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Kongsted, Anna Hauptoft; Tygesen, M. P.; Husted, Sanne Vinter; Oliver, M. H.; Tolver, Anders; Christensen, Vibeke Grøsfjeld; Nielsen, Jens Høiriis; Nielsen, Mette Olaf

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Programming of glucose–insulin homoeostasis: long-term consequences of pre-natal versus early post-natal nutrition insults. Evidence from a sheep model

A. H. Kongsted,1 M. P. Tygesen,2 S. V. Husted,1 M. H. Oliver,3 A. Tolver,4 V. G. Christensen,1 J. H. Nielsen5 and M. O. Nielsen1

1 Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark
2 Cook Medical Europe APS, Bjaeverskov, Denmark
3 Ngapouri Farm Research Laboratory, Liggins Institute, University of Auckland, Auckland, New Zealand
4 Department of Basic Sciences and Environment, Faculty of Science, University of Copenhagen, Frederiksberg, Denmark
5 Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, København N, Denmark

Abstract

Aim: Exposure to adverse intra-uterine conditions can predispose for metabolic disorders later in life. By using a sheep model, we studied (i) how programming of glucose–insulin homoeostasis during late gestation is manifested later in life depending on the early post-natal dietary exposure and (ii) whether dietary alteration in obese individuals can prevent adverse outcomes of early life programming.

Methods: During late gestation, twin-pregnant sheep were fed 100% (NORM) or 50% (LOW) of energy and protein requirements. After birth, offspring were exposed to a moderate (CONV) or high-carbohydrate–high-fat (HCHF) diet until around puberty. Offspring remaining thereafter (exclusively females) were fed a moderate diet until young adulthood.

Results: LOW lambs had increased insulin secretory responses during intravenous glucose tolerance tests indicative of reduced insulin sensitivity. HCHF lambs were hypertriglyceridaemic, 75% had mild pancreatic collagen infiltration, and their acute insulin secretory response and insulin clearance during intravenous glucose and insulin tolerance tests, respectively, were reduced. However, NORM-HCHF in contrast to LOW-HCHF lambs had normal glucose tolerance, indicating that later health outcomes are highly influenced by pre-natal nutrition. Dietary alteration normalized glucose–insulin homoeostasis in adult HCHF females, whereas late-gestation undernutrition (LOW) permanently depressed insulin sensitivity.

Conclusion: Maintenance of glucose tolerance in sheep exposed to pre-natal undernutrition relied on pancreatic hypersecretion of insulin to compensate for reduced insulin sensitivity. A mismatching high-fat diet in early post-natal life interfered with this pancreatic hypersecretion resulting in reduced glucose tolerance. Early post-natal, but not late pre-natal, impacts on glucose–insulin homoeostasis could be reversed by dietary correction later in life.

Keywords high-carbohydrate–high-fat diet, late-gestation undernutrition, obesity, pancreas.
The occurrence of obesity, type 2 diabetes and other associated disorders are part of what is termed the metabolic syndrome. These disorders are increasing in an epidemic-like fashion and represent a serious threat to public health worldwide (Wild et al. 2004, Konnopka et al. 2011). A sedentary lifestyle and an unhealthy diet high in energy and fat have been claimed responsible for the dramatic rise in obesity and metabolic syndrome. However, not all individuals are equally prone to develop the syndrome, indicating that other predisposing risk factors are important (Brochu et al. 2001, Primeau et al. 2011).

In the 1990s, Hales and Barker (Hales & Barker 1992, Ravelli et al. 1998) found a correlation between low birth weight and risk of developing type 2 diabetes later in life. Since then, several animal studies have confirmed that intra-uterine growth restriction (IUGR) and being born small-for-gestational-age increase the risk of metabolic disorders during post-natal life. An altered function of the glucose–insulin axis (Gardner et al. 2005, Husted et al. 2007) appears to be one of the key underlying factors for this adverse association between early life exposures and health outcomes later in life. IUGR has been proposed to induce foetal adaptations, such as asymmetric growth and alterations in peripheral insulin sensitivity favouring the growth of some organs such as the brain at the expense of others, for example the pancreas (Petrlik et al. 1998, Kind et al. 2003, Limesand et al. 2005, 2005, 2005, Barry et al. 2006, Germani et al. 2008), which should increase the chance of survival in a nutrient-deprived environment after birth. However, exposure in post-natal life to a mismatching diet, high in energy and fat, may greatly enhance the risk of developing type 2 diabetes as shown in rodent studies (Vickers et al. 2000, Rueda-Clausen et al. 2011).

Thus, many rodent studies have revealed that pre-natal growth restriction has long-term impacts on glucose–insulin axis function. Also in sheep, it has been shown that insults taking place during different developmental stages during gestation and even prior to conception reduce glucose tolerance in the adult offspring (Todd et al. 2009). However, no one has studied the impact of late-gestation undernutrition combined with a post-natal high-fat feeding in an animal model, which is more comparable to the human with respect to foetal development, offspring number and maturity at birth.

Compared to rodents, human babies as well as lambs are born more physiologically mature (Henning 1981, Metges 2009) and in many respects have comparable body development and tissue maturation in the third trimester of gestation. In comparison, rodent pups are born immature, and their development during the post-natal suckling period may in fact be more comparable to the development taking place pre-natally during the last trimester of gestation in the human and ovine foetus. Thus, differences in timing of the human and rodent peri-natal development, including the endocrine pancreas (Sarkar et al. 2008), impose difficulties in transferring findings from the rodent to the human situation in this particular time window of development.

We have recently developed a new sheep model (Nielsen et al., 2012), in which we can combine late-gestation undernutrition with a high-carbohydrate–high-fat (HCHF) dietary exposure in early post-natal life. Thus, health outcomes later in life in individuals subjected to different combinations of adverse nutritional exposures in late foetal and early post-natal life can be studied in a large animal model with many similarities to early human development. Late gestation is the period, where massive β-cell remodelling takes place in the foetal sheep (Gardner et al. 2005, Limesand et al. 2005, Hay, Jr., 2006). We used this sheep model to test the hypotheses that (i) late-gestation undernutrition in sheep programmes for reduced glucose tolerance and is exaggerated by a post-natal HCHF diet, (ii) Post-natal HCHF diet induces insulin resistance and impairs glucose-facilitated insulin secretion and (iii) dietary alterations and weight reduction later in life can prevent the adverse outcomes induced by early post-natal overnutrition, but not by late-gestation undernutrition.

Improving our understanding of how pre- and post-natal nutrition can programme for health or disease later in life is important in order to be able to develop efficient intervention strategies for low-birth-weight babies. This is especially important in developing countries undergoing rapid economic and lifestyle transitions (Hossain et al. 2007).

Methods

Animals and experimental design

The experimental design and the sheep model have been described in previous studies (Nielsen et al., 2012). All experimental animal handling and procedures were approved by The Danish National Committee on Animal Experimentation and conducted at the experimental farm Rørrendegard of the Faculty of Life Sciences, University of Copenhagen, Denmark. In short, two pre-natal and two post-natal nutritional treatments were applied in a 2 × 2 factorial design. Twenty-one Shropshire twin-pregnant sheep were during the last 6 weeks of gestation (term=147 days) fed diets fulfilling 100% (NORM) or 50% (LOW) of the daily requirements for energy and protein for a normal twin pregnancy. Water and vitamin–mineral supplements were available at all times. The twin lambs were divided and each fed their post-natal diet from...
3 days to 6 months of age: a moderate, conventional hay (CONV) diet supplemented with milk replacer during the first 8 weeks, and adjusted weekly to achieve moderate weight gains (≈225 g per day), or a HCHF diet consisting of dairy cream (38% fat; max 0.5 L per day), popped maize (max 1.0 kg per day) and milk replacer (max 2.0 L per day until 8 weeks of age and 0.5 L per day thereafter). As described previously, a total of four lambs died during the early post-natal life of reasons not related to the feeding regimens or other health problems. All the male offspring and three females were killed at 6 months of age (around puberty), and remaining offspring (females only) were fed the same moderate grass-based diet until 2 years of age (young adulthood) and then killed. As we only had one male in the NORM-CONV group, we slaughtered three females from the NORM-CONV group at 6 months of age together with the males in order to evaluate slaughter weights of the different organs (described in Nielsen et al., 2012). We found that the interpretation of organ weights did not differ whether they were included or not. However, we decided to rule out those females from the pancreas study in order not to disturb the results. Consequently, we could only evaluate the effects of either the pre-natal or the post-natal nutrition. At post-mortem, a pancreas sample (from the body part) from each animal was dissected out immediately after the animal had been killed, fixed in 4% PFA and embedded in paraffin blocks.

Post-prandial plasma profiles
Blood samples were collected from 1-day-old lambs. From 21-day-old lambs with pre-ruminant digestive function and from 42-day-old lambs with emerging ruminant digestive function, blood samples were taken in the morning approx. -30 min before feeding, +1 and +2.5 h after feeding. All samples were taken by venipuncture from the jugular vein and processed as described below.

Glucose, arginine and insulin tolerance tests
Intravenous glucose tolerance tests (IVGTT) were performed in all male and female lambs at 6 months of age and in young females at 1 and 2 years of age. Temporary catheters were inserted into jugular veins under local anaesthesia as previously described (Nielsen et al., 2012, Husted et al. 2007). Food was removed at 4 p.m. in the afternoon, and the following morning animals received an intravenous bolus injection of glucose at time = 0 (0.45 g D-glucose in distilled water kg⁻¹ metabolic body weight (MBW), SAD, Copenhagen, DK) followed by 10 mL saline flush. In 1- and 2-year-old females, an arginine bolus injection (100 mg l-arginine in distilled water kg⁻¹ MBW, pharmacy at Faculty of Life Sciences, University of Copenhagen, DK) was administered 4 h after the glucose bolus. Blood samples were collected at times -5, 2.5, 10, 20, 30 and 60 min after either glucose or arginine injection.

Insulin tolerance tests were performed in 6-month-old (males and females) and 2-year-old (females) offspring on a different day than the IVGTT. A bolus injection of 0.15 U insulin/kg body weight (recombinant human insulin dissolved in distilled water, Eli Lilly, Lyngby, DK) was injected after the morning feeding at time = 0, and blood samples were collected at times -5, 10, 20, 30 and 60 min after injection.

Blood samples were collected in EDTA and heparin vials and kept on iced water until centrifuged (1800×G, 4 °C, 15 min) within 20 min after sampling, and plasma was stored at -20 °C.

Biochemical measurements
Plasma concentrations of glucose, insulin, non-esterified fatty acids (NEFA), β-hydroxybutyrate (BOHB) and triglyceride (TG) were measured as previously described (Husted et al. 2007, Tygesen et al. 2008). The intra- and interassay coefficients of variation were below 5 and 10%, respectively, for all assays.

Immunohistochemistry and morphological stainings of pancreas sections
Pancreas sections (5 μM) were deparaffinized and hydrated in descending washes in ethanol to water. When used for immunohistochemistry, two sections from each block at least 200 μm apart were blocked in 2% bovine serum albumin and incubated overnight at 4 °C with anti-guinea-pig-anti-porcine insulin (Invitrogen, Tåstrup, DK) (1:500) and mouse-anti-porcine glucagon (Sigma, Brøndby, DK) (1:2000). After several washes, sections were incubated with secondary antibodies on the second day: donkey-anti-mouse-alex 594 (1 : 250) and donkey-anti-GP-alex 488 (Invitrogen) for 45 min and mounted in Vectashield mounting media (WVR, Herlev, DK) containing DAPI (1 : 1000) (Sigma, Brondby, DK). Negative controls included sections incubated with IgG1 (Dak, Glostrup, DK) and sections, where primary antibodies were omitted. No unspecific staining was observed on the negative controls (data not shown).

Van Gieson staining was performed by incubating a section from each animal at room temperature in Lilie Weigerts iron-haematin (Th-Geyer, Roskilde, DK) for 5 min, followed by 10 min in running water and a 4-min incubation in Pikrin-acid-fuchsin (WVR). After
subsequent rehydration, the sections were mounted in DPX mounting media (WVR).

All sections were evaluated by bright light microscopy or fluorescence microscopy (Leica Microscope type 020-525.731, Leica, Germany) and analysed. Islet-α/β-cell expression patterns were evaluated under the microscope to search for obvious group differences or outliers. Likewise, collagen infiltration was evaluated on Van Gieson-stained sections. On each of the two sections stained for insulin and glucagon, four fields of view were randomly selected from the four corners (east, west, south and north) and photographs taken with a Video camera (Sony Power HAD 3CCD Color Video Camera DXC.95950P, Leica, Germany). Semi-quantitative measurements of the average area positive for glucagon and insulin per FOV were performed from the 6-month-old lambs and 2-year-old sheep by the use of the Visiopharm integrator system 4.2.3.0 (Visiopharm A/S, Hørsholm, DK).

Statistics

As a preliminary analysis, all outcomes from post-prandial blood samples or from blood samples during tolerance tests were analysed separately at each sampling time using a two-way analysis of variance. We first did an overall test to compare plasma levels of metabolites or insulin between all four treatment groups given as combinations of pre-natal and post-natal treatment. If necessary, additional significance tests were conducted to clarify whether significant differences could be entirely due to pre-natal or post-natal treatment. For a more comprehensive statistical analysis, we used a mixed-effects model for repeated measurements over time for joint analysis of profiles over all sampling points of the tolerance tests. We included fixed effects of pre-natal and post-natal treatments and sampling time as well as their interactions and a random effect of lamb. A suitable correlation structure was used to model dependence within profile. The joint effect of pre-natal and post-natal treatment was assessed using approximate likelihood ratio tests for the interaction between pre-natal or post-natal treatment and sampling time. In case we found a significant effect over the entire sampling profile, post hoc tests contrasting the different treatments at individual sampling times were reported. To study the response during tolerance tests, all statistical analyses were carried out on variables adjusted for baseline levels observed just before the intravenous bolus injection. All variables, except plasma glucose, were log-transformed for normalization before the statistical analysis. Thus, for the analysis of repeated measurements of plasma glucose, the dependent response variables were plasma glucose sampled after injection subtracted plasma glucose level just before injection. For all other variables, the dependent response was the logarithm of the measurement subtracted the logarithm of the baseline sample – which may be regarded as merely the logarithm of the relative change since baseline. A similar mixed-effects model for repeated measurements was used to analyse the glucose-to-insulin ratio during tolerance tests. Area under the curve (AUC) for glucose and insulin measurements from 10 to 60 min after injection was analysed using two-way analysis of variance. Insulin sensitivity index (ISI) during IVGTT was addressed using two-way analysis of variance on a version of the Matsuda and Defronzo index based on AUC from –5 to 60 min after injection by the formula: ISI=10000/(FPG × FPI) × (AUC_IVGTTglucose × AUC_IVGTTinsulin), where FPG is fasting plasma glucose and FPI is fasting insulin. Glucagon- and insulin-staining areas were compared across treatment groups using two-way analysis of variance. \( P < 0.05 \) was considered statistically significant. All analyses were carried out using R: A language and environment for statistical computing (R Development Core Team, 2009).

Results

As described in Nielsen et al., (2012), energy and protein undernutrition (LOW) during the last trimester of foetal development in our sheep model significantly reduced birth weights, increased food preference early in life for high-fat feeds and altered fat deposition pattern towards visceral rather than subcutaneous fat. The post-natal HCHF diet induced obesity with >38% fat in soft tissues in adolescent lambs. Due to logistic reasons (and to avoid fighting), all males were slaughtered at the age of 6 months, and evaluation of gender-specific effects on glucose–insulin homoeostasis was therefore restricted to the time from birth to 6 months of age (around puberty). The effects of diet alteration (and normalization of body fat) after puberty could be evaluated in the female sheep previously fed the HCHF diet, because they were studied throughout the period from birth to young adulthood. There were no significant effects of either gender or age, unless specifically stated in the following.

Post-prandial plasma profiles in pre- (21-day) and post-ruminant (42-day) lambs

There was no influence of pre-natal nutrition on plasma levels of metabolites or insulin in the newborn lambs (Fig. 1a–e).

At 21 days of age prior to feeding, plasma glucose was significantly lower in the LOW-CONV compared with the other lambs, whereas their NEFA levels were
significantly increased. HCHF-fed lambs had higher levels of NEFA and TG prior to feeding and had higher post-prandial increases in BOHB compared with CONV lambs (Fig. 1a–e).

At 42 days of age, HCHF lambs had significantly higher glucose levels prior to feeding and higher post-prandial increases in glucose, insulin and TG compared with CONV lambs (Fig. 1a,b,e). After feeding (1- and 2.5-hour samples), a milky appearance was observed in the plasma obtained from the HCHF lambs, whereas those from the CONV were clear (Fig. 1f).

IVGTT in 6-month-old male and female lambs and 2-year-old female adult sheep

Neither pre-natal nor post-natal diets had any effect on fasting glucose prior to the glucose challenge (Fig. 2a). After the glucose bolus injection and given the same pre-injection levels, the groups between the LOW-HCHF lambs obviously had problems removing the glucose. This was reflected by their high insulin response (Fig. 2b) and high AUCglucose which was significantly increased compared with the other groups (Fig. 2c). When evaluating both LOW groups (LOW-CONV and LOW/HCHF), the acute glucose-stimulated insulin responses (Fig. 2b) and the AUC for insulin AUCinsulin (Fig. 2d) were increased in both male and female lambs. When calculating their insulin sensitivity index (ISI), we also found that both LOW groups were less insulin sensitive compared with the NORM groups (Table 1). However, and as mentioned above, it was only the LOW-HCHF lambs which also had higher AUCglucose. Furthermore, glucose levels remained elevated 1 hour post-injection in these
Figure 2 Changes in metabolites and insulin plasma concentrations during intravenous glucose tolerance tests in 6-month-old adolescent lambs. (a) Glucose, (b) insulin, (c) AUC_glucose 6 months, (d) AUC_insulin 6 months, (e) AUC_glucose 2 years, (f) AUC_insulin 2 years. White box: NORM-CONV (males $n = 2$, females $n = 8$), black box: NORM-HCHF (males $n = 5$, females $n = 4$), white circle: LOW-CONV (males $n = 5$, females $n = 4$) and black circle: LOW-HCHF (males $n = 5$, females $n = 5$): see legends to Fig. 1. A mixed-effects model for repeated measurements over time for joint analysis of profiles over all sampling points of the tolerance tests was used. Included fixed effects were pre-natal and post-natal treatments, sampling time as well as their interactions and a random effect of lamb.

* / † $P < 0.05$, ** / †† $P < 0.01$, *** / ††† $P < 0.001$, where * denotes a difference in the actual concentration and † the response corrected for the baseline value.

Table 1 The Matsuda & Defronzo Insulin sensitivity index

<table>
<thead>
<tr>
<th></th>
<th>NORM-CONV</th>
<th>NORM-HCHF</th>
<th>LOW-CONV</th>
<th>LOW-HCHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>227.0 ± 43.6</td>
<td>257.1 ± 44.0</td>
<td>126.4 ± 17.9**</td>
<td>142.3 ± 28.9**</td>
</tr>
<tr>
<td>6 months</td>
<td>$n = 10$</td>
<td>$n = 9$</td>
<td>$n = 10$</td>
<td>$n = 9$</td>
</tr>
<tr>
<td>Mean</td>
<td>421.0 ± 49.2</td>
<td>310.2 ± 44.4</td>
<td>259.3 ± 85.8</td>
<td>272.8 ± 76.9</td>
</tr>
<tr>
<td>1 year</td>
<td>$n = 4$</td>
<td>$n = 4$</td>
<td>$n = 5$</td>
<td>$n = 4$</td>
</tr>
<tr>
<td>Mean</td>
<td>210.7 ± 33.9</td>
<td>180.9 ± 4.7</td>
<td>152.6 ± 34.8</td>
<td>145.1 ± 34.6</td>
</tr>
<tr>
<td>2 years</td>
<td>$n = 4$</td>
<td>$n = 4$</td>
<td>$n = 5$</td>
<td>$n = 4$</td>
</tr>
</tbody>
</table>

Data are represented as means and SEM. AUC for glucose and insulin measurements from 10 to 60 min after injection was analysed using two-way analysis of variance. Insulin sensitivity index (ISI) during intravenous glucose tolerance tests were addressed using two-way analysis of variance on a version of the Matsuda and Defronzo index based on AUC from $-5$ to 60 min after injection by the formula: 

$$ISI = \frac{10000}{FPG \times FPI} \times (AUC_{IVGTT_{glucose}} \times AUC_{IVGTT_{insulin}}),$$

where $FPG$ is fasting plasma glucose and $FPI$ is fasting insulin. IVGTT, intravenous glucose tolerance tests; AUC, area under the curve.

**$P < 0.01$.**
LOW-HCHF lambs (Fig. 2a) compared with all other groups, and this despite their substantially higher insulin availability (AUC\text{insulin}) during the challenge period (Fig. 2b).

Post-natal HCHF feeding in contrast to the pre-natal LOW diet reduced the glucose-stimulated insulin responses measured 10 minutes after glucose injection (Fig. 2b). Surprisingly, the high-fat-fed NORM-HCHF lambs had in spite of their reduced insulin secretory responses comparable glucose levels as observed for the conventionally fed LOW-CONV and NORM-CONV groups (Fig. 2a + b).

After transfer to a moderate dietary scheme (lasting 1½ years), no effects of neither pre- nor post-natal diet on AUC\text{glucose} could be detected in the 1-year-old and 2-year-old female sheep (Fig. 2e). The insulin levels (AUC\text{insulin}) to a glucose challenge still tended ($P = 0.1$) to be higher in LOW compared with NORM adult female sheep (Fig. 2f).

**Insulin tolerance test in 6-month-old male and female lambs and 2-year-old female adult sheep**

There were no group differences in the initial plasma glucose levels after the insulin injection (Fig. 3a). However, the recovery towards normal glucose concentrations was delayed in the HCHF lambs (Fig. 3a). Following injection of insulin, the HCHF lambs had higher levels of insulin and lower levels of glucose compared to the LOW and NORM groups.

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**Figure 3** Changes in jugular vein plasma concentrations during intravenous insulin tolerance tests in 6-month-old adolescent lambs (Panels a–e) and 2-year-old young adult ewes (Panels f–g). (a) glucose (b) insulin (c) beta-hydroxybutyrate (BOHB) (d) non-esterified fatty acids (NEFA), (e) Triglyceride in 6-month-old adolescent lambs (f) glucose and (g) insulin in 2-year-old adult sheep. Insulin levels were significantly different between the LOW and NORM groups, White box: NORM-CONV (males $n = 2$, females $n = 8$), black box: NORM-HCHF (males $n = 5$, females $n = 4$), white circle: LOW-CONV (males $n = 5$, females $n = 4$) and black circle: LOW-HCHF (males $n = 5$, females $n = 5$): see legends to Fig. 1. A mixed-effects model for repeated measurements over time for joint analysis of profiles over all sampling points of the tolerance tests was used. Included fixed effects were pre-natal and post-natal treatments, sampling time as well as their interactions and a random effect of lamb.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, where * denotes a difference in the actual concentration and † the response corrected for the baseline value.
increased insulin levels at all sampling points as compared to the CONV-fed lambs (Fig. 3b). This was also reflected in their higher AUC_{insulin} during the insulin challenge test (Table 2). The HCHF-fed lambs also had higher plasma levels of NEFA and TG compared but lower BOHB levels than CONV lambs prior to insulin injection (Fig. 3c–e). The BOHB responses after injection of insulin were highest in the HCHF-fed lambs when adjusting for differences in baseline levels (Fig. 3c). The NORM-HCHF lambs responded to the insulin challenge with increases in both plasma TG and NEFA during the first 10 min post-injection, that is, opposite to what was observed in the other groups (Fig. 3d–e).

In the 2-year-old adult female sheep, which had been fed the same moderate diet for 1½ years, none of these post-natal dietary effects could be detected (data not shown except for glucose and insulin graphs, Fig. 3f–g). However, a pre-natal effect became evident at this age. In LOW female adults, insulin levels increased to higher levels and remained elevated for a longer period of time post-injection compared with NORM females (Fig. 3g).

**Arginine tolerance test in 1-year- and 2-year-old female sheep**

The pre-natal and post-natal nutritional treatments did not affect the responses in plasma glucose concentration to a bolus injection of arginine (data not shown).

The only differences in insulin concentrations were observed at 1 year of age, where LOW females had increased insulin levels at times 30, 45 and 60 min after arginine injection compared with NORM females (Fig. 4a). When comparing the increases in insulin relative to baseline levels, the NORM-CONV females had higher insulin responses to an arginine bolus injection compared with the other treatment groups at all times measured during the tolerance test (Fig. 4c–d; P < 0.001). This was mainly a consequence of lower pre-injection baseline levels (Fig. 4a–b), but in the 2-year-old females, insulin concentrations also increased to numerically higher levels in the NORM-CONV compared with the other groups during parts of the tolerance test (Fig. 4b).

**Pancreas morphology and islet structures**

Among the 6-month-old HCHF-fed lambs (all were males), six of eight revealed mild collagen infiltration. This was, however, only seen interlobular and in areas close to the ducts and vessels (Fig. 5b–d). Among the 2-year-old adult female sheep, we only observed interlobular collagen in one animal, and it belonged to the LOW-HCHF group (data not shown).

There is very limited information in the scientific literature on the morphology and expression of endocrine cells in the pancreatic islets of adult sheep (Reddy et al. 1988, Gatford et al. 2008). In this study, we identified distinct islet structures in the pancreas from all lambs and adult female sheep, but islets were highly variable in appearance, structure and composition. Distinct islet structures were observed in particular asymmetric islets with glucagon- and insulin-staining cells intermingled (Fig. 6a–b), but also islets (Fig. 6e) with insulin-positive cells in the core and glucagon in the periphery, which is typical in rodents (Doyle & Sussel 2007). There was sometimes considerable variation in the relative abundance of insulin- and glucagon-staining cells within individual islets and either \( \alpha \)- and \( \beta \)-cells could dominate (Fig. 6a–f). Cells expressing both insulin and glucagon were rare (data not shown). Insulin- and in particular glucagon-staining cells were sometimes observed in the duct epithelium, and this was particularly pronounced in the main ducts (Fig. 6a+f). Interestingly, clusters of glucagon-positive cells were occasionally found adjacent to duct epithelium, and sometimes these clusters contained a few insulin-positive cells (Fig. 6f). It was not possible to point out any group-specific differences by microscopic evaluation. However, a few outliers

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**Table 2** AUC_{insulin} during an insulin challenge

<table>
<thead>
<tr>
<th></th>
<th>NORM-CONV</th>
<th>NORM-HCHF</th>
<th>LOW-CONV</th>
<th>LOW-HCHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>146.1 ± 7.6</td>
<td>279.9 ± 15.3***</td>
<td>135.4 ± 13.5</td>
<td>204.4 ± 14.4***</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Mean</td>
<td>430.8 ± 43.4</td>
<td>516.3 ± 43.6</td>
<td>780.2 ± 88.9***</td>
<td>878.3 ± 74.8***</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

Data are represented as means and SEM. AUC_{insulin} was calculated from baseline (time –5 min before injection) and to 60 min after insulin injection. AUC, Area under the curve.

***P < 0.001.
were observed comprising larger hyperplasia-like insulin-staining islets (a NORM-HCHF male, Fig. 6c) and an adult LOW-HCHF female islets which obviously had islets with more glucagon compared with insulin staining (Fig. 6d). As we only had a limited number of animals and one sampling site per time point, the results are presented as changes in plasma insulin concentration from baseline levels in 1-year-old (a) and 2-year-old young adult ewes (b). The total insulin increase factors from the corresponding baseline levels are also illustrated in the figures from 1-year-old (c) and 2-year-old (d) ewes. White box: NORM-CONV (n = 4), black box: NORM-HCHF (n = 4), white circle: LOW-CONV (n = 5) and black circle: LOW-HCHF (n = 4); see legends to Fig. 1. A mixed-effects model for repeated measurements over time for joint analysis of profiles over all sampling points of the tolerance tests was used. Included fixed effects were pre-natal and post-natal treatments, sampling time as well as their interactions and a random effect of lamb. * P < 0.05, ** P < 0.01, *** P < 0.001, where * denotes a difference in the actual concentration and † the response corrected for the baseline value.

Figure 5 Early life nutrition and interlobular fibrosis in pancreas from 6-month-old adolescent lambs. Pancreas sections 5 μm were stained for collagen by the Van Gieson staining method and representative examples shown from each treatment group. (a) NORM-CONV (n = 1), (b) NORM-HCHF (n = 5), (c) LOW-CONV (n = 5) and (d) LOW-HCHF (n = 3), collagen is shown as the light red fibrotic structures, while the pancreatic tissue is brown, scale bar (200 μm).
animal, we could only perform semi-quantitative evaluations of the pancreatic endocrine cell areas to get an impression if the pre-natal or the post-natal diets had any effects. By the semi-quantitative studies, we did not detect specific differences that could be related to the interaction between pre- and post-natal nutritional exposures. The variations in tissue and islet morphology within and between animals were too large, and in future studies, a larger number of animals and more sampling sites in each treatment group will be needed to allow such an evaluation.

We did, however, find indications that the glucagon-staining area was slightly increased by the HCHF diet ($P = 0.03$) in the adolescent males fed the HCHF diet, and the insulin-staining area was close to significantly reduced in the adult females previously exposed to the HCHF diet (Table 3).

**Discussion**

There are five main findings in this study: (i) Late-gestation undernutrition was associated with peripheral insulin resistance in young lambs, but normal glucose tolerance could be achieved in LOW-CONV adolescents by a compensatory upregulation of the pancreatic insulin secretory response to glucose. (ii) The obesogenic HCHF feeding in early post-natal life caused hypertriglyceridaemia, mild pancreatic collagen infiltration in most adolescent male lambs (no HCHF...
females were studied at this age), and suppressed the pancreatic insulin secretory response to glucose. But NORM-HCHF adolescents had normal glucose tolerance, indicating a compensatory upregulation of peripheral insulin sensitivity. (iii) Glucose tolerance in the lambs that were exposed to undernutrition during late gestation thus relied on a compensatory mechanism (upregulation of pancreatic insulin secretory response), which was interfered with by a subsequent exposure to a high-fat diet in early post-natal life, and vice versa, and glucose tolerance was consequently reduced in LOW-HCHF lambs. (iv) Most adverse effects induced by the HCHF diet in early post-natal life could be reversed upon dietary correction later in life, whereas insulin resistance induced by pre-natal undernutrition persisted into adulthood (this could only be evaluated for females). (v) The descriptive data we have for sheep on the pancreatic islet structures revealed similarities to the human pancreas. Pancreatic a-cell area tended to be slightly enhanced by the HCHF diet in the 6-month-old male lambs.

**Post-natal high-fat feeding during early post-natal life results in hypertriglyceridaemia and reduced insulin secretion**

In the young lambs fed the HCHF diet, very high NEFA and TG levels as high as what has been claimed responsible for the induction of fatty liver disease and pancreatic fibrosis in other species (Van-Saan et al. 2009, Zhang et al., 2008) were obtained after feeding. We also observed mild interlobular pancreatic collagen in 75% of the HCHF male lambs slaughtered (no females were studied), which could be indicative of the progression towards development of pancreatic fibrosis and pancreatitis. As also discussed below, such pancreatic modifications induced by a high-fat post-natal diet have not been described in ruminants before. We have therefore been successful in making a new animal model to humans which show some of the same post-natal effects as seen in other species fed a high-fat diet. At the same time, we take advantage of the similarities between sheep and humans in respect to the number of offspring (1–2), birth weight (~3–4 kg), adult body weight (~50–80 kg), long gestation period and maturity at birth.

When subjecting the 6-month-old HCHF lambs (around puberty) to an intravenous glucose challenge, their insulin responses were decreased compared with the CONV lambs. This agrees with studies in other species showing that prolonged high-fat feeding can inhibit insulin secretion (Zhang et al. 2010) and interfere with β-cell function, resulting in a gradual loss of sensitivity towards secretagogues as glucose (Cerf 2007). Therefore, assumed differences between ruminant and non-ruminant species with respect to the development of glucose–insulin homoeostasis and pancreas dysfunctions may be related more to the patterns of absorbed nutrients under normal feeding conditions than to inherent species differences *per se*.

The HCHF-fed lambs in this experiment could be categorized as obese with widespread ectopic fat deposition and a total fat content in soft tissues >38% (Nielsen et al. 2012). Ectopic fat deposition has been linked to reduced insulin secretory capacity in β-cells, as we also observed in our study, but it has also been linked to reduced insulin signalling in muscles (Weiss et al. 2003, Frangoudakis et al. 2005). In contrast to the LOW-HCHF lambs, we did not find any indications of reduced insulin sensitivity in the NORM-HCHF lambs as they cleared the injected glucose just as efficient as the CONV lambs during the IVGTT. Strikingly, they were in fact able to remove the glucose in spite of the reduced initial insulin as seen 10 min after injection. Could it be possible that a transient increase in insulin sensitivity occurs prior to the development of insulin resistance, thus being a forerunner to the pre-diabetic stage, and as such, alterations in glucose regulation would not be detected by conventional tests (Buysschaert & Bergman 2011, Sjaarda et al. 2012). In this respect, it is interesting to note that a transient increase in insulin sensitivity also

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**Table 3 : Average glucagon- and insulin-positive area (μm²) per FOV on pancreas sections from HCHF- and CONV-fed lambs.**

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<th>CONV</th>
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<th>HCHF</th>
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<tr>
<td></td>
<td>Means</td>
<td>SEM</td>
<td>Means</td>
<td>SEM</td>
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<tr>
<td>Insulin area</td>
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<tr>
<td>Males 6 months</td>
<td>30.696</td>
<td>±5583</td>
<td>54.529</td>
<td>±14.005</td>
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<tr>
<td>Glucagon area</td>
<td>11.235</td>
<td>±4334</td>
<td>20.878</td>
<td>±2454</td>
</tr>
<tr>
<td>Males 6 months</td>
<td>26.319</td>
<td>±1570</td>
<td>17.567</td>
<td>±3378</td>
</tr>
<tr>
<td>Insulin area</td>
<td>14.641</td>
<td>±3377</td>
<td>10.465</td>
<td>±3078</td>
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Data are represented as means and SEM. From each animal, two sections were stained for insulin and glucagon; on each section, four FOV were captured by microscopy (10x) and the area positive for insulin (green) and glucagon (red) was evaluated by the use of the Visiomorph software. CONV 6 months (n = 5 HCHF 6 months (n = 7), CONV 2 years (n = 8), HCHF 2 years (n = 9). Glucagon- and insulin-staining areas were compared across treatment groups using two-way analysis of variance. As there was no influence of the pre-natal diet, only post-natal effects are shown.

*P < 0.05 was considered significant.
Late-gestational undernutrition combined with HCHF feeding decreased glucose tolerance in lambs

In the 6-month-old lambs exposed to late-gestational undernutrition, clear signs of insulin resistance were manifested. This was revealed as increased insulin secretion and AUC$_{\text{insulin}}$ during the intravenous glucose challenge indicative of compensatory $\beta$-cell hypersecretion to maintain normoglycaemia. This was also clearly reflected by the Matsuda & Defronzo index pointing at the LOW offspring having reduced insulin sensitivity. Our study therefore agrees with many other human and animal studies and confirms that pre-natal dietary restriction/low birth weight programmes for insulin resistance later in post-natal life (Chamson-Reig et al. 2009, ???, Song et al. 2008, Ravelli et al. 1998).

On the other hand, it was obvious that the ability to compensate for reduced insulin sensitivity (as in LOW-CONV offspring) was lost in the lambs exposed to the LOW-HCHF diet in early post-natal life, as they had high AUC$_{\text{insulin}}$ and AUC$_{\text{glucose}}$ during the glucose challenge, and in spite of that failed to maintain normal glucose tolerance. The inability to increase insulin secretion sufficiently to compensate for reduced peripheral insulin sensitivity has been linked to the development of type-2 diabetes (Asghar et al. 2006) and the hypersecretion of insulin in the LOW lambs could potentially expose $\beta$-cells to greater stress with advancing age. Maintenance of glucose tolerance in response to pre-natal undernutrition thus relied on compensatory mechanisms in the pancreas, which were interfered with by high-fat feeding in early post-natal life, and vice versa.

The divergent results obtained from the LOW-HCHF and NORM-HCHF offspring are interesting and indicate that early post-natal dietary exposures and weight gain can lead to completely different outcomes depending on the pre-natal exposures.

Long-term impacts of early life nutritional insults after dietary correction later in life

After being fed the same moderate diet from 6 months to 1½ years of age, it was mainly effects of the pre-natal undernutrition (LOW) that were evident in the adult female sheep. The adult LOW sheep had a reduced ability to clear insulin (higher insulin levels after intravenous administration of exogenous insulin) compared with NORM sheep and also tended to have higher AUC$_{\text{insulin}}$ and reduced insulin sensitivity index (Matsuda & Defronzo index) during the intravenous glucose challenge, indicating reduced insulin sensitivity compared with NORM sheep. The reason why the insulin sensitivity index did not turn out significant after 2 years could be the reduced number of animals compared with 6 months of age.

Furthermore, we found that the ability to respond to arginine was reduced in the sheep which had been exposed to both pre-natal undernutrition (LOW) and post-natal HCHF feeding or both compared with sheep exposed to normal nutrition prior to and after birth (NORM-CONV). Arginine stimulates the release of insulin from secretory vesicles by direct depolarization of the $\beta$-cell membrane, but it does not stimulate insulin synthesis in the ovine $\beta$-cell (Oliver et al. 2001). This indicates that both pre-natal undernutrition and post-natal HCHF feeding can have permanent effects on the ability of the $\beta$-cells to store and release insulin from secretory vesicles.

The reduced glucose tolerance observed in lambs subjected to the LOW-HCHF nutritional combination disappeared upon dietary correction and was no longer evident when the female sheep had become adults. The results support the theory that adverse outcomes of foetal undernutrition, such as glucose intolerance, will not necessarily develop as long as the post-natal nutrition does not diverge from what the individual was predicted to be exposed to (Gluckman & Hanson 2007). But the fundamental mechanisms induced in response to adverse foetal nutritional conditions will persist and may contribute to a permanent predisposition for metabolic disorders.

The endocrine sheep and human pancreas share similarities and a high-fat diet can induce alterations towards the development of pancreatic fibrosis

Several studies have evaluated foetal development of the sheep pancreas, but studies in adult sheep are very scarce (Limesand et al. 2005, Reddy et al. 1988, Green et al. 2010). We are the first to report that a post-natal high-fat diet resulting in elevated levels of plasma NEFA and TG can induce pancreatic alterations towards the development of collagen infiltration in a ruminant animal.

In 75% of the HCHF-fed adolescent lambs (only adolescent males were killed), we observed mild interlobular collagen infiltration. We think that the high-fat feeding could have caused acinar injury, inflammation, activation of stellate cells and consequently stimulation of repair mechanisms which could subsequently lead to fibrosis development (Apte 2012). If the animals had been subjected to even higher cream levels or prolonged crème intakes, the fibrosis development might with time have become even more severe and affected the endocrine part as well. On the other hand, collagen
infiltration was only observed in one adult sheep (female), which had previously been exposed to the HCHF diet, and this particular sheep additionally belonged to the LOW group. Future studies with both genders slaughtered at the same time are required to determine whether the difference in occurrence of pancreatic collagen infiltration between adolescent male lambs and adult female sheep reflects a gender difference with respect to susceptibility to develop pancreatic fibrosis upon exposure to a high-fat diet, or whether the pancreas possesses the capacity to recover after a correction of the diet.

There was a high variability in the structural arrangement and relative expression of α- and β-cells in the pancreatic islets from both lambs and adult sheep. Well-organized mantle-core structures were seen, which resemble the islet structure typically reported for the rodent pancreas (Germani et al. 2008), but islets with a more intermingled structure were very frequent, and they bear closer resemblance to what has been described in humans (Jeon et al. 2009, Green et al. 2010). The sheep and human islet architecture may thus share more similarities compared with rodents, as also suggested by others. We occasionally found glucagon-positive cells in the duct epithelium and glucagon-positive cell clusters right next to the ducts. It requires further investigation to reveal whether these glucagon cells play any specific role for hormone secretion. Likewise, a more comprehensive study with more animals and sampling sites would be required to determine whether a high-fat diet indeed can lead to upregulation of glucagon-positive cells as the semi-quantitative study points at.

In conclusion, we have demonstrated that undernutrition in late foetal life can programme the glucose–insulin axis function in sheep and predispose for reduced glucose tolerance, when a mismatching high-fat diet is fed in early post-natal life. Furthermore, we found that the effects of pre- and post-natal nutrition on insulin secretion and glucose tolerance affected both genders to the same extent, at least until the age of puberty. We are the first to report that an early post-natal high-fat diet can induce pancreatic collagen infiltration in a ruminant animal, just as it can in monogastrics. However, it remains to be revealed whether there are gender-specific differences with respect to the development of pancreatic fibrosis, as only one of the adult females studied had collagen infiltration. The detrimental effects on glucose–insulin axis function, which were caused by introduction to an obesogenic HCHF diet from a few days after birth, could largely be reversed by dietary correction after puberty. On the other hand, long-term effects of the pre-natal diet on the glucose–insulin axis function were still evident in the adult female sheep. This indicates that long-term programming of the glucose–insulin axis function is more sensitive to insults taking place prior to than after the time of birth, which is important in terms of choice of animal model, when late-gestation insults are in focus.

Conflict of interest

None.

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References


Petrik, J., Arany, E., McDonald, T.J. & Hill, D.J. 1998. Apoptosis in the pancreatic islet cells of the neonatal rat is associated with a reduced expression of insulin-like growth factor II that may act as a survival factor. Endocrinology 139, 2994–3004.


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