned out to have equally large depositions of abdominal body fat at slaughter, and we have called them "small skinny-fat" animals (Khanal *et al.* 2014). Additional statistical analyses were performed to elucidate whether there were special metabolic and endocrine characteristics associated with their "small skinny-fat" appearance. The statistical model included *fixed effects* of lamb phenotype (skinny or normal) and time of blood sampling, and *random effects* of individual lambs, and *repeated measurements* of samples in lambs. Lamb birth weight and

sex, and ewe body weight and BCS (body condition score) were used as co-variables in all the statistical analyses. Backward model reductions were performed by sequentially removing the least significant factor. Area under the curve (AUC) and above baseline or area over the curve (AOC) and below baseline depending on the shape of the curve were calculated for glucose and insulin responses as a summary measure, where the model included *fixed effects* of the pre- and postnatal feeding levels, and the time of sampling and *random effects* of individual sheep and lambs within the feeding level. Differences in least square means (LS means) were compared by Tukey's multiple comparison test and presented results are expressed as LS means with standard error of mean (LS means±SEM). The level of significance was set at $P<0.05$.

Prediction of effects of pre-postnatal diet, sex and tolerance test across all tolerance tests

Additional comprehensive tests were conducted including data for all the different tolerance tests to get insights into the overall (predicted) effects or influences of sex, pre- and postnatal diets and tolerance tests on the levels of metabolic parameters in lambs.

A Generalized Linear Mixed Model (GLMM) was fitted as follows:

$$\mathbf{Y = \mu + S + ED + LD + T + L + e}$$

Where **Y** is data for a given "trait" (glucose, insulin, cholesterol, creatinine, TG, BUN, GGT, BOHB, NEFA and lactate), **μ** is an intercept, **S** is fixed effect of sex, **ED** is fixed effect of ewe diet, **LD** is fixed effect of lamb diet, **T** is a tolerance test subjected to lambs, **L** is random effect of lamb and **e** is a random residual. The data in **Y** are thus an average of a given metabolic trait for each animal across all time points. All analyses were implemented in GENSTAT v. 15.2 (http://www.vsn-intl.com, VSN International Ltd, UK) assuming normal distribution and identity link function in GLMM. During implementation, one of the levels within each fixed effect was set to zero due to linear dependencies, hence estimated effects

were originally deviations from that level that was set to zero or equivalently deviation from the model mean.

Results

Unless it is specifically stated in the following, we did not detect any significant effects of prenatal diet, any interactions between pre- and postnatal treatments and between treatments over time (the time course of changes in metabolic and endocrine profiles during different tolerance tests) or effects of sex for the studied parameters. Differences between HCHF lambs with a "small skinny-fat" appearance and the rest of the HCHF lambs are only mentioned if they were found to be significant.

Glucose tolerance test (overnight fasted lambs)

Glucose and Insulin: The overnight fasted plasma levels and post-injection glucose (except at 60 min) and insulin profiles and the AUC for glucose and insulin were not affected by prenatal nutrition (Figure 1a and 1c). The basal glucose and insulin levels were higher in HCHF fed lambs compared to CONV fed lambs, but the peak glucose and insulin levels after glucose injection were generally similar in both groups. The HCHF lambs, particularly NORM-HCHF, had poor glucose clearance and thus higher glucose levels compared to CONV lambs. AUC for glucose was therefore higher for HCHF lambs compared to CONV lambs. Plasma insulin profiles generally followed the changes in glucose levels, but HCHF lambs had delayed insulin clearance after insulin levels had peaked and thus substantially higher insulin levels and higher AUC for insulin (~50% higher) compared to CONV lambs. All treatment groups returned to similar insulin levels within 120 min after the glucose injection. HCHF lambs with a "small skinny-fat" appearance had overall lower glucose levels (6.92±0.36 vs 7.77±0.14 mmol L^{-1}; P=0.0466), and they had higher increases in insulin 10-30 min post-injection compared to the rest of the HCHF animals (P<0.05).

Lactate: The prenatal nutrition had differential impacts on lactate levels during the glucose challenge test in CONV lambs, where LOW-CONV lambs had higher overnight fasted lactate levels and higher lactate levels during the first 20 min after glucose injection compared to NORM- and HIGH-CONV, whereas HIGH-CONV lambs had the lowest lactate levels throughout the glucose tolerance test (P=0.024 for interaction between pre- and postnatal nutrition; Figure 1e). Such a prenatal impact was not evident in lambs fed the HCHF diet. The HCHF fed lambs generally had higher lactate levels prior to and after glucose injection compared to CONV lambs, the main exception being, as already mentioned, the LOW-CONV lambs during the first 20 min after glucose injection.

TG, NEFA and BOHB: The overnight fasted HCHF lambs had appr. 2.5 times higher TG (Figure 2a; P<0.001) and more than 50% higher NEFA levels (Figure 2c; P<0.001) than CONV lambs throughout the glucose tolerance test (P<0.05 – 0.001). The TG and NEFA levels generally increased slightly just after glucose injection followed by a decrease, most markedly for NEFA, until appr. 30 min post-injection, whereafter they increased again and returned to baseline levels within 120 min post-injection. The overnight fasted baseline BOHB levels were (in contrast to TG and NEFA) not affected by the postnatal diet, but BOHB levels decreased over the first hour after glucose injection, and increased thereafter, most rapidly in HCHF lambs (Figure 2e; P<0.05).

Cholesterol, BUN, Creatinine and GGT: The HCHF lambs had markedly higher overnight fasted plasma levels of cholesterol (app. 3-fold higher; P<0.001; Figure 3a), creatinine (app. 50% higher; P<0.0001; Figure 3e) and GGT (>2-fold higher; P<0.001; Suppl. Figure 1a) than CONV lambs, whereas BUN levels in HCHF lambs were 50% lower than in CONV lambs (Figure 3c; P<0.0001). There were quantitatively minor, although significant, changes in plasma levels of cholesterol, BUN, creatinine (except for an increase in CONV lambs) and GGT. The "small skinny-fat" HCHF lambs had lower creatinine levels (102.04±7.01 vs.

123.43±2.78 µmmol L^{-1}, P=0.0011), but appr. 2-fold higher GGT levels (280.90±53.86 vs. 148.71±21.94 U L^{-1}; P=0.0216) compared to the other HCHF lambs.

Insulin tolerance test (non-fasted lambs)

Glucose and Insulin: Non-fasted baseline levels and post-injection levels of glucose and insulin were consistently higher in HCHF than CONV fed lambs (P<0.0001) (Figure 1b and 1d), as also observed during glucose tolerance tests in the overnight fasted condition. Peak insulin levels appr. 50 fold higher than baseline levels were reached within 2.5 min after injection, whereafter insulin levels dropped dramatically over the next 20 min and pre-injection levels were reached within 60 min post-injection. HCHF lambs, however, had poor insulin clearance (as also observed in the glucose tolerance test) during the first 10 min post-injection compared to CONV lambs. Glucose levels changed oppositely to those of insulin with a pronounced drop to nadir levels around (CONV) or just above (HCHF) 2 mmol/L within 20 min after injection and by 60 min after injection pre-injection glucose levels had still not been reached. AOC for glucose (P<0.0001) and AUC for insulin (P=0.0003) were thus higher in HCHF compared to CONV lambs. The "small skinny-fat" HCHF lambs had slightly lower insulin levels (1.14±0.09 µg L^{-1}) than other HCHF lambs (1.37±0.04 µg L^{-1}; P=0.01) throughout the test.

Lactate: The prenatal nutrition exposure impacted on lactate levels in fed lambs during the insulin tolerance test in CONV, but not HCHF lambs (P=0.028 for the pre- and postnatal diet interaction; Figure 1f), just as observed in the glucose tolerance test, i.e. LOW-CONV lambs generally had the highest and HIGH-CONV lambs consistently the lowest lactate levels among treatment groups. Pre-injection lactate levels were highest in lambs fed the HCHF diet postnatally (P<0.0003), as observed in the glucose tolerance test. However, oppositely to what was observed in the glucose tolerance test in overnight fasted lambs, lactate levels generally increased over the entire 60 min sampling period after insulin injection. The

increase was less pronounced in HCHF than CONV lambs, and LOW-CONV and NORM-CONV lambs reached the highest lactate levels from 20 min post-injection and onwards.

TG, NEFA and BOHB: The baseline and post-injection levels were higher in HCHF compared to CONV lambs for TG (almost 5-fold; Figure 2b) and NEFA (almost 4-fold; Figure 2d) ($P<0.0001$), as observed during the glucose tolerance test. Male lambs had higher TG ($P=0.001$) and NEFA levels ($P=0.005$) compared to female lambs. TG levels increased slightly just after insulin injection, but otherwise changed very little, whereas NEFA levels increased throughout the 60-min post-injection sampling period, most pronouncedly in HCHF lambs right after injection. BOHB levels were highest in lambs exposed to the prenatal NORM nutrition and HIGH lambs generally had the lowest levels of BOHB. Lambs fed the CONV diet had appr. 2-fold higher BOHB levels than HCHF fed lambs (Figure 2f), which corresponded to the increase in baseline levels from the overnight fasted (glucose tolerance test) to fed state in CONV lambs. The quantitative changes in BOHB in response to insulin injection were small and unsystematic ($P=0.017$ for three-way interaction of pre- and postnatal diet and time).

Cholesterol, BUN, Creatinine and GGT: For cholesterol (Figure 3b), creatinine (Figure 3f) and GGT (Suppl. Figure 1b) the overall plasma levels and minimal post-injection changes were equivalent to what was observed during the glucose tolerance test. The prenatal nutrition had an impact on cholesterol levels within the HCHF fed lambs ($P=0.05$ for interaction between pre- and postnatal diets), where LOW-HCHF lambs had substantially higher cholesterol levels than HIGH- and NORM-HCHF lambs. BUN levels (Figure 3d) were higher in CONV lambs than HCHF lambs, but the difference was much less pronounced compared to what was observed during the glucose tolerance test. The "small skinny-fat" HCHF lambs had lower cholesterol (3.84±0.97 mmol L^{-1} vs 6.19±0.39 mmol L^{-1}; $P=0.014$),

but higher GGT levels (the latter was also observed during the glucose tolerance test) (305.06±59.42 U L^{-1} vs 158.59±24.26 U L^{-1}; P=0.0235) than the rest of the HCHF lambs.

Propionate tolerance tests (fasted and non-fasted lambs)

The basal levels of plasma metabolites and insulin during propionate tolerance tests generally reflected the acute nutritional state of the animal (fed or fasted) and were thus comparable to what has been described above for insulin (fed state) and glucose (overnight fasted) tolerance tests, although the responses to a 2-day period of fasting obviously were more pronounced than after just an overnight fasting period. The following presentation will therefore focus on the acute impacts of an injection with a gluconeogenetic precursor (propionate) depending on whether lambs were fed or fasted.

Glucose and Insulin: Peak glucose levels (15 min after propionate injection) were higher and clearance of glucose thereafter was slower in HCHF as compared to CONV lambs (Figures 4a and 4b). In CONV lambs, neither prenatal nutrition exposure nor the acute nutritional status of the lambs (fed or fasted) had any influence on peak glucose values or AUC for glucose, and glucose levels normalized to pre-injection levels within 60 min post-injection. In HCHF lambs, however, the peak levels and patterns of change depended on both the prenatal nutrition and the acute nutritional state of the lambs (fed or fasted) (Figures 4a and 4b and Suppl. Table 1; interactions of pre- and postnatal diet and time: fasted, P=0.0015; fed, P=0.0084). The HIGH-HCHF lambs obtained higher peak glucose levels (Figures 4b and 4b) compared to NORM- and LOW-HCHF lambs. Both HIGH- and LOW-HCHF lambs had reduced glucose peak responses and clearance of glucose when the tolerance test was conducted in the fasted (Figure 4a) compared to the fed state (Figure 4b), which was not the case for NORM-HCHF lambs. Despite higher blood glucose levels, HCHF lambs failed to secrete insulin as efficiently as CONV lambs (Figures 4c and d), which interestingly contrasts observations during the glucose tolerance tests, where HCHF lambs had the highest insulin

responses. Peak levels of insulin were appr. 2-fold higher in response to a propionate injection in fed lambs (Figure 4d) as compared to a glucose injection in overnight fasted lambs (Figure 1c). Fed HCHF lambs had slower insulin secretary responses with lower peak insulin levels and also delayed normalization of insulin levels after propionate injection as compared to CONV lambs (Figure 4d), but AUC for insulin were not significantly different between fed HCHF and CONV lambs. When the propionate tolerance test was conducted in fasted lambs (Figure 4c), the insulin secretory response to propionate was virtually abolished in HCHF lambs (peak levels <1 μg L^{-1}; Figure 4c). In CONV lambs, however, the peak response and AUC for insulin in response to propionate was increased several fold during fasting as compared to the fed condition (Figures 4c versus 4d), except in HIGH-CONV lambs, where the upregulation of insulin response during fasting was less pronounced. Consequently, when propionate tolerance tests were conducted in the fasted state, it was revealed that HIGH lambs (1.94±0.19) had significantly higher glucose/insulin ratios as compared to LOW lambs (1.25±0.19; P=0.04), with NORM lambs (1.56±0.23) in between, and HCHF lambs (2.48±0.17; P<0.0001) had much higher glucose/insulin ratios as compared to CONV lambs (0.68±0.16). Such clear pre- and postnatal nutrition impacts on insulin secretory responses and glucose clearing ability were not exposed during the glucose and insulin tolerance tests.

Lactate: Clear and pronounced increases in plasma lactate levels were observed in all groups of lambs within the first 20-40 min after the propionate injection, most pronounced when the lambs were fasted (appr. 4-fold increase; Figure 4e), than when they were in the fed state (appr. 2-fold increase; Figure 4f). Lactate levels returned to baseline levels within 120 min after the injection. In fasted lambs, the prenatal nutrition affected the way lactate levels changed over time (P=0.012), which could be ascribed to lower lactate levels including peak levels in LOW animals compared to NORM and HIGH lambs (Figure 4e). There was also a

tendency for different lactate profiles over time depending on the postnatal diet in fasted lambs (P=0.079), since HCHF lambs had higher lactate levels compared to CONV fed lambs during most of the challenge (Figure 4e). HIGH-CONV lambs obtained the highest lactate levels across all groups throughout almost the whole challenge, whereas they obtained the lowest lactate levels among all groups during the glucose and insulin challenges. The reverse was true for the LOW-CONV lambs. When the propionate challenge was conducted in the fed condition, HCHF lambs had much more pronounced increases in lactate in response to propionate injection than CONV lambs, but any signs of a prenatal nutrition effect had disappeared (Figure 4f).

TG, NEFA and BOHB: TG levels were generally substantially higher in HCHF lambs (2-fold), when they were fed compared to when they were fasted (Figures 5a and b). TG levels increased markedly in fed HCHF lambs after propionate injection with higher peak TG levels (~7-fold) compared to CONV lambs 10 (CONV) or 15 (HCHF) min after propionate injection (Figure 5b), whereas only modest increases were observed when the challenge was conducted in fasted lambs. Within 60 min after the injection, TG levels had returned to the baseline values (Figures 5a and b).

The baseline NEFA levels after 2-days of fasting were high (>1.0 mmol L^{-1}; Figure 5c) and not significantly different between HCHF and CONV lambs. Within 5 min after the propionate injection, a small and transient drop in NEFA levels was observed in HCHF lambs, whereas NEFA levels continued to decrease in CONV lambs to become more than halved over the first 36 min after the injection followed by an almost 4-fold increase in NEFA levels over the rest of the sampling period. CONV lambs thus ended up having significantly higher NEFA levels than HCHF lambs at 120 min post-injection (P<0.0001). In the fed state, the NEFA response to propionate injection was completely different compared to in the fasted state. All fed lambs increased their NEFA levels within the first 10-15 min

after propionate injection, but HCHF fed lambs had a much more pronounced increase and greater peak NEFA levels (>2.0 mmol L^{-1}) compared to CONV lambs (0.5 mmol L^{-1}), and in all lambs NEFA levels had returned to their pre-injection levels by the end of the sampling period.

During the fasted state BOHB levels were higher in HCHF lambs compared to CONV lambs (Figure 5e; $P<0.0001$), whereas CONV lambs had higher BOHB levels than HCHF lambs during the fed state (not significantly different at time points 36 and 60 min) (Figure 5f; $P=0.0097$). After propionate injection, a small and transient increase in BOHB levels was obtained in HCHF lambs only, but only during the fasted state. In all other cases, BOHB levels decreased over the first 60 min (fasted lambs) or 20-40 min (fed lambs), whereafter BOHB increased again to the respective pre-injection levels.

Cholesterol, BUN, Creatinine and GGT: There were no major quantitative changes in cholesterol levels during the propionate tolerance tests, except for a small decrease within the first 5 min after propionate injection in fed HCHF lambs (Figure 6b). HCHF lambs generally had much higher cholesterol levels (>1.5-2-fold in the fasted state; Figure 6a; and >3-fold in the fed state; Figure 6b) compared to CONV lambs, as was found in the glucose and insulin tolerance tests, but HIGH-HCHF and NORM-HCHF had a clear reduction in cholesterol levels after 2 days of fasting ($P=0.015$ for interaction between pre- and postnatal diet), which was not observed in LOW-HCHF lambs.

BUN levels in all lambs increased after 2 days of fasting (Figure 6c) compared to their levels in the fed (Figure 6d and insulin tolerance test) or overnight fasted state (glucose tolerance test). In fasted lambs, there was a slight increase in BUN over the first 15 min after propionate injection, and the highest BUN levels were observed in the nutritionally mismatched groups, i.e. HIGH-CONV had the highest levels within CONV lambs, and LOW-HCHF had the highest levels within HCHF lambs ($P=0.056$ for the pre- and postnatal

nutrition interaction). Such an interaction between pre- and postnatal diet was not observed in the fed state.

Creatinine levels were markedly higher in fed HCHF compared to fed CONV lambs (Figure 6f; appr. 1.5-fold) ($P<0.0001$), as also observed in glucose and insulin tolerance tests, but this difference was diminished after 2 days of fasting (to ~1.2-fold) due to increased creatinine levels in CONV lambs. There was a small transient drop in creatinine levels 5 min after propionate injection except in fasted HCHF lambs, followed by a slight increase until 36 min after propionate injection (Figures 6e and 6f). For GGT levels in plasma (Suppl. Figures 1c and 1d), no major changes were observed in response to propionate except perhaps for a small transient drop 5 min after injection.

Discussion

The major findings of this study were that i) late gestation over- and undernutrition have differential impacts on postnatal glucose-insulin regulation, lactate, cholesterol, and urea metabolism, and the liver and pancreas appear to be key targets for differential foetal programming, ii) the postnatal expression of prenatal programming was revealed predominantly in combination with exposure to the postnatal HCHF diet, and a history of prenatal overnutrition did not provide protection against the hyperglycaemic effects of such a diet, iii) foetal programming outcomes were more clearly manifested during tolerance tests with the gluconeogenetic precursor and insulin secretagogue, propionate, particularly in the fasted state, as compared to glucose and insulin tolerance tests.

Late gestation over- and undernutrition have differential impacts on adaptability of glucose-insulin regulation, lactate, cholesterol and urea metabolism

Glucose and insulin tolerance tests have been the traditional *in vivo* methods employed in studies regarding metabolic outcomes of foetal programming. In diagnostic terms it is problematic that the programmed phenotype is not clearly exposed in such tests until the

individual approaches adulthood. Our aim was to search for a broader spectrum of metabolic adaptations under fed and fasted conditions, including hepatic gluconeogenesis and glucose homeostasis in response to provision of propionate. Propionate is a normal end-product from fermentation in the gut and a potent insulin secretagogue in ruminant animals. Propionate is cleared very efficiently from portal blood during the first pass of the liver, where it is a primary gluconeogenetic precursor (Bruss *et al.* 1986, Grohn *et al.* 1985, Larsen & Kristensen 2013). Propionate is also a major end-product from microbial fermentation in the human gut (Hosseini *et al.* 2011).

As suspected, we found the clearest expression of foetal programming under conditions where metabolism in the body was most challenged, namely in response to propionate in the fasted state. Interestingly the implications of prenatal over- as compared to undernutrition were revealed most consistently as differential adaptations in plasma lactate followed by (in decreasing order of importance) glucose, cholesterol, BUN, BOHB, and insulin. The foetal nutrition history had no impact in any of the tolerance tests on TG, NEFA, creatinine or GGT levels or responses.

Impacts of prenatal nutrition on glucose-insulin regulation in the adolescent lambs were revealed only during propionate tolerance tests conducted after two days of fasting, where lambs with a history of prenatal overnutrition (HIGH) and additionally exposed to the obesogenic HCHF diet postnatally became more hyperglycaemic than LOW-HCHF and NORM-HCHF lambs. This shows that different nutritional exposures in late gestation may give rise to differential programming of glucose homeostatic mechanisms, and foetal overnutrition does not, in contrast to what we hypothesized, provide protection against the hyperglycaemic impacts of a postnatal high fat diet.

Lactate was the only metabolite for which prenatal nutrition effects were detected across all three types of tolerance tests and, to the best of our knowledge, no previous reports have

included studies on adaptations in lactate profiles during such tolerance tests. We did not perform isotopic tracer studies, and can therefore not track changes to individual metabolic pathways or organs with certainty, but we can speculate on the nature of possible underlying adaptations. In the following, numbers in parentheses refer to the metabolic pathways depicted in Figure 7. Glucose injection induced pronounced increases in plasma lactate levels in all lambs in addition to the expected marked increases in glucose and insulin. However, the lactate levels did not begin to increase until 20 min after the glucose injection, which strongly suggests that the elevated lactate levels in response to glucose could not be ascribed to insulin stimulated glucose uptake in muscle or other extra hepatic tissues (5) with a resulting release of lactate (24->21->20). Instead the lactate response must have been derived from the liver via shunting of part of the injected propionate (32) via phosphoenolpyruvate (15->16) into lactate (10->12) instead of completing the final steps of hepatic gluconeogenesis (9). During the insulin tolerance test, lactate levels increased steadily from 5 min and onwards after the injection, i.e. even after plasma glucose had dropped to very low levels. This supports the assumption that insulin stimulated glucose uptake in extrahepatic tissues did not account for the observed lactate increases, pointing again to the liver as the most probable site of lactate release also in response to insulin injection. The lambs were fed just prior to the conduct of the insulin tolerance test, and the initial small increase in lactate 5-20 min after insulin injection can probably be explained simply by entry of propionate absorbed from the gut into the gluconeogenetic pathways and subsequent shunting to lactate (14->15->16->10->12) due to insulin suppression of the final steps of gluconeogenesis (9). The more pronounced increases in lactate in the last part of the insulin tolerance test, when lambs became hypoglycaemic and insulin levels had normalized, may however have a different explanation. There is no way of knowing to what extent changes in liver glycogen metabolism (2+3) may have contributed, induced by increased pancreatic secretion of glucagon in response to either

the hypoglycaemia in the fasted lambs or to propionate itself. Further studies are required to shed light on these issues.

Assuming that the liver was the major organ accounting for changes in lactate profiles observed during the different tolerance tests, it is tempting to speculate that the impacts of different prenatal nutrition exposures on lactate profiles could also be ascribed to altered hepatic metabolic function. Lower lactate responses to a propionate injection without increased glucose outputs would thus indicate a reduced hepatic gluconeogenetic capacity in LOW as compared to NORM and HIGH lambs, and also an altered hepatic C-metabolism, resulting in lowered hepatic output of lactate relative to glucose. Conversely, in HIGH lambs, higher plasma lactate and glucose responses to propionate during fasting may reflect an increased hepatic gluconeogenetic capacity or altered metabolic priorities resulting in increased release of lactate (19) as well as glucose (4) to the blood. This may contribute to explain the greater predisposition for hyperglycaemia in the HIGH animals in response to a postnatal obesogenic diet in contrast to our hypothesis. Results from another recent study support this, since maternal high-fat feeding during gestation and lactation in mice led to development of impaired glucose tolerance associated with up-regulation of gluconeogenesis (increased hepatic expression of the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase) (Rother *et al.* 2012). Other previous studies have also pointed to the liver as a key target organ of abnormal maternal nutrition during the critical period of foetal development (Cianfarani *et al.* 2012), and in a previous sheep study we have found that prenatal undernutrition is associated with increased hepatic TG content, alteration in phospholipids fatty acid composition in liver and upregulation of key hepatic targets involved in insulin signalling and carbohydrate and lipid metabolism, such as INSRβ (insulin receptor beta subunit) and PI3K-p110 (phosphatidylinositol 3-kinases p110 beta catalytic subunit) protein in offspring (Hou *et al.* 2013). Foetal programming may have targeted also a

wider spectrum of hepatic metabolic features, since the prenatal nutrition exposures had differential impacts on changes in BUN and plasma cholesterol. The BUN results and their implications are difficult to interpret, and we cannot explain why HIGH lambs on the CONV diet had the largest increases and reached the highest levels of BUN after 2 days of fasting compared to all other groups, whereas it was the HCHF fed lambs among LOW lambs that had the largest increases in BUN levels after fasting. In addition to hepatic ureagenesis, BUN patterns are influenced also by renal function, and BUN is considered a rough index of renal dysfunction (Finco & Duncan 1976). It has been reported previously that high-fat diet during pregnancy is associated with renal injury (Armitage *et al.* 2005, Jackson *et al.* 2012) although underlying mechanisms are not quite clearly understood. However, we have not in previous studies found any indications to suggest that renal urea excretion should be influenced by the foetal nutrition exposure in late gestation (Nyberg *et al.* 2010).

The liver is the major site also for synthesis of cholesterol (Trapani *et al.* 2012) (28), and cholesterol is released from the liver into blood incorporated into lipoproteins (29), and blood cholesterol is considered a major risk factor for cardiovascular diseases (Stamler *et al.* 1993). Previous human epidemiological and animal studies have demonstrated an adverse association between impaired foetal growth and hypercholesterolaemia in postnatal life (Barker *et al.* 1993, Kind *et al.* 1999). Our study confirmed that prenatal undernutrition predisposed for development of hypercholesterolaemia in LOW lambs when fed an obesogenic diet after birth, and the hypercholesterolaemia was observed both in the fed (Figure 3b) and fasted (Figure 6a) states. Our study further demonstrated that exposure to a LOW plane of nutrition also reduced the ability to lower plasma cholesterol during fasting (Figure 6a versus 6b), which was not the case in lambs that had been exposed to a HIGH plane of nutrition prenatally.

The detailed mechanisms through which malnutrition before birth may influence postnatal cholesterol metabolism are, as yet, not well understood. A previous study has shown that alterations in expression of key genes such as LDLr (LDL receptor) could be a potential mechanism behind the increased risk of atherosclerosis by maternal undernutrition (Yates *et al.* 2009). Recent studies also emphasize that the adverse association between maternal undernutrition and long-term cholesterol dysregulation could be explained by epigenetic mechanisms, since protein restriction during pregnancy and lactation increases cholesterol levels in rat offspring due to repressive posttranslational histone modification at the promoter of 7α-hydroxylase (Sohi *et al.* 2011). Hepatic 7α-hydroxylase, the rate-limiting enzyme of bile acid synthesis, alters cholesterol homeostasis by catabolizing cholesterol to bile acids (Jelinek *et al.* 1990). Finally, we have recently found evidence to suggest that late gestation undernutrition can permanently programme the way in which polyunsaturated fatty acids (PUFA) are prioritized when incorporated into structural lipids (Hou *et al.* 2013), which potentially can have major implications for the whole range of functions of cellular membranes and hence metabolic functions of the liver. Future studies are obviously needed to reveal the underlying mechanisms behind the differential foetal programming of hepatic functions related to glucose-lactate metabolism, amino acid oxidation-urea synthesis and cholesterol metabolism, and to establish the long-term implications. However, this study has quite convincingly demonstrated that individuals are differentially programmed in response to over- and undernutrition during late foetal development, since LOW individuals became predisposed for development of hypercholesterolaemia and reduced hepatic gluconeogenetic capacity and adaptability, whereas HIGH lambs had increased gluconeogenetic capacity with reduced plasticity of insulin secretion. Thus, the liver and pancreatic β-cells appear to be central targets for differential programming in late foetal life depending on the nutritional exposure.

An early postnatal obesogenic diet exaggerates many of the adverse prenatal nutrition outcomes and interferes with pancreatic insulin secretion by non-glucose dependent signalling pathways

We observed a number of adverse impacts of the early postnatal HCHF diet on glucose-insulin axis function and lipid metabolism as expected, such as hyperglycaemia, reduced glucose clearance, delayed insulin clearance and sensitivity (as shown in glucose and insulin tolerance tests), reduced pancreatic insulin secretory response to secretagogues (in this case propionate), hypercholesterolaemia, hypertriglyceridaemia, and increased plasma levels of the liver enzyme GGT, which was associated with hepatic fat infiltration (results not shown). These impacts of the high-fat feeding on metabolic and endocrine function have previously been in reported in human, rodent and sheep studies (Brons *et al.* 2009, Kongsted *et al.* 2014, Panchal *et al.* 2011). Our study showed that expression of some of the programming outcomes of prenatal nutrition on metabolic and endocrine adaptations depended upon the postnatal nutrition exposure, suggesting that early postnatal conditions can be critical for determining the expression of the foetally programmed phenotype, and thereby the susceptibility of an individual to developing chronic diseases later in life (Martin-Gronert & Ozanne 2012).

The high-fat obesogenic diet induced a more pronounced hyperglycaemic condition in response to provision of a gluconeogenetic substrate (propionate) in the prenatally overnourished HIGH lambs compared to LOW and NORM lambs irrespective of whether they were fed or fasted during the tolerance test. In the LOW lambs, the HCHF diet induced a general hypercholesterolaemic condition compared to NORM and HIGH lambs (significantly higher cholesterol levels in LOW-HCHF lambs throughout the insulin and propionate fasted tolerance tests). These results do not support our hypothesis that individuals with a history of overnutrition before birth should be superior in their ability to cope with the hyperglycaemic

effect of an early postnatal obesogenic diet; on the contrary they become more predisposed for postnatal hyperglycaemia due to an increased hepatic gluconeogenetic capacity as discussed above. Our results do indicate, however, that individuals exposed to late gestation overnutrition could have superior ability to cope with the hypercholesterolaemic effects of such an obesogenic diet as compared to prenatally undernourished individuals.

An unexpected and interesting finding was the almost complete failure of propionate (and the associated increase in plasma glucose) to increase plasma insulin levels in 2-day fasted HCHF lambs, whereas more pronounced insulin responses were observed in the CONV fed lambs (except for HIGH-CONV) during fasting as compared to when fed. The overall consistently higher insulin responses to propionate in CONV compared to HCHF lambs were in contrast to what was observed during the glucose tolerance test, where HCHF lambs had the highest (although delayed) insulin responses. This shows that the high-fat, obesogenic HCHF diet must interfere with insulin secretory function of the pancreatic β-cell by pathways both depending on glucose as well as by pathways independent of glucose. The latter appear to be the most susceptible towards prenatal nutrition exposures, since prenatal impacts on insulin secretion were only revealed during the propionate and not during glucose tolerance tests. We cannot rule out, however, that a prenatal programming of glucose-dependent pathways could have occurred as well, but masked by the impact of the postnatal diet, or such a programing may only gradually become manifested with age, as we have observed in a previous study focussing on late-gestation undernutrition and with a similar postnatal feeding protocol (Kongsted *et al.* 2014).

The mechanisms whereby propionate stimulates insulin secretion from the pancreas are to the best of our knowledge not known, but older *in vitro* studies (Hertelendy *et al.* 1968) have in support of the above-mentioned deductions demonstrated that glucose and propionate stimulate insulin secretion from sheep pancreas explants by separate and apparently additive

pathways. However, in isolated pancreatic islets from rats, propionate has been shown to inhibit rather than stimulate glucose-induced insulin secretion (Ximenes *et al.* 2007). The non-ruminant and ruminant pancreas may have developed different regulatory features (Sano *et al.* 1999) according to the differences in patterns of absorbed nutrients (glucose versus short chained fatty acids). But our findings should irrespective of this definitely encourage further studies on how non-glucose dependant regulatory pathways are involved in the adaptation of pancreatic β-cell function in response to adverse nutrition exposures pre- and postnatally, as this could be important for both design of diagnostic tools as well as interventions to prevent the development of β-cell dysfunction in subjects who have undergone adverse programming during foetal life.

The high NEFA levels observed in the HCHF fed animals were in the same range or higher than the levels of free fatty acids which have been reported to induce cytostatic (reduced glucose stimulated insulin secretion) and proapoptotic (caspase mediated) events in the human pancreatic β-cell (Lupi *et al.* 2002). Induced β-cell dysfunction at some stage due to very high NEFA levels can therefore most probably provide part of the explanation as to why insulin secretory responses were delayed in HCHF as compared to CONV fed lambs during both glucose and propionate tolerance tests, and again, the glucose independent regulatory pathways would appear to be more affected than the insulin dependent ones. But the very peculiar and unexplainable changes in NEFA concentrations during the propionate challenge suggest that another player may also be involved, namely glucagon. After two days of fasting, NEFA levels in all lambs increased as would be expected, when insulin levels are decreased and the balance of adipose lipid metabolism consequently becomes more negative. Higher insulin levels within the normal physiological limits can reversely explain the clear drop in NEFA during the first phase of the glucose tolerance test. But unexpectedly, NEFA levels did not decrease in the fasted HCHF lambs (except for a transient small drop) or in

either group of fed lambs during propionate tolerance tests, in fact NEFA levels were increased despite the increased insulin levels. Propionate has been reported to be a stimulator not only of pancreatic insulin, but also of pancreatic glucagon secretion in ruminants (Sano *et al.* 1999). We have indeed preliminary results indicating that glucagon increased dramatically in response to propionate (assay remains to be validated for ruminants). Stimulation of glucagon secretion could explain why much more dramatic increases were observed in lactate as compared to glucose levels during propionate challenges especially in fasted lambs, since glucagon inhibition of the final steps in hepatic gluconeogenesis (9) could redirect propionate-C to be released as lactate (19) rather than glucose (4). Glucagon receptors have been identified in ruminant adipose tissue, but its quantitative role as a lipolytic hormone is considered insignificant in mammals (Vernon 1980). If glucagon levels during propionate tolerance tests increased to sufficiently high levels, induction of adipose lipolysis could, however, explain the peculiar increases in NEFA, co-existing with increases in glucose and insulin during this test. There is a remarkable absence of studies on glucagon and the pancreatic α-cell in relation to foetal programming. But we suspect that glucagon in our study may also have been involved in modulating the insulin responses during propionate fasting tolerance tests depending on the prenatal nutrition history of the lambs, since glucagon is also known to be an insulin secretagogue (Kawai *et al.* 1995). Glucagon is most certainly a hormone which deserves to receive more attention in future (foetal programming) research.

In conclusion, prenatal over- and undernutrition differentially programmed priorities in hepatic C-shunting between glucose and lactate synthetic pathways as well as cholesterol homeostasis. LG-ON did not provide protection against the hyperglycaemic impacts of a postnatal high-fat diet. In fact, LG-ON predisposed for hyperglycaemia and hyperlactataemia, presumably due to increased hepatic gluconeogenetic capacity. LG-UN on the other hand predisposed for hypercholesterolaemia, which became expressed upon exposure to a

postnatal obesogenic diet. LG-ON (but not LG-UN) interfered with pancreatic insulin secretory plasticity by non-glucose dependent pathways. Our studies thus point to the liver and endocrine pancreas as major targets of foetal programming and to a potential role of glucagon in foetal programming, which should be addressed in future studies. The propionate tolerance tests particularly during fasting were most efficient in revealing phenotypic traits related to different foetal nutrition exposures. The most sensitive plasma parameters across the conducted tolerance tests were (in decreasing order of importance): lactate, cholesterol, glucose, BUN, BOHB and insulin. The issue of foetal programming of non-glucose dependent regulatory pathways of insulin secretion deserved to be addressed in other non-ruminant model studies.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends:

Figure 1:

Changes in blood metabolites during glucose and insulin tolerance tests. a) plasma glucose (inserted Area Under the Curve and above baseline for glucose from time point 0 to 120; glucose tolerance test) b) plasma glucose (inserted Area Over the Curve and below baseline from time point 0 to 60; insulin tolerance test) c) plasma insulin (inserted Area Under the Curve and below baseline for insulin from time point 0 to 120; glucose tolerance test) d) plasma insulin (inserted Area Under the Curve and above baseline; insulin tolerance test) e) plasma lactate (glucose tolerance test) f) plasma lactate (insulin tolerance test). When significant interactions were found between the pre- and postnatal diet, data have been shown for each combination of pre- and postnatal diet: LOW-HCHF (N=13; 7 male, 6 female; dash line; ▽), LOW-CONV (N=13; 6 male, 7 female; solid line; ▼), HIGH-HCHF (N=13; 5 male, 8 female; dash line; △), HIGH-CONV (N=13; 5 male, 8 female; solid line; ▲) NORM-HCHF (N=9; 6 male, 3 female; dash line; □), and NORM-CONV (N=9; 5 male, 4 female; solid line; ■). When significant effects were found only of the postnatal diet, data have been shown for postnatal dietary group only: CONV (N=35; 16 male, 19 female; solid line; ●) and HCHF (N=35; 18 male, 17 female; dash line; ○). The values are presented as LS means ± SEM and the letters E and L denote pre- (**E**we diet) and postnatal diet (**L**amb diet) effects, respectively. The effects were significant at ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; #, $P<0.1$. The LOW, NORM and HIGH refer to the prenatal nutrition offered to the twin-pregnant dams and fulfilling 50/50%, 100/100% and 150/110%, respectively, of daily requirements for energy/protein. The CONV and HCHF refer to a moderate or obesogenic, high-carbohydrate-high-fat diet, respectively, fed during the first 6 months of postnatal life. The glucose tolerance test was conducted in the morning in overnight fasted lambs whereas the insulin tolerance test was conducted in the afternoon after the normal afternoon meal, except that

HCHF lambs were not fed their milk-cream mix to avoid interference in plasma hormone assays from very high levels of plasma lipids.

Figure 2:

Changes in blood metabolites during glucose and insulin tolerance tests a) plasma TG (triglycerides; glucose tolerance test) b) plasma TG (insulin tolerance test) c) plasma NEFA (non-esterified fatty acids; glucose tolerance test) d) plasma NEFA (insulin tolerance test) e) plasma BOHB (β-hydroxy butyrate; glucose tolerance test) f) plasma BOHB (insulin tolerance test). When significant interactions were found between the pre- and postnatal diet, data have been shown for each combination of pre- and postnatal diet: LOW-HCHF (N=13; 7 male, 6 female; dash line; ▽), LOW-CONV (N=13; 6 male, 7 female; solid line; ▼), HIGH-HCHF (N=13; 5 male, 8 female; dash line; △), HIGH-CONV (N=13; 5 male, 8 female; solid line; ▲) NORM-HCHF (N=9; 6 male, 3 female; dash line; □), and NORM-CONV (N=9; 5 male, 4 female; solid line; ■). When significant effects were found only of the postnatal diet, data have been shown for postnatal dietary group only: CONV (N=35; 16 male, 19 female; solid line; ●) and HCHF (N=35; 18 male, 17 female; dash line; ○). The values are presented as LS means ± SEM and the letters E and L denote pre- (**E**we diet) and postnatal diet (**L**amb diet) effects, respectively. The effects were significant at ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; #, $P<0.1$. The glucose tolerance test was conducted in the morning in overnight fasted lambs whereas the insulin tolerance test was conducted in the afternoon after the normal afternoon meal, except that HCHF lambs were not fed their milk-cream mix to avoid interference in plasma hormone assays from very high levels of plasma lipids. LOW, NORM, HIGH, CONV and HCHF: See legend to Figure 1.

Figure 3:

Changes in blood metabolites during glucose and insulin tolerance tests a) cholesterol (glucose tolerance test) b) cholesterol (insulin tolerance test) c) plasma BUN (blood urea

nitrogen; glucose tolerance test) d) plasma BUN (insulin tolerance test; insulin tolerance test). When significant interactions were found between the pre- and postnatal diet, data have been shown for each combination of pre- and postnatal diet: LOW-HCHF (N=13; 7 male, 6 female; dash line; ▽), LOW-CONV (N=13; 6 male, 7 female; solid line; ▼), HIGH-HCHF (N=13; 5 male, 8 female; dash line; ∆), HIGH-CONV (N=13; 5 male, 8 female; solid line; ▲) NORM-HCHF (N=9; 6 male, 3 female; dash line; □), and NORM-CONV (N=9; 5 male, 4 female; solid line; ■). When significant effects were found only of the postnatal diet data have been shown for postnatal dietary group only: CONV (N=35; 16 male, 19 female; solid line; ●) and HCHF (N=35; 18 male, 17 female; dash line; ○). The values are presented as LS means ± SEM and the letters E and L denote pre- (**E**we diet) and postnatal diet (**L**amb diet) effects, respectively. The effects were significant at ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; #, $P<0.1$. The glucose tolerance test was conducted in the morning in overnight fasted lambs whereas the insulin tolerance test was conducted in the afternoon after the normal afternoon meal, except that HCHF lambs were not fed their milk-cream mix to avoid interference in plasma hormone assays from very high levels of plasma lipids. LOW, NORM, HIGH, CONV and HCHF: See legend to Figure 1.

Figure 4:

Changes in blood metabolites during propionate tolerance tests in 44-hour fasted and fed lambs a) plasma glucose (inserted Area Under the Curve for glucose and above baseline from time point 0 to 120 min; fasted state) b) plasma glucose (inserted Area Under the Curve for glucose and above baseline from time point 0 to 120 min; fed state) c) plasma insulin (inserted Area Under the Curve for insulin and above baseline from 0 to 120; fasted state) d) plasma insulin (inserted Area Under the Curve for insulin and above baseline from 0 to 120; fed state) e) plasma lactate (fasted state) f) plasma lactate (fed state). When significant interactions were found between the pre- and postnatal diet, data have been shown for each

combination of pre- and postnatal diet: LOW-HCHF (N=13; 7 male, 6 female; dash line; ▽), LOW-CONV (N=13; 6 male, 7 female; solid line; ▼), HIGH-HCHF (N=13; 5 male, 8 female; dash line; ▵), HIGH-CONV (N=13; 5 male, 8 female; solid line; ▲) NORM-HCHF (N=9; 6 male, 3 female; dash line; □), and NORM-CONV (N=9; 5 male, 4 female; solid line; ■). When significant effects were found only of the postnatal diet data have been shown for postnatal dietary group only: CONV (N=35; 16 male, 19 female; solid line; ●) and HCHF (N=35; 18 male, 17 female; dash line; ○). The values are presented as LS means ± SEM and the letters E and L denote pre- (**E**we diet) and postnatal diet (**L**amb diet) effects, respectively. The effects were significant at ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; #, $P<0.1$. Both fed and fasted propionate tolerance tests were conducted in the morning. The fasted test was conducted after the normal lambs had been subjected to a 44-hour fasting period. The fed test was conducted after the normal morning meal, except that HCHF lambs were not fed their milk-cream mix to avoid interference in plasma hormone assays from very high levels of plasma lipids. LOW, NORM, HIGH, CONV and HCHF: See legend to Figure 1.

Figure 5:

Changes in blood metabolites during propionate tolerance tests in 44-hour fasted and fed lambs a) plasma TG (triglycerides; fasted state) b) plasma TG (fed state) c) plasma NEFA (non-esterified fatty acids; fasted state) d) plasma NEFA (fed state) e) plasma BOHB (β-hydroxy butyrate; fasted state) f) plasma BOHB (fed state). When significant interactions were found between the pre- and postnatal diet, data have been shown for each combination of pre- and postnatal diet: LOW-HCHF (N=13; 7 male, 6 female; dash line; ▽), LOW-CONV (N=13; 6 male, 7 female; solid line; ▼), HIGH-HCHF (N=13; 5 male, 8 female; dash line; ▵), HIGH-CONV (N=13; 5 male, 8 female; solid line; ▲) NORM-HCHF (N=9; 6 male, 3 female; dash line; □), and NORM-CONV (N=9; 5 male, 4 female; solid line; ■). When significant effects were found only of the postnatal diet data have been shown for postnatal

dietary group only: CONV (N=35; 16 male, 19 female; solid line; ●) and HCHF (N=35; 18 male, 17 female; dash line; ○). The values are presented as LS means ± SEM and the letters E and L denote pre- (**E**we diet) and postnatal diet (**L**amb diet) effects, respectively. The effects were significant at ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; #, $P<0.1$. Both fed and fasted propionate tolerance tests were conducted in the morning. The fasted test was conducted after the normal lambs had been subjected to a 44-hour fasting period. The fed test was conducted after the normal morning meal, except that HCHF lambs were not fed their milk-cream mix to avoid interference in plasma hormone assays from very high levels of plasma lipids. LOW, NORM, HIGH, CONV and HCHF: See legend to Figure 1.

Figure 6:

Changes in blood metabolites during propionate tolerance tests in 44-hour fasted and fed lambs a) plasma cholesterol (fasted state) b) plasma cholesterol (fed state) c) plasma BUN (blood urea nitrogen; fasted state) d) plasma BUN (fed state) e) plasma creatinine (fasted state) f) plasma creatinine (fed state). When significant interactions were found between the pre- and postnatal diet, data have been shown for each combination of pre- and postnatal diet: LOW-HCHF (N=13; 7 male, 6 female; dash line; ▽), LOW-CONV (N=13; 6 male, 7 female; solid line; ▼), HIGH-HCHF (N=13; 5 male, 8 female; dash line; △), HIGH-CONV (N=13; 5 male, 8 female; solid line; ▲) NORM-HCHF (N=9; 6 male, 3 female; dash line; □), and NORM-CONV (N=9; 5 male, 4 female; solid line; ■). When significant effects were found only of the postnatal diet data have been shown for postnatal dietary group only: CONV (N=35; 16 male, 19 female; solid line; ●) and HCHF (N=35; 18 male, 17 female; dash line; ○). The values are presented as LS means ± SEM and the letters E and L denote pre- (**E**we diet) and postnatal diet (**L**amb diet) effects, respectively. The effects were significant at ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; #, $P<0.1$. Both fed and fasted propionate tolerance tests were conducted in the morning. The fasted test was conducted after the normal

lambs had been subjected to a 44-hour fasting period. The fed test was conducted after the normal morning meal, except that HCHF lambs were not fed their milk-cream mix to avoid interference in plasma hormone assays from very high levels of plasma lipids. LOW, NORM, HIGH, CONV and HCHF: See legend to Figure 1.

Figure 7:

Model of the integration of different metabolic processes in the liver and extrahepatic tissues. 1, glucose entering from gut through portal blood to the liver; 2, use of glucose for glycogen synthesis; 3, release of glucose from degradation of glycogen in liver; 4, glucose release from the liver to the blood; 5, uptake of blood glucose by muscle and other extrahepatic tissues; 6, glycogen synthesis from glucose in muscle; 7, glycogen breakdown to glucose-P in muscle; 8; conversion of glucose to phosphoenol pyruvate in the glycolytic pathway; 9, production of free glucose from phosphoenol pyruvate via a series of steps in gluconeogenesis; 10, conversion of phosphoenol pyruvate into pyruvate; 11, conversion of pyruvate to oxaloacetate prior to entry into gluconeogenesis; 12, conversion of pyruvate to lactate; 13, conversion of lactate to pyruvate; 14, propionate entering from the gut through the portal blood to the liver; 15, conversion of propionate to oxaloacetate in the liver; 16, conversion of oxaloacetate to phosphoenol pyruvate; 17, conversion of lactate to propionate in fermentation processes in the gut; 18, entry of lactate from the gut through portal blood to the liver; 19, release of lactate from the liver to the blood; 20, uptake of lactate in the liver from the blood; 21, release of lactate from muscle to the blood; 22, uptake of lactate from the blood in muscle and other extrahepatic tissues; 23, conversion of glucose to pyruvate (glycolysis); 24, conversion of pyruvate to lactate; 25, conversion of lactate to pyruvate; 26, pyruvate entering the tricarboxylic acid cycle in extrahepatic tissues; 27, decarboxylation of pyruvate to acetyl CoA in the liver; 28; cholesterol synthesis from acetyl CoA; 29, release of cholesterol into the blood as part of lipoproteins (VLDL, very low density lipoprotein; LDL, low density

lipoprotein); 30, ketone body synthesis from acetyl CoA; 31, β-oxidation of fatty acids and synthesis of acetyl CoA; 32, blood propionate injected intravenously and transported to the liver.

Supplementary Figure 1:

Changes in plasma GGT levels during glucose, insulin and propionate fasted (44-hour) and fed tolerance tests a) plasma GGT (gamma-glutaryl transferase; glucose tolerance test) b) plasma GGT (insulin tolerance test) c) plasma GGT (propionate fasted tolerance test) d) plasma GGT (propionate fed tolerance test). Since significant effects were found only of the postnatal diet data have been shown for postnatal dietary group only: CONV (N=35; 16 male, 19 female; solid line; ●) and HCHF (N=35; 18 male, 17 female; dash line; ○). The values are presented as LS means ± SEM and the letter L denotes postnatal diet (**L**amb diet) effects. The effects were significant at ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; #, $P<0.1$. The CONV and HCHF refer to a moderate or obesogenic, high-carbohydrate-high-fat diet, respectively, fed during the first 6 months of postnatal life. The glucose tolerance test was conducted in the morning in overnight fasted lambs whereas the insulin tolerance test was conducted in the afternoon after the normal afternoon meal, except that HCHF lambs were not fed their milk-cream mix to avoid interference in plasma hormone assays from very high levels of plasma lipids. Both fed and fasted propionate tolerance tests were conducted in the morning. The fasted test was conducted after the normal lambs had been subjected to a 44-hour fasting period. The fed test was conducted after the normal morning meal, except that HCHF lambs were not fed their milk-cream mix to avoid interference in plasma hormone assays from very high levels of plasma lipids.

Figure 1:

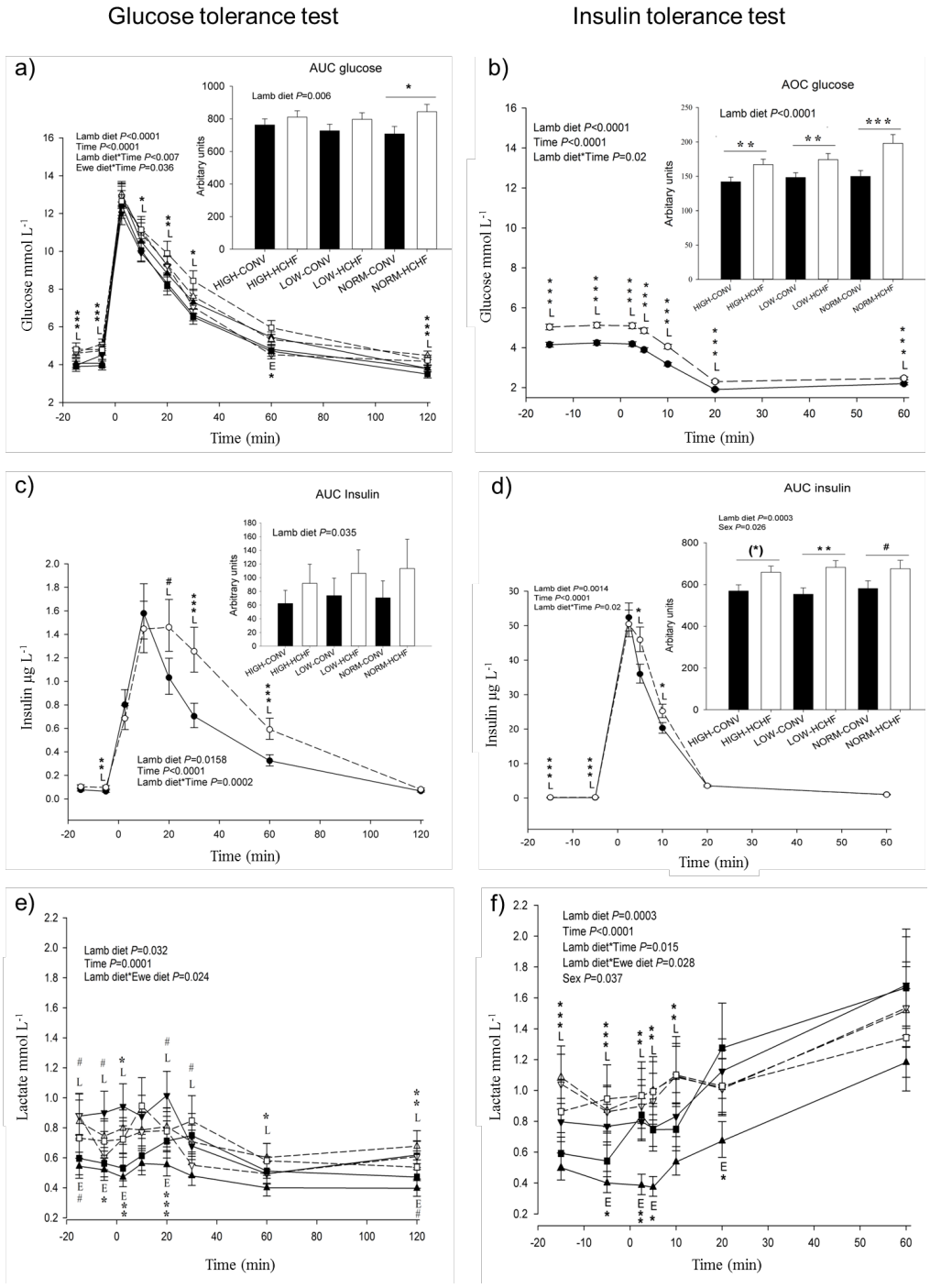

Glucose tolerance test
Insulin tolerance test
a)
AUC glucose
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P<0.007
Ewe diet*Time P=0.036
Lamb diet P=0.006
Arbitary units
HIGH-CONV
HIGH-HCHF
LOW-CONV
LOW-HCHF
NORM-CONV
NORM-HCHF
Glucose mmol L-1
Time (min)
b)
AOC glucose
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P=0.02
Glucose mmol L-1
Time (min)
c)
AUC Insulin
Lamb diet P=0.035
Lamb diet P=0.0158
Time P<0.0001
Lamb diet*Time P=0.0002
Insulin µg L-1
Time (min)
d)
AUC insulin
Lamb diet P=0.0003
Sex P=0.026
Lamb diet P=0.0014
Time P<0.0001
Lamb diet*Time P=0.02
Insulin µg L-1
Time (min)
e)
Lamb diet P=0.032
Time P=0.0001
Lamb diet*Ewe diet P=0.024
Lactate mmol L-1
Time (min)
f)
Lamb diet P=0.0003
Time P<0.0001
Lamb diet*Time P=0.015
Lamb diet*Ewe diet P=0.028
Sex P=0.037
Lactate mmol L-1
Time (min)

Figure 2:

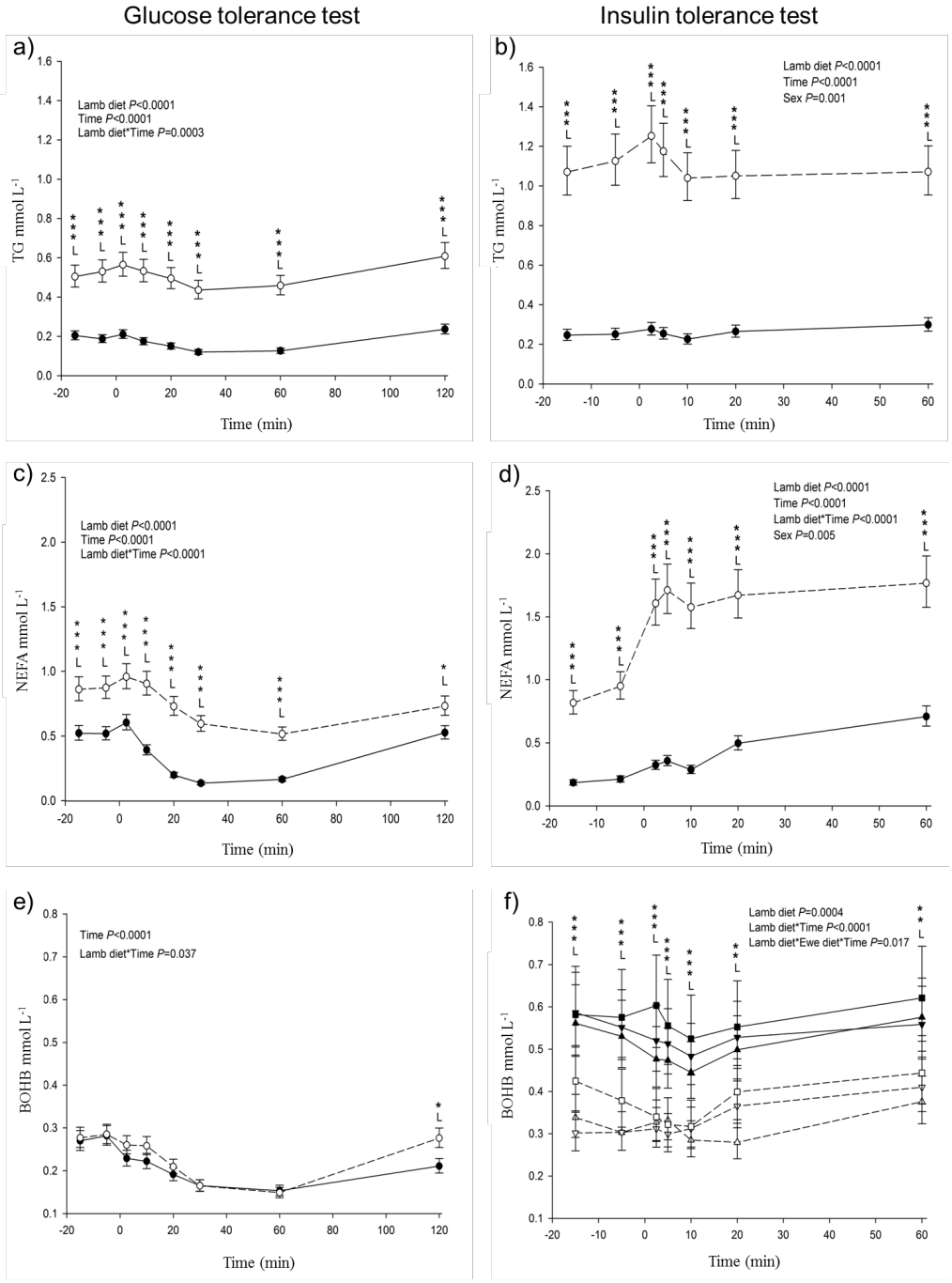

Glucose tolerance test
Insulin tolerance test
a)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P=0.0003
TG mmol L-1
Time (min)
b)
Lamb diet P<0.0001
Time P<0.0001
Sex P=0.001
TG mmol L-1
Time (min)
c)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P<0.0001
NEFA mmol L-1
Time (min)
d)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P<0.0001
Sex P=0.005
NEFA mmol L-1
Time (min)
e)
Time P<0.0001
Lamb diet*Time P=0.037
BOHB mmol L-1
Time (min)
f)
Lamb diet P=0.0004
Lamb diet*Time P<0.0001
Lamb diet*Ewe diet*Time P=0.017
BOHB mmol L-1
Time (min)

Figure 3:

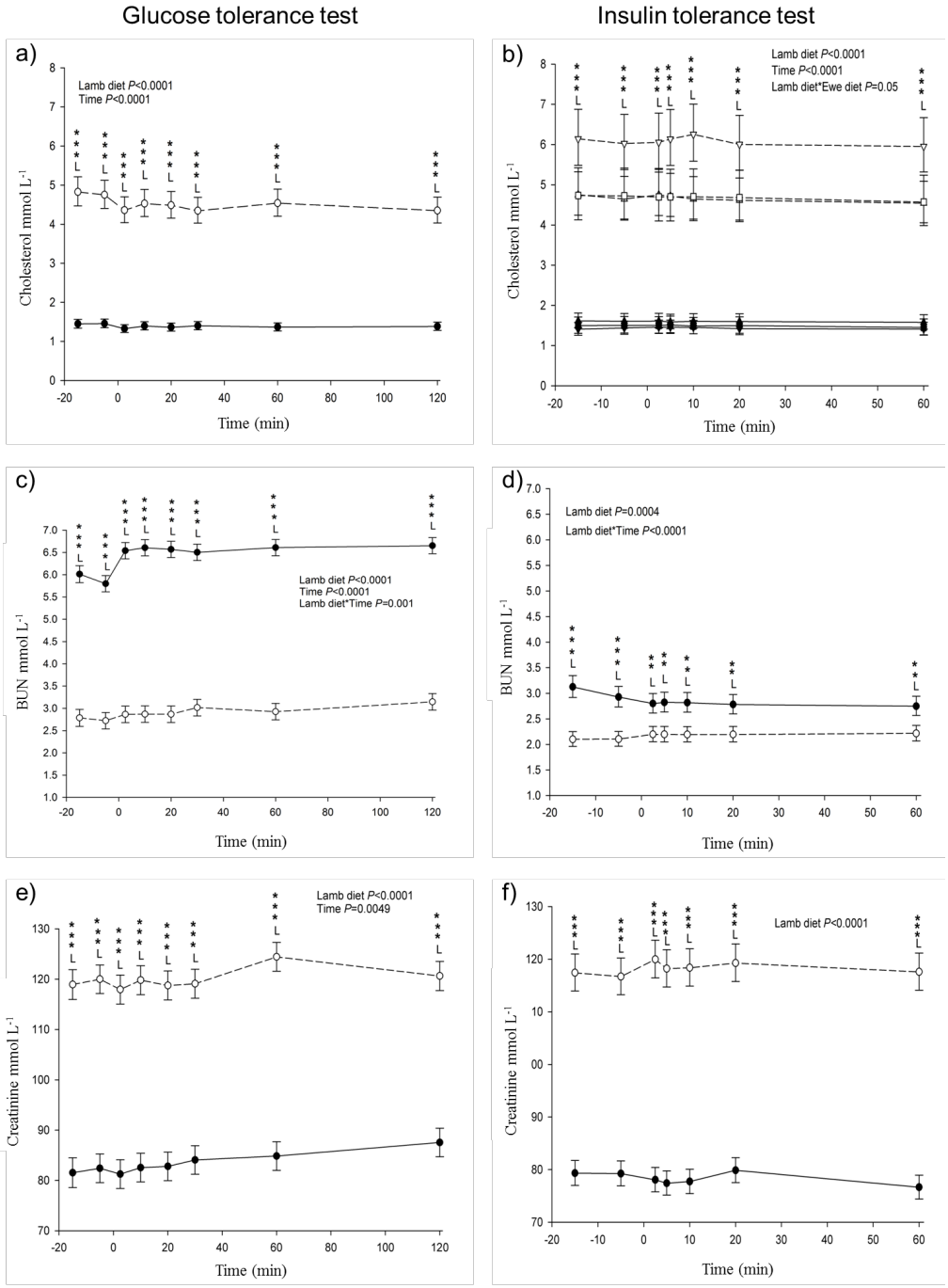

Glucose tolerance test
Insulin tolerance test
a)
Lamb diet P<0.0001
Time P<0.0001
Cholesterol mmol L-1
Time (min)
b)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Ewe diet P=0.05
Cholesterol mmol L-1
Time (min)
c)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P=0.001
BUN mmol L-1
Time (min)
d)
Lamb diet P=0.0004
Lamb diet*Time P<0.0001
BUN mmol L-1
Time (min)
e)
Lamb diet P<0.0001
Time P=0.0049
Creatinine mmol L-1
Time (min)
f)
Lamb diet P<0.0001
Creatinine mmol L-1
Time (min)

Figure 4:

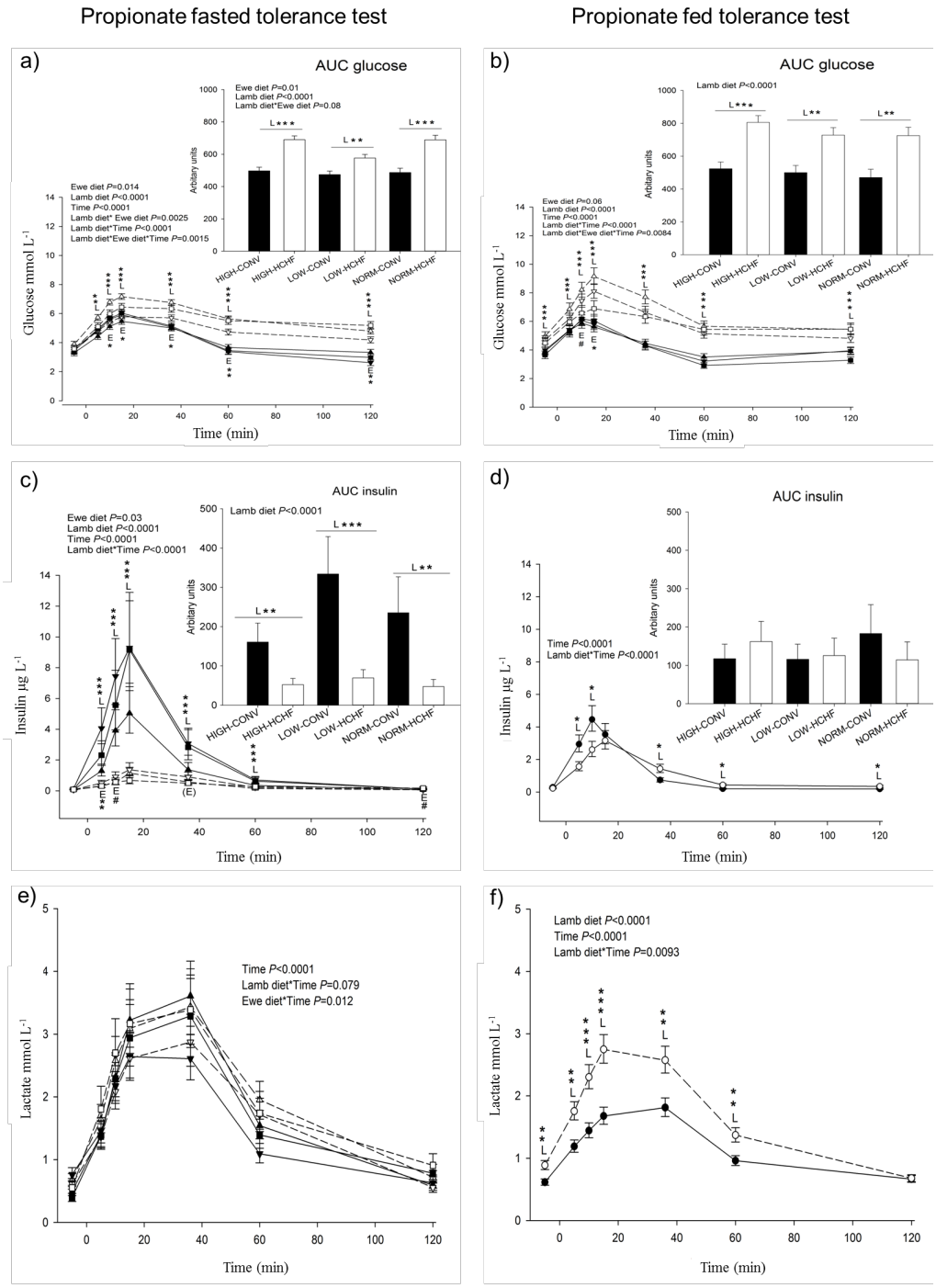

Propionate fasted tolerance test
Propionate fed tolerance test
a)
AUC glucose
Ewe diet P=0.01
Lamb diet P<0.0001
Lamb diet*Ewe diet P=0.08
Ewe diet P=0.014
Lamb diet P<0.0001
Time P<0.0001
Lamb diet* Ewe diet P=0.0025
Lamb diet*Time P<0.0001
Lamb diet*Ewe diet*Time P=0.0015
Arbitary units
HIGH-CONV
HIGH-HCHF
LOW-CONV
LOW-HCHF
NORM-CONV
NORM-HCHF
Glucose mmol L-1
Time (min)
b)
AUC glucose
Lamb diet P<0.0001
Ewe diet P=0.06
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P<0.0001
Lamb diet*Ewe diet*Time P=0.0084
c)
AUC insulin
Ewe diet P=0.03
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P<0.0001
Lamb diet P<0.0001
Insulin µg L-1
d)
AUC insulin
Time P<0.0001
Lamb diet*Time P<0.0001
e)
Time P<0.0001
Lamb diet*Time P=0.079
Ewe diet*Time P=0.012
Lactate mmol L-1
f)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P=0.0093

Figure 5:

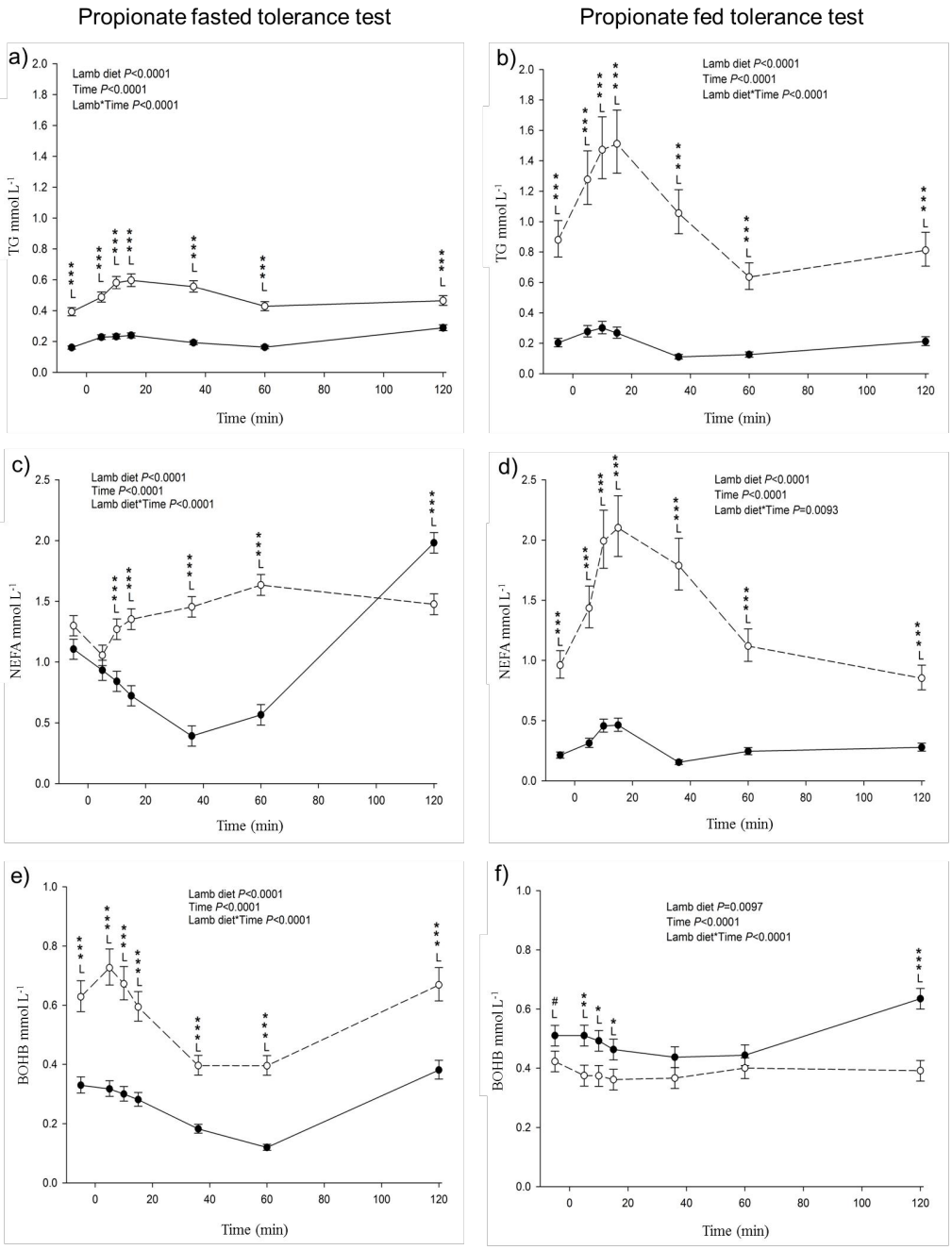

Propionate fasted tolerance test
Propionate fed tolerance test
a)
Lamb diet P<0.0001
Time P<0.0001
Lamb*Time P<0.0001
TG mmol L-1
b)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P<0.0001
TG mmol L-1
c)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P<0.0001
NEFA mmol L-1
d)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P=0.0093
NEFA mmol L-1
e)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P<0.0001
BOHB mmol L-1
f)
Lamb diet P=0.0097
Time P<0.0001
Lamb diet*Time P<0.0001
BOHB mmol L-1
Time (min)

Figure 6:

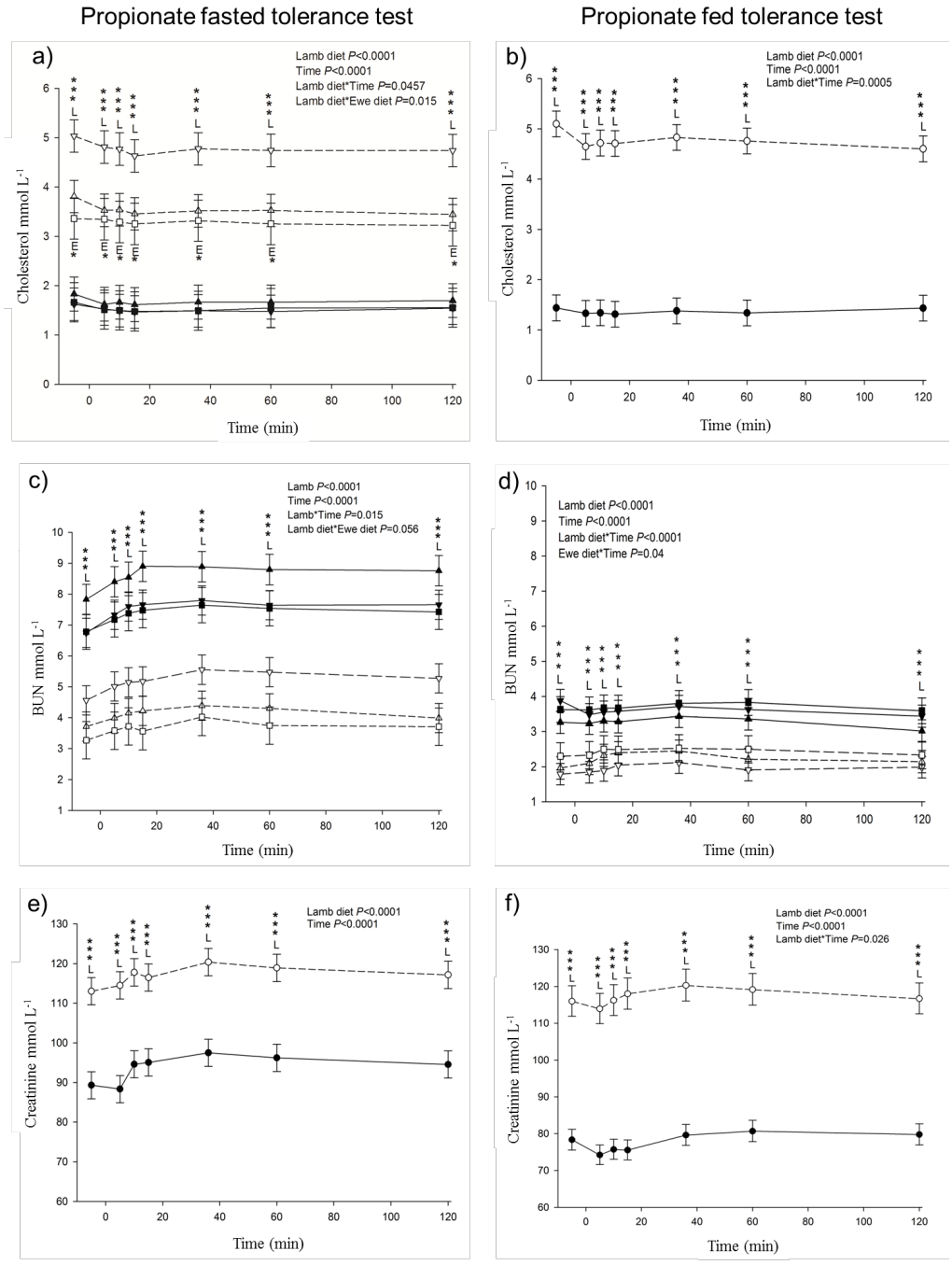
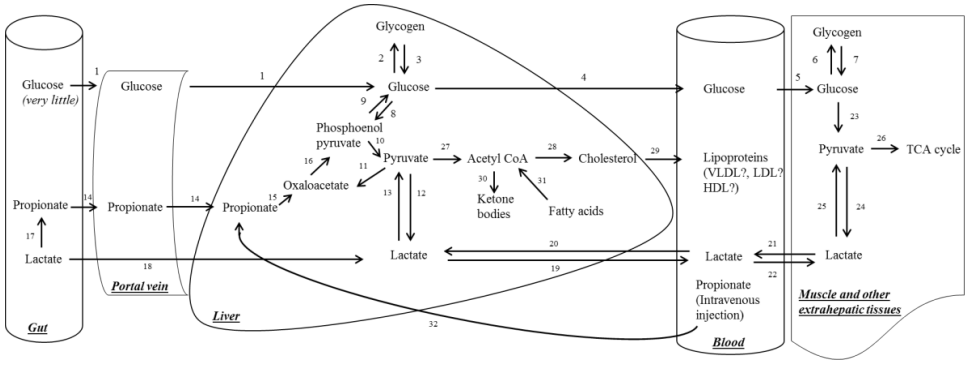
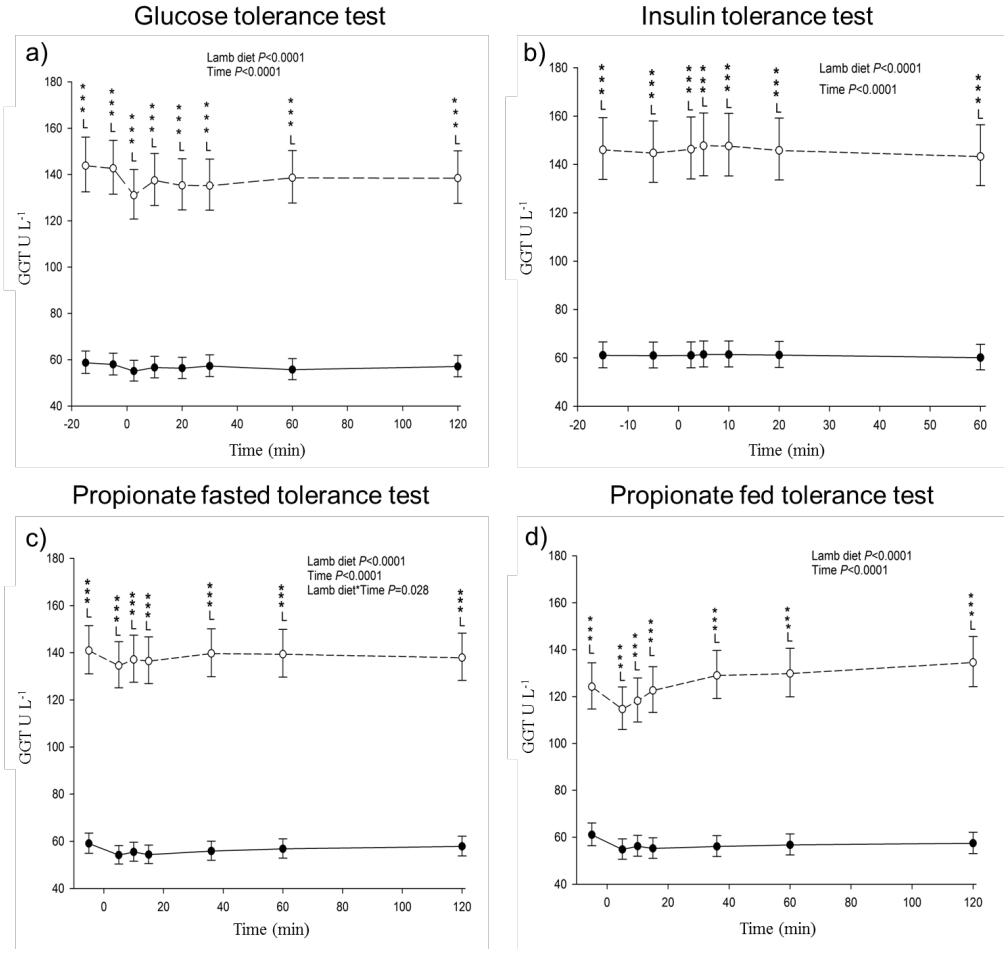

Propionate fasted tolerance test
Propionate fed tolerance test
a)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P=0.0457
Lamb diet*Ewe diet P=0.015
Cholesterol mmol L-1
Time (min)
b)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P=0.0005
Cholesterol mmol L-1
Time (min)
c)
Lamb P<0.0001
Time P<0.0001
Lamb*Time P=0.015
Lamb diet*Ewe diet P=0.056
BUN mmol L-1
Time (min)
d)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P<0.0001
Ewe diet*Time P=0.04
BUN mmol L-1
Time (min)
e)
Lamb diet P<0.0001
Time P<0.0001
Creatinine mmol L-1
Time (min)
f)
Lamb diet P<0.0001
Time P<0.0001
Lamb diet*Time P=0.026
Creatinine mmol L-1
Time (min)

Figure 7:


Glycogen
Glucose
(very little)
Glucose
Glucose
Glucose
Glucose
Glycogen
Glucose
Phosphoenol
pyruvate
Pyruvate
Acetyl CoA
Cholesterol
Lipoproteins
(VLDL?, LDL?
HDL?)
Pyruvate
TCA cycle
Oxaloacetate
Ketone
bodies
Fatty acids
Propionate
Propionate
Propionate
Lactate
Lactate
Lactate
Lactate
Portal vein
Propionate
(Intravenous
injection)
Muscle and other
extrahepatic tissues
Gut
Liver
Blood

Supplementary Figure 1:



Supplementary Table 1. Predicted effects of pre- and postnatal nutrition, sex and tolerance tests on plasma metabolite and insulin levels

Parameter	Prenatal nutrition				Lamb diet			Sex			Challenge				
	HIGH	NORM	LOW	Average SE*	HCHF	CONV	SE	Male	Female	Average SE*	Glucose	Insulin	Prop-fasted	Prop-fed	Average SE*
Glucose (mmol L^{-1})	5.584	5.394	5.168	0.1279	5.869	4.895	0.098	5.329	5.436	0.1017	7.254	3.814	4.875	5.585	0.1397
Insulin (µg L^{-1})	5.952	6.479	5.874	0.5202	6.226	5.978	0.3986	6.131	6.073	0.4107	1.126	17.935	2.361	2.986	0.5623
Lactate (mmol L^{-1})	1.397	1.460	1.314	0.0116	1.520	1.262	0.0827	1.341	1.440	0.0851	0.791	1.130	2.012	1.629	0.1167
GGT (U L^{-1})	112.2	134.3	95.4	12.67	164.6	63.3	9.803	108.7	119.2	10.08	116.2	125.0	110.1	104.5	13.84
TG (mmol L^{-1})	0.642	0.860	0.566	0.258	1.194	0.186	0.221	0.905	0.474	0.213	0.565	0.992	0.646	0.554	0.040
NEFA (mmol L^{-1})	0.751	0.842	0.828	0.123	1.246	0.368	0.105	0.967	0.647	0.106	0.702	1.156	0.699	0.6722	0.033
Cholesterol (mmol L^{-1})	3.164	3.051	3.398	0.2611	4.922	1.487	0.2007	3.349	3.060	0.2066	3.312	3.676	2.752	3.078	0.2831
BOHD (mmol L^{-1})	0.4097	0.4306	0.4161	0.0372	0.4462	0.3914	0.0285	0.4078	0.4298	0.0293	0.2508	0.4961	0.4838	0.4445	0.04025
BUN (mmol L^{-1})	4.093	3.910	4.182	0.2175	2.918	5.206	0.1677	4.006	4.117	0.1725	4.647	2.699	6.091	2.810	0.2366
Creatinine (µmol L^{-1})	103.1	101.1	100.3	2.921	119.4	83.6	2.238	101.7	101.3	2.306	101.5	100	105.1	99.4	3.157

Predicted effects of the pre- and postnatal diet, sex and tolerance tests on metabolic and endocrine function based on all the data from all tolerance tests conducted in the experiment. HIGH, (N=13) 150% of daily requirements for digestible energy and 110% of daily requirements for protein during late gestation, respectively); LOW, (N=14) 50% of requirements for energy and protein during late gestation); NORM (N=9), 100% of requirements for energy and protein during late gestation); HCHF, (N=35; 18 males; 17 females) lambs fed a postnatal obesogenic, high-carbohydrate high-fat diet consisting of a cream-milk replacer mix in a 1:1 ratio supplemented with rolled maize) or CONV, (N=35; 16 males, 19 females) lambs fed a moderate, conventional postnatal diet; SE, standard errors for estimates of difference; GGT, Gamma-glutaryl transferase; TG, Triglycerides; NEFA, Non-esterified fatty acids. BOHD; Beta-hydroxy butyrate; BUN, Blood urea nitrogen. *Average SE is the average of standard errors of the difference between estimated effects within a given fixed effect (e.g. for two levels sex and four level tolerance effects, there are one and three SE of differences, respectively).

PAPER III

Khanal, P., Pedersen, K.L., Pandey, D., Safayi, S., Hou, L., Birtwistle, M., Symonds, M., Kadarmideen, H.N., & Nielsen, M.O. Differential impacts of late gestational under- and overnutrition on adipose tissue development and function and abdominal adiposity risk upon exposure to a postnatal high-fat diet in adolescent sheep. Manuscript.

Differential impacts of late gestational under- and overnutrition on adipose tissue development and function and abdominal adiposity risk upon exposure to a postnatal high-fat diet in adolescent sheep

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Running head: Maternal malnutrition and adipose tissue development and function

Abstract

Aim: To test the following hypotheses: 1) both late gestational over- and undernutrition interfere with subcutaneous adipose tissue development and function, thereby predisposing for visceral adiposity when combined with an early postnatal high-fat diet, and 2) adverse prenatal as well as postnatal nutrition predispose for visceral adiposity, but targeting different adipose tissues.

Methods: A 3×2 factorial design experiment was conducted using twin-lambs born to dams fed HIGH (150% and 110% of required energy and protein, respectively), NORM (100% of required energy and protein) or LOW (50% of NORM) diets during the last trimester. After birth, lambs were raised on moderate (CONV) or obesogenic (HCHF) diets. Sub-groups of animals were sacrificed at 6-months of age. Adipocyte cross-sectional area, cellularity in subcutaneous (SUB), perirenal (PER), mesenteric (MES) and epicardial (EPI) adipose tissues, and gene expressions for lipid metabolism, angiogenic and inflammatory markers were studied.

Results: HIGH and LOW compared to NORM lambs had 1) reduced non-obese cellularity in SUB and MES (especially LOW) and reduced obesity-induced hyperplasia in SUB, MES and PER (especially HIGH except for MES), associated with increased obesity-induced hypertrophy in MES (especially LOW) and PER (especially HIGH) and SUB (not LOW), 2) reduced gene expressions for markers involved in lipid metabolism (SUB and EPI), angiogenesis (SUB), adipose development (EPI) and inflammation (PER; only in LOW). The general hyperplasia (not in HIGH in SUB) and hypertrophy induced by the obesogenic HCHF diet was associated with increased and decreased expressions of genes related to inflammation and lipid metabolism, respectively, in all adipose tissues.

Conclusion: Both prenatal over- and undernutrition predisposed for development of abdominal adiposity and extreme PER hypertrophy by depressing SUB and MES non-obese cellularity and

obesity-induced hyperplasia in SUB, MES and PER. The prenatal programming did not target inflammatory marker gene expressions in contrast to the postnatal obesogenic diet.

Key words: *adipose tissue development, inflammatory responses, foetal malnutrition, postnatal overnutrition*

Introduction

The increasing prevalence of obesity in the modern world is associated not only with a genetic predisposition, sedentary life style or over-nutrition, but also with exposure to an improper nutritional environment before birth (Dyer & Rosenfeld 2011). It is now well accepted that maternal nutrient restriction during gestation and subsequent overnutrition in early life is associated with increased obesity risk (Budge *et al.* 2004). The underlying mechanisms whereby maternal undernutrition can contribute to offspring obesity have been studied in different animal models, and in recent years it has become recognized that adipose tissue must be one of the major targets of foetal programming (Desai & Ross 2011) linking adverse prenatal nutrition to increased prevalence of obesity in postnatal life.

Although, studies on programming effects due to intrauterine overnutrition are scare, some evidence do exist suggesting that maternal overnutrition can have similar adverse impacts as maternal undernutrition on adiposity risk and fat deposition patterns later in life (Ford & Long 2011). From studies in sheep, which have a foetal third trimester development resembling that of humans (Nielsen *et al.* 2012), we have observed that both maternal over- and undernutrition during late foetal life alter fat deposition patterns in an unfavourable way later in life, thereby predisposing for visceral adiposity (Khanal *et al.* 2014). This appeared in our studies to be associated with a reduced expandability of subcutaneous adipose tissue, whereas visceral adiposity in response to a postnatal high-fat diet appeared to have perirenal fat as a more specific target. Subcutaneous fat is considered

as a ‘healthy’ fat depot, which can act as a nutrient ‘sink’ in situations of excessive energy availability. Poor expandability of subcutaneous fat may therefore increase the risk of nutrient overflow and excess accumulation of abdominal fat (Lemieux 2004). The (molecular-) biological mechanisms underlying these tissue specific alterations of adipose tissue development and function in response to early life nutrition are not well-known.

A previous study in rats has shown that maternal protein restriction throughout the gestation and lactation period can increase the expression of glucose transporter-4 gene (GLUT-4), which plays a key role in glucose update by the adipocyte, and expressions of fatty acid synthase (FAS) and CCAAT box enhancer-binding protein (C/EBP)-β genes were also upregulated in visceral adipose tissue from the restricted offspring (Guan *et al.* 2005). Other studies have demonstrated that late gestational nutrient restriction in sheep causes overexpression in adipose tissue of the offspring of inflammatory markers such as cluster of differentiation 68 (CD 68), and toll-like receptor (TLR4) (Sharkey *et al.* 2009), and in rodents it has been shown that high-fat feeding triggers up-regulation of monocyte chemoattractant protein-1 (MCP-1) and CD 68 (Xu *et al.* 2003). These studies indicate that glucose and lipid metabolism pathways as well as inflammatory processes in adipose tissue may be possible targets of foetal programming thereby potentially contributing to explain the link to development of obesity later in life. Although gestational overnutrition and being born large for gestational age are now increasingly recognised as predisposing factors for adiposity later in life, the mechanisms behind the association between maternal overnutrition and adipose tissue development and inflammation remain to be explored.

In this study, we used the Copenhagen sheep model (Nielsen *et al.* 2013, Khanal *et al.* 2014) to test the hypotheses that 1) both late gestational under- and overnutrition interfere with subcutaneous adipose tissue development and function, thereby predisposing for visceral adiposity when

combined with an early postnatal high-fat diet, and 2) adverse prenatal as well as postnatal nutrition predispose for visceral adiposity, but targeting different adipose tissues (subcutaneous versus renal).

Methodology

Experimental animals and treatments

All the experimental animal handling protocols were approved by the Danish National Committee on Animal Experimentation. The animal experiments were conducted at the experimental facilities on the farm Rosenlund, Lynge, Denmark under the auspices of the Faculty of Health and Medical Sciences, University of Copenhagen, Denmark. The experimental animals, dietary treatments, and their management have previously been described in detail (Khanal *et al.* 2014). In short, a 3x2 factorial design experiment was conducted, where 36 twin pregnant ewes were subjected to three different diets in the late gestation: overnutrition (HIGH; fulfilling 150% and 110% of requirements for energy and protein, respectively), moderate nutrition (NORM; fulfilling requirements for energy and protein), and undernutrition (LOW; fulfilling 50% of energy and protein requirements). From 3 days after birth until six months of age (just after puberty), the twin lambs were assigned to either a high-carbohydrate-high-fat diet (HCHF; max. 2 L/d of a milk replacer-dairy cream mix and max. 2 kg/d of rolled maize) or a moderate conventional diet (CONV; hay supplemented with milk replacer during the first 8 weeks of life and fed restricted to achieve moderate growth rates of appr. 225 g/d). Thus six different treatment groups exposed to matched or mismatched nutrition pre- and postnatally were created: HIGH-HCHF, HIGH-CONV, NORM-HCHF, NORM-CONV, LOW-HCHF and LOW-CONV).

Tissue sampling and processing:

At six months of age, sub-groups of animals from each treatment group were slaughtered in such a way that there would be a uniform distribution of sex as possible in slaughtered and surviving animals to assess the long-term consequences of early life exposures in both sexes except that only

males were slaughtered from the NORM-CONV and NORM-HCHF groups due to the smaller group size. Therefore when evaluating results from the present study, it should be borne in mind that sex effects could only be evaluated for the LOW and HIGH prenatal groups as reported earlier (Khanal *et al.* 2014). Tissues samples were randomly sampled from subcutaneous (above the muscularis longissimus dorsii at the level of the last rib), mesenteric, perirenal and epicardial adipose tissues. For gene expression studies, pieces of tissues were immediately fixated in RNA*later* (RNA*later*® Solution, Ambion, The RNA Company, USA) for 24 hours and then all samples were stored at -80ºC pending analyses. For histological evaluations, adipose tissue samples were fixated in 4% paraformaldehyde (PFA) for 24 hours, and afterwards in 1% PFA for a week until paraffin embedding.

Histology

The PFA-fixed tissue samples embedded into paraffin blocks were used for tissue sectioning. Five µm thick sections were cut using a Leica sliding microtome (Leica Microsystems, Ballerup, Denmark) and mounted on a superfrost glass slide (SuperFrost® WHITE; Hounsen Laboratorieudstyr; Århus, Denmark). In every tissue blocks, 300 µm were cut off after the first collection and this was repeated twice yielding sections from three different collection sites within each tissue sample. To ensure unbiased results, each slide was assigned a number to mask its identity throughout the analysis process. After sectioning, tissue slides were put in a heating oven at 50ºC for 40 min and thereafter stored at room temperature until staining. The slides were stained according to the protocol by van Gieson as reported previously (Kongsted *et al.* 2014). Tissue sections were immersed three times in xylene for 10 min to deparaffinise and were then rehydrated with a decreasing gradient of ethanol solutions (from 99% to 70%). Then, they were immersed in a solution of Lillie Weigerts ironhematein (15 min). The sections were washed under running tap water, and then placed in pikrinacid-acidfuchsin (4 min). Thereafter sections were dehydrated with

an increasing gradient of ethanol solutions (from 70% to 99%), cleared with xylene and mounted with DPX Mountant (Fluka, Switzerland).

The stained tissue slides were scanned by a Pannoramic MIDI whole slide scanner (3DHISTECH Ltd., Konkoly-Thege M. str., Budapest, Hungary) and a total of five pictures were randomly taken from the section at 20X magnification, providing a total of fifteen pictures for histology analyses from each adipose tissue depot per animal. The relative proportion of different tissue structures in the slides were evaluated using ImageJ software (Abramoff *et al.* 2004) by randomly applying a 28-points transparent grid on each picture as described by Safayi *et al.* (2010). The tissue structures were classified as adipocyte, collagen fibre or micro-vessels, and proportions of these structures refer to the number of hits on a given cell structures relative to the total number of hits in the whole picture. The counting of coinciding points (420 hits per sample) with the mentioned desired structures allowed unbiased estimation (Gundersen *et al.* 1988) of volume fractions for each animal. Adipocyte cross sectional area was determined manually and adipocytes used for cross sectional area measurements were chosen by randomly assigning a 15-points transparent grid. A cell number index (CNI) was calculated for subcutaneous, mesenteric and perirenal adipose tissues as: adipocyte mass (total fat mass (kg) multiplied by the percentage of adipocytes in the tissue) divided by the cell volume of a spherical adipocyte with a radius derived from the measured mean cross-sectional area (CSA): $CSA=\pi * r^2$. The CNI is obviously not a reliable estimate of the total number of adipocytes in the respective tissues, since adipocytes are not spherical but have an angular shape (particularly in HCHF fed lambs; see Figures 1, 2 and 3), and the mean cross-sectional area determined in the slides will expectedly be lower than the actual cross-sectional area at the centre of the adipocyte, since the cells counted obviously will represent cells cut at different distances relative to the centre. However, the calculated CNI should allow us to evaluate, whether differences in fat depositions in

response to different pre-and postnatal nutritional exposures would be a result of changes in adipocyte cell size or also potentially involving changes in adipocyte cell numbers.

Quantitative real-time PCR (qPCR)

RNA extraction and cDNA synthesis

To extract total mRNA, about 300 mg of adipose tissue was homogenized in 2000 µL TRIzol® reagent (Invitrogen, Life Technologies Europe BV, Nærum, Denmark) on a gentlemacs homogenizer and about 600 µL supernatant solution was used for phase separation by using 120 µL 1-Bromo-3-Chloropropane (Sigma-Aldrich). Then the upper colourless aqueous phase was for precipitation of RNA with 500 µL of isopropanol (Sigma-Aldrich) for 1 hour at -20ºC. RNA was subsequently purified using the Promega RNA Total Isolation kit (Promega Corportation, WI, USA), and RNA concentrations and integrity of isolated RNAs were analysed as previously described (Hou *et al.* 2013). To synthesize cDNA synthesis, 0.5 µg of total RNA was used for reverse transcription and performed as previously described (Hou *et al.* 2013). All cDNA samples were stored at -20ºC until further analysis.

Quantitative real-time PCR (qPCR)

The mRNA expression levels of target genes in different adipose tissue depots were determined by qPCR. The cDNA was pooled to make standard curves and calibrator for each plate. Calibrator, samples, and negative controls were performed in triplicate. Standard curves were made using serial dilutions of cDNA (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64) to determine the efficiency of each primer set within the resulting linear regression. Efficiencies of primers were between 1.85 and 1.92 (this equals an increase between 85 % and 92 % of target nucleic acid in each amplification cycle) (Table 1). The mRNA expression levels of selected target genes for lipid metabolism (FASN, FABP4, LPL, HSL), glucose transport (GLUT4), angiogenesis (VEGFA), adipose tissue differentiation (PPAR-γ, C/EBP-β) and inflammation (TNF-α, MCP-1, TLR4, CD68, and IL-6) were determined

using a LightCycler® 480 SYBER Green I Master (Roche Applied Science, Penzberg, Germany) and LightCycler 480 system (Roche Applied Science). The reaction volume of each well was 10 µl, which contained 2 µl ten-times diluted cDNA, 5 µl 2 x SYBR Green I master mix (Roche Applied Science), 1 µl 10 µM forward primer (TAG Copenhagen, Copenhagen, Denmark), 1 µl 10 µM reverse primer (TAG Copenhagen), and 1 µl nuclease-free water (Roche Applied Science). Samples and negative controls (no cDNA template) were run in triplicate in an amplification cycle program including denaturation (95ºC for 10 sec), annealing (58 – 60ºC for 10 sec) and elongation (72ºC for 20 sec) and were repeated 45 times in each qPCR reaction. Melting curves of PCR products were analyzed by a LightCycler 480 instrument ver. 2.0 software (Roche Applied Science) to ensure that a single product was obtained, and PCR-product size was confirmed by agarose gel electrophoresis. Data were analysed using the advanced relative quantification method provided by the LightCycler 480 instrument ver. 2.0 software. GAPDH was used as a reference gene to normalize the expression levels of target genes. The primer sequences are listed in Table 1.

Protein-protein interaction (PPI) networks analyses for candidate genes

As mentioned earlier, the thirteen candidate genes studied here are known gene targets/biomarkers of adipose tissue metabolism, development and inflammation. Pre- and postnatal nutritional insults potentially could potentially modify expression levels of other genes/markers, which are closely co-regulated along with the target genes. This would result in corresponding changes in protein/metabolite abundance in different tissues. In order to reveal such regulatory and interaction networks including our target genes, protein-protein interaction (PPI) networks were built by anchoring target genes and derived known and predicted interactions by running the network analyses STRING (Search Tool for the Retrieval of Interacting Genes/Proteins; http://string.embl.de/ (Jensen *et al.* 2009) in the same ways as described prevously (Kadarmideen & Janss 2007, Kadarmideen 2008), which is a database of known and predicted protein interactions.

STRING uses one protein per gene. If there is more than one isoform per gene, the longest isoform is selected, unless there is information to suggest that another isoform is better annotated. The interactions revealed in STRING include direct (physical) and indirect (functional) associations derived from four sources: Genomic context, High-throughput experiments, Co-expression databases and previous knowledge (text mining).

Statistical analyses

Statistical evaluation of data was performed in the SAS software (v.9.2; SAS Institute, USA) and JMP (version 10.0; SAS Institute, Inc., Cary, NC). Homogeneity of variance was evaluated by visual inspection of residuals plots, and normality of residuals was tested by means of quantile-quantile plots. The data were analysed by the GLIMMIX procedure. Prior to analysis, suitable transformation of data was performed when needed to obtain normal distribution of residuals. The models included *fixed effects* of pre- and postnatal nutrition and their interaction. Within the postnatal HCHF group, a few animals (one from the NORM, two from the HIGH and two from the LOW prenatal groups) had poorer growth rates compared to other animals in their groups (body weight <30 kg vs >35 kg at six months of age) but despite the skinny appearance, such lambs slaughtered at 6-months of age had extensive abdominal fat deposition (Khanal *et al.* 2014). In the statistical analyses, we therefore decided also to test if these "small-skinny-fat" animals showed any indications of being different from the rest of the animals within their group with "normal" appearances and growth trajectories for the parameters studied. Ewe body weight and body condition score were used to block ewes at the start of the experiment to ensure even distribution of these traits, when ewes were assigned to each of the three different maternal feeding levels. Lamb sex (first priority) and birth weight (second priority) were used to allocate lambs to each of the two different postnatal treatment groups to ensure as even a sex and birth weight distribution as possible in the two postnatal treatment groups arising from a given prenatal treatment. Therefore, to avoid

any biases in variance parameters, lamb birth weight and sex, and ewe body weight and BCS were used as co-variables. Backward model reductions were performed by sequentially removing the least significant factor. Contrast analysis was performed for the comparison of individual treatments. Results were expressed as least square means with standard error of least square means (LS means±SEM) unless otherwise stated. The level of significance was set at $P<0.05$.

Results

There were no significant effects of sex, prenatal nutrition or any significant interactions between pre- and postnatal nutrition exposures for any of the studied parameters unless explicitly stated.

Birth weight, growth and fat deposition

Results for birth weight, growth characteristics and fat deposition and distribution in the experimental animals have been reported elsewhere (Khanal *et al.* 2014). A summary of the main findings are given here, as it is considered necessary to allow interpretation of the results from the present study. In short, the prenatally undernourished LOW lambs had reduced birth weights compared to lambs born to HIGH and NORM dams, whereas birth weights for NORM and HIGH lambs did not differ significantly. The lambs fed the obesogenic HCHF diet postnatally attained significantly higher body weights at six months of age than the CONV fed lambs. The subcutaneous fat deposition was higher in NORM lambs compared to HIGH and LOW lambs (both absolute weight and expressed relative to body weight), although significant difference appeared only between NORM and HIGH lambs. HIGH and LOW lambs had decreased deposition ratios of subcutaneous fat relative to both mesenteric and perirenal fat, but the prenatal diet did not influence deposition ratios of mesenteric relative to perirenal fat. The HCHF lambs had significantly higher depositions in absolute weights of subcutaneous (~5.5 fold), mesenteric (~5 fold) and especially of perirenal (~9 fold) fat compared to CONV lambs also after correction for body weight. Thus, lambs fed the HCHF diet had higher deposition ratios as compared to lambs fed the CONV diet of

perirenal fat relative to both subcutaneous and mesenteric fat. The postnatal diet did not influence deposition ratios of subcutaneous relative to mesenteric fat.

Histology

Adipose morphology, adipocyte area and adipose cellularity

Subcutaneous adipose tissue: HIGH-CONV lambs had higher proportions of adipocyte and lower proportions of collagen infiltration compared to LOW- and NORM-CONV lambs. NORM-CONV had most regular cell size and shape compared to rest of the groups. Upon exposure to a high-fat diet postnatally, NORM-HCHF lambs had more regular cell size of all HCHF-fed animals and HIGH-HCHF lambs had the largest variation between and within animals with respect to cell size and they had quite irregular adipocytes of all HCHF-fed animals. Both HIGH- and LOW-HCHF lambs had lower proportions of adipocyte and higher proportions of collagen infiltration compared to NORM lambs (interaction of pre- and postnatal nutrition: P=0.0005 and P=0.001 for proportions of adipocyte and collagen, respectively).

The HIGH-CONV lambs had the smallest cells and lowest collagen infiltration of all groups (see Table 2). LOW-CONV lambs had slightly higher cell size than NORM-CONV lambs and the NORM-CONV lambs had intermediate cell size. HIGH animals had higher hypertrophy upon exposure to a HCHF diet compared to LOW and NORM lambs whereas cell sizes were similar in NORM and LOW lambs upon exposure to the HCHF diet (interaction of pre- and postnatal nutrition P<0.0001; Figure 1; Table 2). Both HIGH- and LOW lambs had reduced non-obese cellularity indicated by lower CNIs compared to NORM lambs and upon exposure to the postnatal high-fat diet, HIGH lambs did not increase CNIs whereas CNIs were increased in LOW lambs but were not up to the levels achieved in NORM lambs, thus both HIGH and LOW lambs had reduced obesity-induced hyperplasia compared to NORM lambs (interaction of pre- and postnatal nutrition P<0.0001; Table 2). Male lambs had lower proportions of adipocyte (P=0.02), higher proportions of

collagen infiltration (P=0.04) and had higher CNIs (P<0.0001) compared to female lambs. Small skinny obese lambs had reduced adipocyte area (4906.2±120.36 vs 6755.19±81.06 μm^2; P<0.0001) and higher proportions of micro-vessels (5.7±0.67 vs 3.5±0.44%; P=0.03) compared to other HCHF-fed lambs.

Mesenteric adipose tissue: The NORM-CONV lambs had very regular adipocyte size of all CONV lambs. LOW-CONV lambs also had quite regular sized cells whereas HIGH-CONV lambs had the largest variation between animals among the CONV-fed and they had regular cell size within an animal. In response to an early postnatal HCHF diet, all animals had more irregular cell sized cells compared to their respective CONV-fed animals. LOW-CONV lambs had lower proportions of adipocyte compared to HIGH- and NORM-CONV lambs and HIGH-CONV lambs had lower proportions of micro-vessels compared to LOW- and NORM-CONV lambs. Upon high-fat feeding, both HIGH and LOW lambs had higher proportions of adipocyte area and lower proportions of micro-vessels compared to NORM lambs (interaction of pre- and postnatal nutrition: P<0.0001 and P=0.002 for proportions of adipocyte and micro-vessels, respectively).

All CONV lambs regardless of prenatal levels had similar adipocyte size (Figure 2). After early postnatal high-fat feeding, all lambs increased their adipocyte size, however, LOW-HCHF lambs had higher adipocyte size compared to HIGH- and NORM-HCHF whereas adipocyte sizes in HIGH- and NORM-HCHF were similar (interaction of pre- and postnatal nutrition P=0.0007; Table 2). Both HIGH- and LOW lambs had lower non-obese and obesity-induced (upon exposure to an early postnatal HCHF diet) CNIs compared to NORM lambs. Additionally, HIGH lambs had increased non-obese and obesity-induced CNIs compared to LOW lambs (interaction of pre- and postnatal nutrition P<0.0001; Table 2). Male lambs had higher lower adipocyte area (P<0.0001), lower proportions of adipocyte (P<0.0001) and higher proportions of collagen (P=0.0004) and micro-vessels (P=0.002) compared to female lambs. Small skinny obese lambs had higher

adipocyte area (9074.13±103.64 vs 8583.9±103.64 μm^2; P<0.001) lower proportions of adipocyte (88.9±1.41 vs 92.67±0.85%; P=0.006) and higher proportions of collagen (4.27±0.96 vs 1.61±0.58%; P=0.013) compared to other HCHF lambs.

Perirenal adipose tissue: In general perirenal adipose tissue had the least collagen content compared to other adipose tissues studied. The NORM-CONV lambs had most uniform cell size among CONV lambs. HIGH-CONV lambs had irregular size and shape within animal. The LOW-HCHF lambs had most irregular sized cells of HCHF-fed group both within and between animals whereas The NORM-HCHF lambs had most regular cells with respect to size and shape of HCHF-fed lambs. Both HIGH and LOW lambs had lower non-obese adipocyte size compared to NORM lambs. Upon exposure to an early postnatal HCHF diet, both HIGH and LOW lambs had a huge increase in obesity-induced adipocyte size compared to NORM lambs (interaction of pre- and postnatal nutrition P<0.0001; Figure 3; Table 2). The HIGH lambs had higher non-obese and obesity-induced adipocyte size compared to LOW lambs. LOW lambs had slightly higher non-obese hyperplasia followed by NORM and HIGH lambs. Upon exposure to the early postnatal HCHF diet, all lambs had increased hyperplasia but both HIGH and LOW had reduced obesity-induced hyperplasia as indicated by lower CNIs compared to NORM lambs (3-fold vs HIGH; 2-fold vs LOW). The LOW lambs had higher obesity-induced hyperplasia that the HIGH lambs. Like in subcutaneous fat, male lambs had higher CNI compared to female lambs (P<0.0001). Small skinny obese lambs had lower adipocyte area (11453.52±108.24 vs 12100.92±108.24 μm^2) compared to the rest of HCHF lambs (P<0.0001).

Epicardial adipose tissue: All CONV lambs had quite uniform adipocytes with respect to cell size and shape within their prenatal feeding levels. Upon exposure to high-fat diet, LOW animals had larger variability in cell size within and between animals whereas NORM had quite regular in size and shape. Both HIGH and LOW lambs had higher non-obese adipocyte size compared to NORM

lambs and HIGH lambs had lower non-obese adipocyte size compared to LOW lambs. Upon exposure to the early postnatal obesogenic high-fat diet, all lambs increased their adipocyte size but HIGH and LOW lambs had lower adipocyte area compared to NORM lambs (interaction of pre- and postnatal nutrition $P<0.0001$; Suppl. Figure 1; Table 2). Small skinny obese lambs had lower adipocyte area (5593.38±120.47 vs 7758.27±84.04 μm^2) compared to the rest of HCHF lambs ($P<0.0001$).

qPCR

Subcutaneous adipose tissue

Prenatal effects: In lambs exposed to prenatal over- and undernutrition, expression was reduced (and to similar extent) for genes associated with lipid metabolism compared to NORM lambs, namely HSL ($P=0.0008$ and $P=0.0493$; respectively) and FABP4 ($P=0.0864$ and $P=0.0076$; respectively; Figure 4A). A similar trend was also obtained for the angiogenic factor, VEGFA ($P=0.0969$).

Postnatal effects: The postnatal HCHF diet decreased the expression of most of the studied genes with relation to lipid metabolism and adipose development (Figure 4B). Thus, HCHF lambs had decreased mRNA expressions of FASN (~70% reduction; $P=0.0086$), GLUT4 (~70% reduction; $P<0.0001$), HSL ($P=0.0013$), PPAR-γ ($P=0.0002$), C/EBP-β ($P=0.0762$), and VEGFA ($P=0.0075$) compared to CONV lambs (Fig. 1D). However, for inflammatory genes, the opposite pattern was observed, since the postnatal HCHF diet increased mRNA expressions of TNF-α ($P=0.072$), IL-6 (~2 fold, $P=0.0658$), MCP-1 ($P=0.006$), and CD68 (>2 fold, $P=0.028$) compared to CONV lambs (Figure 4C).

Sex effects: Female lambs had higher mRNA expression of LPL (0.77±0.16 vs 0.41±0.06; $P=0.007$) and TNF-α genes (2.4±0.23 vs 1.30±0.18; $P=0.001$) compared to male lambs.

Mesenteric adipose tissue

Prenatal effects: None of the studied genes were affected by the prenatal nutrition, except that LOW lambs exposed to the HCHF diet (1.95±0.27) had higher mRNA expression of the TLR4 gene (relative to the reference gene) compared to LOW lambs fed the CONV diet (0.79±0.27; P=0.006), resulting in a significant interaction of pre- and postnatal diet for this gene (P=0.037).

Postnatal effects: As for subcutaneous adipose tissue, the postnatal HCHF diet decreased the mRNA expression of most of the studied genes associated with lipid metabolism and adipose tissue development (Suppl. Figure 2A). The HCHF lambs had reduced mRNA expression of FASN (~50% reduction; P=0.006), FABP4 (P<0.0001), GLUT4 (~70% reduction; P<0.0001), HSL (~75% reduction, P<0.0001), LPL (P<0.0001), PPAR-γ (P<0.0001), C/EBP-β (P=0.092), and VEGFA (~70% reduction; P<0.0001) as compared to CONV fed lambs. And the postnatal HCHF diet increased mRNA expressions of the inflammatory markers TNF-α (P=0.0004), IL-6 (~4.5 fold, P=0.0004), MCP-1 (~3.5 fold, P<0.0001), and CD68 (~3.5 fold, P=0.0004) (Suppl. Figure 2B) just as in subcutaneous fat.

Perirenal adipose tissue

Prenatal effects: The prenatal diet overall affected the mRNA expressions of IL6 and MCP-1 in perirenal adipose tissue (P=0.001) (Figure 5A). Thus, LOW lambs had significantly higher mRNA expression of MCP-1 compared to HIGH (P=0.016) and NORM lambs (P=0.005), which had similar expression levels of MCP-1. A similar tendency for upregulation of mRNA expression of the IL6 gene (P=0.058) was also observed in LOW compared to HIGH and NORM lambs.

Postnatal effects: The postnatal HCHF diet decreased the mRNA expressions for most of the genes studied related to lipid metabolism and adipose development. As in subcutaneous and mesenteric fat, the HCHF diet decreased mRNA expression in perirenal fat of FASN (P=0.052), GLUT4 (~50% reduction; P<0.0001), HSL (~50% reduction; P=0.0006), LPL (~50% reduction; P<0.0001), PPAR-γ (P<0.0001), C/EBP-β (P=0.0096) and VEGFA (~75% reduction; P<0.0001) (Figure 5B),

but increased expressions of the inflammatory markers TNF-α (P=0.0032), IL-6 (~11.5 fold, P<0.0001), MCP-1 (~4.5 fold, P<0.0001), and CD68 (~3 fold, P=0.0004) (Figure 5C).

Sex effects: Female lambs (1.71±0.39) had higher mRNA expression of the FASN gene compared to male lambs (1.01±0.2; P=0.0873), whereas male lambs (3.01±0.38) had higher mRNA expression of the VEGFA gene compared to female lambs (2.0±0.43; P=0.059).

Epicardial adipose tissue

Prenatal effects: Epicardial adipose tissue was the most sensitive one to prenatal diet effects followed by subcutaneous adipose tissue. The prenatal diet affected the mRNA expression of a range of genes involved in lipid metabolism and adipose tissue development namely FABP4 (P=0.038), GLUT4 (P=0.048), HSL (P=0.035) and PPAR-γ (P=0.033) (Figure 5A). Both lambs exposed to HIGH and LOW levels of nutrition prenatally had reduced mRNA expressions of FABP4, GLUT4, HSL, PPAR-γ compared to NORM lambs, but significant differences were obtained only between LOW (P=0.03, P=0.017, 0.064 and P=0.02, respectively) and NORM lambs. Similar expression levels were observed for all the studied genes in HIGH and LOW lambs except for the HSL gene, where HIGH lambs tended to have higher mRNA expression compared to LOW lambs (P=0.077).

Postnatal effects: Similar to the subcutaneous, mesenteric and perirenal tissue depots, the postnatal HCHF diet decreased the mRNA expressions for most of the genes studied with relation to lipid metabolism and adipose tissue development (Figure 6B). The expression levels of GLUT4 (P=0.0002), LPL (P=0.0266) and VEGFA (~2 fold, P=0.001) were decreased in HCHF compared to CONV lambs (Fig 7A). However, the postnatal HCHF diet showed opposite trend in terms of inflammatory profiles also in the epicardial fat as in other adipose tissue depots studied, since the HCHF diet increased the expression of TNF-α (P=0.0308), IL-6 (~3 fold, P=0.0009), MCP-1 (~3.5

fold, $P<0.0001$), TLR4 ($P=0.0008$) and CD68 ($P=0.0127$) levels as compared to CONV-fed lambs (Figure 6C).

Sex effects: Female lambs had higher mRNA expressions of FASN (1.17±0.16 vs 0.60±0.14; $P=0.015$) and LPL genes (1.08±0.11 vs 0.65±0.10; $P=0.013$) compared to male lambs. On the other hand, male lambs had higher mRNA expressions of MCP-1 (1.13±0.17 vs 0.68±0.12; $P=0.0464$) and IL-6 genes (1.42±0.12 vs 0.59±0.13; $P=0.0047$) as compared to female lambs.

Protein-protein interaction networks analyses

The results of the PPI networks are given in two formats: a network with varying levels of confidence (Suppl. Figure 3) and a network showing various evidences that support interactions (Suppl. Figure 4). The input nodes are coloured and nodes of a higher iteration/depth are in white. Each family of proteins is assigned a different colour. The STRING analyses revealed many (more than 20) other candidate genes/molecular markers very strongly interact with the target genes/markers studied and they are thus also likely to be involved in the foetal and postnatal nutrition programming of adipose tissue function. The genes affected by foetal malnutrition in our study included lipid metabolism markers, which interacted with other lipid metabolism markers such as monoacylglycerides lipase (MGLL), perilipin 1 (PLIN1), acetyl co-enzyme A carboxylase (ACCCA), with markers of adipose development such as sterol regulatory binding protein-1 (SREBP1) and with a family of apolipoproteins, such as apolipoprotein E (APOE), apolipoprotein A1 (APOA1) and apolipoprotein C3 (APOC3). Other markers such as colony stimulating factor 2 (CSF2, involved in cell proliferation and differntiation) and nitric oxide synthase 2 (NOS2) interact with our target inflammatory markers, which were targets of the postnatal HCHF diet.

Discussion

The major findings of this study were both prenatal over- and undernutrition predisposed for development of visceral adiposity by depressing non-obese cellularity in subcutaneous and mesenteric fat and obesity-induced hyperplasia in subcutaneous, mesenteric and perirenal fat and increasing obesity-induced hypertrophy in perirenal adipose tissue. The postnatal HCHF diet induced general hyperplasia and hypertrophy in all adipose tissues and which was associated with increased and decreased expressions of genes related to inflammation and lipid metabolism, respectively. In our study we evaluated only a selection of molecular markers reported to be well-correlated with adipose tissue development, lipid metabolism and inflammatory responses in adipose tissue depots. However, we have demonstrated that a range of other markers involved in the regulation of adipose tissue development and lipid metabolism such as MGLL, ACCCA, SREBP-1 and a family of apolipoproteins which may strongly interact with our target markers, and it is therefore highly likely that they could be targets of pre- and/or postnatal dietary treatments as well. Future studies are needed to evaluate how these makers may contribute to the developmental and functional adaptations in different adipose tissues depending on the pre- and postnatal nutrition exposures.

Exposure to over- or undernutrition in late gestation can lead to similar changes in cellularity and hypertrophy and lower the mRNA expression of lipid metabolism-related genes in subcutaneous and epicardial fat

We have previously reported that both late gestation over- and undernutrition can predispose for visceral adiposity in sheep associated with a reduced capacity for fat deposition in subcutaneous adipose tissue (Khanal *et al.* 2014). In this study, we tried to investigate the molecular and histological mechanisms underlying altered fat deposition patterns in different adipose tissues in response to matching or mismatching pre- and postnatal nutrition exposures. Subcutaneous fat is

considered a relatively healthy fat due to a specific function in preventing lipid-overflow to visceral areas and non-adipose tissues related to its expandability, and due to unique intrinsic properties related to e.g. improvement of insulin sensitivity (Tran *et al.* 2008). It has earlier been reported the adipose tissue overflow hypothesis regarding higher susceptibility of South Asians in developing central obesity and its atherogenic consequences compared to Caucasians, highlighting that a reduced capacity of superficial subcutaneous compartment to store fatty acids in South Asians would result earlier utilization of visceral compartments and then central obesity (Sniderman *et al.* 2007). In the current study, both prenatal over- and undernutrition reduced non-obese cellularity in subcutaneous and mesenteric adipose tissues and obesity-induced hyperplasia in subcutaneous and perirenal fat but not in the mesenteric fat and this was co-existed with an extreme obesity-induced perirenal hypertrophy in LOW and particularly HIGH lambs. It seems that HIGH lambs are not protected against adverse effects of obesity but they have perhaps healthier fat in the non-obese state than LOW due to higher cellularity which is reduced particularly in LOW lambs. In humans, we do not know about the functional/developmental difference between mesenteric and perirenal adipose tissues and their implications.

Alterations in nutritional exposure during foetal and postnatal life can also modify gene expression in adipose tissue after birth (Bol *et al.* 2009). Therefore, along with changes in cellularity and hypertrophy in non-obese and obese situations, we attempted to investigate any molecular mechanisms responsible for similar alteration in fat deposition and distribution pattern in response to prenatal nutrition exposures. In this study, prenatal malnutrition both in the form of over- and undernutrition led to downregulation of genes associated with lipid metabolism namely HSL, FABP4 and VEGFA in the subcutaneous adipose tissue of adolescent lambs, and that these changes co-existed with the development of visceral obesity. This may indicate that both prenatal over- and undernutrition programs the subcutaneous adipose tissue in such a way that it may lead to poor

angiogenesis and reduced ability for uptake of fatty acids and mobilization of fat store in response to hormonal stimuli affecting normal growth, development and metabolism of subcutaneous fat which may lead to increased fat deposition toward visceral compartments. It has been previously reported that growth of perirenal-abdominal adipose tissue commences at about day 70 days of gestation in sheep and rapid increase in fat mass along with parallel increase in lipid content occurs until 110-120 days of gestation (Alexander 1978). In the same study, it has been reported that growth of subcutaneous fat commences 2-3 weeks later than perirenal-abdominal fat depots and a significant regression of subcutaneous fat occurs about day 115, and it virtually disappears by full term. Another study also confirms that both perirenal and subcutaneous adipose tissue in foetal sheep begins to grow at about day 70 of gestation and a marked increase in proliferation of mitochondria and development of nerves were visible in the perirenal fat depot during late gestation (Gemmell & Alexander 1978). These studies reveal that a nutritional insult potentially can have different impacts on development of adipose tissues, since the timing of their development are different in foetal life. Since a considerable part of development and maturation of subcutaneous and perirenal adipose tissues takes place in late gestation and insults at this stage could therefore potentially interfere with such functional maturation, cellularity and expandability, suggesting these depots as potential targets late gestational nutrition.

In spite of similar obesity-induced hypertrophy in perirenal adipose tissue, prenatal malnutrition show differential impacts on inflammatory responses as expression of MCP-1 and IL-6 genes were enhanced by maternal undernutrition but not overnutrition. Very few studies have previously reported that nutritional environment during pregnancy can alter adipose tissue inflammatory profile in offspring. For example nutrition restriction during late gestation enhanced the expression of inflammatory genes such as Chemokine receptor-2, whose main legend is MCP-1, in perirenal

adipose tissue in sheep offspring (Sharkey *et al.* 2009), however, detailed mechanisms are yet to be investigated.

Unlike other tissues, epicardial behaves differently in response to late gestation malnutrition as both over- and undernutrition increase non-obese adipocyte area and reduce obesity-induced hypertrophy. Such different impacts are also observed with respect to sex influences as unlike other adipose tissues, higher expandability of adipocytes are found in males compared to female in epicardial adipose tissue. Furthermore, changes in gene expression associated with lipid metabolism and adipocyte development due to prenatal over- and undernutrition are more pronounced in the epicardial adipose tissue suggesting that epicardial fat is another most susceptible tissue along with subcutaneous and perirenal adipose tissue. Epicardial adipose tissue is associated with the development of coronary atherosclerosis, dangerous types of plaques (Alexopoulos *et al.* 2010) and unfavourable cardiometabolic risk factors in children (Schusterova *et al.* 2014). Epicardial fat has the greatest capacity for fatty acid release among fat depots studied, and it may act as a local energy supply for cardiac muscle and has a protective role or acts as a buffer against elevated levels of free fatty acids in the coronary circulation (Marchington *et al.* 1989; Marchington & Pond 1990). The reduced expression of FABP4, GLUT4 and PPAR-γ genes in prenatally malnourished individuals and along with decreased expandability of epicardial adipocyte in response to a postnatal high-fat diet may indicate the poor adipocyte growth and development and lipogenesis in the epicardial adipose tissue which might lead to reduced ability to store fatty acids and to act as a buffer against toxic levels of those fatty acids to protect adjacent myocardium. The underlying mechanisms are not understood behind such impaired ability of epicardial fat to expand in respond to the high-fat diet and reduced expression of lipogenic markers in prenatal malnourished animals, however it indicates that along with other adipose depots epicardial fat is certainly one of the major targets of prenatal nutrition.

Hence this study demonstrates that both prenatally over- and undernutrition reduce the non-obese cellularity in subcutaneous and mesenteric and depress ability to undergo hyperplasia in response to high-fat diet in especially in perirenal adipose tissue. Additionally, a depression in lipid metabolism and angiogenic gene expression in subcutaneous adipose tissue may explain the more pronounced reduction in expandability of subcutaneous fat leading to increased abdominal adipocyte. There might be limitations in the ability of other adipose tissues to hypertrophy thus leading to extremely hypertrophy in perirenal adipose tissue but underlying mechanisms remain to be established. In the current study, the small skinny obese HCHF-fed animals had reduced cell size in all adipose tissues studied except the mesenteric adipose tissue in which they had increased adipocyte area compared to rest of the HCHF-fed animals. Further studies are needed to evaluate why the animals with skinny obese phenotype targets the mesenteric adipose tissue with higher capacity to expand and then predisposing the risks of abdominal obesity and associated metabolic disorders.

A postnatal obesogenic diet targets all visceral depots and down-regulates the expression of genes related to lipid metabolism and is a major risk factor for adipose tissue inflammation

Postnatal diet increased the expandability of adipocytes in all adipose tissues studied as indicated by increased adipocytes area and this was co-existed with depressed expression of markers for fatty acid synthesis and transport and increased inflammation. Despite lower gene expression of adipose development and lipid metabolism per unit of tissue, the quantitative fat deposition could be higher in HCHF than CONV animals due to larger fat depots as the HCHF lambs had excessive fat accumulation in all of these fat depots studied (Khanal *et al.* 2014). Our study very convincingly demonstrated that an early postnatal unhealthy HCHF diet is a major factor contributing adipose tissue inflammation in early age and this applies to the ruminant lamb just as it has been observed in monogastric animal models and in humans. The high-fat energy dense diet significantly up-regulated the expression of almost all proinflammatory markers studied irrespective of the adipose

tissue depots compared to the lambs exposed to CONV diet. We observed marked increases in TNFα and IL-6 genes due to the postnatal high-fat feeding and it has been postulated that the proportion of TNFα and IL-6 in adipose tissue is solely determined by the macrophage population in the tissue (Fried *et al.* 1998, Weisberg *et al.* 2003). Our data also illustrates a marked upregulation of CD 68 and MCP-1 in all adipose depots studied, which are markers of infiltration of non-resident macrophages and monocytes into those tissues. A high-fat diet in mice also increased the expression of inflammatory genes, especially macrophage specific genes such as MCP-1, MIP-1α and CD68 indicating macrophage infiltration as obesity progressed (Xu *et al.* 2003). Influx of non-resident macrophages or switching of anti-inflammatory macrophages into pro-inflammatory macrophages in adipose tissue are among the signs of progression of obesity (Weisberg *et al.* 2003, Xu *et al.* 2003), and this increased macrophage infiltration is responsible for the chronic low grade inflammation of adipose tissues (Chawla *et al.* 2011).

Such enhanced inflammatory responses in HCHF-fed lambs were associated lower expression of markers responsible for lipid metabolism, angiogenesis and adipose development. It has earlier been reported that increased pro-inflammatory responses are associated with reduced activity of regulatory enzymes in adipocyte metabolism (Perry *et al.* 2001). The HCHF-fed lambs also had higher body weights and huge increase in adipocyte size which might be contributing factors to enhanced inflammation in adipose tissues as previous studies mice and human also indicate a positive relationship between adipose tissue inflammation and adipose tissue size and body mass (Weisberg *et al.* 2003). In response to prenatal malnutrition, HCHF fat diet showed differential impacts on hyperplasia and hypertrophy. In subcutaneous fat, both HCHF fat increased adipocyte size irrespective of prenatal nutrition, although more increase in adipocyte size was obtained in HIGH-HCHF lambs. Regardless of increased adipocyte size in HIGH-HCHF lambs, they failed to increase cell number as indicated by lower CNI levels. However, LOW-HCHF lambs were able to

increase both cell size and cell numbers. On the other hand in perirenal fat, both HIGH and LOW lambs had huge increase adipocyte area and lower hyperplasia in response to an early postnatal high-fat diet. As we earlier showed that perirenal fat may be a major target of an early postnatal high-fat diet (Khanal *et al.* 2014), in the current study both HIGH and LOW lambs preferred to deposition more fat in perirenal area in response to HCHF diet which is mainly through hypertrophy. Hence, the HCHF diet had adipose depot-specific implications for lambs depending on their prenatal nutrition and this indicates that adverse prenatal as well as postnatal nutrition may predispose for visceral adiposity, but targeting different adipose tissues.

In conclusion both prenatal over- and undernutrition predisposed for development of abdominal adiposity and extreme perirenal hypertrophy by depressing non-obese cellularity in subcutaneous and mesenteric fat and obesity-induced hyperplasia in subcutaneous, mesenteric and perirenal adipose tissues. The prenatal programming did not target inflammatory marker gene expressions except that late gestation undernutrition, but not overnutrition, enhanced inflammatory responses in perirenal adipose but how this relates to perirenal and visceral adiposity risks remains to be established. The early postnatal HCHF diet reduced mRNA expressions for a range of genes associated with lipid metabolism, adipocyte development and vascularization and increased inflammatory makers in all the adipose tissues studied and these were associated general hypertrophy and hyperplasia.

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Figure legends

Figure 1:

Histological evaluation of adipocytes in subcutaneous adipose tissue. Five µm thick tissue sections were stained by van Gieson staining and the tissue slides were scanned by a panoramic whole slide scanner and the pictures were taken at 20X magnification. Scale bar is 100 µm for all pictures. HIGH (N=10; four males, six females; diet fed to twin-pregnant dams during the last trimester and

fulfilling 150% of daily energy and 110% of daily protein requirements); LOW (N=10; 5 males, 5 females; diet fed to twin-pregnant dams during the last trimester and fulfilling 50% of their energy and protein requirements); NORM (N=6; six males, 0 female; diet fed to twin-pregnant dams and fulfilling 100% of their daily energy and protein requirements); HCHF (N=13; 8 males, 5 females; high-carbohydrate-high-fat postnatal diet fed to lambs and consisting of cream-milk replacer mix in a 1:1 ratio supplemented with rolled maize) and CONV (N=13; 7 males, 6 females; conventional postnatal diet fed to lambs and consisting of milk replacer and hay until 8 weeks of age and hay only thereafter and adjusted to achieve moderate and constant growth rates of approx. 225 g/day).

Figure 2:

Histological evaluation of adipocytes in mesenteric adipose tissue. Five µm thick tissue sections were stained by van Gieson staining and the tissue slides were scanned by a panoramic whole slide scanner and the pictures were taken at 20X magnification. Scale bar is 100 µm for all pictures. HIGH, LOW, NORM, HCHF and CONV: See legends to Figure 1.

Figure 3:

Histological evaluation of adipocytes in perirenal adipose tissue. Five µm thick tissue sections were stained by van Gieson staining and the tissue slides were scanned by a panoramic whole slide scanner and the pictures were taken at 20X magnification. Scale bar is 100 µm for all pictures. HIGH, LOW, NORM, HCHF and CONV: See legends to Figure 1.

Figure 4:

mRNA expression of genes in subcutaneous adipose tissue as affected by prenatal (A) and postnatal nutrition (B and C). HSL, hormone sensitive lipase; FABP4, fatty acid binding protein 4; VEGFA, vascular endothelial growth factor A; FASN, fatty acid synthase; GLUT4, glucose transporter 4; PPAR-γ, peroxisome proliferator-activated receptor- γ; C/EBP-β, CAAT enhancer binding protein-β; TNF-α, tumour necrosis factor-alpha; IL-6, interleukin-6; MCP-1, monocyte chemoattractant

protein-1; CD68, cluster of differentiation 68. HIGH (N=10; four males, six females; diet fed to twin-pregnant dams during the last trimester and fulfilling 150% of daily energy and 110% of daily protein requirements); LOW (N=10; 5 males, 5 females; diet fed to twin-pregnant dams during the last trimester and fulfilling 50% of their energy and protein requirements); NORM (N=6; six males, 0 female; diet fed to twin-pregnant dams and fulfilling 100% of their daily energy and protein requirements); HCHF (N=13; 8 males, 5 females; high-carbohydrate-high-fat postnatal diet fed to lambs and consisting of cream-milk replacer mix in a 1:1 ratio supplemented with rolled maize) and CONV (N=13; 7 males, 6 females; conventional postnatal diet fed to lambs and consisting of milk replacer and hay until 8 weeks of age and hay only thereafter and adjusted to achieve moderate and constant growth rates of approx. 225 g/day). Data represent expression ratios relative to glyceraldehyde 3-phosphate dehydrogenase and values presented as least square (LS) means±SEM. Vertical bars indicate significant differences between treatment groups at # $P<0.1$; * $P<0.05$; *** $P<0.0001$.

Figure 5:

mRNA expression of genes in perirenal adipose tissue as affected by prenatal (A) and postnatal nutrition (B and C). LPL, lipoprotein lipase. IL-6, MCP-1, FASN, GLUT4, HSL, PPAR-γ, C/EBP-β, VEGFA, TNF-α, IL6, MCP-1, C68, HIGH, NORM, LOW, HCHF and CONV: see legends to Figure 4. Data represent expression ratios relative to glyceraldehyde 3-phosphate dehydrogenase, and values are presented as least square (LS) means±SEM. Vertical bars indicate significant differences between treatment groups at # $P<0.1$; * $P<0.05$; *** $P<0.0001$.

Figure 6:

mRNA expression of genes in epicardial adipose tissue as affected by prenatal (A) and postnatal nutrition (B and C). TLR4, toll like receptor 4. FABP4, GLUT4, HSL, PPAR-, FABP4, GLUT4, LPL, VEGFA, TNF-, IL6, MCP-1, CD68, HIGH, NORM, LOW, HCHF and CONV: see legends to

Figures 4 and 5. Data represent expression ratios relative to glyceraldehyde 3-phosphate dehydrogenase, and values are presented as least square (LS) means±SEM. Vertical bars indicate significant differences between treatment groups at # $P<0.1$; * $P<0.05$; *** $P<0.0001$.

Supplementary Figure 1:

Histological evaluation of adipocytes in epicardial adipose tissue. Five μm thick tissue sections were stained by van Gieson staining and the tissue slides were scanned by panoramic whole slide scanner and pictures were taken at 20X magnification. Scale bar is 100 μm for all pictures. HIGH (N=10; four males, six females; diet fed to twin-pregnant dams during the last trimester and fulfilling 150% of daily energy and 110% of daily protein requirements); LOW (N=10; 5 males, 5 females; diet fed to twin-pregnant dams during the last trimester and fulfilling 50% of their energy and protein requirements); NORM (N=6; six males, 0 female; diet fed to twin-pregnant dams and fulfilling 100% of their daily energy and protein requirements); HCHF (N=13; 8 males, 5 females; high-carbohydrate-high-fat postnatal diet fed to lambs and consisting of cream-milk replacer mix in a 1:1 ratio supplemented with rolled maize) and CONV (N=13; 7 males, 6 females; conventional postnatal diet fed to lambs and consisting of milk replacer and hay until 8 weeks of age and hay only thereafter and adjusted to achieve moderate and constant growth rates of approx. 225 g/day).

Supplementary Figure 2:

mRNA expression of genes in mesenteric adipose tissue as affected by postnatal nutrition. No impacts of the prenatal nutrition history were observed in this adipose tissue. FASN, fatty acid synthase; FABP4, fatty acid binding protein 4; GLUT4, glucose transporter 4; HSL, hormone sensitive lipase; LPL, lipoprotein lipase; PPAR-γ, peroxisome proliferator-activated receptor- γ; C/EBP-β, CAAT enhancer binding protein-β; VEGFA, vascular endothelial growth factor A; TNF-α, tumour necrosis factor-α; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; CD68, cluster of differentiation 68. HIGH, NORM, LOW, HCHF and CONV: see legends to Figure

1. Data represent expression ratios relative to glyceraldehyde 3-phosphate dehydrogenase, and values are presented as least square (LS) means±SEM. Vertical bars indicate significant differences between treatment groups at # $P<0.1$; * $P<0.05$; *** $P<0.0001$.

Supplementary Figure 3:

Confidence view of protein-protein interaction network. The confidence score for each interaction is the approximate probability that a predicted link exists between two nodes in the same metabolic map in the KEGG database; the thickness of the blue line between two nodes indicates the confident level of the association of these two nodes. The thicker the blue line is, the higher the confidence level that the two nodes are associated with each other.

Supplementary Figure 4:

Evidence view of protein-protein interaction network; the edges, i.e. predicted functional links, consist of up to seven lines: one colour for each type of evidence; a red line indicates the presence of fusion evidence; a green line - neighbourhood evidence; a blue line - co-occurrence evidence; a purple line - experimental evidence; a yellow line – textmining evidence; and a light blue line - database evidence

Figure 1 (Subcutaneous fat):

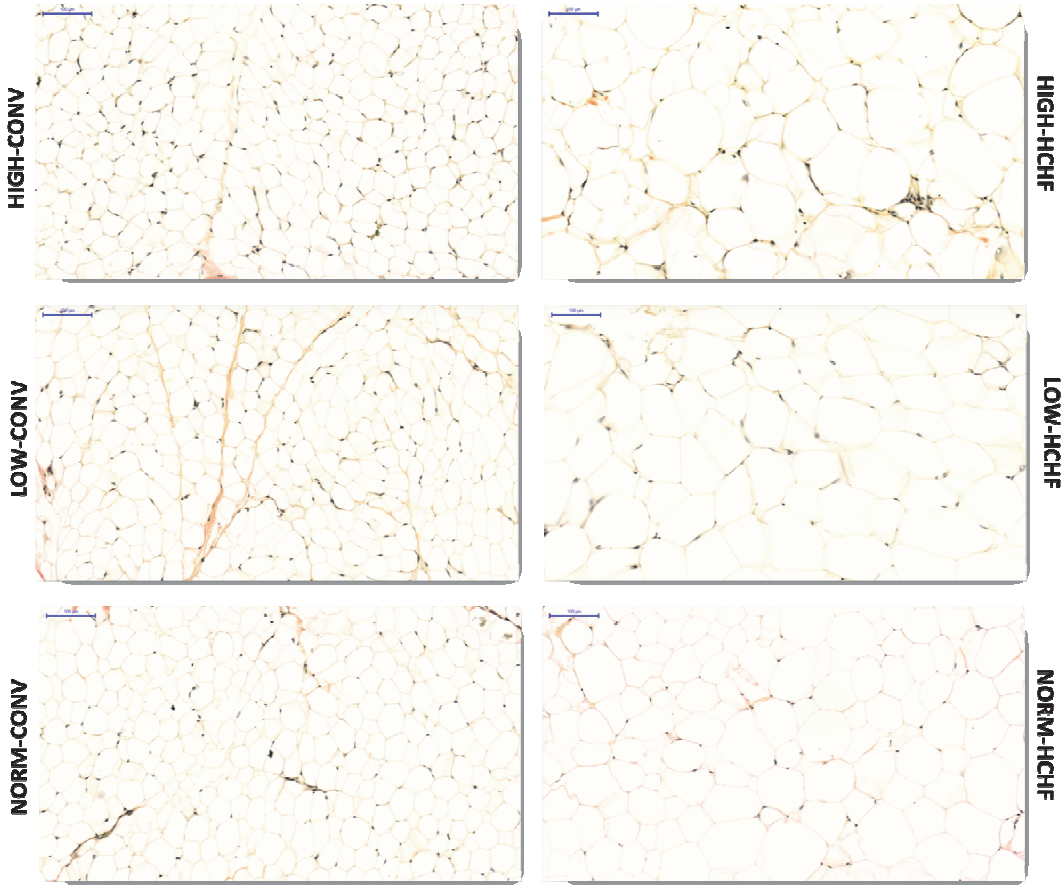


Figure 2 (Mesenteric fat):

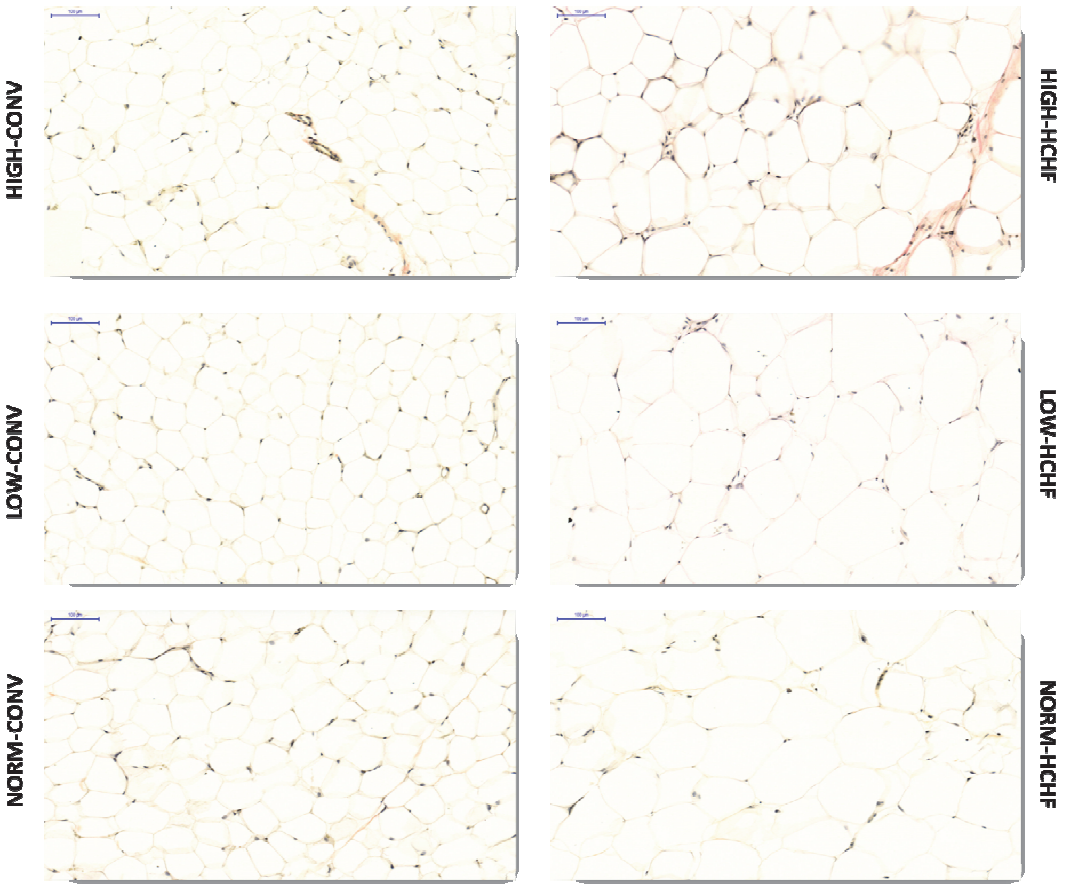


Figure 3 (Perirenal fat):

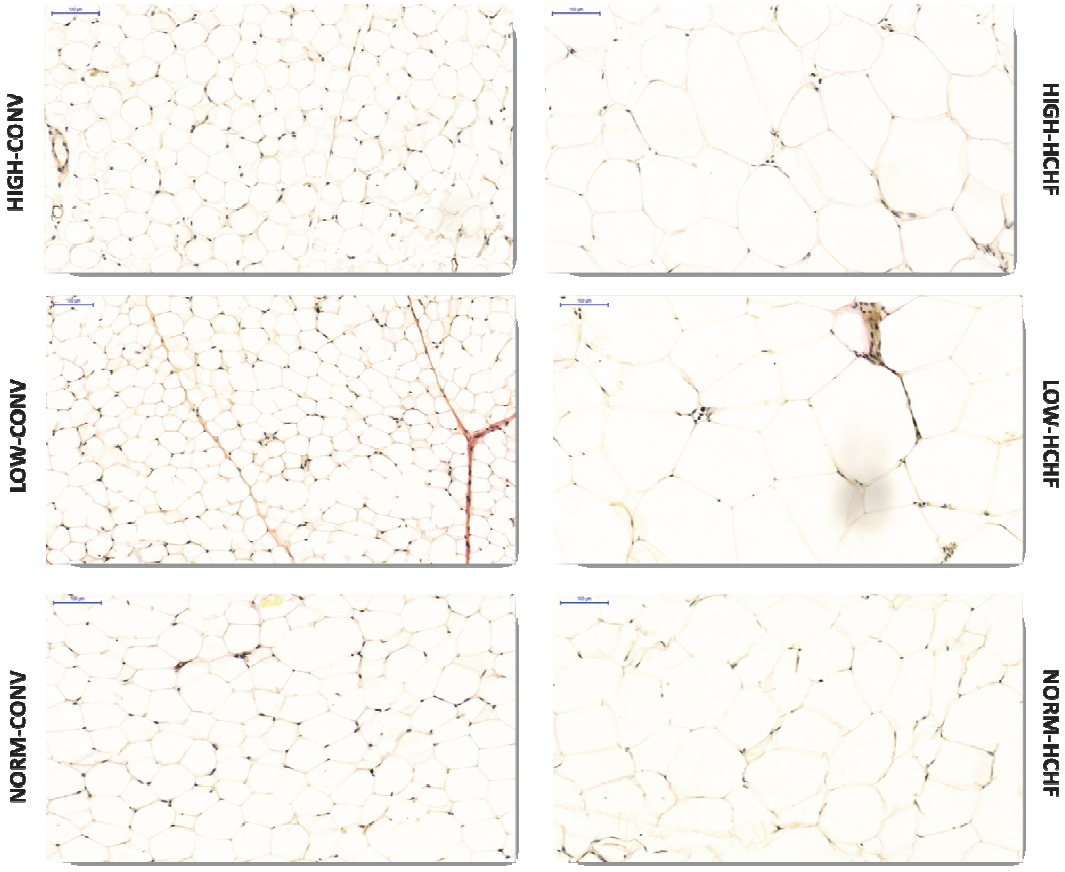


Figure 4 (Subcutaneous fat):

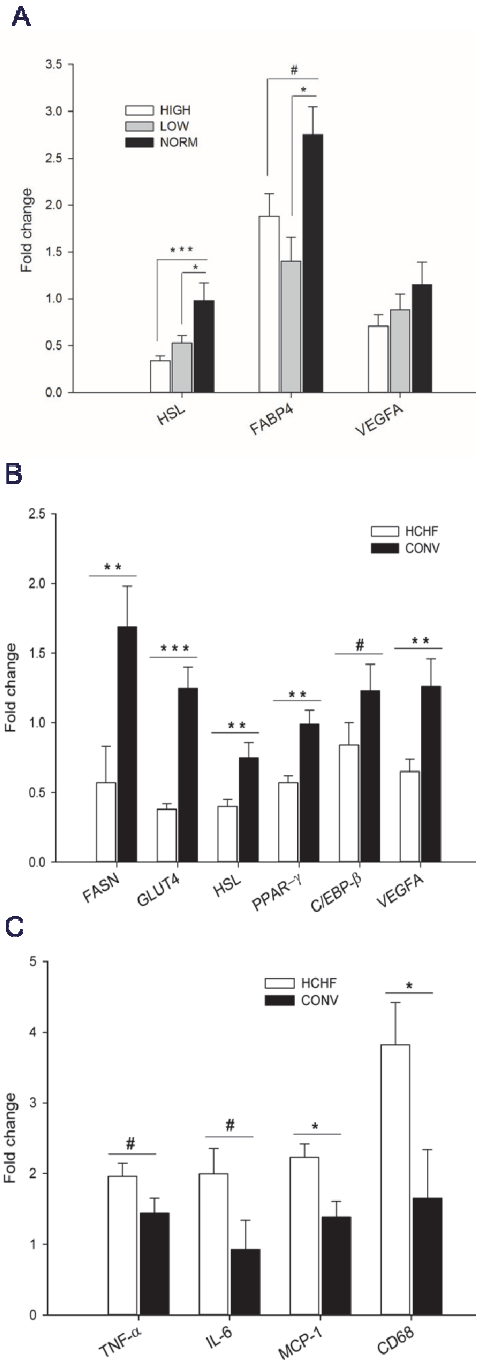


Figure 5 (Perirenal fat):

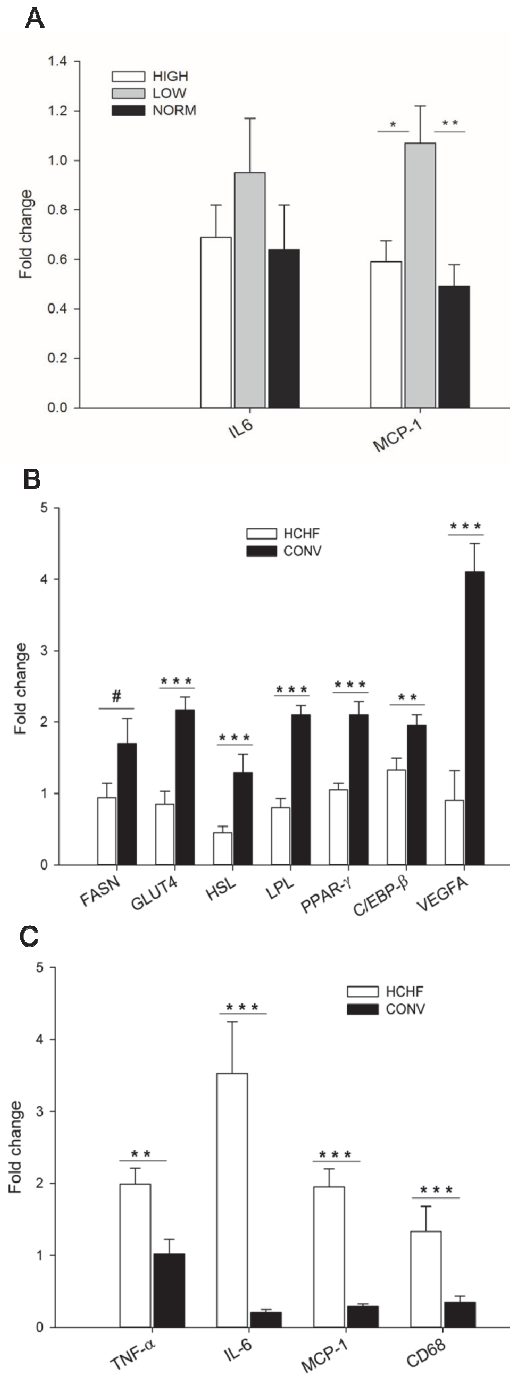
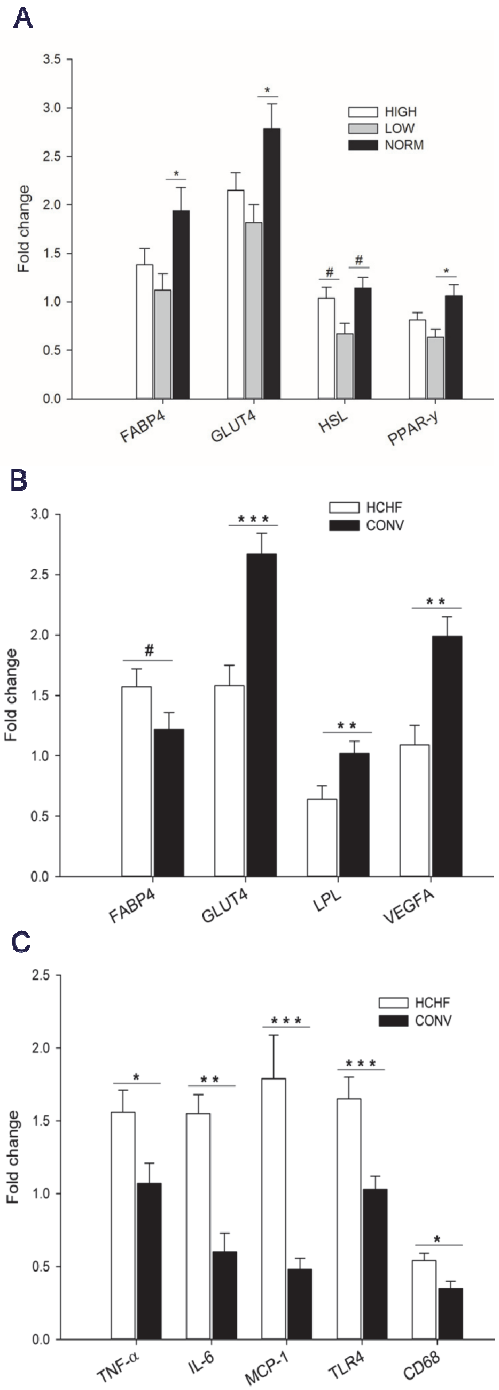
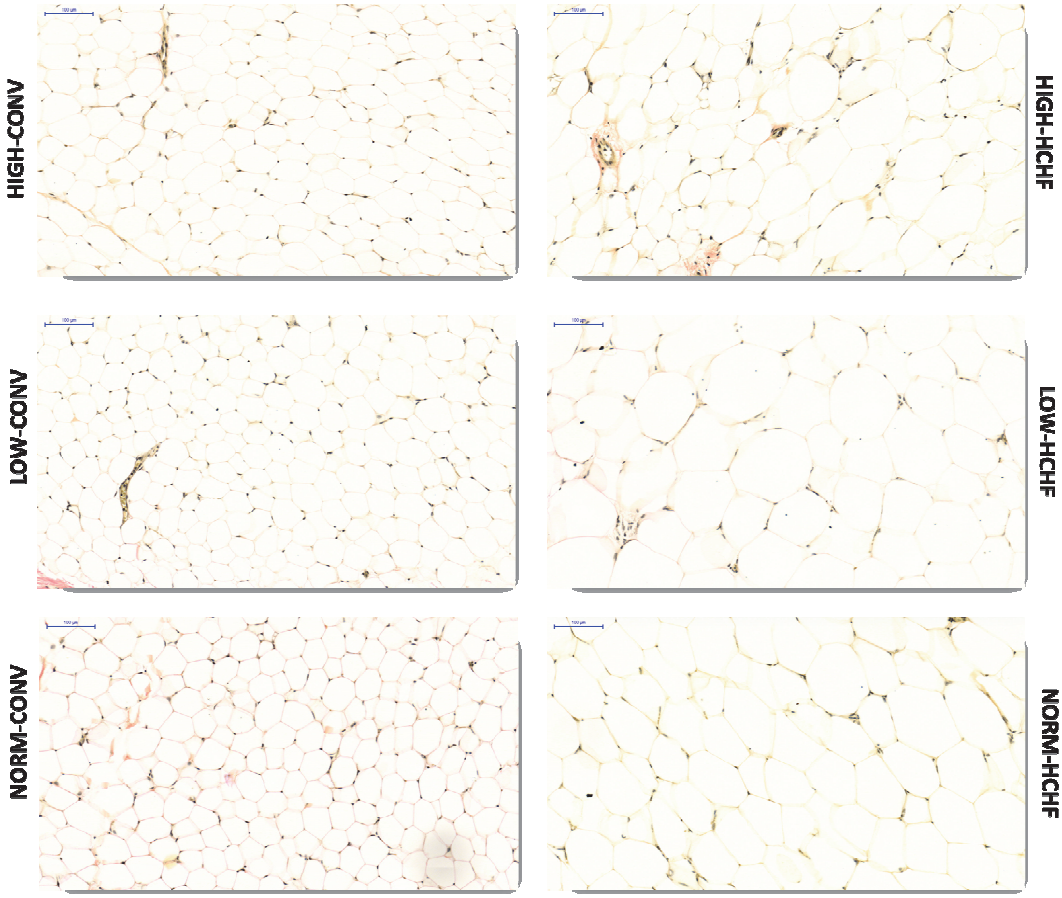


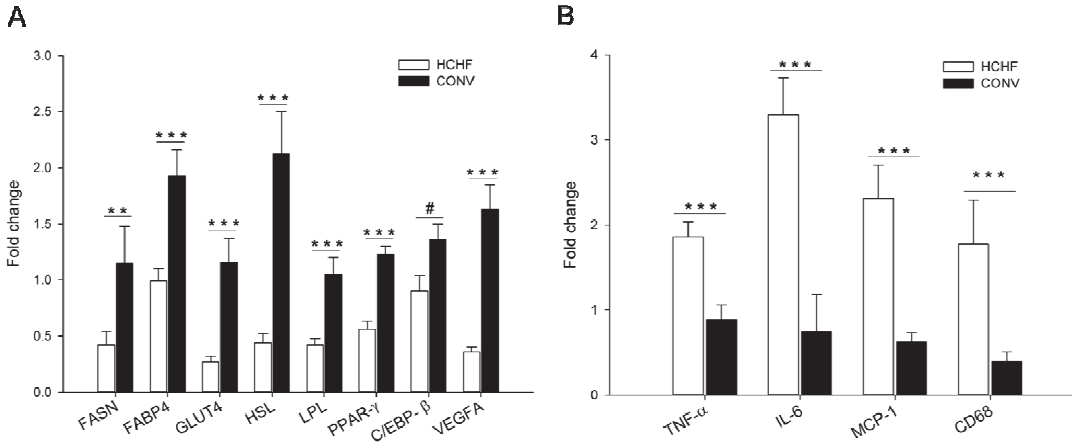
Figure 6 (Epicardial fat):



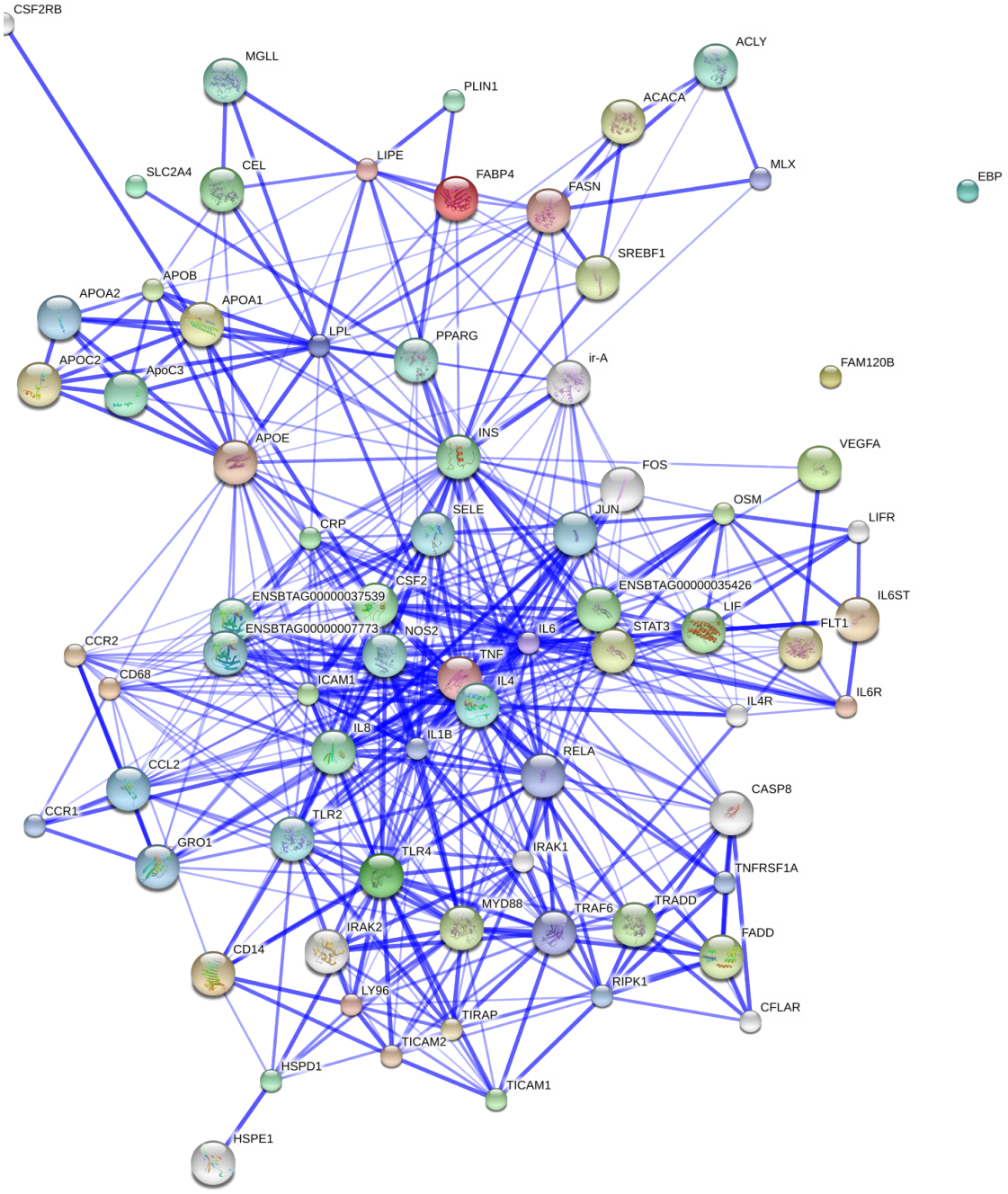
Supplementary Figure 1 (Epicardial fat):



Supplementary Figure 2 (Mesenteric fat):



Supplementary Figure 3 (PPI – Confidence view):



Supplementary Figure 4 (PPI – Evidence view):

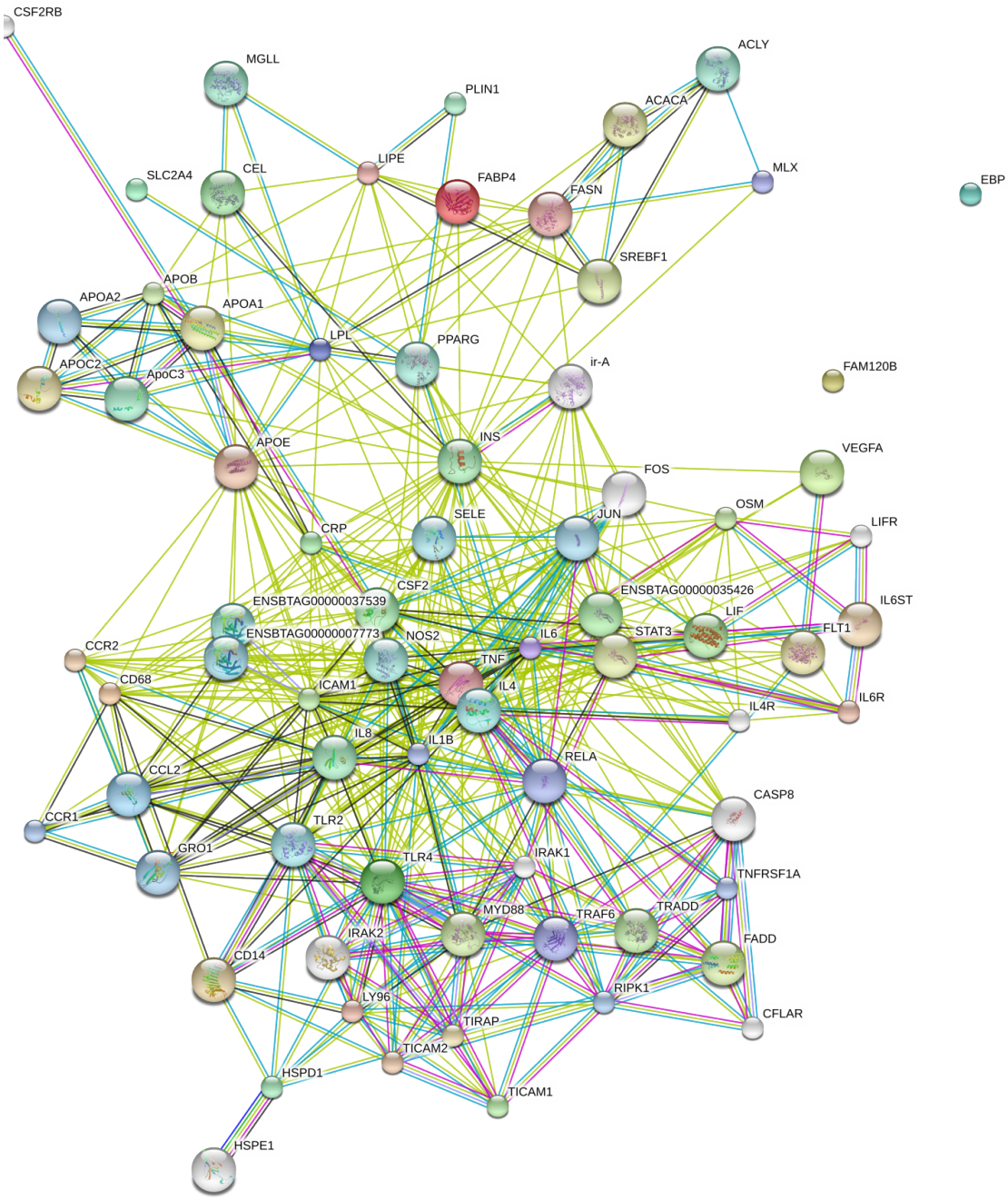


Table 1: Primer sequences used in RT-PCRAT

Gene	Forward primer	Reverse primer	PE	Product size (bp)
FASN	5'-CCCAGCTCAACGAAACCA-3'	5'-GACGAGGTCAACACCCTTCC-3'	1.850	95
FABP4	5'-CAT CTT GCT GAA AGC TGC AC-3'	5'-AGC CAC TTT CCT GGT AGC AA-3'	1.846	160
GLUT4	5'-AGT ATG TGG CGG ATG CTA TGG G-3'	5'-CGG CGG AAG ACG GCT GAG-3'	1.918	139
HSL	5′-GGTAATTGCCGACTTCCTACGA-3′	5′-TGGCAGGTGTGAACTGGAAA-3′	2	89
LPL	5′-CGGCTTTGATATTGGGAAGA-3′	5′-CCGGGAACAGAAGATCACC-3′	2	70
PPAR-γ	5'-ACGGGAAAGACGACAGACAAATC-3	5′-CACGGAGCGAAACTGACACC-3′	1.91	159
C/EBP-β	5'-ACGACTTCCTCTCCGACCTC-3'	5'-CCCAGACTCACGTAGCCGTA-3'	1.918	85
VEGFA	5'-GCC TTG CCT TGC TGC TCT AC-3'	5'-GGT TTC TGC CCT CCT TCT GC-3'	1.860	75
GAPDH	5′-GTCGGAGTGAACGGATTTGG-3′	5′-AACGATGTCCACTTTGCCAGTA-3′	1.904	86
TNF-α	AACAGGCCTCTGGTTCAGACA	CCATGAGGGCATTGGCATAC	1.927	136
IL-6	GACACCACCCCAAGCAGACTA	TGCCAGTGTCTCCTTGCTGTT	1.993	144
MCP-1	GCTGTGATTTTCAAGACCATCCT	GGCGTCCTGGACCCATTT	1.929	72
CD68	GTCCTGCTACCACCACCAGT	GCTGGGAACCATTACTCCAA	1.903	177
TLR4	CTGAATCTCTACAAAATCCC	CTTAATTTCGCATCTGGATA	1.945	149

PE, primer efficiency; bp; base pairs; FASN, FABP4, fatty acid binding protein 4; GLUT4, glucose transporter 4; fatty acid synthase; HSL, hormone sensitive lipase; LPL, lipoprotein lipase; PPAR-γ, peroxisome proliferator-activated receptor- γ; C/EBP-β, CAAT enhancer binding protein- β;VEGFA, vascular endothelial growth factor A; GAPDH; glyceraldehyde 3-phosphate dehydrogenase; TNF-α, tumour necrosis factor-alpha; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; CD68, cluster of differentiation 68; TLR4, toll-like receptor 4.

Table 2: Effects of pre- and postnatal nutrition and sex on adipocyte cross-sectional area, tissue composition and cell number index in different adipose tissues

Adipocyte CSA or Proportion (%)	HIGH-HCHF	HIGH-CONV	LOW-HCHF	LOW-CONV	NORM-HCHF	NORM-CONV	Male	Female	*P values*			
Subcutaneous fat									**SD**	**LD**	**SD*LD**	**Sex**
Adipocyte CSA (µm2)	6535±78^{a}	1600±101^{e}	6088±94^{b}	2426±90^{c}	6088±139^{b}	2107±111^{d}	4098±51	4184±67	0.09	<0.0001	<0.0001	0.37
Adipocyte (%)	85.3±2^{ab}	90±2.6^{a}	81.8±2.3^{bc}	79.2±2.3^{c}	90±3.5^{a}	74.5±2.9^{c}	80.6±1.3^{b}	86.6±1.7^{a}	0.01	0.1	0.0005	0.02
Collagen (%)	9.8±1.8^{bcd}	5.7±2.4^{d}	13.5±2.1^{ab}	14.7±2.1^{abc}	6.2±3.2^{cd}	19.5±2.6^{a}	13.8±1.2^{a}	9.3±1.6^{b}	0.01	0.17	0.001	0.04
Micro-vessels (%)	5.0±0.6	4.4±0.9	4.6±0.8	6.1±0.8	3.7±1.2	6±0.9	5.5±0.4	4.4±0.6	0.64	0.24	0.17	0.18
CNI	35.8±0.8^{c}	36.1±0.8^{c}	51.5±1.2^{b}	27.6±0.8^{d}	62.5±1.8^{a}	48.5±1.4^{b}	44.4±0.6^{a}	35.8±0.6^{b}	<0.0001	0.40	<0.0001	<0.0001
Mesenteric fat												
Adipocyte CSA (µm2)	7994±112^{b}	4543±138^{c}	8567±146^{a}	4233±111^{c}	7949±207^{b}	4251±137^{c}	5176±69^{b}	7336±90^{a}	0.11	<0.0001	0.0007	<0.0001
Adipocyte (%)	92.6±1.3^{ab}	96.3±1.7^{a}	89±1.5^{bc}	89.7±1.3b^{c}	86.3±2.1^{c}	95.4±1.6^{a}	88.2±0.7^{b}	94.9±1.1^{a}	0.004	0.007	0.02	<0.0001
Collagen (%)	2.2±1^{ab}	0.6±1.4^{ab}	3.9±1.2^{a}	3.9±1.1^{a}	4.2±1.7^{ab}	0.008±1.3^{b}	4.6±0.6^{a}	0.3±0.9^{b}	0.1	0.15	0.22	0.0004
Micro-vessels (%)	5.2±0.7^{bcd}	3.1±0.9^{d}	7.1±0.8^{b}	6.4±0.7^{bc}	9.5±1.2^{a}	4.6±0.9^{cd}	7.2±0.4^{a}	4.8±0.6^{b}	0.003	0.004	0.02	0.002
CNI	61.7±1.4^{b}	40.4±0.8^{e}	53.2±1.3^{c}	34.8±0.7^{f}	84.5±2.7^{a}	46.3±1.2^{d}	45.2±0.7	49.9±0.7	<0.0001	0.03	<0.0001	0.5
Perirenal fat												
Adipocyte CSA (µm2)	13786±120^{a}	3816±150^{e}	11980±149^{b}	2991±131^{f}	7354±214^{c}	5338±176^{d}	7152±77^{b}	7936±99^{a}	<0.0001	<0.0001	<0.0001	<0.0001
Adipocyte (%)	96±0.9^{ab}	90.3±1.1^{c}	97.2±1.0^{a}	93.5±1.0^{b}	95.7±1.6^{ab}	94.5±1.2^{ab}	94.4±0.6	94.7±0.7	0.1	0.003	0.13	0.77
Collagen (%)	0.6±0.7^{b}	3.4±0.9^{a}	0.6±0.8^{b}	1.3±0.8^{ab}	1.3±1.2^{ab}	0.8±1^{b}	1.5±0.4	1.2±0.6	0.41	0.28	0.15	0.7
Micro-vessels (%)	3.4±0.5^{cd}	6.3±0.6^{a}	2.2±0.6^{d}	5.2±0.5^{ab}	3±0.8^{cd}	4.6±0.7^{bc}	4.1±0.30	4.1±0.4	0.12	<0.0001	0.49	0.96
CNI	41.7±0.9^{c}	30.2±0.6^{e}	57.9±1.3^{b}	32.4±0.7^{d}	112.6±3.2^{a}	31.5±0.9^{de}	46.3±0.6^{a}	37.4±0.5^{b}	<0.0001	<0.0001	<0.0001	<0.0001
Epicardial fat												
Adipocyte CSA (µm2)	6462±83^{ab}	4244±111^{c}	6345±93^{b}	3912±81^{d}	6668±145^{a}	2572±141^{e}	5139±51^{a}	4929±66^{b}	<0.0001	<0.0001	<0.0001	0.024
Adipocyte (%)	91.3±1.1^{b}	92.9±1.6^{ab}	94.7±1.3^{ab}	92.3±1.2^{ab}	93.3±1.9^{ab}	95.2±1.5^{a}	92.8±0.7	93.8±1	0.39	0.81	0.14	0.42
Collagen (%)	5.8±1^{a}	4.3±1.3^{ab}	2.3±1.1^{b}	5.5±1^{a}	2.4±1.6^{ab}	1.8±1.3^{b}	4.4±0.6	3±0.8	0.08	0.77	0.04	0.19
Micro-vessels (%)	2.9±0.5	2.8±0.7	3±0.6	2.2±0.6	4.3±0.9	3.1±0.7	2.8±0.3	3.2±0.4	0.26	0.31	0.65	0.51

Data are presented as least square means±SEM. Effects of prenatal nutrition, postnatal nutrition or sex were significant $P<0.05$ if the data within a row and within the respective columns are marked by different superscripts. CSA, cross-sectional area; CNI, cell number index calculated as adipocyte mass (total fat mass (kg) multiplied by the %'age of adipocytes in the tissue) divided by the volume of a spherical adipocyte with a

radius derived from the measured mean cross-sectional area and expressed as $\times 10^8$; SD, sheep diet; LD, lamb diet; HIGH (N=10; 4 males, 6 females): diet fed to the twin-pregnant dam fulfilling 150% of requirements for energy and 100% of their requirements for protein; LOW (N=10; 5 males, 5 females): diet fulfilling 50% of requirements for energy and protein; NORM (N=6; 6 males, 0 female): diet fed to the twin-pregnant dam in the last trimester fulfilling their requirements for energy and protein; HCHF (n=13; 8 males, 5 females) , high carbohydrate-high fat diet; CONV (N=13; 7 males, 6 females) conventional diet to achieve moderate and constant growth rates of appr. 225 g day^{-1}.

PAPER IV

Khanal, P., Axel, A.M.D., Johnsen, L., Hansen, P.W., Kongsted, A.H., Lyckegaard, N.B. & Nielsen, M.O. Long-term consequences of late gestation malnutrition and early postnatal high-fat diet on growth characteristics and metabolic adaptations to fasting in adult sheep. Manuscript.

Long-term consequences of late gestation malnutrition and an early postnatal high-fat diet on growth characteristics and fasting metabolism in adult sheep

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Running head: Prenatal malnutrition, early postnatal high-fat diet and dietary correction

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Abstract

Aim: To investigate long-term consequences of late gestation over- versus undernutrition combined with an early postnatal high carbohydrate, high fat diet on growth characteristics, fat deposition patterns and fasting metabolism in adult sheep.

Methods: Twin-pregnant ewes were fed a NORM (fulfilling 100% of daily requirements for energy and protein), LOW (50% of NORM) or HIGH (150% of energy and 110% of protein requirements) diet during the last six weeks of gestation (term=147days). Postnatally, twin-lambs were fed a high-carbohydrate-high-fat (HCHF) or moderate (CONV) diet until six months of age (around puberty). Thereafter, all animals received the same moderate diet low in dietary fat until 2½ years of age (adulthood). Metabolic responses during 68-hour fasting were then examined and animals sacrificed.

Results: LOW-HCHF sheep had the highest plasma levels of creatinine, cholesterol and the highest fasting induced blood urea-nitrogen among all groups. LOW and NORM, but not HIGH, sheep previously fed the HCHF diet tended to have higher mesenteric fat deposition than the CONV fed. Sheep previously fed the HCHF diet had increased subcutaneous to perirenal and mesenteric to perirenal fat deposition ratios as adults. These long-term impacts of early life nutrition were found among both males and females.

Conclusion: Late gestation undernutrition predisposed for adult hypercholesterolaemia, hypercreatinaemia and hyperureamia, but development of these adverse features could be prevented by feeding a moderate diet in early postnatal life. However, individuals exposed to a history of late gestation overnutrition and developing obesity in early postnatal life were capable of restoring normo-adipocity and normal metabolic features upon dietary correction later in life.

Key words: fat deposition, foetal malnutrition, metabolic profiles, obesity, postnatal overnutrition

Introduction

Maternal undernutrition can lead to permanent changes in metabolism and endocrine function, thus increasing the risks of metabolic disorders later in life (Hales & Barker 1992). Recently, it has been suggested that prenatal overnutrition may also have similar disease risks in offspring revealing a 'U' – shaped curve relating birth weight to the risk of adult obesity (Curhan *et al.* 1996, Godfrey & Barker 2000). This suggests that widely different nutritional insults (over- versus undernutrition) during gestation, resulting in individuals being born at the extremes of the birth weight spectrum, may predispose for similar adverse outcomes on health and disease later in life. We have previously shown that prenatal undernutrition as well as overnutrition history during late gestation in lambs could predispose for development of abdominal obesity in lambs in response to an obesogenic diet after birth. This coexisted with differential physiological adaptations as prenatally over- and undernourished lambs were hyperglycaemic and hypercholesterolaemic upon exposure to an early postnatal obesogenic diet (Khanal *et al.* 2014a). These results indicate that manifestation of adverse outcomes of prenatal malnutrition may depend upon early postnatal dietary exposure. Moreover, we also observed that the early postnatal obesogenic diet induced metabolic characteristics resembling the metabolic syndrome in humans with hyperglycaemia, hypercholesterolaemia, hyperlipidaemia and abdominal obesity. It has previously been reported that dietary correction later in life can reverse the adverse impacts of early postnatal high-fat diet but late gestational undernutrition can permanently depress insulin sensitivity (Kongsted *et al.* 2014). However, it is not known whether programming induced by widely different nutritional insults during late foetal life would be evident later in life. Such studies are required to reveal, whether the adverse impacts of early development of adiposity in individuals with different nutritional histories in late foetal life are, at least partially, reversible upon dietary correction later in life. This knowledge is important in order to be able to

design efficient and targeted strategies for intervention for individuals exposed to different kinds of malnutrition during foetal life.

In this context, we hypothesized that maternal malnutrition in the form of under- or overnutrition would have similar long-term impacts on development and metabolic adaptability to fasting in adult sheep. Moreover, we aimed to investigate if early-life obesity followed by a diet correction later in life could reverse adverse outcomes of prenatal malnutrition and the early postnatal high-fat diet, such as altered metabolic adaptions and visceral adiposity in adulthood. To test this hypothesis, we conducted a study, with the Copenhagen sheep model (Khanal *et al.* 2014a) using 2½-year old adult sheep with histories of late gestation under- (LOW) or overnutrition (HIGH) combined with subsequent exposure to a moderate (CONV) or high-carbohydrate-high-fat (HCHF) diet from 3-days until 6-months of age (around puberty). We subjected the 2½-year old lambs to a 68-hour fasting period to elucidate metabolic adaptability since fasting is an important nutritional challenge during which a number of metabolic adaptations occur in the body. Thereafter the animals were slaughtered, and organ/tissue weights determined.

Materials and methods

The present experiment was conducted at the experimental facilities on the farm Rosenlund, Lynge, Denmark under the auspices of the Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark. All experimental procedures were approved by the National Committee on Animal Experimentation, Denmark.

Experimental animals and design

The Copenhagen sheep model was used in this experiment and details about the experimental animals used in this study, experimental design, feeding, care and management of the animals until 6-months of age (around puberty) have been described previously (Khanal *et al.* 2014a). In short, the experiment was a 3×2 factorial design where twin-pregnant Texel ewes were assigned to one of

three feeding treatments during the last six weeks of gestation (term=147 days): NORM (N=9; fulfilling 100% of required digestible energy and protein), HIGH (N=13; 150% of digestible energy and 110% of protein provided to NORM), LOW (N=14; 50% of digestible energy and protein provided to NORM). After lambing, each of the twin lambs were assigned to either a conventional (CONV; N=35; 16 males, 19 females) or an obesogenic high-carbohydrate-high-fat (HCHF; N=35; 18 males, 17 females) diet from 3-days until 6-months of age. The CONV diet consisted of milk replacer and hay until weaning at 8-weeks of age and from 8-weeks to 6-months of age, green hay only. Daily allowances of milk and hay were adjusted weekly to ensure a constant and moderate daily live weight gain (appr. 225 g/day). The HCHF lambs were fed a mixture of 50% milk replacer and 50% dairy cream (ad libitum; daily max: 2.5 L) and rolled maize (ad libitum; max: 1 kg/day). From 6-months of age all lambs regardless of their previous pre- and early postnatal dietary treatments, were fed the same diet consisting of artificially dried green hay of high quality *ad libitum,* supplemented with barley and they were managed as sex divided flocks. All animals received water *ad libitum*. An additional eight (4 males; 4 females) age-matched lambs were purchased from the same Danish commercial sheep farm that delivered the pregnant dams giving birth to the other experimental lambs, and the extra lambs were included in this experiment as undisturbed external controls (EC) and raised in the same flocks with the other experimental animals from 6-months until 2½ years of age. This resulted in seven different groups: HIGH-HCHF, HIGH-CONV, LOW-HCHF, LOW-CONV, NORM-HCHF, NORM-CONV, EC. Body weights were recorded monthly from six months until 2½ years of age. Body proportions (crown-rump length, girth circumference, height over withers, head width) were measured at 6-months, 1½- and 2½-years of age.

Fasting tolerance test

At 2½-years of age, the lambs were subjected to a 68-hour fasting period. They had free access to drinking water during that time. Permanent indwelling catheter tubes were placed in jugular veins at least one day prior to the test to facilitate repeated collection of blood samples as described by Khanal *et al.* (2014b) except that a silicone tubing (Silicone Rubber Tube, 1.2 mm ID × 0.4 mm wall; Silex Ltd., Lindford, Bordon Hampshire UK) was used as the indwelling permanent catheter material. The first blood sample was taken prior to removal of the food (zero sample; at appr. 13:00) and thereafter at times 24 (at appr. 13:00), 48 (at appr. 13:00) and 68 hours (at appr. 09:00) after onset of the fasting period. The samples collected in EDTA coated tubes were stored on ice until centrifugation ($1800 \times g_{av}$ at 4 ^{O}C for 15 min). After centrifugation, plasma samples were transferred to labelled cryo tubes (Th. Geyer Danmark Aps, Roskilde, Denmark) and stored at -20 ^{O}C until later analysis.

Laboratory analyses

Glucose, non-esterified fatty acids (NEFA), triglycerides (TG), blood urea nitrogen (BUN), creatinine, lactate, β-hydroxy butyrate (BOHB), γ-glutamyltransferase (GGT) and cholesterol levels were determined in plasma samples as previously described (Khanal *et al.* 2014a, Khanal *et al.* 2014b). The intra- and interassay coefficients of variation were below 5 and 10%, respectively, for all assays.

Slaughtering

Forty four sheep in total [NORM-CONV: 6 (2 males, 4 females); NORM-HCHF: 4 (2 males, 2 females); HIGH-CONV: 5 (2 males, 3 females); HIGH-HCHF: 6 (3 males, 3 females); LOW-CONV: 9 (4 males, 5 females); LOW-HCHF: 7 (4 males, 3 females); EC: 7 (3 males, 4 females)] were slaughtered at 2½ years of age. Just before slaughtering, animals were anaesthetized with

Propofol (5-6 mg i.v. kg^{-1} body weight; B. Braun, Melsungen, Germany) and thereafter sacrificed by decapitation. Immediately after decapitation, different organs were excised and weighed.

Statistical analyses

Statistical evaluation of all data was performed in SAS (v.9.2; SAS Institute, USA). Homogeneity of variance was evaluated by visual inspection of residual plots and normality of residuals was tested by means of quantile-quantile plots. Log transformations were applied when it was needed to obtain normal distribution of residuals. Analyses for body weights and measurements, different organ or tissue weights or for different parameters during the fasting challenge test were performed in two different ways: either including all animals divided in seven individual experimental groups, or excluding the EC animals to be able to compare separate effects of pre- and postnatal nutrition and animal sex by using the generalized linear mixed model (PROC GLIMMIX procedure). For the repeated measurements, different correlation structures between measurements and inhomogeneous variances were tested and the structure yielding the best fit was chosen. The model included *fixed effects* of feeding level, sex and time of sampling; individual sheep and lambs within feeding level were included as *random effects*, and samples within sheep and lambs were considered *repeated measurements*. Within the postnatal HCHF group, a few animals (one from the NORM, two from the HIGH and two from the LOW prenatal groups) had poorer growth rates compared to other animals in their groups, which was evident from 4-6-weeks of age, and they reached substantially lower body weights at 6-months of age (all <30 kg) compared to the rest of the HCHF fed animals (all >35 kg). They attained a characteristic skinny appearance not observed in any of the other animals irrespective of postnatal feeding, but despite the skinny appearance, such lambs slaughtered at 6-months of age had extensive abdominal fat deposition. In the statistical analyses, we therefore decided also to test if these "small-skinny-fat" animals, although few in number, showed any indications of being different from the rest of the animals within their group with "normal"

appearances and growth trajectories for the parameters studied but no such impacts of phenotype were found on any of the parameters studied. The model for this test had included *fixed effects* of phenotype, age of animal or time of sampling and phenotype ("small-skinny-fat" or "normal"). Individual sheep and lambs were included as *random effects,* and parameters within sheep and lambs were treated as *repeated measurements.* Difference in least square means (LS means) were compared by Tukey's multiple comparison test and presented results are expressed as LS means with standard error of mean (LS means±SEM). The level of significance was set at $P<0.05$.

Results

Unless it is specifically stated in the following, we did not for the studied parameters detect any significant effects of the prenatal nutrition, the interactions between pre- and postnatal diets, interactions with time (age of the animal during development or the time course of changes in metabolic and endocrine profiles during the fasting exposure), effects of sex, or indications of differences between the "small-skinny-fat" versus "normal" HCHF animals.

Body weight and body proportions

Sheep fed the HCHF diet in early postnatal life generally reached higher body weights compared to CONV fed sheep, except for HIGH sheep, where HIGH-CONV reached higher body weights than HIGH-HCHF sheep although the HIGH-HCHF had been heavier than the HIGH-CONV earlier in life. The highest body weights were reached in the undisturbed EC animals and the LOW-CONV sheep were consistently the lightest ($P<0.05$; interaction for prenatal nutrition and age; Figure 1). Male animals grew faster from earlier in life and they had consistently higher body weights compared to female animals ($P<0.05$). At six months of age, HCHF lambs had higher BCS than CONV lambs, but after transfer to the moderate diet at that time, HCHF sheep attained BCS similar to those of CONV animals from ~12 months of age ($P<0.0002$ for the postnatal diet and time

interaction). Overall, female sheep had higher BCS (3.2±0.05 vs. 2.8±0.05; $P<0.0001$) and smaller head circumference compared to male sheep ($P<0.0001$).

Metabolic adaptations during fasting

Lactate ($P=0.0002$ or 0.01 with or without EC included; Figure 2b), NEFA ($P<0.0001$ with or without EC included; Figure 2c), BOHB ($P<0.0001$ with or without EC included; Figure 2d), creatinine ($P=0.05$ with or without EC included) and TG (less pronounced; $P=0.006$ or $P=0.007$ with or without EC included; Figure 2e) levels in plasma increased steadily during the 68 hour fasting period. BUN levels increased by 20 hours after onset of fasting, but returned to baseline levels after 68 hours of fasting ($P<0.0001$) except in LOW-HCHF sheep, which had greater increases in BUN during fasting than the other groups ($P=0.02$ or <0.05 with or without EC included; Figure 3a). The BUN levels in general were highest in the LOW sheep followed by HIGH as compared to the NORM ($P<0.05$) and EC ($P=0.05$) whereas the levels in HIGH, NORM and EC sheep were similar. LOW-HCHF sheep also had distinctly higher plasma levels of cholesterol ($P=0.01$ or 0.04 for the pre- and postnatal nutrition interaction with or without EC included; Figure 1f) and creatinine ($P=0.005$ for the pre- and postnatal nutrition interaction; Figure 3b) compared to all other groups. Finally, the HCHF diet depressed GGT levels in LOW and NORM but not in HIGH sheep ($P=0.03$ for the pre- and postnatal nutrition interaction; Figure 3c). Female sheep had higher plasma levels of glucose (when EC included: $P=0.04$), TG ($P=0.02$) and cholesterol ($P=0.0003$ or 0.0004 with or without EC sheep) than males.

Fat deposition patterns and organ weights

Fat deposition and distribution

The distribution of fat in the body in adulthood depended on nutrition in both late gestation and early postnatal life. Sheep with a history of late gestation overnutrition had decreased depositions of pericardial fat when exposed to the obesogenic HCHF as compared to CONV diet in early postnatal

life (P=0.02; when EC was included; P=0.06), whereas pericardial fat deposition was unaffected by the early postnatal diet in LOW and NORM sheep. In subcutaneous fat, HIGH sheep exposed to the HCHF diet in fact had a decreased deposition of fat as compared to the HIGH-CONV sheep, whereas the LOW and NORM sheep previously exposed to the HCHF diet tended to have increased subcutaneous fat deposition compared to those fed the CONV diet, and greater increases were observed in NORM than LOW sheep (P=0.1 for the pre- and postnatal nutrition interaction). Deposition of mesenteric fat also tended to be unaffected by the early postnatal diet in HIGH sheep, whereas LOW and NORM sheep had increased depositions of mesenteric fat in absolute (P=0.08; when EC was included: P=0.08) and body weight-corrected terms (P=0.07; when EC was included: P=0.07) if they had been fed the HCHF as compared to CONV diet in early postnatal life (Table 1). Overall though, all groups of sheep that were previously fed the HCHF diet had higher ratios of subcutaneous to perirenal (P=0.004; when EC animals included: P=0.009) and mesenteric to perirenal fat (P<0.0001; when EC animals included: P<0.0001) deposition than CONV fed sheep irrespective of the prenatal diet. The ratio of subcutaneous to mesenteric fat was not related to nutrition history in neither pre- nor postnatal life. Female animals had higher absolute and body weight corrected depositions of subcutaneous (>2.5-fold; P=0.0001), mesenteric (~2-fold; P<0.0001), perirenal (2.5-fold; P<0.0001), pericardial (P=0.007) and sternal fat (>2-fold; P<0.0001) and lower mesenteric to perirenal (P=0.003; when EC animals included: P=0.002) and higher subcutaneous to mesenteric (P=0.01; when EC animals included: P=0.02) fat deposition ratios compared to male sheep (Table 1).

Other organ and tissue weights

There were no impacts of pre- or postnatal nutrition on gross or body weight corrected weights of any of the other organs or tissues studied except for the thyroids and adrenals, where HCHF sheep tended to have lower gross weights of thyroids (P=0.06; when EC animals included: P=0.08) and

gross (only when EC animals included, P=0.08) and body weight corrected weights of adrenals (P=0.08; when EC animals included: P=0.08) compared to CONV sheep (Table 1). Males compared to females had higher gross weights of liver (P<0.0001; when EC animals included: P<0.0001), kidney (P=0.003; when EC animals included: P=0.0005), heart (P=0.004; when EC animals included: P=0.003), *longissimus dorsii* (P=0.0006; when EC animals included: P<0.0001) and *biceps femoris* (P=0.08; when EC animals included: P<0.01). Only gross adrenal weights were higher in females compared to males (P=0.02; when EC animals included: P=0.03). After correction for body weight the sex effects disappeared for *biceps femoris*, and for kidney and heart they were found only when EC control animals were included and excluded, respectively, in the statistical analyses (Table 1).

Discussion

We aimed to evaluate whether maternal malnutrition in the form of under- or overnutrition would have similar long-term impacts on development and metabolic adaptability to fasting in adult sheep. Moreover, we hypothesized that transfer to a normal low-fat diet later in life would, at least partially, reverse the adverse outcomes such as altered metabolic adaptations and increased visceral adiposity due to prenatal malnutrition and early postnatal high-fat diet The major findings of this study were that 1) exposure to a high-fat diet in early postnatal life had long-term implications for fat deposition patterns even after two years of dietary correction, but this was not associated with adverse metabolic features *per se*; 2) Late-gestation undernutrition predisposed for development of hypercholesterolaemia, hypercreatinaemia and more pronounced fasting-induced hyperureamia, and this became evident in sheep exposed to a mismatching high-fat diet in early postnatal life, and 3) Individuals with a history of late gestation overnutrition may have superior ability compared to others to recover normo-adipocity and normal metabolic function after development of obesity in

early postnatal life by correcting their diet later in life. The observed long-term implications of foetal malnutrition were not restricted to any specific sex.

Exposure to maternal malnutrition in late gestation can lead to differential long-term metabolic adaptations in adult life

We have previously shown that the LOW female and male lambs included in this study were predisposed already at 6-month of age for development of hypercholesterolemia and hyperuraemia (higher plasma BUN levels in response to intravenous injection of propionate) after feeding the HCHF diet during the first 6-month of postnatal life, whereas HIGH animals became hyperglycaemic compared to other groups upon exposure to the HCHF diet (Khanal *et al.* 2014b). There were no signs of hyperglycaemia in the HIGH-HCHF sheep after they had been transferred to a normal, low-fat diet for two years. However, once the foetal predisposition for hypercholesterolaemia and hyperuraemia became expressed in individuals exposed to prenatal undernutrition by feeding the HCHF diet postnatally, it was retained into adulthood, and not even 2 years of diet correction with a normal, low-fat diet could reverse these traits in the LOW-HCHF sheep. Studies in humans have also demonstrated that impaired growth during late gestation is associated with raised blood cholesterol levels in adult life, and altered liver growth has been suggested as a responsible factor for such permanent alterations in cholesterol metabolism (Barker *et al.* 1993). Plasma levels of reduced cholesterol levels in adult animals subjected to moderate diet during postnatal life suggesting that hypercholesterolaemic impact of maternal undernutrition depends upon early postnatal dietary provisions. We have in a previous sheep study found indications pointing to the liver as a key target organ for programming in response to maternal undernutrition during late gestation (Hou *et al.* 2014), but detailed information is missing with regards to what alterations in cholesterol metabolism in the liver or other tissues are responsible for the sustained elevations of cholesterol levels throughout early and adult life.

In this study, the LOW-HCHF animals had higher levels of plasma BUN and creatinine in adult life after correction of early postnatal obesogenic high-fat diet. The LOW-HCHF sheep in addition to hyperchoelsterolaemia also developed hyperuraemia and hypercreatinaemia. We had earlier shown that within LOW-HCHF fed animals were hyperureamic characterized by higher plasma BUN levels in response to propionate after fasting at six months of their age (Khanal *et al.* 2014b). Although extra-renal factors may alter BUN as well as creatinine, these are generally considered to be rough indices of renal function (Finco & Duncan 1976). Future studies are obviously needed to reveal, if the development of hyperuraemia and hypercreatinaemia in LOW-HCHF sheep observed in this study were caused by disturbance of renal function in response to different types of nutrition during late foetal and early postnatal life, or whether other organs were involved. Our previous studies suggest that skeletal and cardiac muscle are not likely to be candidate organs, since they do not appear to be major targets of programming in response to nutritional insults occurring in the last trimester (Hou *et al.* 2013).

An important observation in our study was that the expression of adult hypercholesterolaemia, hypercreatinaemia and hyperuraemia in the predisposed LOW sheep could be prevented by feeding a moderate, low-fat diet throughout postnatal life. The long-term foetal imprinting on metabolic features were apparently not related to changes in fat deposition patterns as such, although we did observe a tendency for less of an increase in subcutaneous fat deposition in LOW as compared to NORM sheep when they had been exposed to the HCHF rather than CONV diet in early postnatal life, and HIGH sheep previously fed the HCHF diet did not have larger deposits at all of subcutaneous fat compared to those that had been fed the CONV diet. This agrees with previous studies showing that both late gestation undernutrition and overnutrition can predispose for reduced expandability of subcutaneous fat in adolescent lambs (Khanal *et al.* 2014, Nielsen *et al.* 2013) and

thus increasing the risk of lipid overflow associated with excessive abdominal and ectopic fat deposition when an obesogenic diet is fed.

Few long-term effects on development and fat deposition patterns of an obesogenic diet in early postnatal life, and they depended on the prenatal nutrition exposure

Sheep fed the HCHF diet as lambs generally reached higher body weights than those previously fed the CONV diet, except if they had also been prenatally overnourished, and HIGH-HCHF unexpectedly reached lower body weights than the HIGH-CONV fed. We have no explanation for that and it was not possible to assess feed intake since all animals were managed in a single flock (male and female separately). Thus, we cannot rule out that HIGH-HCHF sheep in our experiment may have had different feed intakes, which might explain why body weight gains were reduced and fat deposition restored completely in these sheep after dietary correction.

As adolescents, the HCHF sheep had significantly higher depositions of fat in all the studied depots, and extensive accumulation of perirenal fat resulted in decreased subcutaneous to perirenal and mesenteric to perirenal fat deposition ratios compared to the CONV-fed (Khanal *et al.* 2014a). The mesenteric adipose tissue was shown to be the major adipose tissue affected by the HCHF diet in the adolescent lambs with respect to gene expression of markers for lipid metabolism and inflammatory response (Khanal *et al.* 2014c). After 2-years of dietary correction, mesenteric fat deposition still tended to be higher in HCHF compared to CONV sheep, but accumulation of fat in all other depots was normalized. The sheep previously fed the HCHF diet thus ended up having lower ratios of subcutaneous to perirenal and mesenteric to perirenal fat deposition ratios than CONV sheep, i.e. opposite to what was observed in adolescence when they were being fed the HCHF diet.

In humans, the site of fat accumulation has received particular attention since it can play a central role regarding health risks and development of disorders later in life associated with obesity. Thus,

alterations in gene expressions in mesenteric adipose tissue in humans have been suggested to play a central role in development of insulin resistance associated with type 2 diabetes (Yang *et al.* 2008). Abdominal fat is considered to be an important risk factor for the development of obesity-related complications such as the metabolic syndrome (Wajchenberg 2000), and our study indicates that excessive accumulation of mesenteric fat in early postnatal life in response to a high-fat diet may still persist to adulthood even after 2 years of dietary correction. We are not aware of studies in humans or other species that have looked into the differential features of perirenal as compared to mesenteric fat, but this is definitely an issue which deserves further attention.

As adolescents, the HCHF fed sheep irrespective of the prenatal nutrition were hyperglycaemic, hypercholesterolaemic, hyperlipidaemic, had reduced glucose tolerance and delayed insulin clearance (Khanal *et al.* 2014a, Khanal *et al.* 2014b). It was remarkable that all of these adverse metabolic features had completely disappeared in the adult sheep after 2 years of dietary correction, except in LOW sheep as discussed above. This means that development of obesity in early life *per se* may not be as closely associated to the development of metabolic disorders in adulthood as hitherto anticipated, and dietary and body fat correction may be an efficient instrument to uncouple such an association except in individuals who have been exposed to prenatal undernutrition. In this study, female animals had higher glucose, cholesterol and triglyceride levels compared to male lambs in response fasting and they also had higher fat deposition in all depots studied compared to male animals, but the long-term consequences of early nutrition were not restricted to a particular sex.

In conclusion, our study has shown that late gestation undernutrition predisposed for development of hypercholesterolaemia, hypercreatinaemia and more pronounced fasting-induced hyperureamia, but in adult sheep these traits were evident only if the sheep in addition had been exposed to a mismatching high-fat diet in early postnatal life. Individuals with a history of late gestation

overnutrition, however, may have improved tolerance towards development of obesity, since they were able to restore normo-adipocity and normal metabolic function upon dietary correction later in life. Thus, although exposure to a high-fat diet in early postnatal life had long-term implications for fat deposition patterns even after 2 years of dietary correction, this was not associated with adverse metabolic features *per se* in adulthood, except in prenatally undernourished individuals. The long-term consequences of early nutrition were not restricted to a particular sex.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1: Changes in body weights of sheep from 8 months until 2 years of age. NORM (N=10; 4 male, 6 female), normal diet fulfilling requirements for energy and protein; HIGH (N=11; 5 male, 6 female), 150% of requirements for energy and 110% of requirements for protein; LOW (N=16; 8 male, 8 female), 50% of requirements for energy and protein; HCHF (n=13; 8 male, 5 female) , high carbohydrate-high fat diet from birth until six months of age and hay-based normal diet thereafter until 2½ years of age; CONV (N=13; 7 male, 6 female) conventional diet to achieve moderate and constant growth rates of appr. 225 g day-1 from birth until six months of age and hay-based normal diet thereafter until 2½ years of age; EC, external controls (N=7; 3 male, 4 female). NORM-CONV (N=6; 2 male, 4 female); NORM-HCHF (N=4; 2 male, 2 female); HIGH-CONV (N=5; 2 male, 3 female); HIGH-HCHF (N=6; 3 male, 3 female); LOW-CONV (N=9; 4 male, 5 female); LOW-HCHF (N=7; 4 male, 3 female); EC (N=7; 3 male, 4 female). Values are LS means ± SEM represented by vertical bars. Within week, LS means were significantly different at $P<0.05$.

Figure 2: Changes in plasma levels during a 68-hour fasting period of (a) glucose (b) lactate (c) NEFA (non-esterified fatty acids) (d) BOHB (β-hydroxy butyrate) (e) TG (triglycerides), and (f) cholesterol. HIGH, NORM, LOW, HCHF, CONV: see legends to Figure 1. Values are LS means ± SEM represented by vertical bars.

Figure 3: Changes in plasma levels during a 68-hour fasting period of (a) BUN (blood urea nitrogen); (b) creatinine; (c) GGT (γ-glutaryl transferase). HIGH, NORM, LOW, HCHF, CONV: see legends to Figure 1. Values are LS means ± SEM represented by vertical bars.

Figure 1:

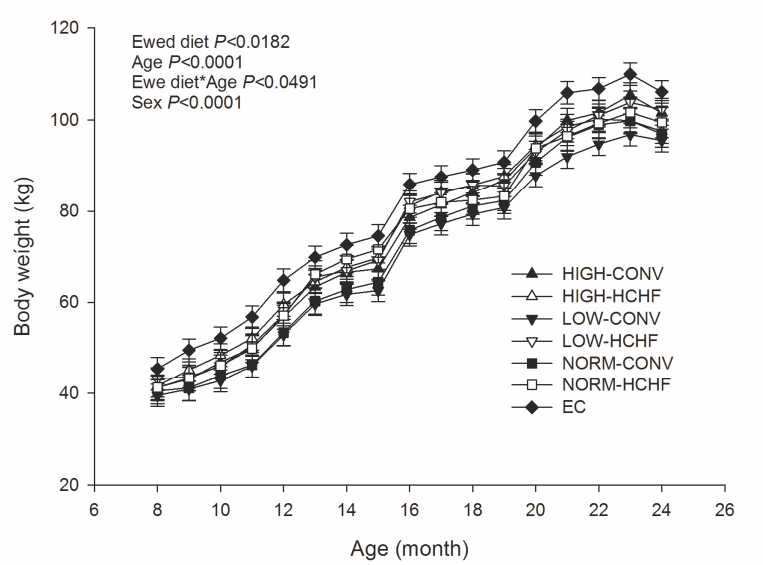
Ewed diet P<0.0182
Age P<0.0001
Ewe diet*Age P<0.0491
Sex P<0.0001
Body weight (kg)
Age (month)
HIGH-CONV
HIGH-HCHF
LOW-CONV
LOW-HCHF
NORM-CONV
NORM-HCHF
EC

Figure 2:

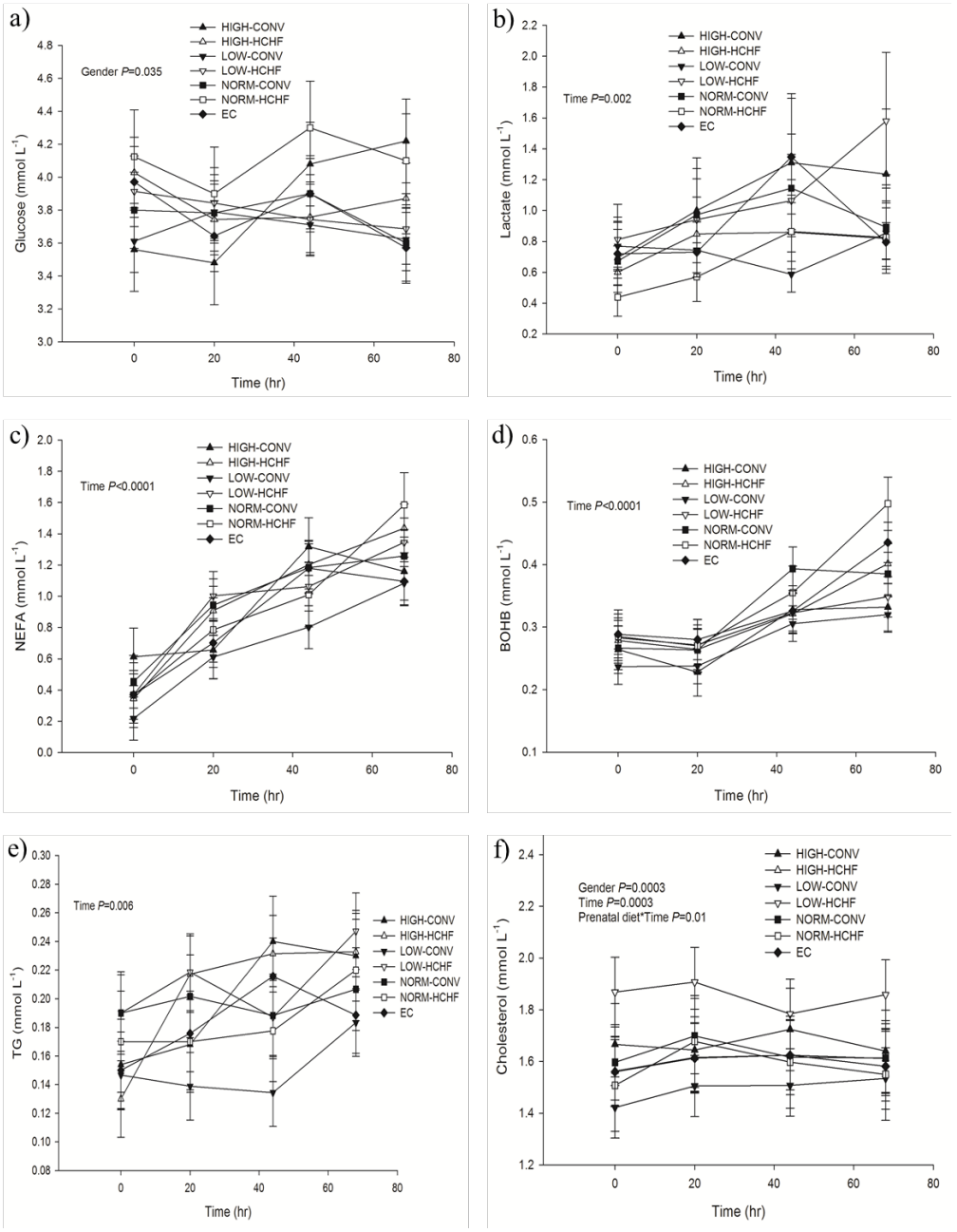
a)
Gender P=0.035
Glucose (mmol L-1)
Time (hr)
b)
Time P=0.002
Lactate (mmol L-1)
Time (hr)
c)
Time P<0.0001
NEFA (mmol L-1)
Time (hr)
d)
Time P<0.0001
BOHB (mmol L-1)
Time (hr)
e)
Time P=0.006
TG (mmol L-1)
Time (hr)
f)
Gender P=0.0003
Time P=0.0003
Prenatal diet*Time P=0.01
Cholesterol (mmol L-1)
Time (hr)
HIGH-CONV
HIGH-HCHF
LOW-CONV
LOW-HCHF
NORM-CONV
NORM-HCHF
EC

Figure 3:

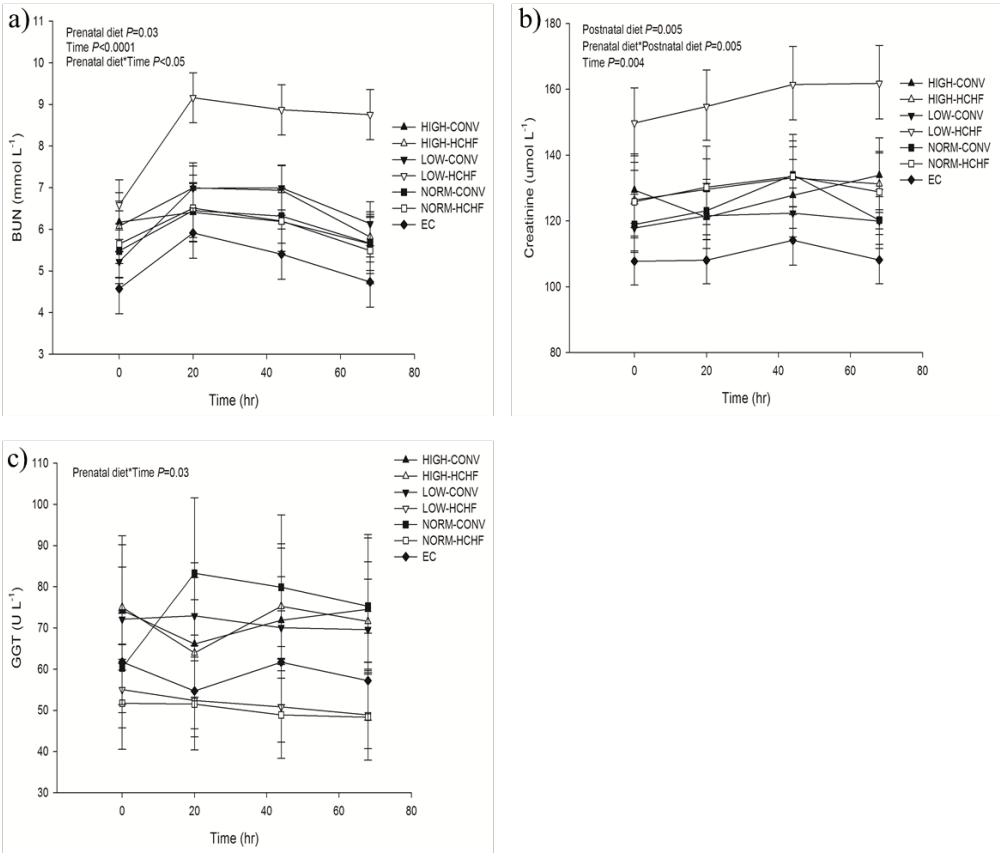
a)
Prenatal diet P=0.03
Time P<0.0001
Prenatal diet*Time P<0.05
BUN (mmol L-1)
Time (hr)
b)
Postnatal diet P=0.005
Prenatal diet*Postnatal diet P=0.005
Time P=0.004
Creatinine (umol L-1)
Time (hr)
c)
Prenatal diet*Time P=0.03
GGT (U L-1)
Time (hr)
HIGH-CONV
HIGH-HCHF
LOW-CONV
LOW-HCHF
NORM-CONV
NORM-HCHF
EC

Table 1: Effects of pre- and postnatal nutrition and sex on organ and adipose tissue weights in 2½ years animals

Parameters (actual weight, g or % of total body weight)	Treatments groups based on the combinations of pre- and postnatal nutrition*							Sex		*P* values			
	HIGH-HCHF	HIGH-CONV	LOW-HCHF	LOW-CONV	NORM-HCHF	NORM-CONV	EC	Male	Female	SD	LD	Sex	SD*LD
Body weight, kg	94.7±2.9	98.1±3	99±2.7	91.2±2.4	92.1±3.5	91.13±2.9	103.3±2.7	$99.4±1.2^{a}$	$92.9±1.4^{b}$	0.14	0.92	0.006	0.12
Subcutaneous fat, g	540±96	725.2±105.2	688.3±88.9	546.9±78.4	725.5±117.6	535.3±96	832.6±88.9	$357.5±52.6^{b}$	$891.6±48^{a}$	0.92	0.14	<0.0001	0.1
Subcutaneous fat %	0.59±0.09	0.74±0.11	0.69±0.09	0.60±0.08	0.78±0.12	0.60±0.1	0.82±0.09	$0.36±0.05^{b}$	$0.95±0.05^{a}$	0.98	0.15	<0.0001	0.19
Mescenteric fat, g	3736±524	3802±574	3698±486	2845±428	3939±642	3019±524	3899±485	$2632±287^{b}$	$4227±262^{a}$	0.71	0.08	0.0001	0.55
Mescenteric fat %	4.04±0.52	3.80±0.57	3.69±0.48	3.17±0.43	4.30±0.64	3.37±0.52	3.76±0.48	$2.6±0.3^{b}$	$4.6±0.26^{a}$	0.75	0.07	<0.0001	0.76
Renal fat, g	1551±263.4	2548.8±288.5	1415.1±243.8	2191.8±215	1887.5±322.5	1830.7±263.4	2585.7±243.8	$1252±144^{b}$	$2724±132^{a}$	0.63	0.2	<0.0001	0.14
Renal fat %	1.67±0.27	2.60±0.29	1.71±0.25	2.44±0.22	2.07±0.33	2.04±0.27	2.52±0.25	$1.2±0.2^{b}$	$2.9±0.1^{a}$	0.82	0.16	<0.0001	0.17
Sternal fat, g	253.3±47.7	265.2±52.3	242.9±44.2	199.1±39	212±58.5	214.7±47.8	315.7±44.2	$142±26^{b}$	$327±24^{a}$	0.6	0.39	<0.0001	0.79
Sternal fat %	0.27±0.05	0.27±0.05	0.24±0.05	0.22±0.04	0.23±0.06	0.24±0.05	0.31±0.05	$0.14±0.03^{b}$	$0.35±0.02^{a}$	0.68	0.41	<0.0001	0.97
Pericardial fat, g	165.7±20.4	236±22.3	200.6±18.9	178±16.6	217.8±25	212.3±25.1	218.3±18.9	$178.2±11.2^{b}$	$220.4±10.8^{a}$	0.59	0.62	0.01	0.06
Pericardial fat %	0.18±0.02	0.23±0.02	0.20±0.02	0.20±0.02	0.24±0.02	0.24±0.03	0.21±0.02	$0.18±0.01^{b}$	$0.24±0.10^{a}$	0.32	0.59	0.0008	0.26
Thyroids, g	4.73±0.81	6.36±1.0	4.89±0.75	5.96±0.66	5.19±0.99	6.15±0.81	5.89±0.81	5.6±0.5	5.6±0.4	0.94	0.08	0.78	0.92
Thyroids %	0.0053±0.001	0.0064±0.001	0.005±0.001	0.0067±0.001	0.0056±0.001	0.0068±0.001	0.0056±0.001	0.0057±0.001	0.006±0.005	0.93	0.12	0.77	0.94
Adrenals, g	3.09±0.5	4.14±0.4	4.09±0.5	4.14±0.4	3.02±0.6	3.99±0.5	4.72±0.5	$3.6±0.3^{b}$	$4.4±0.3^{a}$	0.42	0.08	0.03	0.29
Adrenals %	0.0033±0.0005	0.0046±0.0006	0.004±0.0005	0.0046±0.0004	0.0034±0.0007	0.004±0.0005	0.0045±0.0005	$0.004±0.0003^{b}$	$0.005±0.0003^{a}$	0.5	0.07	0.01	0.66
Pancreas, g	71.4±11	65.1±12	68.3±10.2	73±9.6	83.2±13.5	86±11	69.5±10.2	79.4±6.2	68.2±5.5	0.31	0.83	0.15	0.85
Pancreas %	0.077±0.012	0.062±0.013	0.069±0.01	0.08±0.01	0.09±0.015	0.094±0.012	0.067±0.011	0.080±0.007	0.07±0.006	0.19	0.9	0.4	0.53
Liver, g	807.7±35.5	844.4±38.9	845.7±32.9	788.3±30.8	745±43.5	805±35.5	931.9±35.5	$902.4±20^{a}$	$765.3±18.2^{b}$	0.48	0.29	<0.0001	0.2
Liver %	0.85±0.03	0.83±0.03	0.86±0.03	0.86±0.03	0.81±0.04	0.88±0.03	0.89±0.03	$0.9±0.02^{a}$	$0.8±0.01^{b}$	0.88	0.2	0.0004	0.29
Kidney, g	160.3±8.9	157.6±9.8	169.4±8.2	173.8±7.3	155.5±10.9	164.3±8.9	176.8±8.9	$180.8±4.9^{a}$	$155.4±4.6^{b}$	0.37	0.3600	0.0005	0.78
Kidney %	0.17±0.01	0.15±0.010	0.17±0.01	0.19±0.01	0.17±0.01	0.18±0.01	0.17±0.01	$0.18±0.01^{a}$	$0.17±0.01^{b}$	0.15	0.3000	0.03	0.15
Heart, g	347±19.1	332.4±20.9	326.6±17.6	297.3±15.6	320.4±27.1	340±19.1	328.6±17.6	$350.3±10.8^{a}$	$305.1±9.5^{b}$	0.23	0.900	0.003	0.46
Heart %	0.37±0.02	0.33±0.02	0.33±0.02	0.33±0.02	0.34±0.03	0.37±0.02	0.32±0.02	0.35±0.01	0.33±0.009	0.36	0.920	0.09	0.22
Longissimus dorsi, g	1110±59.9	1241.2±65.7	1192.6±55.5	1081.6±48.9	1088±73.4	1036.7±59.9	1165.7±55.5	$1250±33^{a}$	$1029±30^{b}$	0.27	0.71	<0.0001	0.13
Longissimus dorsi %	1.18±0.06	1.22±0.06	1.21±0.05	1.20±0.05	1.18±0.07	1.14±0.06	1.13±0.05	$1.3±0.03^{a}$	$1.1±0.3^{b}$	0.8	0.75	0.003	0.85
Biceps femoris, g	666.3±37.1	748.8±40.6	722.6±34.3	679.8±30.3	624.5±45.4	687.7±37.1	743.4±34.3	$734.4±20.3^{a}$	$669.1±18.5^{b}$	0.44	0.17	0.01	0.17
Biceps femoris %	0.71±0.04	0.74±0.04	0.73±0.03	0.76±0.03	0.68±0.04	0.75±0.04	0.72±0.03	0.74±0.01	0.72±0.01	0.73	0.12	0.45	0.76

Data are presented as least square means±SEM. Effects of prenatal nutrition, postnatal nutrition or sex were significant ($P<0.05$) if the data within a row and within the respective columns are marked by different superscripts. SD, sheep diet; LD, lamb diet; NORM (N=10; 4 males, 6 females), normal diet fulfilling requirements for energy and protein; HIGH (N=11; 5 males, 6 females), 150% of requirements for energy and 110% of requirements for protein; LOW (N=16; 8 males, 8 females), 50% of requirements for energy and protein; HCHF (n=13; 8 males, 5 females), high carbohydrate-high fat diet from birth until six months of age and hay-based normal diet thereafter until 2½ years of age; CONV (N=13; 7 males, 6 females) conventional diet to achieve moderate and constant growth rates of appr. 225 g day-1 from birth until six months of age and hay-based normal diet thereafter until 2½ years of age; EC, external controls (N=7; 3 males, 4 females). NORM-CONV (N=6; 2 males, 4 females); NORM-HCHF (N=4; 2 males, 2 females); HIGH-CONV (N=5; 2 males, 3 females); HIGH-HCHF (N=6; 3 males, 3 females); LOW-CONV (N=9; 4 males, 5 females); LOW-HCHF (N=7; 4 males, 3 females); EC (N=7; 3 males, 4 females). The subcutaneous fat represents the fat layer above the *longissimus dorsii* from the right side of the animal.

Table 2: Effects of pre- and postnatal nutrition and sex on fat ratios in 2½ years animals

Fat ratios	Treatments groups based on pre- and postnatal nutrition							Sex		*P* values			
	HIGH-CONV	HIGH-HCHF	LOW-CONV	LOW-HCHF	NORM-CONV	NORM-HCHF	EC	M	F	SD	LD	Sex	SD*LD
SC:PF	0.27±0.06	0.36±0.05	0.25±0.045	0.39±0.05	0.27±0.05	0.42±0.06	0.31±0.05	0.31±0.03	0.33±0.027	0.87	0.009	0.4578	0.83
MF:PF	1.50±0.22	2.48±0.20	1.42±0.17	2.39±0.19	1.79±0.20	2.49±0.25	1.52±0.19	2.23 ± 0.114^{a}	1.62 ± 0.40^{b}	0.4	<0.0001	0.002	0.740
SC:MF	0.19±0.05	0.15±0.047	0.19±0.038	0.18±0.0438	0.15±0.047	0.17±0.058	0.28±0.04	0.14 ± 0.025^{b}	0.23 ± 0.023^{a}	0.8	0.96	0.02	0.79

Data are presented as least square means±SEM. Effects of sex were significant ($P<0.05$) if the data within a row are marked by different superscripts. SC:PF, ratios of subcutaneous to perirenal fat weights; MF:PF; ratios of mesenteric to perirenal fat weights; SC:MF, ratios of subcutaneous to mesenteric fat weights; SD, sheep diet; LD, lamb diet; NORM (N=10; 4 males, 6 females), normal diet fulfilling requirements for energy and protein; HIGH (N=11; 5 males, 6 females), 150% of requirements for energy and 110% of requirements for protein; LOW (N=16; 8 males, 8 females), 50% of requirements for energy and protein; HCHF (N=13; 8 males, 5 females), high carbohydrate-high fat diet from birth until six months of age and a moderate, low-fat hay-based diet thereafter until 2½ years of age; CONV (N=13; 7 males, 6 females) conventional diet to achieve moderate and constant growth rates of appr. 225 g day^{-1} from birth until six months of age and the moderate hay-based diet thereafter until 2½ years of age; EC, external controls (N=7; 3 males, 4 females). NORM-CONV (N=6; 2 males, 4 females); NORM-HCHF (N=4; 2 males, 2 females); HIGH-CONV (N=5; 2 males, 3 females); HIGH-HCHF (N=6; 3 males, 3 females); LOW-CONV (N=9; 4 males, 5 females); LOW-HCHF (N=7; 4 males, 3 females); EC (N=7; 3 males, 4 females). The subcutaneous fat represents the fat layer above the *longissimus dorsii* from the right side of the animal.

Chapter 7: GENERAL DISCUSSION AND CONCLUSION

In this study, it was aimed to evaluate whether maternal malnutrition in the form of over- or undernutrition leads to similar impacts on growth and development, metabolic and endocrine function and adipose tissue development and function in sheep offspring. Furthermore, we hypothesized that alterations in metabolic function induced by different prenatal malnutrition exposures would be further exacerbated when combined with an obesogenic diet in early postnatal life, and individuals with a history of prenatal overnutrition are programmed to have superior ability to cope with an early postnatal obesogenic diet. Finally, it was aimed to investigate whether dietary correction would reverse the adverse impacts of late gestation malnutrition and an early postnatal obesogenic diet on growth and organ development, fat deposition patterns and metabolic adaptability during fasting in adult sheep.

The major findings of this study were that: a) exposure to widely different types of malnutrition (either overnutrition or undernutrition) in late gestation depressed gene expressions of makers involved in lipid metabolism (subcutaneous and perirenal fat), reduced non-obese cellularity (subcutaneous and mesenteric fat), reduced obesity-induced hyperplasia (subcutaneous, mesenteric and perirenal fat) and markedly increased obesity-induced hypertrophy (perirenal life) in postnatal life thereby predisposing for development of visceral adiposity later in life upon exposure to an obesogenic diet, b) prenatal under- and overnutrition had differential impacts on postnatal glucose-insulin regulation, lactate, cholesterol, and urea metabolism, and the liver and pancreas appear to be key targets for differential foetal programming, c) individuals with a history of prenatal overnutrition did not have superior ability to cope with an early postnatal obesogenic diet, and d) foetal programming outcomes were most clearly manifested during tolerance tests with the gluconeogenetic precursor and insulin secretagogue, propionate, particularly in the fasted state.

In this section, it will be discussed how the prenatal malnutrition either in the form of over- or undernutrition during late gestation impact on postnatal phenotypic characteristics, metabolic and endocrine adaptability, adipose tissue development and function in young and adult individuals.

Late gestational malnutrition and visceral obesity

Changes in foetal nutrient supply due to altered maternal nutrition during specific time periods of pregnancy can increase the risks of developing obesity and type 2 diabetes later in life (Budge *et al.* 2005). More than 30 years ago, Ravelli and colleagues studied the impacts of nutrient deprivation in 19-year old men exposed to the Dutch famine 1994-95 and found that nutrient insufficiency during the first half of pregnancy resulted in higher rates of obesity whereas such exposure during the last trimester and the first months of age actually lowered the obesity rates (Ravelli *et al.* 1976). Later, retrospective data from the Dutch Hunger Famine (1944-45) revealed that exposure to famine during early gestation influences cardiovascular system (increased risk of coronary heart disease) in adult life (Roseboom *et al.* 2000) whereas prenatal exposure to famine during late gestation is associated with adverse impacts on glucose-insulin homeostasis (increased risk of type 2 diabetes) (Ravelli *et al.* 1998). These studies indicate that the timing of abnormal nutritional exposure during pregnancy may have differential impacts on physiological and metabolic functions thus contributing to the increased risks of obesity and metabolic disorders later in life. As reviewed earlier, the majority of adipose tissue deposition occurs during late gestation in species born precocial, such as humans and sheep (Symonds & Lomax 1992) and adipose tissue is considered as an important target of foetal programming due to maternal malnutrition (Desai & Ross 2011, Symonds *et al.* 2012). We have earlier shown that maternal undernutrition during late gestation in sheep reduced the subcutaneous fat deposition and increased visceral to subcutaneous fat ratios in offspring (Nielsen *et al.* 2013) supporting the adipose tissue overflow hypothesis that poor expandability or reduced ability of subcutaneous fat to store excess lipid may lead to an early utilization of visceral

compartments to store fatty acids thus increasing risks of central obesity (Sniderman *et al.* 2007). Along with maternal undernutrition, evidence is accumulating to suggest that maternal malnutrition also in the form of overnutrition may have similar adiposity risks later in life (Ford & Long 2011). In this study, we showed that both prenatal over- and undernutrition predisposed for early development of visceral adiposity by altering adipose deposition patterns away from subcutaneous and towards the mesenteric and perirenal adipose compartments. This was associated with reduced gene expression of lipid metabolism markers (subcutaneous fat), reduced non-obese cellularity (subcutaneous and mesenteric fat) and reduced obesity-induced hyperplasia (subcutaneous, mesenteric and perirenal fat) and increased obesity-induced hypertrophy (perirenal fat). Thus both prenatal over- and undernutrition during late gestation predispose for development of visceral adiposity through similar but depot-specific changes in cellularity, hyperplasia and lipid metabolism.

Maternal malnutrition and metabolic and endocrine function

This study has shown that widely different nutritional insults i.e. over- and undernutrition during late gestation may lead to some of the similar phenotypic outcomes such as adipose distribution patterns after birth. However, it is not known whether maternal over- and undernutrition *in utero* impacts development of organs and body functions through similar physiological mechanisms and whether prenatally over- or undernourished individuals have different susceptibilities towards an obesogenic high-fat diet in postnatal life. In this study, we found that although prenatal malnutrition may lead to similar impacts on fat deposition and obesity risk, the underlying changes in metabolic and endocrine functions are not identical. Findings from different metabolic tolerance tests demonstrated that prenatal malnutrition differentially impacted adaptations of particularly plasma lactate followed by glucose, cholesterol, BUN and insulin and this was most clearly expressed during the propionate tolerance test in fasted lambs and less convincingly during insulin and

glucose tolerance tests. Prenatally overnourished lambs were hyperlactataemic and became hyperglycaemic in response to early postnatal high-fat whereas prenatal undernutrition in combination with an early postnatal obesogenic diet resulted in hypercholesterolaemia. These results do not support our hypothesis that individuals exposed to overnutrition before birth would have superior capability to cope with the hyperglycaemic effect of an early postnatal obesogenic diet however they did appear to be able to resist the maintain hypercholesterolaemic effect of the obesogenic diet.

In humans elevated lactate levels are often associated with liver dysfunction (Jeppesen *et al.* 2013). In this study, we found indications that lactate kinetics in response to propionate administration during fasting is altered in individuals exposed to late gestation malnutrition, and we have argued that the most plausible explanation for this is altered priorities in hepatic C-metabolism in prenatally programmed individuals. Lower lactate levels in response to propionate in prenatally undernourished animals may be associated with reduced hepatic gluconeogenetic capacity whereas higher plasma lactate and glucose responses in prenatally overnourished animals may reflect an increased hepatic gluconeogenetic ability. Although underlying mechanisms behind this are not yet understood a previous study has also shown that maternal high-fat feeding during gestation and lactation in mice led to development of impaired glucose tolerance associated with up-regulation of gluconeogenesis (increased hepatic expression of the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase) (Rother *et al.* 2012). Our study reveals that prenatal undernutrition predisposed for development of hypercholesterolaemia upon exposure to an obesogenic diet after birth which might also be related to impaired hepatic function since the liver is the major site for synthesis of cholesterol (Trapani *et al.* 2012). Moreover, prenatally undernourished and postnatally HCHF fed lambs also had higher BUN levels during propionate tolerance test. Such altered BUN levels could be associated with impaired hepatic or renal function

(Finco & Duncan 1976). Other previous studies have also pointed to the liver as a key target organ of maternal undernutrition during the critical period of foetal development (Cianfarani *et al.* 2012; Hou *et al.* 2013). Findings from this study demonstrate that the liver is a major target also of maternal overnutrition and hepatic function may be differentially regulated by widely different prenatal nutritional insults during late gestation.

Prenatally overnourished lambs had reduced pancreatic insulin secretory responses compared to undernourished lambs although this was evident only during the propionate tolerance test conducted when animals were fasted. A previous study in sheep indicated that maternal overnutrition at conception and throughout pregnancy depressed foetal pancreatic growth and β-cell numbers and increased β-cell apoptotic rate in late gestation (Zhang *et al.* 2011). We have not done detailed studies on pancreatic function and structure in our study, but our findings indicate that prenatal over- and undernutrition have differential impacts on propionate-induced insulin secretion in offspring. Irrespective of prenatal nutrition, propionate-induced insulin secretion was virtually abolished in HCHF-fed lambs in the fasted state, whereas the insulin secretory response in CONV-fed lambs was increased when the propionate was administered in the fasted compared to the fed state. The underlying mechanisms for this difference are not known, but it is evident that the postnatal HCHF diet interfered with adaptability to fasting in non-glucose dependant regulatory pathways in the pancreatic β-cell.

Long-term impacts of prenatal malnutrition

In this study, we found that most of the metabolic features of the early postnatal high-fat diet resembling the metabolic syndrome (as it manifests in humans) disappeared in adults after dietary correction except for the fat distribution patterns as the HCHF-fed animals still increased mesenteric fat depositions and had higher ratios of subcutaneous to perirenal and mesenteric to perirenal fat deposition as adults. The hyperglycaemic, hypercholesterolaemic and hyperlipidaemic

impacts of the early postnatal high-fat diet were disappeared when the animals were transferred to a moderate low-fat diet later in life except that LOW-HCHF adult animals were hypercholesterolaemic and had higher levels of plasma BUN during fasting. On the other hand, the hyperglycaemic impacts of prenatal overnutrition had disappeared upon dietary correction in adult life. Our previous study also shows that adverse impacts of an obesogenic diet in early postnatal life could be reversed after diet (and body fat) correction for 1½ years, whereas reduced insulin sensitivity due to late gestational undernutrition were still evident in adult life (Kongsted *et al.* 2014). This indicates that prenatal undernourished animals may have lower ability to recover when they have been exposed to such a mismatching nutrition in early life. This points towards a need for differential nutritional strategies targeting such individuals, where a recommendation for moderate weight gain and prevention of development of adiposity during early life is crucial particularly in individuals exposed to prenatal undernutrition.

In conclusion, this study evaluated the impacts of prenatal over- and undernutrition during late gestation on adipose tissue deposition and lipid metabolism and metabolic and endocrine function in both young and adult offspring. Both prenatal under- and overnutrition have similar impacts on fat deposition, distribution, cellularity and obesity-induced hypertrophy and hyperplasia resulting in similar phenotypic manifestation and visceral-obesity risks. However, underlying physiological mechanisms might be different. In response to an early postnatal obesogenic high-fat diet, late gestational overnutrition induced hyperglycaemia in young offspring and elevated BUN and cholesterol levels were found in prenatally undernourished young offspring. Upon dietary correction of the early postnatal high-fat diet, such hyperglycaemic impacts disappeared in prenatally overnourished adult animals whereas hyperureamic and hypercholesterolaemic impacts of prenatal undernutrition were still evident. This suggests that prenatally overnourished animals were not protected from the hyperglycaemic effect of a high-fat diet in early life as such, but they

did appear to have superior ability compared to the other groups to recover from the adverse consequences when they underwent dietary correction and body fat loss. This shows that the potentials for recovery later in life upon dietary correction appear to be more favourable in individuals exposed to late gestation overnutrition compared to undernutrition. Thus, although some of phenotypic features may be similar in the individuals exposed to prenatal over- or undernutrition during late gestation, differential nutritional strategies are needed for those individuals as their metabolic and endocrine function may be differentially programmed.

Chapter 8: FUTURE PERSPECTIVES

1. Glucagon is an important regulator of blood glucose levels and plays a central role in diabetes and metabolic syndrome. Using our Copenhagen sheep model, we have evaluated the impacts of abnormal pre- and postnatal nutrition on changes in a series of metabolites and hormones during fasting and various intravenous tolerance tests such as glucose, insulin, and propionate (fed and fasted conditions). In this study, we have not been able to investigate the dynamics of glucagon levels during the above-mentioned tolerance tests and to the best of our knowledge, current studies are extremely limited behind the regulation of glucagon in prenatally nutritionally programmed individuals. Future studies should be directed towards evaluating the role of prenatal malnutrition on glucagon plasticity in offspring which would allow us to further understand the complexity of altered metabolic and endocrine function in the individuals exposed to abnormal early life nutrition.
2. In this study, we have been able to show, particularly during propionate tolerance test, alterations in insulin plasticity due to prenatal malnutrition and early postnatal high-fat diet exposures but further studies are needed to understand the mechanisms behind the programming of the endocrine pancreas and potential non-glucose dependent pathways involved. Moreover, it is necessary to understand in future how adverse early life nutrition affects the programming of key regulators involved in pancreatic mass and function such as α- ad β-cell proliferation and apoptosis.
3. Findings of this study demonstrate that adipose tissue, liver and pancreas may be crucial targets of early life nutrition. Future studies are needed to further understand the molecular biological mechanisms behind the programing of these organs and tissues and hypothesis-free advanced approaches such as microarray techniques could be useful to identify novel and a wide range of key markers involved in foetal metabolic programming.

4. The current study investigated the impacts of maternal malnutrition during late gestation. Late gestation is certainly an important critical time window during foetal life and a significant part of organ development and maturation take place during this stage and contributes the most to birth weight. However, there are other critical time windows during pregnancy such as early- and mid-gestation and even prior to conception in which abnormal maternal nutrition may have long-term metabolic and physiological impacts on the offspring. Comparative studies regarding impacts of maternal over- versus undernutrition during early- and mid-gestation are limited. Future studies focusing on impacts of early-or mid-gestational nutrition on the status of health and disease risks in offspring may provide valuable information to design proper nutritional strategies throughout the pregnancy.

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APPENDICES

Appendix I: List of animals and their experimental groups

Prenatal Group	Sex	Postnatal group			Slaughtered animals (at 6 months of age)			Surviving animals (from six months onward)			Loss of animals due to sickness (from 6 months until 2½ years of age)			Total animals at 2½ years of age		
		HCHF	CONV	Total	HCHF	CONV	Total	HCHF	CONV	Total	HCHF	CONV	Total	HCHF	CONV	Total
NORM	Males	6	5	**11**	3	3	**6**	3	2	**5**	1	0	1	2	2	**4**
	Females	3	4	**7**	0	0	**0**	3	4	**7**	1	0	1	2	4	**6**
HIGH	Males	5	5	**10**	2	2	**4**	3	3	**6**	0	1	1	3	2	**5**
	Females	8	8	**16**	3	3	**6**	5	5	**10**	2	1	3	3	4	**7**
LOW	Males	7	6	**13**	3	2	**5**	4	4	**8**	0	0	0	4	4	**8**
	Females	6	7	**13**	2	3	**5**	4	4	**8**	1	0	1	3	4	**7**
EC	Males									**4**			**1**			**3**
	Females									**4**						**4**
Sum		**35**	**35**	**70**	**13**	**13**	**26**	**22**	**22**	**52**	**5**	**2**	**8**	**17**	**20**	**44**

NORM, normal diet fulfilling requirements for energy and protein; HIGH, 150% of requirements for energy and 110% of requirements for protein; LOW, 50% of requirements for energy and protein; HCHF, high carbohydrate-high fat diet from birth until six months of age and hay-based normal diet thereafter until 2½ years of age; CONV, conventional diet to achieve moderate and constant growth rates of appr. 225 g day-1 from birth until six months of age and hay-based normal diet thereafter until 2½ years of age; EC, external controls

Appendix II: Determination of plasma insulin concentration by using Mercodia Ovine Insulin ELISA Kit

Preparations

- Bring all samples, calibrators and reagents to room temperature before use and vortex
- Prepare washing buffer (1X solution): Dilute 50 ml washing buffer 21 X (1 bottle) with 1000 ml redistilled water
- Prepare protocol (plate set up) in the KC4 program (calibrator 0 = blank, calibrator 1 = std. 1 ect.). Perform each determination in duplicates

Test procedure

- Add 25 µl sample, calibrator and reference to the coated plate according to protocol/set up
- Mix enzyme conjugate 11x and enzyme conjugate buffer (table 1) and add 100 µl of this mixture to each well (use a channel pipette)

Table 1. Enzyme conjugate 11x/enzyme conjugate buffer mixture.

Number of strips	Enzyme conjugate 11x	Enzyme conjugate buffer
12 (full plate)	1 vial (1.3 ml)	1 vial (13 ml)
8	700 µl	7 ml
6	500 µl	5 ml
4	400 µl	4 ml

- Incubate the plate on a plate shaker (700 – 900 rpm) for 2 hours at room temperature

- After end incubation, place the plate on an absorbent paper and remove liquid from the wells (use the automatic plate washer)
- Use the automatic plate washer with overflow-wash function to fill the wells with washing buffer (with top, app. 700 µl) and subsequently suck up the washing buffer (filling and removal of washing buffer = 1 washing step)*. Repeat 5 times (resulting in a total of 6 washes)

  * Be aware not to scratch the wells and perform the washing steps as uniform as possible

- After the final wash, invert and tap the plate against the absorbent paper to remove excess liquid
- Add 200 µl substrate TMB to each well (use a channel pipette) and afterwards shake the plate on the place shaker at app. 800 rpm for 5 seconds
- Incubate for 15 minutes (in darkness)
- Turn on the ELISA reader
- After incubation, add 50 µl Stop Solution to each well (use a channel pipette) and afterwards shake the plate on the place shaker at app. 800 rpm for 5 seconds.

The wells will change color from blue to yellow (no blue tint/tone is supposed to be present after shaking)

- Measure absorbance at 450 nm (within 30 min)

Data evaluation (in KC4 program)

- Check the standard curve (click on "Biograph" buttom). Calculation std. 1-5: LIN-LIN Curvefit: SPLINE (cubic), a.o.b. log-log)
- Check reference samples (intra/inter controls)
- Check samples
- Save data:
    1. Press on "Date" → "Save as" and save the file as a "KC4 Data (*.PLA)" file
    2. Press on "Date" → "Save as" and save the file as a "KC4 Data + Prtcl (*.GLB)" file
    3. Press on "Date" → "Export" → Save as "TXT" file. Open Excel → Press "Open file" and open the previously saved TXT-file → Choose "afgrænset" and press "næste" → Choose "Tabulator" and "Semikolon" and press "næste" → Press "Udfør" → Save the data as an Excel file

Redo analyses if:

- CV % > 10 % (between duplicates)
- Concentration of a sample exceeds the concentration of the highest standard (calibrator 5).
    - Samples exceeding the concentration of the highest standard can be diluted with calibrator 0 and after analyses of diluted samples, the concentration of these can be multiplied with the dilution factor (to calculate the concentration)
- Anything seems incorrect/strange

Practical information

Order number: 10-1202-01 (same number is used for bovine, equine, porcrine and canine. When ordering, identify what animal it is going to be used for).

Address: Mercodia AB Sylveniusgatan 8A SE-754 50 Uppsala, Sweden

Plate setup for Mercodia Ovine Insulin Elisa

	1	2	3	4	5	6	7	8	9	10	11	12
A	Calibrator 0	1	1	9	9	17	17	25	25	33	33	Calibrator 0
B	Calibrator 1	2	2	10	10	18	18	26	26	34	34	Calibrator 1
C	Calibrator 2	3	3	11	11	19	19	27	27	35	35	Calibrator 2
D	Calibrator 3	4	4	12	12	20	20	28	28	36	36	Calibrator 3
E	Calibrator 4	5	5	13	13	21	21	29	29	37	37	Calibrator 4
F	Calibrator 5	6	6	14	14	22	22	30	30	38	38	Calibrator 5
G	REF. SHEEP	7	7	15	15	23	23	31	31	39	39	REF. SHEEP
H	REF. SHEEP	8	8	16	16	24	24	32	32	40	40	REF. SHEEP

Appendix III: van Gieson's staining of adipose tissue

Opdateret af Mari-Louise D. Andersen d.02-02-2011

Van Gieson:

Lillie Weigerts iron hematein

Solution A:	
2,5g $FeCl_3$, $6H_2O$ (2,5%)	Th-geyer, cat nr.: BK/00000119
4,5g $FeSO_4$, $7H_2O$ (4,5%)	Th-geyer, cat nr.:ME/00103965
2ml concentrated HCl	Th-geyer, cat nr.: SA/00007102
298ml Milli Q water	in lab B105

Solution B:	
1g Hematoxylin	Th-geyer, cat nr.: SA/H9627
100ml 99% EtOH	from anobis

Mix:
3 parts of solution A
1 part of solution B

0,5% Acidfuchsin

0,5g Acidfuchsin	Th-geyer, Cat nr.: SE/00034597
100ml Milli Q water	in lab B105

Pikrinacid-acidfuchsin:

360ml Pikrinacid solution, saturated	VWR, Cat nr.: 84512.260
30ml 0,5% Acidfuchsin	

Pikrinacid-acidfuchsin[1] is to be filtrated before use, and when finished for the day, pour it into the chemical waste container marked:**H**

The jars with xylen and EtOH have to be changes when needed. If you have finish you analyses, and nobody is using the deparafination jars it have to be emptied into the chemical waste container marked:**C**

Lillie Weigerts ironhematein is to be filtrated before use, and when finished for the day, pour it into the chemical waste container marked:**C**

[1] **Can be used again and again, so do not through out until the colour gets unusable!!!**

Van Gieson:

Use gloves at all time!

1. deparafination:

3x10 min in Xylen
3x5 min in 99% EtOH
2x5 min in 96% EtOH
5 min in 70% EtOH

2. rinse in running water, 5 min
3. Lillie Weigerts ironhematein,15 min
4. rinse in running water, 5-10 min
5. Pikrinacid-acidfuchsin, 4 min

1x 70% EtOH
1x 96% EtOH
4x 99% EtOH
1x Xylen

6. Mount with DPX[2] mounting medium, Use only a few drops on each slide. Carefully press down onto the coverslip, so there are no air bubbles.
7. leave in the hood for 24 hours.

Results:

Nuclei	brown
Collagen	red
Erythrocytes	yellow
Muscle	yellow

[2] DPX is toxic!

Appendix IV: Evaluation of proportions and adipocyte size using ImageJ software

Steps for proportional/ratio measurements:

1) Open ImageJ
2) File/Open
3) Plugins/Grid
4) Grid Type: Crosses; Area per Point: 50000 pixels^2; Color: Blue; Random Offset: Yes! → OK
5) Plugins/Analyze/Cell Counter → click on keep original → Initialize → Counter Window
6) Type 1 (Adipocyte); Type2 (Collagen) and Type3 (Micro-vessel)
7) Results → right click (copy) → paste in excel
8) Cell Counter → Export Image → File/Save (as Jpeg in another folder than the original image!)

Steps for measuring the adipocytes area:

1) Open ImageJ
2) File/Open
3) Plugins/Grid
4) Grid Type: Crosses; Area per Point: 100000 pixels^2; Color: Blue; Random Offset: Yes! → OK
5) Anylyze/Set Measurements: select "Area", "Perimeter" and "Feret's diameter"
6) Edit/options/colors…/Foreground: black; Background: White; Selection: blue
7) Freehand selections (4^{th} icon from left) → circle around the adicpocyte
8) Plugins/Analyze/Measure and set label/Set Label (Lable: --; Color: Black; Line width: 3; Font size: 15; Add measurement number: yes)
9) Analyse/Measure
10) Results → right click (select all/copy) → paste in excel
11) File/Save (as TIFE in another folder than the original image!)

Appendix V: RNA extraction

RNA extraction with TRIZOL in gentlemacs homogenizer (Recommended for adipose tissue)

The whole procedure will be performed in the fume hood. You will use the centrifuge 5471R and 5430R, under suction. RNA is highly sensitive to degradation by RNases and care should be taken to avoid any contamination by wearing a new clean lab coat and gloves at all time. Gloves are changes regularly. All surfaces and pipettes should be cleaned with RN'aseAWAY and only new disposable RN'ase free tips and tubes should be used. Trizol is toxic (contains phenol) and should be handled with gloves and in a fume hood at all time. See product sheet for safety notes! You will wear gloves at all time and changes gloves when you suspect they are contaminated. (Gloves are to be disposed in a normal bin, unless they have been contaminated with trizol, 1-Bromo-3chloropropane or isopropanol, then you need to leave them in the hood, until it has evaporated, and then disposed in normal bin. If you are not sure whether it has evaporated, they will be disposed in the transparent bin in the hood. Tubes and tips used with Trizol, have to be disposed in the chemical waste container (for trizol: tubes and tips) marked:C. All other tips and tubes are to be disposed in the transparent bag in the hood. Leave it until the next day, where you close it with tape, and dispose it in the yellow bag in the cupboard.

Equipment:

Tubes to gentlemacs called Dispomix tubes

gentleMACSTM Dissociater(in PCR-lab B104, remember to put it in the hood while in use)

Cooling centrifuge: 5430R eppendorf(in PCR-lab B104)

1.5 and 2.0 ml lobind DNA and RNA eppendorftubes

Cooling centrifuge: 5417R eppendorf(in PCR-lab B104)

2.0 ml Maxtract high density tubes from Qiagen

Reagents:

RNaseAWAY(VWR#13364-1)

Trizol reagent (Invitrogen#15596-018)

1-Bromo-3chloro-propane (Sigma #B9673)

Isopropanol (Sigma #I9516)

75% EtOH in DEPC water (Absolute alcohol 1.00983.1000 MERCK)

DEPC treated water

Homogenization:

1. No more than maximum recommended amount of tissue is transferred to a Dispomix tube with 650µl trizol (For adipose tissue ~300 mg of tissue was transferred to a tube with 2000 µl of trizol due to very poor RNA concentration).
2. Homogenize for 60 seconds (program RNA tube M) (homogenizer in hood)
3. Knock possible unhomogenized tissue to the bottom of the tube and repeat homogenization for 60 sec.
4. Incubate tube for 5 min at room temperature
5. Centrifuges for 10 minuts at 9.000 g at 4 °C (70 00rpm i 5430R) (make sure that the suction is over the centrifuge)
6. Transfer the supernatant to a 2.0 ml pre-centrifuged MaXtract tube (~600 µl)
(Disposal of the dispomix tubes in chemical waste container (for tubes and pipettetips- marked: C)

Phase separation

1. Ad 120µl 1-Bromo-3chloropropane (or chloroform)
2. Shake the tubes vigorously for 15 seconds
3. Incubate on ice for 10 min.
4. Add 240 µl of water
5. Centrifuge at 12.000 g for 10 min. at 4°C (10750r pm i 5417R) (make sure the suction is over the centrifuge)
6. Transfer the upper colourless aqueous phase (~600 µl) to a new 2.0 ml eppendorf tube (Disposal of tubes with the organic phase is in a chemical waste container marked: C)

Precipitation of RNA with isopropanol

1. Ad 500 µL isopropanol per 600 µl aqueous phase (x0,833) (in hood, with gloves)
2. Incubate for 10 min at RT or over night at - 20 °C (in hood)

Proceed with RNA clean-up

RNA Clean-up using Promega Total Isolation
Equipment: Centrifuge 5417R or Minispin eppendorf (in PCR-lab B104)
Spin column from kit
Collection tubes from kit
1.5 ml lobind DNA and RNA eppendorftubes

Reagents:

SV Total Isolation System from Promega
DEPC treated water

RNA Clean-up using Promega Total Isolation

The whole procedure will be performed in the hood, if you have more than 12 samples, you will use the centrifuge 5471R, under suction! You will wear gloves at all time and changes gloves when you suspect they are contaminated. (Gloves are to be disposed in a normal bin, unless they have been contaminated with isopropanol, then you need to leave them in the transparent bin in the hood. Disposal of tubes and tips should be done in the transparent bin in the hood. Leave it in the hood until the next day, and then close it with tape and put it in the yellow bag in the cupboard.

Make DNase Incubation Mix - pr sample

Yellow Core Buffer - 40 µl

MnCl2 0,09M - 5 µl

DNase 1 - 5 µl - mix gently on ice

1. Mix samples by pipitting 2 to 3 times.
2. Transfer 700 µl of the samples to a mini spin column in a collection tube
3. Centrifuge for 15 seconds at max speed in mini spin(RT) or 10500rpm in 5471R discard the flow-through (Disposal of the liquid waste in a chemical waste container marked:C)
4. Transfer the rest of the sample including all sediments to the column and centrifuge for 15 seconds
5. Use the same collection tube after disposal of waste (Disposal of the liquid waste in a chemical waste container marked:C)
6. Add 600 µl SV RNA Wash Solution (w. ethanol added)
7. Centrifuge for 1 min at maximum speed or 10500rpm in 5471R - discard the flow-through (Disposal of the liquid waste in a chemical waste container marked:C)
8. Add 50 µL DNase-mix on the middle of the membrane - incubate for 15 min at room temperature
9. Add 200 µL SV DNase Stop Solution (w. ethanol) - centrifuge 1min, at max speed in mini spin(RT) or 10500rpm in 5471R (Disposal of the liquid waste in a chemical waste container marked:C)
10. Add 600 µL SV RNA Wash Solution - Centrifuge 1 min, at max speed in mini spin(RT) or 10500rpm in 5471R (Disposal of the liquid waste in a chemical waste container marked:C)
11. Add 250 µL SV RNA Wash Solution - centrifuge 2 min, at max speed in mini spin(RT) or

10. 500rpm in 5471R (Disposal of the liquid waste in a chemical waste container marked:C)

12. Transfer Spin Basket to Elution-tube
13. Add 50 µL RNase-free water - incubate for 1 min

14. Centrifuge for 1 minute at max speed or 10500rpm in 5471R
15. Add 50 µL RNase-free water
16. Centrifuger i 1 minutter at max speed or 10500rpm in 5471R
17. Store RNA at -80 °C

Appendix VI: Determination of RNA integrity using bioanlyzer

~~210~~ 2010 expert program

* In plastic - pipette for RNA use it, do not touch the pipettes which are hanging as they are for DNA.

* RNA scp + water chip with in kit & white * simple chip is green

Agilent RNA 6000 Nano Kit Quick Start Guide

* Put 350 ml RNA Scp in chip in one whole (all connected)
* put 350 ml water in another chip (comes with RNA nano chip)

Agilent RNA 6000 Nano Assay Protocol - Edition April 2007

Preparing the Gel

1 Pipette 550 µl of RNA 6000 Nano gel matrix (red ●) into a spin filter.
2 Centrifuge at 1500 g ± 20 % for 10 minutes at room temperature.
3 Aliquot 65 µl filtered gel into 0.5 ml RNase-free microfuge tubes. Use filtered gel within 4 weeks.

1500g ≈ 4,5 rpm on the mini spin

In computer 1) first RNA scp (1 min)

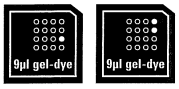
Preparing the Gel-Dye Mix

1 Allow the RNA 6000 Nano dye concentrate (blue ●) to equilibrate to room temperature for 30 min.
2 Vortex RNA 6000 Nano dye concentrate (blue ●) for 10 seconds, spin down and add 1 µl of dye into a 65 µl aliquot of filtered gel.
3 Vortex solution well. Spin tube at 13000 g for 10 min at room temperature. Use prepared gel-dye mix within one day. (top speed)

1) Blue cap freezer 2nd run left no 9 RNA ladder first

Loading the Gel-Dye Mix

1 Put a new RNA 6000 Nano chip on the chip priming station.
2 Pipette 9.0 µl of gel-dye mix in the well marked ⊖ 70,9 ml
3 Make sure that the plunger is positioned at 1 ml and then close the chip priming station.
4 Press plunger until it is held by the clip.
5 Wait for exactly 30 seconds then release clip. 0,9 ml
6 Wait for 5 s. Slowly pull back plunger to 1 ml position.
7 Open chip priming station and pipette 9.0 µl of gel-dye mix in the wells marked G.
8 Discard the remaining gel-dye mix.



Loading the Agilent RNA 6000 Nano Marker (buffer)

1 Pipette ~~9.0~~ 6µl of RNA 6000 Nano marker (green ●) in all 12 sample wells and in the well marked ◈.

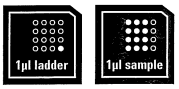
and green

bad ladder RNA ladder

Denaturate the samples at 70°C for 2min

Loading the Ladder and Samples

1 Pipette 1 µl of prepared ladder in well marked ◈.
2 Pipette 1 µl of sample in each of the 12 sample wells. Pipette 1 µl of RNA 6000 Nano Marker (green ●) in each unused sample well.
3 Put the chip horizontally in the adapter of the IKA vortexer and vortex for 1 min at ~~2400~~ rpm. 2000rpm
4 Run the chip in the Agilent 2100 bioanalyzer within 5 min.



Spin quickly before use and RNA ladder as well

Technical Support In the U.S./Canada: 1-800-227-9770 (toll free); lsca-ibs-support@agilent.com. In Europe: call your local Customer Care Center; bio_solutions@agilent.com. In Japan: 0120 477 111; yan_ccr@agilent.com. In Asia Pacific: call your local Customer Care Center; Bioanalyzer_ap@agilent.com

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76337 Waldbronn, Germany

RIN should be not below 6.5

* Just water in between samples

* same as pdf file but you go through print as pdf.

* Both scp + water in the begining each for 1 min, first scp + then water

* only water in between samples.

Appendix VII: cDNA synthesis

RNA is highly sensitive to degradation by RN'ases and care should be taken to avoid any contamination by wearing a new clean lab coat and gloves at all time. Gloves are changes regularly. All surfaces and pipettes should be cleaned with RN'aseAWAY and only new disposable RN'ase free tips and tubes should be used. Disposal of tubes and tips should be done in the transparent bags on the tables. The enzymes: M-MLV RT and RN'ase inhibitor should be kept in the container at all times, and put it back in the freezer straight after use. Do not add enzyme until the end of the mixing procedure. And keep the mastermix on ice at all times.

Equipment:

G-storm PCR
PCR tubes
1.5 ml lobind DNA and RNA eppendorf tubes
Container with ice

Reagents:
The entire reagent below **(all reagents are to be found in the freezer in PCR-lab B104)**
DEPC treated water (ddH_2O)

cDNA for 1 sample with and without enzyme:

5x M-MLV RT buffer:	5 µl
dNTP 10µM	1.3 µl
Random hex 2µg/µl	0.2 µl
oligo dT 0,5µg/µl	0.4 µl
RN'ase inhibitor	0.8 µl
MMLV enzym	1 µl
Master mix	**8.7 µl**
RNA: max	2µg
dH20	16.3 µl
Sum	**25 µl**

cDNA for 1 sample with enzyme:

5x M-MLV RT buffer:	5 µl
dNTP 10µM	1.3 µl
Random hex 2µg/µl	0.2 µl
oligo dT 0,5µg/µl	0.4 µl
RN'ase inhibitor	0.8 µl
H2O	1 µl
Master mix	**8.7 µl**
RNA max.	2µg
dH20	16.3 µl
Sum	**25** µl

RT-PCR program:

i) 25°C 10min ii) 42°C 60min iii) 95°C 5 min iv) 4°C and then to freezer

PCR apparatus: G-storm in PCR-lab

Quick manual:
1. Turn it on at the back of the apparatus.
2. Run program
3. Put in the PCR tubes
4. Choose the program: CDNA.scr
5. Run selected
6. Enter sample volume: 25µl
7. Press ok!
8. When the program is finished (when it reaches 4°C), press stop and ok!
9. Put the tubes in the freezer
10. Turn apparatus off in the back

Appendix VIII: qPCR

Opdateret af Mari-Louise D. Andersen d. 22-09-2010

qPCR on LightCycler 480

Equipment:
0,5ml lobind DNA and RNA eppendorftubes
1,5ml lobind DNA and RNA eppendorftubes
96well(white) or 384well(transparent) plates (Roche)
Cooling box that fits the plates
Container with ice
Whirlmixer(PCR-Lab B104)
Mini table centrifuge for both PCR tubes and eppedorftubes (PCR-Lab B104)
Centrifuge for the plates (anatomy building?)
LightCycler480 (anatomy building?)

Reagents:
DEPC treated water (ddH_2O)
Forward and reverse primer for both target and reference gen
Sybr green mastermix (Roche)

Protocol:
When the cDNA is made, you need to calculate how much pool and Calibrator that you need for you analysis.

You will make a triplet on each sample. Apart from the samples there has to be a negative control which is water and a Calibrator (this has to be the same all through the analysis, so make sure that you have enough and then some) on each plate.

There will be room for 30 samples pr 96well plate and 119 samples pr 384well plate(if you have the standard curve on the same plate as your samples)

The Calibrator can be made out of the pool, or if you got some tissue that you know the gen is well expressed in, as long as you use the same all the way through the analysis.

You also have to make at least 6 different dilutions of the pool(for each gen). This is for the standard curve, so that you can find the primers efficiency. If you have never done this before, there is a big possibility, that it will go wrong the first couple of times, ***so make sure you got enough pool!***

When you have calculated how much pool and calibrator that you need, start making the pool:

Ex.: Take 5µl from each sample and pool them!

If you expect the gen to be well expressed in your control group, you will need to dilute the samples and calibrator **1:10**

But we have had experiment where the control group has a low expression, and then we have diluted **1:4**

Mastermix:

	1 x	n x
Sybr green	5µl	n x 5µl
Forward primer 10µM	1µl	n x 1µl
Reverse primer 10µM	1µl	n x 1µl
ddH_2O	1µl	n x 1µl
SUM	**8µl**	**n x 8µl**

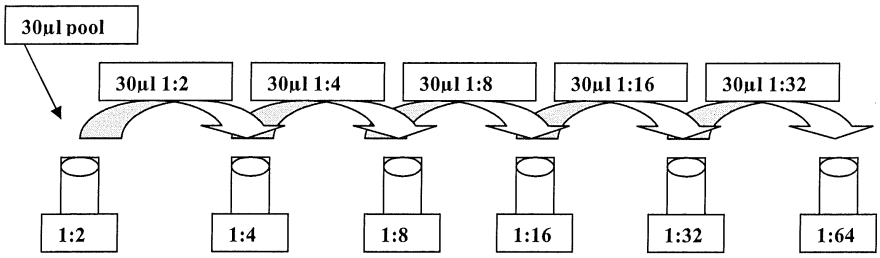
Use the stepper to ad the 8µl mastermix to each well.

Ad 2µl of template into each well (remember triplet). Allways whirlmix the sample before you pipette it up.

Do not put the pipette all the way down into the diluted sample, but try to keep the pipette tip in the surface of the diluted sample, but still long enough down, so you do not get air up into the pipette tip.

Standard curve:

Example on a dilution row:



In each tube contains 30µl ddH_2O

Because the analysis is relative quantification, it does not matter what value the dilution are set as in the software, as long as they have the right ratio from one dilution to another. So the standard values on the dilution like the dilution row above will be written like this in the software:

1:2	1:4	1:8	1:16	1:32	1:64
0,5	**0,25**	**0,125**	**0,0625**	**0,03125**	**0,015625**

When there is a good efficiency (between 1,7 and 2,0), slope (Between -3,5 and -3,10 or close), a link close to 0,000, as small an error as possible and a good enough standard deviation (0-0,4) on the triplets, it shows that the primers are good and you are able to make the dilution row properly.

If you use a 96well plates, there is room for 4 standard curves (4 genes) on a plate like this:

cali	**cali**	**cali**	**1:2**	**1:2**	**1:2**	**1:4**	**1:4**	**1:4**	**1:8**	**1:8**	**1:8**
1:16	**1:16**	**1:16**	**1:32**	**1:32**	**1:32**	**1:64**	**1:64**	**1:64**	**neg**	**neg**	**neg**
cali	**cali**	**cali**	**1:2**	**1:2**	**1:2**	**1:4**	**1:4**	**1:4**	**1:8**	**1:8**	**1:8**
1:16	**1:16**	**1:16**	**1:32**	**1:32**	**1:32**	**1:64**	**1:64**	**1:64**	**neg**	**neg**	**neg**
cali	**cali**	**cali**	**1:2**	**1:2**	**1:2**	**1:4**	**1:4**	**1:4**	**1:8**	**1:8**	**1:8**
1:16	**1:16**	**1:16**	**1:32**	**1:32**	**1:32**	**1:64**	**1:64**	**1:64**	**neg**	**neg**	**neg**
cali	**cali**	**cali**	**1:2**	**1:2**	**1:2**	**1:4**	**1:4**	**1:4**	**1:8**	**1:8**	**1:8**
1:16	**1:16**	**1:16**	**1:32**	**1:32**	**1:32**	**1:64**	**1:64**	**1:64**	**neg**	**neg**	**neg**

Now you can run your samples!

If you use a 384well plate you can make an in run standard curve (both the dilution row and your samples). But remember always to use a calibrator!

If you use a 96well plate you will ad the samples like this:

cali	**cali**	**cali**	**1**	**1**	**1**	**2**	**2**	**2**	**3**	**3**	**3**
4	**4**	**4**	**5**	**5**	**5**	**6**	**6**	**6**	**7**	**7**	**7**
8	**8**	**8**	**9**	**9**	**9**	**10**	**10**	**10**	**11**	**11**	**11**
12	**12**	**12**	**13**	**13**	**13**	**14**	**14**	**14**	**15**	**15**	**15**
16	**16**	**16**	**17**	**17**	**17**	**18**	**18**	**18**	**19**	**19**	**19**
20	**20**	**20**	**21**	**21**	**21**	**22**	**22**	**22**	**23**	**23**	**23**
24	**24**	**24**	**25**	**25**	**25**	**26**	**26**	**26**	**27**	**27**	**27**
28	**28**	**28**	**29**	**29**	**29**	**30**	**30**	**30**	**Neg**	**neg**	**neg**

The numbers 1-30 are the samples.

Appendix IX: Recommended amount of tissue for RNA extraction

Tissue	*Maximum weight (mg)*
Brain	*60*
Heart	*60*
Skeleton muscles	*30*
Small intestine	*60*
Large intestine	*40*
Thymus	*30*
Spleen	*15*
Kidney	*20*
Liver	*30*
Lung	*60*
Skin	*60*
Lymph node	*30*
Testicle	*30*
Ovary	*30*
Mammary glands	*30*
Stomach	*30*
Thyroid	*30*
Salivary gland	*10*
Uterus	*60*
Pancreas	*5-10*
Adrenal gland	*30*
Aorta	*60*
*Adipose tissue**	*60*

*, Since very poor RNA concentration of adipose tissue was obtained with 60 mg tissue, ~300 mg of adipose tissue was used for RNA extraction.

Additional publications during PhD

L. Hou (侯蕾), L. Johnsen, **P. Khanal**, A.M.D Axel, C.J. Leidesdorff, A.H. Kongsted, L.I. Hellgren, A. Vaag and M.O. Nielsen. Long-term effects of late-gestation undernutrition and a high-fat diet in early postnatal life on functional development of different adipose tissues in sheep *(Manuscript to be submitted shortly in the International Journal of Obesity)*.

M.N. Haque, M. Roggenbuck, **P. Khanal**, M.O. Nielsen and J. Madsen. Development of methane emission from lamb fed milk replacer and cream for a prolonged period *(Submitted to the Journal Animal Feed Science and Technology)*.

A.M.D. Axel, **P. Khanal**, A.H. Kongsted, L. Johnsen, S. Husted, L. Hou, K.L. Pedersen and M.O. Nielsen. Fetal metabolic programming of the small intestine in a Copenhagen sheep model. Abstract. J Diabetes 2013; 5: Suppl.1:72.

A.H. Kongsted, **P. Khanal**, A.M.D. Axel, L. Johnsen and M.O. Nielsen. Perinatal programming in sheep and susceptibility to development of metabolic syndrome. Acta Obstet Gynecol Scand 2013; 92: Supplement 160: 22.

P. Khanal, A.M.D. Axel, A. Kongsted, S. Husted, K.L. Pedersen, L. Johnsen and Nielsen MO. Prenatal over- and undernutrition leads to similar fat deposition pattern in offspring: Evidence from Copenhagen sheep model. Abstract. The PhD Day, Faculty of Health and Medical Sciences, Panum Building, University of Copenhagen, May 16, 2013.

P. Khanal, A.M.D. Axel, S. Husted, A. Kongsted, L. Hou and M.O. Nielsen. Programming effects of prenatal and early postnatal nutrition on growth and fat deposition in young lambs. Abstract. 1st Pears Alumni Workshop in Nutritional Sciences, Robert H. Smith Faculty of Agriculture, Food & Environment, Rehovot, Israel, September 2-8. 2012.

P. Khanal, S. Husted, A.M.D. Axel, A. Kongsted, L. Hou and M.O. Nielsen. Effects of prenatal diets on growth and birth weight in sheep. Abstract. Danish PhD School of molecular metabolism, University of Southern Denmark (SDU), Odense, Denmark, September 7-10, 2011.

Nielsen MO, P. **Khanal**, L. Johnsen and C.G.R. García Díaz. (2014): Malnutrition during fetal life, fetal programming and implications for farm animal productivity. VI Edition of International Conference on Agricultural Development and Sustainability AGROCENTRO 2014. ISBN 978-959-250-973-3 (DVD).

Activities in relation to the PhD period:

1. **PhD course:** Foetal metabolic programming. *Steno-Diabetes Centre; 1 to 4th February 2011.* Gentofte, Denmark
2. **PhD course:** PhD introduction course. *University of Copenhagen, Faculty of Life Sciences; 28th February to 4th March 2011.* Lyngby, Denmark
3. **Meeting:** Scientific meeting in Centre for Foetal Programming (CFP). *CFP, Statens Serum Institute; 6th May 2011.* Copenhagen, Denmark.
4. **Workshop:** Workshop on Computer-assisted Analysis of Digital Images. *Visiopharm; 15th June 2011.* Frederiksberg Campus, Frederiksberg, Denmark.
5. **Summer meeting:** CRAFT (Centre for Reproduction and Foetal Development) – Early Life Nutrition. *CRAFT, 25th August 25 2011.* Frederiksberg Campus, Frederiksberg, Denmark.
6. **Summer school (PhD course):** Summer school 2011 (A2) on molecular metabolism. *Danish PhD School of molecular metabolism, University of Southern Denmark (SDU); 7th to 10th September 2011.* Odense, Denmark.
7. **PhD course:** Laboratory animal science (requirement for category C license to work on animal experimentation). *Department of Veterinary Disease Biology, Faculty of Life Sciences, University of Copenhagen; 26th September to 7th October 2011.* Frederiksberg Campus, Frederiksberg, Denmark.
8. **Training/Course:** Hands-on qPCR and Experimental design and statistical analysis for qPCR. *TATAA Biocenter;* 5th to 9th December 2011. Goteborg, Sweden.
9. **PhD course:** Academic writing. *University of Copenhagen, 20 to 23th February 2012.* Frederiksberg Campus, Frederiksberg, Denmark.

10. **Statistics course:** **"**Statistics for Veterinarians". *Department of Basic Sciences and Environment – IGM; Faculty of Life Sciences, University of Copenhagen; March to June, 2012.* Frederiksberg Campus, Frederiksberg, Denmark.
11. **Congress**: The new remodelling dimension of the adipose organ: Satellite of the 19th ECO. Centre *de Recherche des Cordelier; 7 to 8th May 2012*. Paris, France.
12. **Seminar**: The PhD day 2013. *Faculty of Health and Medical Sciences, University of Copenhagen, The Panum Building; 16th May 2013*; Copenhagen, Denmark.
13. **Workshop**: Pears foundation alumni workshop in nutritional sciences: Confronting global nutrition challenges. *The Hebrew University of Jerusalem, The Robert H. Smith Faculty of Agriculture, Food and Environment*; *2 to 8th September 2012*. Rehovot Campus, Israel.
14. **PhD course**: CarrerLab. *University of Copenhagen; 22-25th October 2012*. Frederiksberg Campus, Frederiksberg, Denmark.
15. **PhD course**: Quantitative Genetics, Genomics and Breeding. *University of Copenhagen; 19th November, 2102-27th January 2013*. Frederiksberg Campus, Frederiksberg, Denmark

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PRABHAT KHANAL

Impacts of late gestation malnutrition and an early postnatal high-fat diet on metabolic, endocrine and adipose tissue function and development in sheep

Mr. Prabhat Khanal was born in 1983 in the western region of Nepal. He obtained his School Leaving Certificate (SLC, equivalent to class ten) in 1998 and completed his Intermediate of Science (I. Sc.) with major in Biology in 2002. Then he moved from basic science to applied scientific area and completed his Bachelor of Science in Agriculture (B. Sc. Ag.) in 2006 from the Institute of Agriculture and Animal Science (IAAS), Chitwan, Nepal. After the completion of his Bachelor Degree, he moved to Israel as part of the "On the Job Training Program" at the Arava International Centre for Agriculture Training, Arava, Israel for 11 months. After completion of the "On the Job Training Program", he achieved a scholarship to join the M. Sc. program at the Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel and completed his M. Sc. (Agriculture) in 2009 with Prof. Zwi Weinberg (from Volcani Research centre, Bet Degan) as his M. Sc. thesis supervisor. In 2011, he obtained a PhD position at the University of Copenhagen, Denmark under the supervision of Prof. Mette Olaf Nielsen, which mainly dealt with the phenomenon termed Foetal Metabolic Programming in the scientific world, and his contribution to this area were studies on the long-term impacts of pre- and postnatal dietary exposures on biology and health of in relation to obesity, metabolic disorders and type-2 diabetes, using an experimental sheep model.

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