Depot and sex-specific implications for adipose tissue expandability and functional traits in adulthood of late prenatal and early postnatal malnutrition in a precocial sheep model

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Research

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Abstract

Background Early life malnutrition in the prenatal and postnatal life is well recognized to have long-term implications on the development of offspring and neonate key metabolic organs and their function including adipose tissue later in life. Therefore, the aim of this study was to evaluate the effect of malnutrition in prenatal and early postnatal life on the expandability and functional traits of adipose tissues in adulthood. Twin-pregnant ewes were fed NORM (~ requirements), LOW (50% of NORM) or HIGH (150% and 110% of energy and protein, respectively) diets the last 6 weeks pre-partum (term ~ 147-days). Lambs received moderate, low fat (CONV) or high-carbohydrate-high-fat (HCHF) diets from 3-days until 6-months of age and thereafter CONV diet. At 2½ years of age, histomorphometric and gene expression patterns were characterized in subcutaneous (SUB), perirenal (PER), mesenteric (MES) and epicardial (EPI) adipose tissue.

Results SUB had sex-specific (males < females) upper-limits for adipocyte size and cell-number-indices, irrespective of early life nutrition. PER mass and contents of adipocytes were highest in females and HIGH males while adipocyte cross sectional area was lowest in LOW males. Pre- and postnatal nutrition affected gene expression sex-specifically in SUB and PER, but unrelated to morphological changes. In PER, LOW/LOW males were specific targets of gene expression changes. The EPI was affected by postnatal nutrition as HCHF sheep had enlarged adipocytes and upregulated expressions for adipogenic and lipogenic genes.

Conclusion Upper-limits for SUB expandability were markedly lower in males. Major targets for prenatal malnutrition were PER and males. LOW males had the lowest PER expandability, whereas HIGH males had an adaptive advantage observed as increased hypertrophic ability equivalent to females. Fixed expandability in SUB meant PER became a determining factor for ectopic fat deposition, rendering LOW males particularly predisposed for obesity-associated metabolic risks. EPI, in contrast to other adipose tissues, was a particular target of early postnatal obesity, resulting in adipocyte hypertrophy in adulthood.

Background

Pre- and postnatal malnutrition have distinct impacts on key metabolic organs [1, 2] and adverse consequences for health during the entire lifespan. White adipose tissue formation and differentiation take place during fetal development and continues into the early postnatal period [3]. Previous studies have demonstrated that nutritional perturbations at different stages of gestation can alter adipocyte morphology and key genes involved in the regulation of adipose tissue development, leading to postnatal alterations in the functional properties and accumulation of body fat [2, 4]. We have previously shown that both maternal over- and undernutrition (HIGH and LOW, respectively) during late gestation alters fat deposition patterns as well as adipose gene expression patterns in adolescent sheep and rats [5–8]. This could be ascribed to suppressed expandability of subcutaneous adipose tissue (SUB), resulting in a predisposition for visceral adiposity upon early postnatal development of obesity.

SUB is believed to play a key role in the partitioning of fat deposition [9], and it counteracts fat deposition elsewhere by acting as an energy ‘sink’ in periods of excess energy intake [10]. However, the storage capacity of a single adipocyte appears to be finite (8). According to the adipose tissue expandability hypothesis [11], once the limit for SUB expandability is exceeded, there is therefore an increased risk of redirection of lipid deposition towards other adipose tissues and/or non-lipocyte cell types. As reviewed by Tan and Pidal-Pulg [12], the risk of metabolic disturbances associated with obesity is not so much linked to the amount of fat deposited in the body per se, but primarily to the expandability of adipose tissues and hence capacity for uptake and storage of excess nutrients.

Sex is known to have a major impact on body fat distribution, and males are more susceptible for visceral adiposity and obesity-related diseases than females [13], although the underlying mechanisms for this gender differences are not well understood. It has been shown that sex-specific differences in expression of molecular markers of fat tissue differentiation and/or function appear to emerge already in utero [14, 15], and hence the sex-specific phenotypic manifestation of traits could potentially be sensitive to nutritional insults during gestation. A study in baboons (Papio sp.), demonstrated that maternal suboptimal nutrition during mid-gestation suppressed the growth of male but not female offspring and led to adipocyte hypertrophy accompanied by increased markers of white and brown-type adipogenesis in omental fat [14]. Another study in sheep also showed that young males are at greater risk than females to the onset of comorbidities associated with juvenile-onset obesity, as they had a higher storage capacity of lipids within perirenal-abdominal adipocytes and exhibited raised insulin levels and upregulation of inflammatory markers [13]. Similarly, in sheep, a low birth weight combined with a high fractional growth rate in early postnatal life was associated with an increased expression of lipogenic genes in males only [4].
It is noteworthy that the majority of rodent and human studies addressing long-term implications of fetal nutrition have involved male individuals only, and the female "side of the story" is much less elucidated. In addition, the vast majority of experimental animal studies pertaining to fetal programming have been conducted in altricial species, such as rodents, where the offspring do not undergo an intra-uterine development equivalent to third trimester development in humans.

In the present study, we aimed to test the hypotheses that mismatching combinations of late gestation and early postnatal malnutrition have adverse implications for adipose expandability and functional traits in adulthood, which are differentially manifested in SUB, mesenteric (MES), perirenal (PER) and epicardial (EPI) adipose tissues, respectively and in a sex-specific way. To test this hypothesis, we used a well-documented precocial animal model, the Copenhagen sheep model, for fetal programming [5, 16, 17]. The sheep had been exposed to LOW, adequate (NORM) or HIGH levels of nutrition during the last trimester of fetal development, followed by a restricted CONV or obesogenic, high-fat HCHF diet from 3-days until 6 months of age (right after puberty), and finally, a CONV diet fed ad libitum during the next 2 years. Adipose tissues were sampled at autopsy from the adult sheep when they reached the age of 2½ years.

Material And Methods

Experimental design, animals and diets

The Copenhagen sheep model, experimental design and dietary interventions have been described in detail previously [5, 16]. All the experimental animal handling procedures were approved by the Danish National Committee on Animal Experimentation. In short, a 3 (prenatal nutrition) x 2 (early postnatal nutrition) factorial design experiment was conducted, where 36 twin pregnant multiparous ewes were allocated to one of the following diets during the last 6 weeks of gestation (term~147 days): NORM (N=9, 3 male (♂):6 female (♀))- fulfilling 100% of daily energy and protein requirements, LOW (N=15, 8♂:7♀)- providing 50% of NORM, or HIGH (N=12, 5♂:7♀)- providing 150% of daily energy and 110% of daily protein requirements. From 3 days after birth until 6 months of age (i.e. after puberty), one twin lamb from each dam was fed a low-fat, moderate CONV (N=20, 8♂:12♀) diet (hay supplemented during the first 8 weeks of life with milk replacer; amounts were adjusted to ensure moderate growth rates of appr. 225 g/d). The other twin lamb was fed an obesogenic, high-carbohydrate-high-fat HCHF (N=17, 8♂: 8♀) diet (37% fat dairy cream with milk replacer in a 1:1 ratio (max. 2½ l/d) supplemented with rolled maize (max. 2 kg/d) and barley straw). Subgroups of lambs from each of the 6 treatment groups exited the experiment at 6 months of age. Remaining sheep (N=36) included in this study were from 6 months until 2½ years of age (adulthood) fed the same low-fat hay-based diet (hay ad libitum; supplemented until approx. 1 year of age with barley). Details on the chemical compositions of all feed ingredients used and daily intake of digestible energy and digestible crude protein were tabulated in Supplementary Table 1 and 2, respectively. All animals had ad libitum access to water and a vitamin-mineral supplement at all times. At 2½ years of age (adulthood), all sheep had developed adiposity, and were euthanized by exsanguination following intravenous administration of Propofol (B. Braun, Melsungen, Germany; 5-6 mg/kg body weight [BW]). SUB (above the m. logissimus dorsii), MES, PER, and EPI were immediately isolated, total tissue weight determined (except for EPI) and samples were taken for histology and genes expression analysis.

Tissue preparation and histology

Adipose tissue specimens were fixed in 4% paraformaldehyde (PFA) solution for 24 h followed by 2% PFA for one week. Before embedding, tissues were trimmed and immersed in a 70% ethanol solution for 24 h. Sections were cut at 5 µm and subsequently stained. Epicardial adipose tissue sections were hematoxylin and eosin (HE) stained, which gave the best color differentiation of individual adipocytes in this tissue, whereas the other adipose tissues were stained with iron-hematoxylin to provide a more marked coloring of cell membranes. The slides were scanned at 5x magnification using an automated slide scanner for bright field and fluorescence (AXIO Scan.Z1; Zeiss; Germany).

Histomorphometric and adipocyte size distribution analysis

The cell membrane, shape, and size of adipocytes were identified and measured using a specially designed morphometric application, Iron Hematoxylin Adipose Tissue (APP ID 10113; Visiopharm®, Hoersholm, Denmark), (Additional file 2: Fig. S1). This application
consists of 3 protocols, where the first calculates the relative percentages of different tissue structures in the slide, i.e. adipocytes, cell membranes and undefined area. The second protocol classifies the shape of individual adipocytes according to a 'Form Factor' (Additional file 2: Fig. S1A) ranging from 0 (being a straight line) to 1 (being a perfect circle). The third protocol calculates the cross-sectional area (CSA) of individual adipocytes (Additional file 2: Fig. S1B) and automatically categorizes adipocytes into cell size classes ranging from 0-40, 40-200, 200-400, 400-6400, 6400-12800, 12800-26500, 26500-36000 and >36000 \( \mu \text{m}^2 \). The cell size class >36000 \( \mu \text{m}^2 \) was not included in calculations, since it contained a high proportion of cells with inadequately stained membranes, i.e. the program could not identify the individual cells. The average CSA for cells determined on the slides will expectedly be lower than the average diameter of cells measured at their centers, because cells in slides were cut at varying distance from their center. However, changes in distribution patterns are expected to reflect differences in the size of cell populations. A cell number index (CNI; arbitrary units) was calculated as previously described [8]:

\[
\text{CNI} = \frac{\text{adipose mass (kg) x percentage adipocyte coverage in tissue slides}}{\text{volume of an average spherical adipocyte}}.
\]

The volume of spherical adipocyte was calculated using the formula:

\[
V = \frac{4}{3} \pi r^3
\]

Where the radius, \( r \), was derived from a circle with the same area as the average CSA of adipocytes. Cell size distribution patterns, average CSA and CNI allowed us to evaluate whether differences in fat deposition resulted from changes in adipocyte numbers or size.

**Gene expression analysis**

Samples of adipose tissues were preserved in RNA*late®* Solution (Ambion, The RNA Company, Austin, Texas, USA) for 24 hours, thereafter the solution was discarded and tissue samples then stored at -80 °C pending mRNA expression analysis, conducted as previously described [8]. In short, total RNA was isolated by homogenizing approximately 150 mg tissue (TissueLyser II, QIAGEN, Hilden, Germany) in 1000 \( \mu \text{l} \) TRItol® reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA), and cDNA synthesized from purified RNA was then used for analyses of gene expression by real-time qPCR using a LightCycler 480 SYBR Green I Master (Roche Diagnostic GmbH, Mannheim, Germany) and using \( \beta \)-actin (\( \text{ACTB} \)) as a reference gene. The genes examined involved target genes for adipogenesis (\( \text{ADIPOQ, CD34, CD44, CEBPB, PGC1A, PPARA, PPARG, PREF1, TGF\beta1, and WNT5A} \)), angiogenesis (\( \text{VEGF, and VEGFA} \)), lipid (\( \text{ADRA1, ADR\beta1, ATGL, CGI58, FABP4, FAS, HSL, LPL, and PLIN1} \)) and glucose (\( \text{FBPASE, GAPDH, GLUT1, and GLUT4} \)) metabolism, hormone signaling (\( \text{Gcr, IGF1R, IRS1, and LEPTIN} \)), energy homeostasis (\( \text{FTO} \)), mitochondrial-derived reactive oxygen species synthesis (\( \text{UCP2} \)), as well as markers for inflammation (\( \text{CD68, IL6, MCP1, TLR4, and TNFA} \)). The primer sequences and efficiencies are listed in Additional file 1: Table S3.

**Statistical analysis**

Data were analyzed separately for each adipose tissue by linear mixed effects (nlme) (version 3.1-137), and emmeans (v1.3.3 and v1.4.5) [18] procedures of the R studio (R Core Team, 2017) software package using the following overall model:

\[
Y = \text{PreN} + \text{PostN} + \text{Sex} + \text{PreN*PostN} + \text{PreN*Sex} + \text{PostN*Sex} + BW + BW6 + BW2\frac{1}{2} + BW\text{ewe}
\]

Where \( Y \) is the observed dependent variables, \( \text{PreN} \) is the fixed effect of prenatal nutrition (NORM, LOW, HIGH), \( \text{PostN} \) is the fixed effect of postnatal nutrition (CONV, HCHF), \( \text{Sex} \) is the fixed effect of sex (\( m \)=males, \( f \)=females) on adipose morphological traits, adipocyte size distribution, and mRNA expression. Body weights of the dams at the onset of the experiment 6 weeks pre-partum (\( BW\text{ewe} \)) and of the experimental sheep at birth (\( BW \)), at six months of age, i.e. when the differential postnatal feeding ended (\( BW6 \)), and by the end of the experiment at 2½ years of age (\( BW2\frac{1}{2} \)) were included as covariates in the models.

The normality of the model was examined using the Shapiro-Wilk's test and qqnorm plots of the residuals. If the model did not follow a normal distribution, the model was normalized by log-transformation. The stepwise model reduction was further performed using package MASS (v7.3-51.5) and the model having the lowest AIC was selected for the best-fit model. *Post hoc* analysis was performed using Tukey's multiple comparison tests when one of the main effects or their interactions were significant. Data are presented as
emmean ± SEM, and P-values provided are based on ANOVA. Correlations between different adipocyte size classes were examined using R package “corrplot” (Version 0.84) [19], and Pearson correlation coefficients were derived from correlation plots.

Results

Adult sheep characteristics

As previously reported (10), LOW sheep were born with lower birth weights compared to HIGH and NORM. Compared to NORM, both LOW and HIGH sheep deposited a greater amount of MES and PER than SUB mass, when they became obese as adolescents upon exposure to the HCHF diet. At the age of 2½ years, all sheep had been exposed to the same low-fat hay-based diet for 2 years; LOW-HCHF sheep (99.0 kg) became heavier as adults compared to LOW-CONV and NORM-CONV sheep (91.1-91.2 kg) with others groups in between. Males were heavier (99.4 kg) than females (92.9 kg). The adult LOW-HCHF sheep had markedly increased plasma levels of cholesterol, urea, creatinine, and lactate compared to other groups [16].

Overall tissue and sex-specific differences in adipose cell size distribution

Distribution of adipocytes on size classes followed a unimodal pattern in SUB and EPI (Fig. 1A and 1B), whereas a clear bimodal pattern was observed in PER with the second peak falling in in different cell-size classes depending on treatment group (Fig. 1C). In MES, there was a plateau across the lower (<800 µm²) cell-size classes, which continued in NORM (unimodal pattern) or was followed by a more or less clear second peak in the other groups (bimodal pattern) (Fig. 1D).

As shown in Table 1-2 and Fig. 1, females had the highest fat mass (not determined in EPI), adipocyte CSA (except for HIGH in PER), CNI (PER only), and proportions of very small (<40 µm²; PER and MES only) and large adipocytes (SUB: >3200 µm²; MES and PER: >6400 µm²), but lowest proportions of smaller-medium sized adipocytes (SUB: 40-1600 µm²; PER: 800-6400 µm²; MES: 800-3200 µm²). However, in EPI, only few sex effects were seen, and only as interactions with the postnatal nutrition (see below).

In all tissues, positive correlations existed between adipocytes numbers within the smaller cell-size classes (SUB and MES: 200-1600 µm², P<0.001 and P<0.001-0.05; PER: 200-3200, P<0.01-0.001 µm²; EPI: 40-1600 µm², P<0.001-0.05) and within the large cell-size classes (SUB: 3200-36000 µm², P<0.001-0.01; PER and MES: >12800 µm², P<0.001-0.01; EPI: >6400 µm², P<0.001-0.05). Correlations between the small and large cell size classes were generally negative (Additional file 2: Fig. S2A-2E), except for the very smallest (<40 µm²) adipocytes in PER and MES, which (unlike other small cells) were positively correlated with numbers of the largest adipocytes (PER: 12800-36000 µm², P<0.001; MES: >36000 µm², P<0.05) (Additional file 2: Fig. S2B and S2C).

Long-term impacts of early nutrition history on adipose tissue histology

Implications of malnutrition in early life on adipose morphology and gene expression patterns were, in general, more predominant in males than females, but in a tissue dependent way.

Subcutaneous adipose tissue

There were generally no prolonged effects of early life nutrition history on SUB mass, CNI, average adipocyte CSA (Table 1) or adipocyte size distribution (Fig. 1A). The only exceptions were for very small adipocytes (<40 µm²), where NORM and HIGH had a higher percentage compared to NORM and with other groups in between (P=0.01). The proportion of 40-200 µm² adipocytes was increased by HCHF compared to CONV in HIGH, but decreased by HCHF in NORM and LOW sheep (Additional file 1: Table S4; P=0.02).

Perirenal adipose tissue
This was the adipose tissue most affected by the prenatal nutrition history, but in a sex-specific way. LOW♀ had the highest and LOW♂ (followed by NORM♀) had the lowest PER mass, average CSA of adipocytes and adipose cell coverage (P=0.01, 0.002, and 0.01 respectively) with other groups in between.

In the first cell size peak (40-400 µm²), NORM♀ had the highest and NORM♂ the lowest ratio of adipocytes, (P<0.01). In the second cell size peak, the NORM♀ and LOW♀ peaked earlier and had a higher ratio of cells in the 1600-6400 µm² classes and lowest proportions of cells in the largest adipocyte classes, whereas HIGH♀ together with all females peaked in the 6400-12800 µm² cell size classes (P<0.01). LOW♂ had a markedly higher proportion of large adipocytes (>12800 µm²) (P<0.001) compared to other groups. Interestingly, HIGH♂, unlike NORM♀ and LOW♀, had a phenotype similar to female sheep for most of the studied parameters (adipocyte CSA, tissue composition, and cell size distribution).

Surprisingly, sheep fed the HCHF diet in early postnatal life had a lower adult PER mass than CONV sheep (P=0.02) (Table 2). This was associated with a slightly smaller adipocyte average CSA and a lower proportion of the largest (25600-36000 µm²), but increased proportion of medium sized adipocytes (3200-6400 µm²) in HCHF compared to CONV (P=0.03 and 0.04, respectively; Supplementary Table 3). For medium-large adipocytes (6400-25600 µm²), the proportion was increased by HCHF in NORM and LOW sheep, but decreased by HCHF in HIGH sheep (P=0.01 and 0.005; Additional file 1: Table S4). The CNI was not affected by early life nutrition history (Table 1).

Mesenteric adipose tissue

There were no systematic long-term impacts of the early postnatal nutrition exposure in this tissue. Impacts of the prenatal nutrition history were sex-specific. Hence, LOW♀ and NORM♀ had clear peaks in the lower cell size classes (1600-3200 and 3200-6400 µm²) and lowest proportion of the largest adipocytes compared to other animals; the adipocyte distribution pattern for HCHF♀ was very similar to that of females rather than other males, and in the largest cell size classes (>12800 µm²), LOW♀ had by far the highest proportions of adipocytes and with LOW♂ at the other extreme (lowest proportion) (Fig. 1D; P=0.00001-0.008).

Epicardial adipose tissue

The PER was, in contrast to the other tissues, strongly affected by the early postnatal nutrition history with hardly any effects of prenatal nutrition (Table 1). HCHF sheep had larger average adipocyte CSA (Additional file 1: Table S5) and a shift in cell size distribution (Fig. 1B) towards higher proportions of both small (40-200 µm²; P=0.01) and large (>6400 µm²; P=0.003 to <0.001) at the expense of medium sized adipocytes (1600-3200 µm²; P<0.0001). The HCHF diet increased and decreased proportions of medium-large (3200-6400 µm²) and medium (800-1600 µm²) sized adipocytes, respectively, in females, whereas males had the opposite responses to HCHF (P=0.02 and 0.01, respectively).

Correlations between cell-number-index and numbers of very small (<40 µm²) and very large adipocytes (25000-36000 µm²) across tissues

Within SUB and MES, CNI was negatively correlated to adipocytes numbers in the largest cell size class (25600-36000 µm²) (r=-0.39 and -0.31, respectively; P<0.05) (Additional file 2: Fig. S2E). Across tissues, CNI in PER was positively correlated to numbers of very small adipocytes in SUB (r=0.37, P<0.05) and to numbers of very large adipocytes in SUB (r=0.42, P<0.05) and MES (r=0.48, P<0.01). Numbers of very large adipocytes in MES were positively correlated to numbers of very small adipocytes in PER (r=0.28, P<0.05) and to very large adipocytes in SUB (r=0.33, P<0.05) and PER (r=0.50, P<0.01).

Systematic sex differences in mRNA expression levels
Across the four tissues studied, males had consistently and markedly higher expression levels than females for almost all genes in all adipose tissues (Additional file 2: Fig. S3), although males only had around half or less fat mass in these tissues (not determined in EPI) compared to females (Table 2). The only genes, where females had the highest expression level, were \textit{LPL} in SUB and EPI, \textit{LEPTIN} and \textit{CD68} in PER and \textit{CGI58} in EPI.

\section*{Long-term impacts of early nutrition history on mRNA expression levels}

The \textit{ADRB1}, \textit{FTO}, and \textit{LEPTIN} were the only genes, for which expression levels were unaffected by the early life nutrition history.

\subsection*{Subcutaneous adipose tissue}

\textit{LPL} was the only gene affected independently of other factors by the prenatal nutrition, and LOW had the lowest expression levels followed by NORM and HIGH (Fig. 2A; \textit{P}=0.031). The mRNA expression of 17 genes involved in adipose development and metabolism were affected by the prenatal nutrition in a sex-specific way (Fig. 2B). For all except one gene (\textit{ATGL}), NORM had higher mRNA expression levels (Fig. 2B; \textit{P}<0.0001-0.05), whereas for \textit{ATGL}, the expression level was higher in HIGH and LOW than other groups. In females, if anything, expression levels for several genes were consistently different (higher or lower) in LOW compared to NORM and HIGH.

Regarding early postnatal nutrition, HCHF sheep had decreased expression levels for \textit{GLUT1}, \textit{PGC1A}, and \textit{TNFA}, and increased \textit{PPARG} expression (Additional file 2: Fig. S4A; \textit{P}=0.004-0.03). For 5 other genes (\textit{CD44}, \textit{CEBPB}, \textit{FBPASE}, \textit{IL6}, and \textit{MCP1}), higher expression levels were observed in CONV compared to other groups. For 15 genes (\textit{ADIPOQ}, \textit{CD44}, \textit{CEBPB}, \textit{CGI58}, \textit{FBPASE}, \textit{GADPH}, \textit{HSL}, \textit{IGF1R}, \textit{IL6}, \textit{IRS1}, \textit{MCP1}, \textit{PLIN1}, \textit{PGC1A}, \textit{VEGF}, and \textit{VEGFA}) expression levels depended on the pre- and postnatal interaction, and highest expression levels were observed in NORM-CONV compared to all other groups (\textit{P}=0.003-0.05). The only deviation from this pattern was that HIGH-HCHF had the highest \textit{LPL} expression (\textit{P}=0.02).

\subsection*{Perirenal adipose tissue}

In PER, 17 genes were affected by the prenatal nutrition and 6 of them independently of other factors: LOW had the highest expression levels compared to other groups for \textit{CGI58}, \textit{FABP4}, \textit{GLUT1}, \textit{IRS1}, and \textit{VEGFA}, or compared to HIGH for \textit{IGF1R} (Fig. 3A; \textit{P}=0.0.03-0.03). For 6 genes, the prenatal impacts were sex-specific (Fig. 3B), where the general pattern was that LOW attained the highest expression levels compared to other groups (\textit{CD44}, \textit{GcR}, \textit{HSL}, and \textit{TGF\beta1}) except that NORM achieved the highest levels for \textit{CEBPB} and \textit{GLUT4} (Fig. 3B; \textit{P}=0.0001-0.02). For 5 genes (\textit{HSL}, \textit{IL6}, \textit{MCP1}, \textit{UCP2}, and \textit{VEGF}), significant pre- and early postnatal nutrition interactions were found, but no consistent patterns of changes could be deciphered across all groups (Fig. 3C).

Only 3 genes were affected by the early postnatal nutrition independently of other factors, and HCHF sheep had the highest expression levels for \textit{CEBPB} and \textit{PLIN1}, but lowest for \textit{CD68} (Additional file 2: Fig. S4B; \textit{P}=0.01-0.02). Similar to SUB, expression levels were highest in CONV compared to other groups for three genes, \textit{FABP4}, \textit{GLUT4}, and \textit{VEGF} (Additional file 2: Fig. S4B; \textit{P}=0.0007-0.3). Females, if anything, tended to have the opposite response to the postnatal diet compared to males.

\subsection*{Mesenteric adipose tissue}

The MES was the depot least affected by early life nutrition history in terms of gene expression patterns. Expression levels of only 4 genes were affected by the prenatal nutrition in either a sex-specific way (\textit{CD34}, and \textit{CEBPB}; \textit{P}=0.01) or depending on the subsequent early postnatal nutrition exposure (\textit{CD34}, \textit{FABP4}, and \textit{PPARG}; \textit{P}=0.02-0.03), but it was difficult to discern any systematic patterns (results not shown). Furthermore, postnatal nutrition had an impact on expression levels of only one gene, namely \textit{ADRA1}, where expression levels were highest in HCHF sheep (Additional file 2: Fig. S4C; \textit{P}=0.03).
Epicardial adipose tissue

The prenatal nutrition history affected the expression of 14 genes in EPI, but only independently of other factors for two genes. Thus, ADR47 expression levels were reduced in HIGH compared to NORM with LOW in between, and GLUT4 expression levels were reduced in LOW compared to HIGH and NORM (Fig. 4A; P=0.04 and 0.02, respectively). For another 7 genes (ATGL, IGF1R, Gcr, LEPR, TGFB1, UCP2, and VEGF), the prenatal impact on expression levels was sex-dependent due to opposite responses in LOW compared to LOW in the prenatal nutrition (Fig. 4B).

Of all the adipose tissues, EPI was affected most by the early postnatal nutrition history. HCHF compared to CONV sheep had increased expression levels for 6 genes (ADIPOQ, FABP4, FAS, HSL, PLIN1, and PPARG) and reduced for 2 (CD68, and PPARA) (Additional file 2: Fig. S4D). For 5 genes (ATGL, CD68, CGI58, GLUT1, and TLR4) postnatal nutrition impacts depended on the prenatal nutrition history, and the general pattern was that LOW-CONV had the highest expression levels for these, and the changes in expression levels induced by HCHF were opposite in LOW compared to CONV and HIGH sheep (Fig. 4C; P=0.05-0.05). CONV had higher expression levels compared to other groups for two genes, namely CD34, and GLUT1 (Additional file 2: Fig. S4D; P=0.04 and 0.01, respectively).

Discussion

To be able to put the findings of this study in perspective, a summary will be given of findings relating to adipose tissue in the subgroup of lambs from the same experiment, which were studied at 6 months of age. The LOW and HIGH nutrition in late fetal life reduced intrinsic cellularity (CNI) in both SUB and MES and reduced obesity-induced hyperplasic ability in both SUB, MES, and PER. Consequently, when LOW and HIGH lambs were fed the HCHF diet and became obese, expansion of their fat mass in these tissues relied more on hypertrophic rather than hyperplasic growth. Subcutaneous adipocytes appeared to have a fixed upper limit for hyperplasic expansion irrespective of the overall degree of adiposity as observed across groups of CONV and HCHF fed lambs. In HIGH and LOW lambs fed the HCHF diet as compared to CONV-HCHF lambs, a larger proportion of fat deposition was directed towards PER and MES rather than SUB [8]. An approximately 9-fold increase in PER fat mass in HCHF compared to CONV fed lambs was associated with 1/3 reduction in kidney size [5].

Persistent effects of fetal programming on functional traits of adipose tissues in adulthood

Findings in the adult sheep confirmed that subcutaneous adipocytes have a quite fixed, but sex-specific, upper limit for hypertrophic growth. The impacts of late gestation nutrition on adipose cellularity in SUB and MES observed in lambs were not evident in adulthood. PER was the primary target, followed by SUB, of prenatal nutritional programming with persistent and sex-specific implications in adulthood for expandability capacity and other functional traits. Any differences in phenotypic traits in MES that could be related to the early nutrition history appeared to be primarily indirect consequences of changes in PER and SUB. In contrast to the other tissues, differences in the adult phenotype of EPI was mainly related to the early postnatal nutrition. This will be discussed in greater detail in the following.

Early life nutrition impacts on subcutaneous, perirenal and, mesenteric and their integrated functions

Adipocyte size distribution had a unimodal appearance in SUB and bimodal appearance in PER and MES. From studies in humans, a bimodal pattern has been reported for all of these tissues [20, 21], suggesting two separate cell populations. The discrepancy for SUB may be due to species or sampling site-specific differences [22] or degree of adiposity. Subjects included in the two human studies were in contrast to our sheep morbidly obese, and we actually did observe a second “shoulder” in the cell size distribution, which together with correlation structures between cell size classes indicated that two distinct cell size populations may indeed exist in all of these three tissues also in sheep.

Adipose tissue growth occurs by hyperplasia and hypertrophy, where the former normally occurs only until a certain age is reached, and thereafter, adipose expansion relies on hypertrophy of existing adipocytes (22–28). However, in the case of extreme obesity, hyperplasic cellular expansion can be reactivated (29). The CSA determined in the histological assessments is expected to underestimate the actual size of adipocytes, as already mentioned, but the maximum cell sizes observed should be indicative of the CSA of the largest cells, when cutting at their mid-plane. Thus, in SUB there appeared to be quite fixed, but sex-specific, upper limits for adipocyte size, irrespective of the early nutrition history, as we also observed in the younger lambs. This distinguished SUB from PER and MES. Subcutaneous adipocytes appear to have fixed upper limits for hypertrophy also in humans, and metabolically healthy obese and
metabolically unhealthy obese patients have very similar average subcutaneous adipocyte sizes [23], despite different fat distribution patterns in the body.

The previously reported [8] quite marked reduction in intrinsic, non-obese cellularity (CNI) in SUB and MES in the 6 months old LOW and HIGH compared to NORM lambs were not evident in the adult sheep. This indicates there must have been a time window for compensatory hyperplastic expandability in these tissues after puberty, which was uncoupled from the development of obesity. The male sheep had a substantially lower SUB accumulating capacity as also observed in humans [21–23]. This can explain the greater propensity in males for redirection of fat deposition in situations of excess nutrient intakes towards visceral adipose tissues and the accumulation of triglycerides in non-adipocyte cell types [22]. In agreement with this, human studies have suggested that SUB may be more of an initiating factor in the process of redistribution of fat overflow for deposition at other sites rather than being directly implicated in the development of the metabolic dysfunctions relating to obesity [23].

Visceral adipose tissues are located in the thorax and abdomen and consists of fat deposits in e.g. the omentum, mesenterium, retroperitoneum, pericardium and around the kidneys [24, 25]. Fat accumulation around and within the renal sinus is known to play a crucial role for kidney functions [26, 27]. However, contrary to SUB, very little is known about the specific role of PER in relation to development of obesity and associated disorders in humans due to issues of accessibility, where indirect measurement using ultrasound and other non-invasive approaches are the most commonly used in humans [28]. In rodents, most studies on visceral adipose tissue have been conducted on epididymal fat, which does not exist in humans and sheep, and although it is presumed to have similar characteristics to omental fat [22], it has been reported that it is dissimilar to visceral tissues found in humans [25].

The present study points to PER as a major target of nutritional programming, both in late gestation and early postnatal life, a finding in line with rapid maturation and deposition of PER in these stages of life [3]. PER appears to be an even more important target for early life nutrition than SUB and may be an important determinant for disadvantageous intra-abdominal and ectopic fat deposition. Excessive PER adipocyte hypertrophy during early postnatal life appeared to compromise the expandability of this tissue later in life. PER was the only of a large number of tissues studied [16] that did not increase, but rather decreased its gross weight from adolescence to adulthood in HCHF sheep, and this was associated with redirection of intra-abdominal fat deposition towards MES. The underlying reason for the loss of, or collapse in, PER expandability in the previously obese sheep is unknown, and it could not be related to changes in expression of the genetic markers examined in the present study.

A range of genes involved in adipose development and metabolisms was upregulated in LOW and particularly LOW↓ sheep, but despite this, these expression changes were poorly related to changes observed in cell size distribution and adipocytes expandability. Similarly, poor association also existed between gene expression and expandability traits in SUB. Other genetic markers than the ones included in the present study may have been responsible for observed morphological changes in both tissues.

An intriguing finding was that a HIGH level of nutrition during fetal life appeared to offer some protection towards adverse changes in PER expandability in males, and HIGH↓ in fact, attained a more female phenotype with respect to adipocyte size distribution and had twice as much PER fat compared to LOW↓ and NORM↓ (Tables 1 and 2).

Apparently, the lack of expansion of PER fat mass from adolescence into adulthood, allowed for compensatory kidney growth in HCHF sheep, since no differences were observed in kidney weight between CONV and HCHF sheep. However, it cannot be ruled out that the suppression of kidney growth in obese lambs may have had implications for kidney function in adulthood. Previous studies (reviewed in [26]) demonstrated that the infiltration of adipocytes into the renal sinus could compress the renal vein and artery, leading to increased interstitial pressure, and excessive PER fat deposition could similarly modify kidney function simply due to compression [27]. In a cross-sectional study performed on type-II diabetic patients [27] and in the Framingham Heart Study [26], PER fat thickness was found to be a determining factor for kidney dysfunction, and individuals with “fatty kidneys” (high amount of renal sinus fat) had increased risk of hypertension and chronic kidney diseases.

In this context, it is interesting that the nutritionally mismatched LOW-HCHF sheep became hypercholesterolemic, hyperuricemic and hypercreatinemic in adulthood compared to all other groups (12). Hypertrophic expansion of adipocytes increases the risk for the overflow of fatty acids and cholesterol into non-adipocyte cell types [29], whereas adipose expansion by formation of larger numbers of smaller adipocytes offers protection from metabolic disorders associated with obesity. Within PER, and to a lesser extent MES, numbers of very large adipocytes were positively correlated to a specific class of very small adipocytes (< 40 µm²), but negatively to other small adipocytes and CNI. In studies with human obese subjects, it has been shown that occurrence of a populations of such very small adipocytes in SUB was associated with development of insulin resistance in equally obese subjects [20, 30]. Future studies are
needed to determine to what extent fetal programming of PER expandability, especially in males, can impact renal function and associated risk of development of such metabolic disorders in adulthood.

It is tempting to speculate that unlike SUB and PER, MES is not a primary target of early nutrition programming, since hardly any effects on gene expression patterns could be detected. Changes in the adipocyte expandability in MES could therefore be indirect consequences of expandability changes in SUB and PER, which agrees with the positive correlations observed between numbers of large adipocytes in SUB and PER with numbers of large adipocytes in MES.

**Epicardial adipose tissue is a target of primarily early postnatal programming**

To date, scientific literature offers very limited information on the ontogenesis of EPI, and not least implications of early life malnutrition on this tissue in comparison with the previously mentioned adipose tissues. In human fetuses, EPI was detected as early as 20–28 weeks of gestation [31, 32].

Fat surrounding the heart is associated with artery diseases independent of amount of visceral adipose tissue [24]. In our study, late gestation malnutrition had negligible impacts on adipose expandability traits and gene expression patterns in EPI in adulthood. Epidemiological studies in humans have shown that a combination of maternal undernutrition in the early stage of pregnancy followed by overnutrition from day 60 up to term promoted increased the risks for adult cardiovascular diseases [33–35]. The discrepancies between human and our sheep study is presumably due to differences in the timing of the nutritional insult.

In humans, exposure to energy dense diets and rapid catch-up growth in the early postnatal period is known to alter the metabolism and functionality of EPI, which may have adverse implications for cardiovascular health later in life [36, 37]. Under pathological conditions, hypertrophy of epicardial adipocytes may be associated with accumulation of lipids in the wall of the proximal coronary arteries [38] and in the left atrium along the adventitia [39], since there is no muscle fascia separating EPI from the myocardium. In agreement with this, the obesogenic HCHF diet fed in early postnatal life in the present study gave rise to adipocyte hypertrophy in EPI, which was associated with upregulation of both adipogenic and lipogenic genes. This demonstrates that EPI behaves distinctly different compared to SUB, PER, and MES, being the main target of early postnatal but not late gestation nutritional programming. Early postnatal obesity gave rise to enlarged epicardial adipocytes, and this could not be completely reversed by dietary correction and associated weight loss later in life.

**Conclusion**

SUB had upper-limits for hypertrophy, which were unrelated to early nutrition history. Adipose cellularity, adipocyte size and hence expandability capacity of SUB, PER, and MES were markedly lower in males than in females. PER was the major target of prenatal malnutrition with altered expression levels for a range of genes in LOW and specifically LOW!, which also had reduced adipocyte hypertrophic ability in PER. Prenatal HIGH nutrition, on the other hand, offered an adaptive advantage for males, by increasing PER hypertrophic ability in HIGH! towards the level observed in females. Other genes than the ones included in the present study may have been responsible for these morphological adaptations. With fixed upper-limits for expandability in SUB, the PER expandability appears to be a determining factor for patterns of intra-abdominal fat deposition, rendering LOW and particularly LOW! predisposed for MES and ectopic lipid accumulation with associated metabolic risks in adulthood. Contrary to the other three tissues, EPI was a major target of early postnatal obesity development, which tracked into adulthood as increased expression levels for adipogenic and lipogenic genes and adipocyte hypertrophy.

**Abbreviations**

BW; body weight/birth weight, BWewe; dam body weight at the onset of the experiment 6 weeks pre-partum, BW6; lamb body weight at 6 months of age, BW2½; sheep body weight at 2½ years of age, CONV; low-fat moderate diet, CNI; cell number index, CSA; cross-sectional area, EPI; epicardial adipose tissue, HCHF; high-carbohydrate-high-fat diet, HE; hematoxylin and eosin, HIGH; late gestation overnutrition, LOW; late gestation undernutrition, MES; mesenteric adipose tissue, NORM; late gestation nutrition fulfilling norms dietary recommendations, PFA; paraformaldehyde, PER; perirenal adipose tissue, SUB; subcutaneous adipose tissue

**Declarations**

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Consent for publication

Not applicable

Authors’ contributions

MON was the principle investigator of this project. SA and MON interpreted the histomorphometric and gene expression data and were the major contributors in writing the manuscript. LKL and MM carried out the gene expression analysis. SA and MM performed the histomorphometric analysis of the adipose tissue. RD and SA performed the statistical analysis. JSA and PK contributed to the revision of the article. MON had the primary responsibility for the content of the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable

Ethics approval and consent to participate

All of the experimental animal handling procedures were approved by the Danish National Committee on Animal Experimentation.

Competing interests

The authors declare that they have no competing interests.

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Tables

**Table 1** Effects of prenatal nutrition and sex on the morphological composition of adipose tissues in sheep
| Sex | Sex | Lambda | Lambda | sex | Ewed* |
|-----|-----|--------|--------|-----|------|
| 0   | 0   | 0.54   | 1.60   | 0.40| 0.11 |
| 3.5%| 6%  | 0.70   | 0.70   | 0.06| 0.24 |
| 4.7%| 5%  | 0.70   | 0.70   | 0.06| 0.24 |
| 10.2%| 12%  | 0.70   | 0.70   | 0.06| 0.24 |
| 17.1%| 14%  | 0.70   | 0.70   | 0.06| 0.24 |
| 20.1%| 18%  | 0.70   | 0.70   | 0.06| 0.24 |
| 23.3%| 21%  | 0.70   | 0.70   | 0.06| 0.24 |
| 26.3%| 24%  | 0.70   | 0.70   | 0.06| 0.24 |
| 30.0%| 28%  | 0.70   | 0.70   | 0.06| 0.24 |
| 33.1%| 31%  | 0.70   | 0.70   | 0.06| 0.24 |
| 36.2%| 34%  | 0.70   | 0.70   | 0.06| 0.24 |
| 39.3%| 37%  | 0.70   | 0.70   | 0.06| 0.24 |
| 42.4%| 40%  | 0.70   | 0.70   | 0.06| 0.24 |
| 45.5%| 43%  | 0.70   | 0.70   | 0.06| 0.24 |
| 48.6%| 46%  | 0.70   | 0.70   | 0.06| 0.24 |
| 51.7%| 49%  | 0.70   | 0.70   | 0.06| 0.24 |
| 54.8%| 52%  | 0.70   | 0.70   | 0.06| 0.24 |
| 57.9%| 55%  | 0.70   | 0.70   | 0.06| 0.24 |
| 61.0%| 59%  | 0.70   | 0.70   | 0.06| 0.24 |
| 64.1%| 62%  | 0.70   | 0.70   | 0.06| 0.24 |
| 67.2%| 65%  | 0.70   | 0.70   | 0.06| 0.24 |
| 70.3%| 68%  | 0.70   | 0.70   | 0.06| 0.24 |
| 73.4%| 71%  | 0.70   | 0.70   | 0.06| 0.24 |
| 76.5%| 74%  | 0.70   | 0.70   | 0.06| 0.24 |
| 79.6%| 77%  | 0.70   | 0.70   | 0.06| 0.24 |
| 82.7%| 80%  | 0.70   | 0.70   | 0.06| 0.24 |
| 85.8%| 83%  | 0.70   | 0.70   | 0.06| 0.24 |
| 88.9%| 86%  | 0.70   | 0.70   | 0.06| 0.24 |
| 92.0%| 89%  | 0.70   | 0.70   | 0.06| 0.24 |
| 95.1%| 92%  | 0.70   | 0.70   | 0.06| 0.24 |
| 98.2%| 95%  | 0.70   | 0.70   | 0.06| 0.24 |
The values are expressed as emmean ± SEM derived from proportions (%) with 95% confidence interval. The effects were significant P < 0.05. abc Significant differences between groups are denoted by different superscript letters within row.

Number of animals per groups are; SUB: NORM (N=9, 3:6); HIGH (N=12, 5:7); LOW (N=15, 8:7). PER: NORM (N=10, 4:6); HIGH (N=12, 5:7); LOW (N=15, 8:7). MES: NORM (N=10, 4:6); HIGH (N=12, 5:7); LOW (N=15, 8:7). EPI: NORM (N=10, 4:6); HIGH (N=12, 5:7); LOW (N=15, 8:7). Values for effects of the early postnatal nutrition are presented in Supplementary Tables 2 and 3.

**Table 2** Effect of pre- and early postnatal nutrition on the weight of adipose tissues in sheep

| Prenatal nutrition | NORM | HIGH | LOW | P-Value |
|--------------------|------|------|-----|---------|
|                     | N   |     |     |         |
| Ewed*Sex           | 1   |     |     |         |
| Ewed               | 2   |     |     |         |
| Lambd*Sex          | 3   |     |     |         |
| Lambd              | 4   |     |     |         |
| Sex                | 5   |     |     |         |
| Ewed*Lambd         | 6   |     |     |         |
|                     |     |     |     |         |
| ht (g)             | 241±59 | 1242±283 | 292±89 | 0.40 | 0.52 | 0.73 | 0.83 | <.0001 | 0.36 |
|                    | 707±291ab | 2662±246abc | 1708±262abc | 0.008 | 0.81 | 0.97 | 0.02 | <.0001 | 0.10 |
|                    | 2199±533abc | 4719±447abc | 2997±590abc | 0.22 | 0.41 | 0.69 | 0.09 | <.0001 | 0.69 |

NORM, HIGH, LOW, CONV, HCHF, Ewed, Ewed*Sex, Lambd, Lambd*Sex and, Ewed*Lambd: see legends to Figure 1. The values are expressed as emmean ± SEM derived from proportions (%) with 95% confidence interval. abc Significant differences between groups are denoted by different superscript letters within row.

Number of animals per groups are; NORM (N=10, 4:6); HIGH (N=11, 5:6) and; LOW (N=16, 8:8). For the lambd effect, HCHF (1768±145g) animals had less PER mass than CONV (2021±1365g) animals.

**Figures**
Figure 1

Effect of pre- and early postnatal nutrition on the adipocyte size distribution patterns in tissue slides of (A) subcutaneous (SUB), (B) epicardial (EPI), (C) perirenal (PER) and (D) mesenteric (MES) adipose tissue from male (♂) and female (♀) 2½ years old adult sheep. Values are expressed as the proportion (%) of adipocytes found in different cell size classes. Tissue slides were stained with Iron-Hematoxylin except for EPI, which was stained with Hematoxylin and eosin for optimal staining of cell membranes. Cross-sectional area of individual cells was determined in whole tissue scans using the Iron Haematoxylin Adipose Tissue software (APP ID 10113; Visiopharm®, Hoersholm, Denmark). Values are expressed as percentages. All sheep were born as twins from mothers, which during the last 6 weeks of gestation (term~147 days) had been exposed to NORM (fulfilling 100% of daily energy and protein requirements); HIGH (fulfilling 150% of energy and 110% of protein requirements, respectively); or LOW (50% of NORM) levels of nutrition. From 3-days of age until 6 months of age (post-puberty), one twin was fed a CONV diet (milk replacer during the first 8 weeks of life and exclusively hay thereafter, and adjusted in amounts to achieve moderate constant growth rates of approx. 225 g/day) and the other twin a HCHF diet (high carbohydrate (starch)-high-fat diet (37% fat dairy cream mixed with milk replacer in a 1:1 ratio (max. 2½ l/day) supplemented with rolled maize (max. 2 kg/d)). From 6 months until 2½ years of age, all sheep were fed with the same CONV (low-fat hay-based diet). Number of animals in the different groups: NORM (N=9, 3♀:6♂); HIGH (N=12, 5♀:7♂) and; LOW (N=15, 8♀:7♂). Ewed, Lambd, sex, Ewed*sex, Lambd*sex, Ewed*Lambd indicate significant effects found of the late gestation nutrition, early postnatal nutrition, sex of the sheep or any interactions.
Figure 2

Effects of prenatal nutrition (A) and sex-dependent effects of the prenatal nutrition (B) on mRNA expression patterns in subcutaneous adipose tissue (SUB), expressed relative to the reference gene, beta-actin (ACTB). NORM, HIGH, LOW, ♂, ♀, Ewed and sex: See legends to Figure 1. Values are expressed as emmean ± SEM. ab Significant differences between groups are denoted by different superscript letters. Number of animals in the prenatal nutrition groups: a) NORM (N=8, 3♂:5♀); HIGH (N=10, 5♂:5♀) and; LOW (N=11, 6♂:5♀).
Effect of prenatal nutrition (A), prenatal*sex interactions (B) and prenatal*early postnatal nutrition interactions (C) on mRNA expression patterns in perirenal adipose tissue (PER), expressed relative to the reference gene, beta-actin (ACTB). NORM, HIGH, LOW, CONV, HCHF, Ewed, Lambd, Sex, a and b: see legends to Figure 1. Values are expressed as emmean ± SEM. ab Significant differences between groups are denoted by different superscript letters. Number of animals in the prenatal nutrition groups: NORM (N=10, 4:6); HIGH (N=12, 5:7) and; LOW (N=14, 5:6). Number of animals in the pre- and postnatal interaction groups: NORM:CONV (N=6); NORM:HCHF (N=4); HIGH:CONV (N=6); HIGH:HCHF (N=6); LOW:CONV (N=8) and; LOW:HCHF (N=6).
Figure 4

Effect of prenatal nutrition (A), prenatal*sex (B) and pre±postnatal nutrition (C) on mRNA expression patterns of epicardial adipose tissue (EPI), expressed relative to the reference gene, beta-actin (ACTB). Ewed, NORM, HIGH, LOW, Lambd, CONV, HCHF, and &; see legends to Figure 1. Values are expressed as emmean ± SEM. ab Significant differences between groups are denoted by different superscript letters. Number of animals in the prenatal nutrition groups: NORM (N=10, 4:6); HIGH (N=12, 5:7) and; LOW (N=13, 8:7). Number of animals in the pre- and postnatal interaction group: NORM:CONV (N=6); NORM:HCHF (N=4); HIGH:CONV (N=6); HIGH:HCHF (N=6); LOW:CONV (N=8) and; LOW:HCHF (N=7).

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