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ORIGINAL CONTRIBUTION

Hepatic expression of the GH/JAK/STAT/IGF pathway, acute-phase response signalling and complement system are affected in mouse offspring by prenatal and early postnatal exposure to maternal high-protein diet

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Abstract
Purpose Effects of pre- and early postnatal exposure to maternal high-protein diets are not well understood. Transcription profiling was performed in male mouse offspring exposed to maternal high-protein diet during pregnancy and/or lactation to identify affected hepatic molecular pathways.
Methods Dams were fed isoenergetic diets with control (20% w/w) or high protein levels (40%). The hepatic expression profiles were evaluated by differential microarray analysis 3 days (d3) and 3 weeks (d21) after birth. Offspring from three different high-protein dietary groups, HP (d3, high-protein diet during pregnancy), HPHP (d21, high-protein diet during pregnancy and lactation) and CHP (d21, control diet during pregnancy and high-protein diet during lactation), were compared with age-matched offspring from dams fed control diet.
Results Offspring body and liver mass of all high-protein groups were decreased. Prenatal high-protein diet affected hepatic expression of genes mapping to the acute response/complement system and the GH/JAK/STAT/IGF signalling pathways. Maternal exposure to high-protein diet during lactation affected hepatic gene expression of the same pathways but additionally affected genes mapping to protein, fatty acid, hexose and pyruvate metabolism.
Conclusions (1) Genes of the acute response/complement system and GH/JAK/STAT/IGF pathways were down-regulated in offspring of dams exposed to high-protein diets during pregnancy and/or lactation. (2) Genes related to nutrient and energy metabolism, however, were only affected when high-protein diet was administered during lactation. (3) Modulation of the GH/JAK/STAT/IGF pathway might be responsible for reduced body and liver masses by maternal high-protein diet.

Keywords Diet · Early nutrition · Gene expression profiling · High-protein · Lactation · Microarray analysis · Mouse · Neonate · Pregnancy

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Introduction

High-protein diets have been shown to cause body mass loss and changes of body composition, which is thought to be induced by longer postprandial satiety, increased thermogenesis and inhibition of de novo lipogenesis [1, 2]. In obese and diabetic individuals, a higher utilization of fat reserves

and improved glucose homeostasis was reported while subjected to diets containing higher protein contents [3, 4]. Similar results were obtained in rats fed high-protein diets with reduced carbohydrate content [5, 6]. Thus, due to its metabolic effects helping to control body mass gain, high-protein diets might be consumed also in women in child-bearing age. Reports on the impact of an increased dietary protein intake during pregnancy and lactation on maternal and foetal/offspring development and health are scarce, and the results obtained are inconsistent [7–9], although there seems to be a similarity with the effects of a low-protein diet that also causes decreased birth body mass (BM) and altered BM development of the offspring during lactation [10–13]. Lack or excess of nutrients can lead to an altered milk volume [14, 15], lactose [16] as well as milk fat concentration [17, 18] subsequent to changes in mammary gland structure and gene expression [19, 20]. We have previously observed in mice that offspring was negatively affected by maternal high-dietary protein intake during pregnancy and lactation, which might be related to impaired lactational function with altered mammary gland histology and mRNA abundance of several mammary gland key genes [21]. In addition, in response to a high-protein diet, we found that pregnant and lactating mice showed distinct changes in the hepatic proteomic profile related to nutrient metabolism [22]. Others have previously reported the effects of protein restriction in reproducing rodents on the offspring [11, 23]. Following dietary protein restriction of pregnant rats, in 21-day-old foetuses, whole-body and liver growth retardation was associated with a down-regulation of growth factors in liver tissue [24]. Understanding developmental plasticity of the liver is important to elucidate possibly underlying mechanisms linking diet-related growth retardation and later metabolic health [25], considering that the liver plays an important role in the regulation of nutrient metabolism.

The objective of this study was to contribute to this by analysing the effects of a maternal high-protein diet, fed during pregnancy and lactation, on the hepatic gene expression profile of the offspring. We aimed to elucidate (i) affected molecular pathways that might underlie the poor foetal and early postnatal growth related to maternal high-protein intake and (ii) whether there is a difference in the hepatic transcript pattern when offspring was exposed to maternal high-protein diet during lactation only or during pregnancy and lactation.

Materials and methods

Animals and diets

The animal model, housing and diets are described in detail in our previous study [21]. Briefly, unselected nulliparous female mice of the outbred line DUK bred at the Leibniz Institute for Farm Animals Biology (FBN), Dummerstorf, Germany, were mated at 9 weeks of age. Female mice were randomly allocated to two feeding groups receiving one of two experimental isoenergetic diets with control and high-protein levels, respectively. Protein was added at the expense of carbohydrates. The diets were similar to those used earlier [7] and consisted of casein (Molkereigesellschaft Lauingen mbH, Lauingen, Germany; control, 222; high protein 426 g/kg) supplemented with DL-methionine (4.0 g/kg; LAH GmbH & CO. KG, Cuxhaven, Germany), wheat starch (Ferdinand Kreutzer Sabamühle GmbH, Nürnberg, Germany; control, 439.9; high protein 217.9 g/kg), sucrose (Nordzucker GmbH, Hamburg, Germany; 160 g/kg), soy oil (Sedina ADM, Hamburg, Germany; 50 g/kg), microcellulose (50 g/kg), vitamin mixture (20 g/kg; SSNIFF Spezialdiäten GmbH, Soest, Germany), mineral mixture (50 g/kg; SSNIFF Spezialdiäten GmbH) and butylhydroxytoluene (0.1 g/kg; LAH GmbH & CO. KG). Crude protein content in dry matter was 24.8 and 44.2%, and protein to carbohydrate ratio was 0.37 and 1.15 in control and high-protein diets, respectively.

One male and one female were put in the same cage to mate. During this time, they were fed the respective experimental diets to allow adaptation of the dam to the food. Males were withdrawn immediately after the vaginal plug appeared, which was considered day 1 of pregnancy. Pregnant dams were continued on the respective experimental diet and housed individually in plastic cages (Macrolon, Type II, EBECO, Castrop-Rauxel, Germany) in a controlled environment of 21 °C with 12:12 h light/dark cycle. Water was available ad libitum.

At day 1 after parturition, dams were either kept on the diet fed during pregnancy or they were switched to the other diet and continued on this diet until days 3 or 21 (d3 or d21). Litters were standardized to 10 pups immediately after birth and stayed with their mothers. Offspring from three different high-protein groups (HP, high-protein diet during pregnancy, and evaluation at d3; HPHP, high-protein diet during pregnancy and lactation, and evaluation at d21; CHP, control diet during pregnancy, high-protein diet during lactation, and evaluation at d21) were compared with respective, age-matched controls (C, control diet during pregnancy, and evaluation at d3; CC, control diet during pregnancy and lactation, and evaluation at d21) (Fig. 1a).

At d3 or d21, body mass of individual pups was determined, the pups were killed by decapitation, and livers were dissected for liver mass determination and subsequent hepatic RNA preparation. For evaluation at d3 (HP vs. C), three males each from nine different litters (i.e. dams) with $n = 27$ pups per dietary group were randomly selected, whereas at d21 (CHP and HPHP vs. CC) samples were taken from one to two randomly selected male pups from

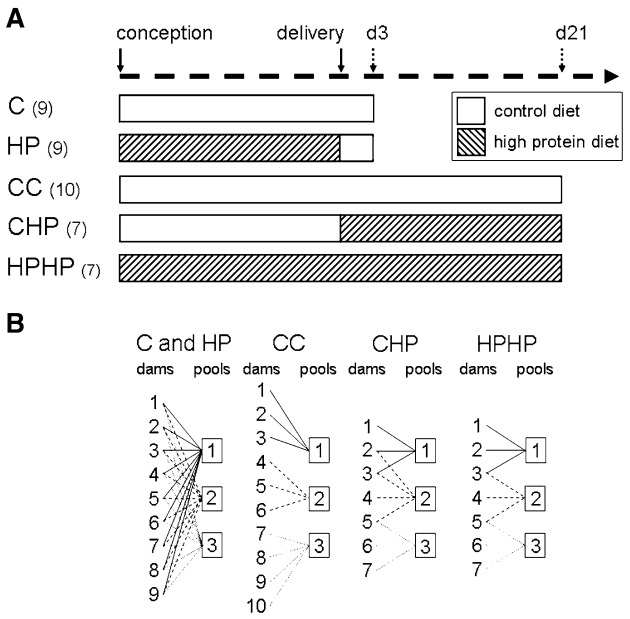

Fig. 1 Different dietary groups and experimental design of microarray expression profiling experiments. **a** Control and/or high-protein diets were fed to dams during pregnancy and/or gestation. Offspring of different dietary groups were analysed three (d3, dietary groups C HP) or 21 (d21, dietary groups CC, CHP, HPHP) days after delivery. Number of dams (i.e. litters) within each dietary group is shown in brackets. **b** For microarray analysis, nine and three to four pups (at d3 and d21, respectively) from seven to ten individual dams (*numbers*) of the different dietary groups were combined to three pools (*framed numbers*) within each dietary group

seven or ten litters (i.e. dams) with $n = 9$ or 10 pups per dietary group (Fig. 1b).

The study followed German guidelines for the protection of animals used for experimental purposes and was conducted with approval of the Animal Care Committee of the Ministry of Nutrition, Agriculture, Forestry and Fishery, State Mecklenburg-Vorpommern, Germany (LALLF M-V/TSD/7221.3-1.1-033/06).

Microarray expression profiling

For differential expression profiling, liver RNA samples were pooled to include as many individuals as possible into the analysis (Fig. 1b). As demonstrated earlier, pooling can be beneficial to accentuate group-specific expression profiles despite of large inter-individual variation [26, 27]. Microarray expression profiling was performed with livers of 3- and 21-day-old male offspring. At d3, liver samples from 27 pups were collected from both dietary groups (C and HP). Samples were combined to three pools with nine individuals per pool for each dietary group. For each pool, pups were selected from nine different litters of each dietary group, in order to reduce individual maternal effects. Samples from individuals were combined to pools by considering pup body mass in order to generate pools with similar mean body and liver mass. One-way analysis of variance (ANOVA) revealed no significant differences of means between pools from the same dietary group (not shown).

At d21, liver samples from nine to ten pups from each of the three dietary groups (CC, CHP and HPHP) were combined to three pools with three to four samples per pool. Animals for each pool were also selected from different litters, and pools from animals with similar (i.e. statistically non significant differences, ANOVA) mean body mass were combined.

Livers were dissected and freed from attached tissues and preserved in RNAlater® (Qiagen, Hilden, Germany). Total RNA was prepared using the RNeasy Mini Kit with simultaneous removal of genomic DNA with RNAse free DNAse (both from Qiagen). RNA was quantified in a NanoDrop 1000 instrument (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Varying amounts of RNA from nine (d3) or three to four samples (d21) were then combined to pools of 5 µg RNA per pool considering equal contributions of individual samples.

Array hybridization was performed according to the supplier's instructions using the "GeneChip^R Expression 3'Amplifikation One-Cycle Target Labeling and Control reagents" (Affymetrix, St. Clara, CA, USA). In detail, the first-strand cDNA was synthesized using 5 µg whole RNA sample and superscript II reverse transcriptase (RNaseH minus) introducing a T7-$(dT)_{24}$ primer. The second-strand synthesis was done as strand replacement reaction using the *E. coli* DNA-Polymerase I complex, hybridstrand-specific RNA degrading RNaseH, a ligase reaction (*E. coli* DNA Ligase), and finally, an endpolishing with recombinant T4-polymerase was performed. Biotin-16-UTP was introduced as label by a linear amplifying in vitro transcription reaction using T7-polymerase overnight (16 h). The required amount of cRNA was fragmented by controlled chemical hydrolysis to release the proportionality of cRNA molecule length and the amount of incorporated biotin derivate. The fragmented cRNA was hybridized overnight (45 °C) in the GeneChip^R Hybridisation Oven 640 (Affymetrix) to the Affymetrix MOE 430_2.0 mouse expression arrays (>45.000 probe sets = mouse gene specificities). Subsequently, washing and staining protocols were performed with the Affymetrix Fluidics Station 450. For a signal enhancement, an antibody amplification was carried out using a biotinylated antistreptavidin antibody (Vector Laboratories, Peterborough, UK), which was cross-linked by a goat IgG (Sigma–Aldrich, Taufkirchen, Germany) followed by a second staining with streptavidin–phycoerythrin conjugate (Molecular Probes, Invitrogen, Karlsruhe, Germany). The scanning of the microarray was done with the GeneChip Scanner 3000 (Affymetrix) at 1.56 micron resolution.

Data analysis was performed with the MAS 5.0 (Microarray Suite statistical algorithm, Affymetrix) probe-level analysis using GeneChip Operating Software (GCOS 1.4), and the final data extraction was done with the Data Mining Tool 3.1 (Affymetrix). 'Comparison expression analysis' of MAS 5.0 was done for all possible pairwise comparisons ($n = 9$) between three pool arrays of the respective control diet groups (C or CC) as baseline and three pool arrays of the HP diet group and the HPHP or CHP diet groups at d3 and d21, respectively. For each of the 45,000 probe sets, this resulted in one of the following statements: increased, decreased, not changed or not detected. Additionally, the common *t* test (MAS5.0) for each gene was performed to get a list of corresponding *p*-values of significance. Distinct thresholds of *p*-values and pairwise comparisons were combined to define differentially expressed probe sets, respectively, transcripts (see Results).

Bioinformatics

Bioinformatic analysis was performed with the Ingenuity Pathway Analysis tool (Ingenuity IPA 8.0, Ingenuity Systems Inc.) to map differentially expressed transcripts to specific canonical pathways. If the calculated *p*-value was below 0.05, mapping of the respective transcript to a specific canonical pathway was considered significant. For clarity, significant canonical pathways were combined to superordinate categories (see Results).

qPCR

At d3, the abundance of selected transcripts, which had been significantly identified by differential microarray expression profiling (*Anxa4*, *Ifitm3*, *Igf1*, *Igtp*, *Mpa2l* and *Stat1*), was re-evaluated by qPCR in ten individual samples of the control and HP dietary group (C and HP) in order to re-evaluate data from microarray analysis after sample pooling and to estimate inter-individual variation of transcript levels. The transcript abundance levels of additional members of the GH/IGF signalling pathway (*Igf2*, *Igfbp2*, *Igfbp3* and *Ghr*) and of *Gapdh* as a housekeeping control were also determined by qPCR. Primers used for reverse transcription and qPCR are shown in Table 1. Primers were designed to bind within or close to the target sequence of the corresponding microarray probe sets and if possible were derived from different exons to avoid amplification of residual genomic DNA. For cDNA synthesis, 0.9 µg total RNA were reversely transcribed in a 25 µL reaction volume using M-MLV reverse transcriptase, RNase H Minus, Point Mutant (Promega, Mannheim, Germany). The freshly synthesized cDNA samples were cleaned with the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and eluted in 50 µL elution buffer.

For qPCR, 0.5 and 0.25 µL of each purified cDNA sample were amplified with the LC 480 SYBR Green I Master Kit (Roche) in 12 µL total reaction volume. Values from both reactions were averaged. Amplification and quantification of PCR product was performed in a LightCycler® 480 instrument (Roche) under the following cycling conditions: Pre-incubation at 95 °C for 10 min, followed by 45 cycles denaturation at 95 °C for 15 s, annealing at 60 °C for 10 s, extension at 72 °C for 10 s and single-point fluorescence acquisition at 83 °C for 6 s.

The identity of the products generated with different primer pairs was initially controlled once by sequencing. The melting peaks of all samples were routinely determined by melting curve analysis in order to ascertain that only the expected products had been generated. Additionally, the length of all PCR products was monitored by agarose gel electrophoresis analysis (3% agarose, ethidium bromide stained). Cloned PCR products of each of the respective transcripts were used to generate external standard curves. Routinely, dilutions of standards covering five orders of magnitude (5×10^{-16} to 5×10^{-12} g DNA/reaction) were freshly diluted from stocks of 10 ng DNA/µL and co-amplified during each run. Transcript abundance was expressed relative to *Gapdh* transcripts.

Plasma insulin-like growth factor 1

At d21, plasma insulin-like growth factor 1 (IGF-I) concentrations were determined in the pups of CC and HPHP groups selected for microarray expression profiling. The analysis was performed in duplicate aliquots of 10 µL using the sandwich mouse IGF-I ELISA Kit "Quantikine" (R&D Systems Inc., Minneapolis, MN, USA) according to the supplier's instructions. Within- and between-assay coefficients of variation for this assay are 3.3 and 4.6%, respectively.

Statistical analysis

Means of body and liver masses, of plasma IGF-I concentrations, and of transcript abundance levels of different dietary groups were statistically analysed by *t* testing or one-way ANOVA (Bonferroni multiple-comparison test). When $p < 0.05$, differences were considered significant. All tests were calculated with the SigmaStat software (Jandel Scientific, San Rafael, CA, USA). Results are expressed as means ± SEM (standard errors of means).

Table 1 Primers used for cDNA synthesis and qPCR

Gene	[a]	Sequence	Accession nos.	bp[b]
Anxa4	rt	AAAAAGGCACAGAGGGGATCAAA	NM_013471	227
	rev	AACCTTGGCAGAGGCACTCTTGA		
	for	GTTGCTGAGTATGGTGGGGCTTG		
Gapdh	rt	AGCCCTTCCACAATGCCAAAGTT	NM_001034034	197
	rev	TTTCTCGTGGTTCACACCCATCA		
	for	TCACCATCTTCCAGGAGCGAGAC		
Ghr	rt	GCTTGTGTGGCCAGCAAAGTACC	NM_010284	190
	rev	CGTGGTCAGCCTATGGTGAAATG		
	for	TTGGGTGTTTAAGGTTCGCCTTG		
Ifitm3	rt	GGACCGGAAGTCGGAATCCTCTA	NM_025378	209
	rev	GCTGAGGACCAAGGTGCTGATGT		
	for	TGATCAACATGCCCAGAGAGGTG		
Igf1	rt	TTCCTTCTCCTTTGCAGCTTCG	NM_010512	241
	rev	TTTGTAGGCTTCAGTGGGGCACA		
		TTTGTAGGCTTCAGTGGGGCACA		
	for	TCCACCACAGCTGGACCAGAGAC		
Igf2	rt	GGGGGCTTGTGCCAATTAAGTTC	NM_010514	171
	rev	TGACATGGGGGACATTTGTGATG		
	for	CCCAGAACCCGAGAAGAAAGGAA		
Igfbp2	rt	CATTTCTGCTGGTGTTCGGGATG	NM_008342	203
	rev	TGGCTGGGGTTTACTGCACACTT		
	for	CAAGCATGGCCGGTACAACCTTA		
Ifgbp3	rt	CAGTCTCCTCTCACCCTGCGTTG	NM_008343	228
	rev	TCCTTGGGCGTGTCTGCAGTTAT		
	for	AGTGGCTTGTCTGAGAGCCTGGT		
Igtp	rt	TTGCACTTTGAAGGAACGGGCTA	NM_018738	162
	rev	GTGAATTTCGGGAGGGAGGACAG		
	for	TCAGGGTTCTCGAAAGCTCATGG		
Mpa2l	rt	GCAGGTGAATACCACATCAACCTCA	NM_194336	159
	rev	CAGAGGATTTCCTGTTCCAACCACCA		
	for	GAAAGCTGACCTGCCAGGCACAA		
Stat1	rt	TTCCCAAAGGCGTGGTCTTTGTC	NM_009283	189
	rev	CACGGCATGGAAGTCAGGTTCAC		
	for	CTGCTGTGCCTCTGGAATGATGG		

[a] *for* forward primer, *rev* reverse primer, *rt* primer used for cDNA synthesis

[b] Lengths of amplicons in base pairs

Results

Body mass, liver mass and plasma IGF-1 concentrations in offspring

Body and liver mass of pups, which had been selected for microarray analysis, were determined after dams had been exposed to control diet or high-protein during pregnancy only (HP) or throughout pregnancy and/or the full lactation period (HPHP, CHP). Three days after birth, offspring of the HP group showed significantly lower body and liver mass than offspring from the control group (C). At age d21, the body mass was also significantly different between offspring of the CC compared with offspring of the CHP and HPHP groups. Also, liver mass was significantly different between the CC and CHP, but not between CC and the HPHP group (Table 2). Liver mass relative to body mass tended to be higher in the HPHP group than in the CC or CHP group (4.04 ± 0.11, 3.76 ± 0.11, and $3.76 \pm 0.12\%$). Plasma IGF-1 concentrations in d21 offspring did not significantly differ between CC and HPHP groups (213.7 ± 15.7 vs. 209.5 ± 22.4 ng/mL).

Table 2 Body and liver mass (means ± SEM) of offspring from different dietary groups that were included in microarray analysis 3 days (d3) and 21 days (d21) after birth

	d3		d21		
	C	HP	CC	CHP	HPHP
Body mass (g)	1.92 ± 0.06 (27)a	1.65 ± 0.04 (27)b	7.94 ± 0.33 (10)A	5.79 ± 0.34 (10)B	6.15 ± 0.44 (9)B
Liver mass (g)	0.06 ± 0.002 (27)a	0.05 ± 0.002 (27)b	0.30 ± 0.02 (10)A	0.22 ± 0.02 (10)B	0.25 ± 0.02 (9)AB

C control diet during pregnancy, *HP* high-protein diet during pregnancy, *CC* control diet during pregnancy and lactation, *CHP* control diet during pregnancy and high-protein diet during lactation, *HPHP* high-protein diet during pregnancy and lactation, *numbers* in brackets indicate numbers of animals, identical and different letters (d3: lower case; d21: upper case) denote similar and significantly different mean values, respectively ($p < 0.05$, d3: *t* test; d21: ANOVA Bonferroni *t* test)

Microarray analysis

For microarray analysis, 27 or 9–10 liver RNA samples were combined to three pools for each dietary group at d3 or d21 (d3: C and HP; d21: CC, CHP and HPHP), respectively (Fig. 1b). After labelling, the cRNA of pooled samples was hybridized to Mouse Genome 430 2.0 arrays, scanned and statistically evaluated. Two statistical criteria were used to decide which of the 45.000 probe sets were differentially expressed comparing the different dietary groups: (i) the mean transcript abundance levels must be significantly different (*t* test; $p < 0.05$) and (ii) at least 78% (seven of nine at d3) or 89% (eight of nine at d21) of the nine pair wise comparisons (each of the three microarrays of the control diet groups were compared with each of the three microarrays of the high-protein diet groups) should lead to the same statement, either increased or decreased.

T testing ($p < 0.05$) indicated 79, 3,284 and 2,129 probe sets with differential mean transcript abundance when comparing C versus HP (d3), CC versus HPHP (d21) and CC versus CHP (d21), respectively (data not shown). These numbers were reduced to 26, 206 and 224 probe sets ($\approx$22, 166 and 138 annotated genes; Table. 3 and Online Resource 1), when the second criterion (pair wise comparison) was applied. Only 47 of the probe sets were identical in the CC versus HPHP and CC versus CHP comparisons. Except for the *Hamp2* probe set that was up-regulated in the HPHP group but largely down-regulated in the CHP group, all other 46 probe sets showed very similar changes in transcript abundance in the HPHP and CHP groups compared to the control. In case of d3, six of the significantly different probe sets were up- and twenty down-regulated (Table 3). At d21, 97 and 156 probe sets showed significantly increased, and 109 and 68 significantly reduced abundance in the CC versus HPHP and the CC versus CHP comparison, respectively (Online Resource 1).

Bioinformatic evaluation

In case of the C versus HP comparison at d3, eight of the annotated genes significantly mapped to canonical pathways of the Ingenuity IPA bioinformatics tool. For the sake of clarity, most of them were combined to two superordinate categories (Fig. 2), acute response/complement and GH/JAK/STAT/IGF signalling. At d21, 50 and 31 of the differentially expressed genes mapped significantly to canonical pathways in case of the HPHP and CHP dietary groups, respectively. These were combined to 10 or 11 superordinate pathway categories, respectively (Table 4 and Fig. 2). As observed at the earlier stage, also at d21 genes of the acute response/complement and GH/JAK/STAT/IGF signalling pathways were significantly affected, but in addition, also genes of the PX/RX/FX/RA/PPAR, aldosterone, glucocorticoid and prolactin signalling pathways. However, the largest number of affected genes mapped to pathways involved in fatty acid, hexose, protein/amino acid and pyruvate metabolism. In both treatment groups (HPHP and CHP), a relatively large number of genes were found consistently mapping to IPA canonical pathways aryl hydrocarbon receptor signalling, metabolism of xenobiotics by cytochrome P450 and xenobiotic metabolism signalling. These were combined to the superordinate pathway category xenobiotic metabolism (Table 4), because it is well known that the aryl hydrocarbon receptor (AhR) binds to a xenobiotic response element of genes of the Cytochrome P450 family, which are induced by toxic environmental pollutants [28].

qPCR analysis

Three days after birth (d3), the transcript abundance of six significantly regulated genes (*Anxa4*, *Ifitm3*, *Igf1*, *Igtp*, *Mpa2l* and *Stat1*) was re-evaluated by qPCR analysis. In addition, also other transcripts of the significantly affected GH/JAK/STAT/IGF signalling pathway (*Ghr*, *Igfbp2*, *Igfbp3*, *Igf2*) were quantified by qPCR. qPCR analysis was applied to liver samples from nine arbitrarily selected individuals from both dietary groups, which were also included in the pools for microarray analysis. Five of the quantified transcripts were significantly down-regulated by the prenatal high-protein diet; additionally, four of them tended to decrease in the HP

Table 3 Offspring hepatic transcripts[a] that were affected by maternal HP diet during pregnancy

Probe set ID[b]	Gene ID	Symbol	Title	FC[c]	Pathways[d]
1428083_at	66961	*2310043N10Rik*	RIKEN cDNA 2310043N10 gene	1.5	
1421223_a_at	11746	*Anxa4*	Annexin A4	−1.4	
1425394_at	207269	*BC023105*	cDNA sequence BC023105	−1.8	
1424041_s_at	50908	*C1s*	Complement component 1, s subcomponent	−1.2	1
1457664_x_at	12263	*C2*	Complement component 2 (within H-2S)	−1.4	1
1451625_a_at	69379	*C8g*	Complement component 8, gamma subunit	−1.2	1
1424638_at	12575	*Cdkn1a*	Cyclin-dependent kinase inhibitor 1A (P21)	−1.4	8
1455393_at	12870	*Cp*	Ceruloplasmin	−1.3	1
1422412_x_at	53876	*Ear3*	Eosinophil-associated, ribonuclease A family, member 3	2.8	
1426906_at	15950	*Ifi203*	Interferon-activated gene 203	2.1	
1423754_at	66141	*Ifitm3*	Interferon-induced transmembrane protein 3	−1.2	
1452014_a_at	16000	*Igf1*	Insulin-like growth factor 1	−1.3	8
1437401_at	16000	*Igf1*	Insulin-like growth factor 1	−1.3	
1417141_at	16145	*Igtp*	Interferon gamma-induced GTPase	−1.4	
1416833_at	64697	*Keg1*	Kidney-expressed gene 1	1.8	
1417079_s_at	107753	*Lgals2*	Lectin, galactose-binding, soluble 2	1.6	
1436531_at	56307	*Metap2*	Methionine aminopeptidase 2	1.6	
1416980_at	71664	*Mettl7b*	Methyltransferase like 7B	−1.4	
1447927_at	100702	*Mpa2l*	Macrophage activation 2 like	−1.5	
1426452_a_at	75985	*Rab30*	RAB30, member RAS oncogene family	−1.5	
1417654_at	20971	*Sdc4*	Syndecan 4	−1.5	
1416625_at	12258	*Serping1*	Serine (or cysteine) peptidase inhibitor, clade G, member 1	−1.3	1
1450033_a_at	20846	*Stat1*	Signal transducer and activator of transcription 1	−1.5	8
1437277_x_at	21817	*Tgm2*	Transglutaminase 2, C polypeptide	−1.3	
14303428_x_at	21817	*Tgm2*	Transglutaminase 2, C polypeptide	−1.4	
1449009_at	21822	*Tgtp*	T-cell-specific GTPase	−1.3	

[a] Transcripts were identified by differential microarray analysis at d3 comparing the C and HP diet groups

[b] ID-numbers of mouse gene specificities on Affymetrix MOE430_2.0 expression array

[c] FC, fold change of transcript abundance in HP versus C

[d] Pathways combined from significantly affected IPA (Ingenuity Pathway Analysis tool) canonical pathways (see Fig. 1): 1 acute response/complement, 8 GH/JAK/STAT/IGF signalling

group. Only *Igf2* transcripts seemed to be completely unchanged (Table 5).

Discussion

Maternal high-protein diet during pregnancy and/or lactation was detrimental to litter growth

Results of the present study clearly demonstrated that maternal high-protein diet significantly impaired early postnatal body mass gain of offspring. We have previously reported in rats, mice and pigs that a maternal high-protein diet fed from mating to parturition can cause intrauterine growth retardation followed by reduced body or total litter mass at birth [7, 13, 21]. Preliminary data in foetal rats and pigs suggest that high-protein diets during pregnancy lead to a lower availability of indispensable amino acids for foetal or placental protein synthesis [29, 30]. Furthermore, it has been reported earlier in rodent and sheep models that an increased concentration of ammonium as well as a high periconceptional protein intake decreased the number of developing blastocysts, perturbed embryonic metabolism, lead to impaired foetal growth and increased early embryonic losses [31–33]. Thus, it is possible that high-protein diets during pregnancy cause ammonia toxicity for the embryo and foetus. In a recent study using the same experimental design, it has been demonstrated that pregnant and lactating dams fed high-protein diet showed a distinct change of hepatic metabolism, which was linked to decreased abdominal fat mass and alterations of plasma amino concentrations [21, 22]. Specifically, the branched-chain amino acids and methionine concentrations were elevated as well as citrulline, arginine and ornithine participating in the interorgan

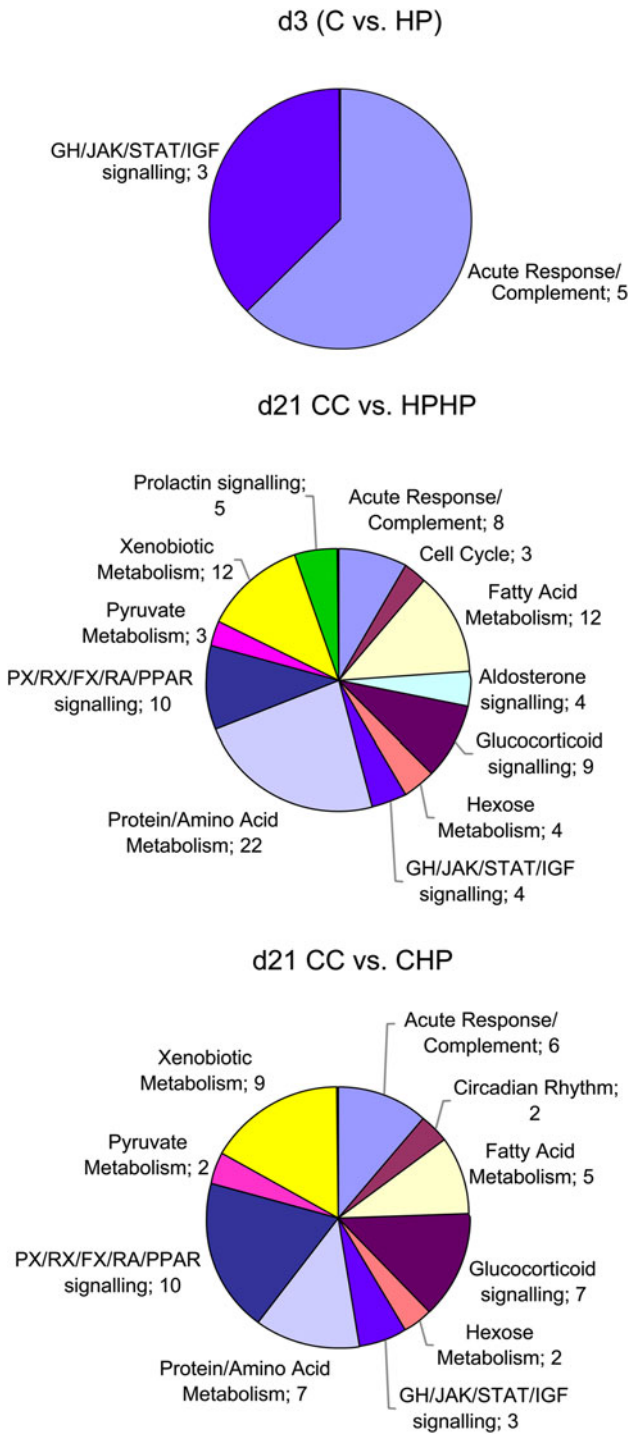

Fig. 2 Hepatic pathways that were affected in offspring of dams exposed to high-protein diet during pregnancy (C vs. HP) and during pregnancy and/or during the full lactation period (CC vs. HPHP and CC vs. CHP). Animals were analysed three and 21 days after birth (d3 and d21). Significantly affected canonical pathways that were identified by the Ingenuity Pathway Analysis tool (IPA) were combined to superordinate categories for the sake of clarity. *Numbers* indicate significantly affected transcripts that were assigned to the respective pathways

nitrogen transfer [21, 22]. Dams fed the HP diet during lactation had lower plasma glucose concentrations at day 3 of lactation (16.4 v. CC 20.7 mmol/L; $p < 0.043$; C. C. Metges et al. (unpublished results)). However, at peak lactation (d14), plasma glucose was not reduced in dams receiving an HP lactation diet, presumably due to the upregulation of hepatic gluconeogenesis [22]. Also, the gene expression in mammary gland parenchyma was affected by high-protein diet with reduced abundance of α-lactalbumin, β-casein, whey acidic protein, xanthine oxidoreductase mRNA [21]. In addition, milk lactose concentration was also reduced in lactating mouse dams fed high protein diet during lactation, suggesting a reduced lactational yield [21]. In line with this, we have recently observed that high-protein diets fed throughout pregnancy tended to reduce colostrum fat and lactose concentrations in sows [13]. As found in other species including humans, increased thermogenesis and satiety, and altered nutrient partitioning are associated with high-protein–low carbohydrate diets [2]. Thus, the experimental high-protein diet very likely caused a state of malnutrition in the offspring via restricted placental nutrient supply during pregnancy and/or via the production of a less nutritious dam's milk during suckling.

HP diet during pregnancy significantly affected genes of the Acute Response/Complement and GH/JAK/STAT/IGF signalling

HP diet during pregnancy significantly impaired growth of offspring. However, the expression of only remarkably few hepatic genes was significantly affected, with most of them only moderately down-regulated. Several of these genes, among them *Igf1*, encoding insulin-like growth factor I (IGF-I), significantly mapped to the GH/JAK/STAT/IGF signalling network. This is consistent with earlier studies reporting that particularly, the IGF-I concentration is highly sensitive to nutritional factors. The liver is the principal source (80-90%) of circulating IGF-I, and it has been shown that the nutritional status and supply of energy and protein are critical regulators of IGF-I and insulin-like growth factor binding proteins (IGFBPs) [34, 35]. Thus, IGF signalling appears to provide an important mechanism linking nutrition and growth. In several species, it has been shown that malnutrition leads to down-regulation of IGF-I, and it has been suggested that changes in serum IGF-I reflect changes in protein metabolism. It is also known that decreased serum IGF-I correlates with reduced hepatic *Igf1* mRNA. So far, down-regulation of hepatic *Igf1* expression has been only reported in the context of maternal low-protein diets [24, 36], As discussed earlier, maternal high-protein diet throughout pregnancy as used in this study causes shifts in

Table 4 Significantly affected IPA[a] canonical pathways in the liver of offspring affected by maternal high-protein diet during pregnancy or lactation and combined superordinate pathways

	Combined Pathways	Groups[b]	IPA canonical pathways
1	Acute Response/Complement	HP	Acute-phase response signalling; complement system
		HPHP	Acute-phase response signalling; complement system
		CHP	Acute-phase response signalling; complement system
2	Cell cycle	HPHP	Cell cycle: G1/S checkpoint regulation
3	Circadian rhythm	CHP	Circadian rhythm signalling
4	Fatty acid metabolism	HPHP	Arachidonic acid metabolism; fatty acid metabolism; glycerophospholipid metabolism; phospholipid degradation
		CHP	Arachidonic acid metabolism; fatty acid metabolism; linoleic acid metabolism
5	Aldosterone signalling	HPHP	Aldosterone signalling in epithelial cells
6	Glucocorticoid signalling	HPHP	Glucocorticoid receptor signalling
		CHP	Glucocorticoid receptor signalling
7	Hexose metabolism	HPHP	Glycolysis/gluconeogenesis
		CHP	Fructose and mannose metabolism; galactose metabolism
8	GH/JAK/STAT/IGF signalling	HP	Growth hormone signalling; JAK/STAT signalling
		HPHP	Growth hormone signalling
		CHP	IGF-1 signalling
9	Protein/amino acid metabolism	HPHP	Alanine and aspartate metabolism; arginine and proline metabolism; cysteine metabolism; D-glutamine and D-glutamate metabolism; glutamate metabolism; glycine, serine and threonine metabolism; methionine metabolism; nitrogen metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; selenoamino acid metabolism; sulphur metabolism; tryptophan metabolism; tyrosine metabolism; urea cycle and metabolism of amino groups; valine, leucine and isoleucine degradation; β-alanine metabolism
		CHP	D-glutamine and D-glutamate metabolism; glutamate metabolism; nitrogen metabolism; tryptophan metabolism
10	PX/RX/FX/RA/PPAR signalling	HPHP	PXR/RXR activation; RAR activation
		CHP	FXR/RXR activation; PPAR signalling; PPARα/RXRα activation; PXR/RXR activation; RAR activation; TR/RXR activation
11	Pyruvate metabolism	HPHP	Pyruvate metabolism
		CHP	Pyruvate metabolism
12	Xenobiotic metabolism	HPHP	Aryl hydrocarbon receptor signalling; metabolism of xenobiotics by cytochrome P450; xenobiotic metabolism signalling
		CHP	Aryl hydrocarbon receptor signalling; metabolism of xenobiotics by cytochrome P450; xenobiotic metabolism signalling
13	Prolactin signalling	HPHP	Prolactin signalling

[a] Ingenuity Pathway Analysis tool

[b] *HP* offspring at d3 after high-protein diet during pregnancy, *CHP* offspring at d21 after control diet during pregnancy and high-protein diet during lactation, *HPHP* offspring at d21 after high-protein diet during pregnancy and lactation

maternal metabolism followed by a state of malnutrition in the foetal offspring. This is in contrast to direct effects of high-protein diets usually observed but somewhat comparable to the effects of a maternal low-protein diet [24, 36]. Thus, we interpret the significant down-regulation of genes of the GH/JAK/STAT/IGF pathway, particularly that of hepatic *Igf1* transcripts, as a response to a restricted macronutrient and energy supply. Thus, the decreased gain of litter mass [21] in the HP group is mediated by decreased levels of liver derived IGF-I.

Surprisingly, also the hepatic expression of a number of genes, which map to the acute response/complement system, was significantly affected (mainly down-regulated) by pre- and postnatal maternal high-protein diet. This is in line with recent observation in mice that high-protein intake without malnutrition can impair resistance to infections [37]. The regulatory context and functional relevance of this and our observation are presently unclear. However, there is certainly a regulatory connection between factors of the GH/JAK/STAT/IGF pathway and elements of the

Table 5 Relative abundance of d3 offspring hepatic transcripts after exposure to maternal control (C) or high-protein (HP) diet during pregnancy

	*Mpa2l**	*Igtp*	*Igfbp3**	*Anxa4**	*Ghr*	*Stat1*	*Igf1**	*Ifitm3**	*Igfbp2*	*Igf2*
C	0.027 ± 0.007	0.425 ± 0.098	1.232 ± 0.141	1.129 ± 0.095	0.869 ± 0.114	0.830 ± 0.188	1.985 ± 0.396	5.129 ± 0.829	49.503 ± 5.939	67.532 ± 4.577
HP	0.013 ± 0.002	0.269 ± 0.026	0.939 ± 0.077	0.784 ± 0.040	0.738 ± 0.053	0.591 ± 0.071	1.090 ± 0.159	3.336 ± 0.457	43.300 ± 5.169	67.486 ± 3.291

Transcript abundance (relative to *Gapdh* transcripts) was determined by qPCR from $n = 9$ animals from each diet group; mean values ± SEM (×100) are shown; Significantly different means between both dietary groups are indicated with *asterisks* ($p < 0.05$, *t* test)

immune system. Several members of this pathway, among them *Igf1* and *Stat1*, showed significant hepatic down-regulation in high-protein diet groups. IGF-I can activate target genes along the JAK/STAT signalling pathway, particularly via STAT1 and STAT3 [38]. Furthermore, the STAT-I factor acts via gamma interferon activation sites (GAS) [39], which are present in many effector genes including those of the immune system. Therefore, one might speculate that the down-regulation of GAS containing target genes, among them possibly genes of the acute response/complement system networks, is a side effect of reduced *Igf1* and/or *Stat1* expression. In any case, this interesting observation certainly deserves further functional elucidation.

We did not find effects on genes of the hepatic protein, amino acid or hexose metabolism in the perinatal period in contrast to what was observed 3 weeks later. This might either be explained by the fact that the hepatic metabolism pathways at d3 are still immature and cannot yet be regulated by the experimental nutritional challenge during pregnancy or alternatively, that the placenta could effectively dampen the nutritional challenge.

qPCR analysis was performed to re-evaluate microarray data and to analyse additional transcripts of the GH/JAK/STAT/IGF pathway. Except for the *Igf2* transcript abundance levels that were similar in the control and HP group, all other transcripts were down-regulated by the dam's high-protein diet, some of them, however, without statistical significance. Thus, a statistically significant impact of the diet on hepatic expression could not be confirmed in case of all re-evaluated genes. This might have been due to large inter-individual variation of transcript abundance, particularly in the control diet group (see Table 5). This would confirm earlier findings that pooling can be beneficial to identify group-specific expression profiles in spite of large inter-individual variation [26]. The fact that *Igf2* transcript abundance was found unchanged is consistent with the overall literature stating that, IGF-II is much less sensitive to dietary factors than IGF-I [35]. Transcript abundance levels of *Igfbp3* were found significantly down-regulated by qPCR analysis. In contrast, by differential microarray analysis the same gene was only found down-regulated after lactation in the HPHP diet group but not at the earlier stage (d3). In any case, this again supports the view that the GH/JAK/STAT/IGF pathway must be involved in high-protein diet–induced growth retardation.

HP diet during lactation mainly affected hepatic metabolic pathways in addition to acute response/complement and GH/JAK/STAT/IGF signalling

Compared to prenatal effects, high-protein diet during the complete lactation period affected a much higher number

of genes in both dietary groups, HPHP and CHP. Thus, also the number of significantly affected hepatic pathways was remarkably increased. In both dietary groups, when compared to the CC control, basically the same hepatic pathways had been affected, although the number of overlapping (i.e. identically affected) genes was only about 20%. Interestingly, also transcript abundance of genes significantly mapping to the acute-phase/complement and GH/JAK/STAT/IGF signalling pathways was found to be changed. Significant effects on hepatic *Igf1* or *Stat1* transcript abundance, however, as observed at d3, were not evident. In line with this, we could not observe differences in plasma IGF-I in 21-day-old offspring exposed to maternal high protein during pregnancy and lactation. Instead, hepatic *Igfbp3* and *Igfbp7* transcript abundance was found decreased. It is well known that IGFBP1 and IGFBP2 abundance is affected by the nutritional status [34, 35] and by amino acid supply in cultured cells [40, 41]. However, effects of nutrients on the abundance of IGFBP3 and IGFBP7 have not been described yet. Also, the putative roles of these factors are less well defined. Nevertheless, the data of the present study strongly suggest that the IGF-axis again was involved in high protein-induced growth retardation of d21 offspring.

In both dietary groups, HPHP and CHP, a relatively large number of genes significantly mapped to xenobiotic metabolism and signalling pathways, among them several members of the cytochrome P450 family. These genes are involved in the biotransformation of xenobiotics and can be activated via the aryl hydrocarbon receptor pathway and xenobiotic response element [28]. It is also known that the hepatic cytochrome monoxygenase system is highly responsive to nutrient and non-nutrient factors present in the diet and that transcription is the principal mechanism by which the expression of cytochromes is controlled. The constitutive expression of several cytochromes in mammalian liver is under the control of GH/JAK/STAT/IGF signalling pathway, but also several nuclear receptors as RXR, PXR, FXR and PPARs control hepatic expression of cytochromes. Particularly, PPAR-α is an important mediator of nutritional regulation of hepatic cytochromes (for review see [28]). The present data clearly demonstrated that high-protein diet during lactation affected the expression of genes involved in xenobiotic pathways. On the other hand, the fact that both GH/JAK/STAT/IGF and PX/RX/FX/RA/PPAR signalling pathways are also significantly affected by maternal high-protein diet clearly suggests that one or both of these signalling pathways might be responsible for the effects on genes of the xenobiotic metabolism. The possible functional role of this pathway during high-protein diet, however, needs further investigation.

By far the largest number of affected genes after exposure to high-protein diet during lactation mapped to different metabolic pathways with a clear focus on the protein/amino acid metabolism. Also the fatty acid metabolism seems to be remarkably affected, in addition to hexose and pyruvate metabolism. These data clearly show that in contrast to the early postnatal d3 animals, the liver metabolism of the 3-week-old animals was significantly affected. Possibly, these effects have been mediated by the inferior amount or composition of the milk during the lactation period [21]. However, another possibility is that older pups (>17 days) start nibbling solid food from the food supply of their dams and were therefore directly exposed to the high-protein diet, although to a very low extent.

In the HPHP diet group aldosterone signalling and in both groups numerous genes involved in the glucocorticoid signalling pathway were significantly affected. Glucocorticoids are involved in the regulation of gluconeogenesis in the liver, thus confirming the observed effects on the hexose metabolism. We have recently reported that high-protein diet in pregnant and lactating mice activates hepatic proteins related to amino acid, carbohydrate and fatty acid catabolism and activates gluconeogenesis [22]. The relevance of the PX/RX/FX/RA/PPAR signalling pathway that was affected in both dietary groups and the role of prolactin and aldosterone signalling pathways is not clear and remains to be elucidated yet.

Expression profiling data of the present study identified several metabolic and signalling pathways that have been significantly affected by high-protein diet during the pregnancy and lactation period. In the future, this will enable more targeted approaches focussing on the specific involvement and functional relevance of these pathways in diet induced effects not only in mice but also other species including humans.

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