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Alteration of the embryonic microenvironment and sex-specific responses of the preimplantation embryo related to a maternal high-fat diet in the rabbit model

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Abstract

The maternal metabolic environment can be detrimental to the health of the offspring. In a previous work, we showed that maternal high-fat (HH) feeding in rabbit induced sex-dependent metabolic adaptation in the fetus and led to metabolic syndrome in adult offspring. As early development representing a critical window of susceptibility, in the present work we aimed to explore the effects of the HH diet on the oocyte, preimplantation embryo and its microenvironment. In oocytes from females on HH diet, transcriptomic analysis revealed a weak modification in the content of transcripts mainly involved in meiosis and translational control. The effect of maternal HH diet on the embryonic microenvironment was investigated by identifying the metabolite composition of uterine and embryonic fluids collected in vivo by biomicroscopy. Metabolomic analysis revealed differences in the HH uterine fluid surrounding the embryo, with increased pyruvate concentration. Within the blastocoelic fluid, metabolomic profiles showed decreased glucose and alanine concentrations. In addition, the blastocyst transcriptome showed under-expression of genes and pathways involved in lipid, glucose and amino acid transport and metabolism, most pronounced in female embryos. This work demonstrates that the maternal HH diet disrupts the in vivo composition of the embryonic microenvironment, where the presence of nutrients is increased. In contrast to this nutrient-rich environment, the embryo presents a decrease in nutrient sensing and metabolism suggesting a potential protective process. In addition, this work identifies a very early sex-specific response to the maternal HH diet, from the blastocyst stage.

Introduction

In recent decades, eating habits have changed with increased fat consumption, exceeding the World Health Organization recommendations of total fat ≤ 30% total calories and saturated fat <10% total calories per day.[1] Associated with a low energy expenditure, excess fat intake contributes to the worldwide increase in metabolic dysfunctions such as overweight, obesity, dyslipidemia and diabetes.[2–4] These chronic diseases now affect young populations in reproductive age. In women, the prevalence of preexisting type 2 diabetes and the incidence of maternal obesity at the start of pregnancy has increased worldwide.[5] Indeed, in the early 2010s, the proportion of obese women of childbearing age was over 30% in the United States and between 7 and 25% among European countries.[5–7] Dietary changes and reduction in physical activity also affect low-income and middle-income countries, with the result that excess weight is now more common than underweight among women of reproductive age in most developing countries.[5] Thus, worldwide, a large proportion of pregnancies take place in a long-standing altered metabolic environment.

Overweight and obesity can be detrimental to fertility and have been associated with fetal defects and congenital abnormalities.[5] Moreover, altered maternal metabolic health can be detrimental to the lifelong offspring cardiometabolic health, increasing the likelihood that they

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will develop obesity and metabolic disease,[8–10] a phenomenon currently known as "Developmental Origins of Health and Disease" (DOHaD).[10,11] Epidemiological and animal models studies have explored consequences of a variety of maternal metabolic alterations, deciphered critical windows of susceptibility and identified the discrepancy between males and females in terms of consequences.[12] Animal models were developed to discriminate effects of maternal metabolic parameters on offspring health independently of other maternal risk factors.[8,9] In rodents, a maternal high-fat diet during pregnancy was shown to induce sex-dependent susceptibility to develop obesity, dyslipidemia, cardiovascular deregulation, impaired liver lipid metabolism and glucose homeostasis in the adult offspring.[9] In rabbits, a maternal hypercholesterolemic diet administered 2 weeks before mating and during gestation induced aortic lesions in offspring.[13] Moreover, a hypercholesterolemic and hyperlipidic diet administered 8 weeks before mating induced intrauterine growth retardation in both sexes with fetal dyslipidemia and led to adult offspring overweight associated with hypertension, with more effects on males than females.[14,15] Using the same diet, lipid droplet accumulation has been observed as early as the preimplantation embryo, in the trophoblastic cells of the blastocyst.[15]

The periconceptional period, which includes ovogenesis, generation of a zygote from two gametes and first stages of embryonic development, represents a high vulnerability time to the maternal metabolic environment.[16,17] In the preimplantation embryo, epigenetic reprograming, embryonic genome activation, differentiation of cell lineages from totipotent cells, X inactivation and first sex-linked differential gene expression occur.[18] Studies combining exposure during the periconceptional period until the end of the preimplantation period and subsequent embryo transfer into a control recipient female have been set up to discriminate the role of the periconceptional vs the post-implantation gestational period in the programing of offspring health. In rabbits, exposure to hyperglycemia during periconception only induced fetal hypotrophy, hyperglycemia and dyslipidemia as well as abnormalities in placental vascularization and nutrient transport functions, close to term.[19] In sheep, exposure to maternal overnutrition during periconception increased body fat mass in female offspring and decreased the expression of insulin signaling molecules in liver and skeletal muscle in both male and female offspring.[20] In contrast, maternal undernutrition during the periconceptional period altered placental and fetal growth dynamics and increased fetal arterial blood pressure.[21] The mechanisms underlying the sex-specific programing of offspring phenotype by periconceptional maternal metabolic environment remains to be elucidated.

Previous studies in our laboratory have shown that a hypercholesterolemic and hyperlipidic diet administered to rabbit does from before puberty induced dyslipidemia and high adiposity but not obesity in females at mating age[14] and induce long-term effects on offspring.[14,15] The objectives of this study were to analyze the effects of the periconceptional maternal hypercholesterolemic and hyperlipidic diet, previously shown to induce long-term effects on offspring, on oocyte transcripts content, uterine fluid composition and preimplantation embryo gene expression in a rabbit model.

Methods

Animals

New Zealand White female rabbits (INRA 1077 Line) were housed individually in one building maintained at 18–20°C. At 10 weeks of age, does were fed ad libitum with either a lipid cholesterol-enriched diet (HH) or a control diet (C). The experimental HH diet, a control diet supplemented with 6% soybean oil and 0.2% cholesterol, contained quantitatively more fatty acids than the C diet (2% of lipids) from each fatty acid class and provided 16% more energy than the C diet. Nutrient and chemical composition of C and HH diets have been previously published.[14,15]

At 18 weeks of age, 8 C and 8 HH does were superovulated as previously described[15] and subsequently mated with either a C vasectomized male for oocytes retrieval or a C male for blastocyst collection. At 16 hours post-coïtum (hpc), 4 C and 4 HH does were euthanized and freshly ovulated metaphase II oocytes were recovered from oviducts by flushing, removed from the cumulus by incubation in PBS/0.5% hyaluronidase and by mechanic treatment then frozen at −80°C until RNA extraction. At 144hpc, 2 C and 2 HH does were euthanized and blastocysts were recovered from uterus by flushing and subsequently dry frozen individually for sex determination, microarray analysis and RT-qPCR experiments. At 168hpc, 4 C and 4 HH does were anesthetized, then laparotomy, externalization of uterine horn and isolation of embryonic vesicle were realized as previously described.[22] The 70 MHz probe (MS-700) of a micro-ultrasound platform (Vevo2100, VisualSonics Inc., Toronto, Ontario, Canada) was positioned on the uterine horn opposite an embryonic vesicle to visualize it in real time and guide the micropuncture of blastocoelic fluid and of uterine fluids as described previously.[22] Blastocoelic and uterine fluids were snap frozen at -80°C until for metabolomics experiments.[22]

Sex determination

DNA from each blastocyst was extracted using the AllPrep DNA/RNA Mini Kit (Qiagen) according to instructions of the manufacturer. Blastocyst' sex was determined by the sex-determining region of the Y chromosome gene (SRY) gene detection on genomic DNA using nested PCR as previously described.[23] Sex determination was confirmed using detection of SRY gene using qPCR.[24]

Genome-wide gene transcription profiling

For the microarray analysis, total RNA was extracted from pools of oocytes ($n = 4$ pools of 20 oocytes C and $n = 4$ pools of 20 oocytes HH) and from individual blastocysts ($n = 15$ C and $n = 12$ HH) using the PicoPure RNA Isolation kit (Invitrogen). Prior to the elution, a purification procedure was performed using DNAse I (Qiagen) treatment at 25°C for 15 min. Total extracted RNAs were stored at − 80°C for further RNA labeling. Transcriptional profiling of oocytes and blastocysts were performed using customized rabbit microarrays (GEO accession GPL21733, Agilent-075973 Rabbit Microarray V3 020908 and GEO accession GPL18913, Agilent-042421 Rabbit BDR version 2, Agilent Technologies, respectively).[25] RNA amplification, labeling and hybridization was performed as previously described.[26]

After hybridization, the scanned images were analyzed using Feature Extraction software (v10.7.3.1; Agilent Technologies). The data were normalized using intra-array median subtraction and log2 transformation. The raw data intensity files were read into R (www.r-project.org). The identification of differentially expressed genes (DEG) from HH and C oocytes and blastocysts was achieved using the Limma package 67. Interaction between diet ad sex was assessed. P values obtained by this analysis were adjusted for

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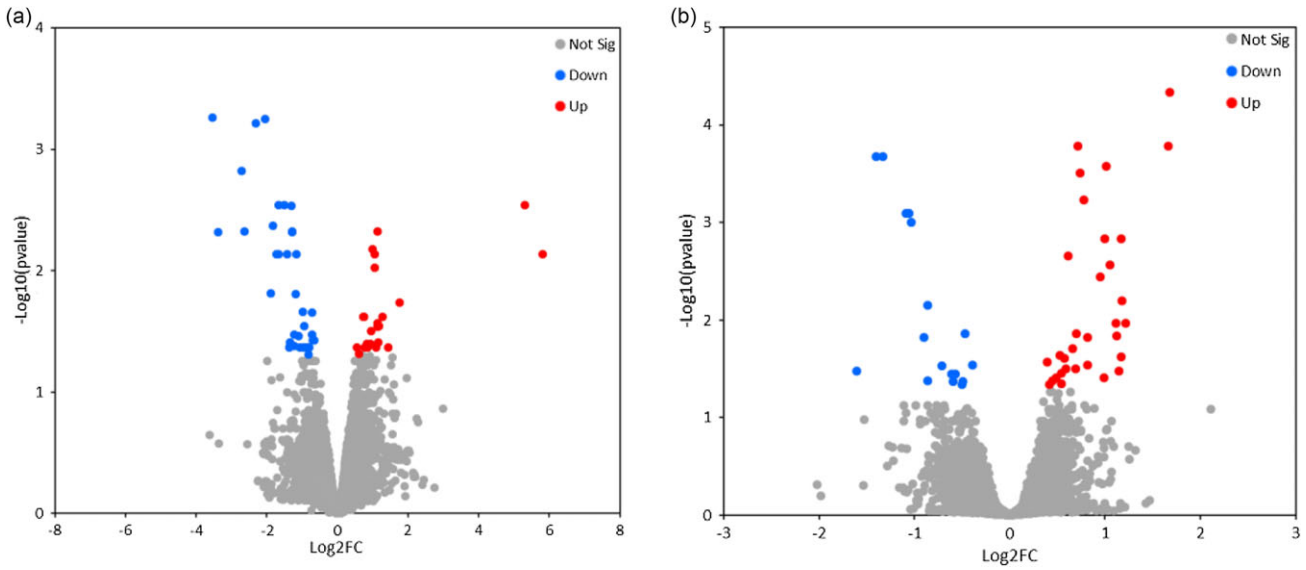

Figure 1. Volcano plot of microarray data of oocytes (***a***) and blastocyts (***b***). The data of all probes are plotted as log2 fold change (Log2FC) versus the -log10 of the adjusted p-value (-Log10(pvalue)). Probes selected as significantly down are highlighted as blue dots and probes selected as significantly up are highlighted as red dots.

multiple testing using the Benjamini-Hochberg procedure. Probes with an adjusted P value < 0.05 were considered significant.

The gene functional classification of DEG was carried out using DAVID bio-informatic database by identifying the first two terms of Gene Ontology (GO) in biological processes (BP) and molecular functions (MF) (DAVID Bioinformatics resources 6.8, NIAID/NIH) (https://david.ncifcrf.gov/).

Pathway analysis of gene transcription data

Transcriptomic data (normalized intensities of annotated probes) were analyzed by Gene Set Enrichment Analysis (GSEA version 4.1.0).[27] The GSEA approach was used on transcriptomic data to systematically identify biological pathways enriched using GO databases (BP and MF) and hallmark gene sets (v7.2), part of Molecular Signatures Database (MSigDB) collections. Hallmark gene sets comprise 50 gene sets that represent well-defined biological states or processes processes. The GSEA-derived normalized enrichment score (NES) was used for the visualization of pathway regulation. The NES was calculated for each gene set and reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes created by GSEA for each gene set according to differential gene expression between HH or C blastocyst. Positive and negative ES indicated that the gene set was overrepresented or under-represented, respectively. Gene set was considered significantly enriched when FDR score was less than 0.25.

Proton nuclear magnetic resonance spectroscopy measurements

Proton nuclear magnetic resonance (^{1}H-NMR) samples were prepared using 20 µl blastocoelic and uterine fluids diluted with 180 µl of 0.2 M potassium phosphate buffer in deuterium oxide. The spectra were recorded at 298 K on a Bruker Ascend 600-MHz spectrometer equipped with a TCI cryoprobe (Triple resonance Cryoprobe for Inverse detection) as previously described.[22] Metabolite quantifications using the Electronic Reference To access In vivo Concentrations peak as a quantitative reference were obtained by the specific subroutine of the Bruker TopSpin 3.2 program as previously described.[22] The metabolite data from blastocoelic and uterine fluids were analyzed by multivariate statistical data analysis, using MetaboAnalyst. Sample normalization was performed using the MetaboAnalyst procedure entitled "A pooled sample from group" using T values to normalize the data set. The data were scaled using auto scaling mode (mean-centered and divided by the standard deviation of each variable) of MetaboAnalyst. Data were analyzed using partial least squares discriminant analysis (PLS-DA). The overall quality of the models was judged by cumulative R 2 defined as the proportion of variance in the data explained by the model and cumulative Q 2, the class prediction ability of the model obtained by cross-validation. Comparison between groups was performed using a t-test. $P < 0.05$ was considered statistically significant and data are expressed as ratio.

Results

HH diet induces few differences in oocyte transcripts content

To determine the effect of HH maternal diet on oocyte transcripts content, 4 C and 4 HH pools of 20 oocytes were profiled using rabbit dedicated microarrays. Differential gene expression analyses between C and HH oocytes transcriptomes revealed 57 differentially expressed probes (Fig. 1a). These probes corresponded to 15 annotated genes, 12 being over-expressed and 3 under-expressed in HH as compared to C oocytes (Table 1 and Supplementary Table S1).

Among these DEGs, 3 were involved in meiosis and cell cycle: zinc finger CW type with PWWP domain 1 (*ZCWPW1*, related to "meiosis I" GO term), Ret finger protein-like 1 (*RFPL1*, related with "cell cycle" GO term) and Cyclin A1 (*CCNA1*, related to "G1/S transition of mitotic cell cycle" GO term) (Supplementary Table S1). Five DEGs were involved in the regulation of gene expression, transcription and translation processes: Arginyl-tRNA synthetase (*RARS*, related to "tRNA binding" GO term), Vav guanine nucleotide exchange factor 1 (*VAV1*, related to "regulation of transcription DNA templated" GO term), RIO kinase 1 (*RIOK1*,

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Table 1. Differential expressed genes in oocytes of females under HH vs C diet

Gene name	Gene Symbol	Log2FC	Adjusted *p* value
Over-expressed in HH ovocytes			
Ret finger protein-like 1	RFPL1	1.75	0.018
Arginyl-tRNA synthetase	RARS	1.44	0.004
Tolloid-like 1	TLL1	1.17	0.029
Vav 1 guanine nucleotide exchange factor	VAV1	1.13	0.027
RIO kinase 1	RIOK1	1.00	0.005
Coiled-coil domain containing 18	CCDC18	1.09	0.043
Transient receptor potential cation channel, subfamily M, member 6	TRPM6	1.05	0.007
Collagen, type XXVIII, alpha 1	COL28A1	0.96	0.031
Cyclin A1	CCNA1	0.93	0.040
Myosin, heavy chain 4, skeletal muscle	MYH4	0.85	0.043
IGF-like family member 1	IGFL1	0.78	0.043
Solute carrier family 36, member 3	SLC36A3	0.74	0.024
Under-expressed in HH ovocytes			
ADP-ribosyltransferase 3	ART3	−1.23	0.042
Zinc finger, CW type with PWWP domain 1	ZCWPW1	−1.42	0.007
Mitochondrial ribosomal protein L55	MRPL55	−1.50	0.003

related to "rRNA processing" GO term), Mitochondrial ribosomal protein L55 (*MRPL55*, related to "translation" GO term) and *ZCWPW1* (related to "methyl-CpG binding" GO term) (Supplementary Table S1). Collagen type XXVIII alpha 1 chain (*COL28A1*) and myosin heavy chain 4 (*MYH4*) were involved in extracellular matrix (Supplementary Table S1).

Despite these gene-by-gene statistical differences of 15 genes, global gene expression analyses using Gene Set Enrichment Analysis (GSEA) on hallmarks gene set collections and Gene Ontology Biological Process and Molecular Function databases did not identify any significant functional gene set enrichment between HH and C oocytes.

HH diet modifies uterine fluid composition

To determine the effect of HH diet on embryo's microenvironment, uterine fluid in the vicinity of the blastocyst was collected *in vivo* using a real-time ultrasound biomicroscopy guided puncture. ^{1}H-NMR metabolomic profiling was conducted on seven uterine fluids collected from four C females and on four uterine fluids collected from two HH females. PLS-DA analysis of metabolites concentrations displayed a slight separation between HH and C uterine fluids (Fig. 2a) (Accuracy = 0.73; R2 = 0.81; Q2 = 0.13). Table 2 shows the ratio between the metabolite concentration in HH and C uterine fluids. Pyruvate was identified more concentrated in HH than in C uterine fluid (Table 2).

HH diet modifies blastocoelic fluid composition

To determine the effect of HH diet on blastocyst's metabolism, real-time ultrasound biomicroscopy guided puncture was used to collect blastocoelic fluid of seven embryos from four C does and five embryos from four HH does.

Metabolomic composition were determined using ^{1}H-NMR. PLS-DA analysis of metabolites concentrations displayed a slight separation between HH and C blastocoelic fluids (Fig. 2b) (Accuracy = 0.67; R2 = 0.899; Q2 = 0.25). Table 2 shows the ratio between HH and C metabolites concentrations of blastocoelic fluids. Alanine, glucose and methionine were less concentrated in HH than in C uterine fluid (Table 2).

Maternal HH diet impaired blastocyst gene expression

The effect of HH maternal diet on blastocyst gene expression was determined by profiling individual blastocysts (15 C and 12 HH) using a rabbit dedicated microarray.

First, the expression data were analyzed regardless of independently of the sex oh the blastocyst sex. Gene-by-gene statistical analysis between C and HH blastocysts transcriptomes revealed 49 differentially expressed probes (Fig. 1b). These significantly differentially expressed probes corresponded to 26 annotated genes (Table 3 and Supplementary Table S2), 16 over-expressed and 10 under-expressed in HH as compared to C blastocysts. To identify coordinated gene expression changes, gene expression datasets were analyzed globally using GSEA on hallmarks gene set collections and Gene Ontology Biological Process and Molecular Function databases (Fig. 3 and Supplementary Table S3). In HH blastocysts, when sex was not considered, GSEA only identified the positive enrichment of one functional GO term and the negative enrichment of 22 functional gene sets (7 hallmarks and 15 GO terms).

Transcriptome analyses of HH blastocysts identified deregulation of metabolic processes. Gene-by-gene statistical analysis identified DEG implied in metabolism as the sterol carrier protein 2 (*SCP2*) and the phosphogluconate dehydrogenase (*PGD*) (Table 3, Supplementary Table S2). Functional analysis identified in HH blastocysts the negative enrichment of several gene sets involved in metabolism as "Adipogenesis" (NES = −1.56), "Fatty Acid Metabolism" (NES = −1.50) or "Mtorc1 Signaling" (NES = −1.71) hallmark gene sets and "Sterol Transfer Activity" (NES = −1.70), "Lipid Transfer Activity" (NES = −1.63) or "Neutral Amino Acid Transport" (NES = −1.97) GO terms (Fig. 3 and Supplementary Table S3).

Transcriptomes of HH blastocysts exhibited also deregulation of the expression of genes involved in transcriptional regulation. HH blastocysts exhibited over-expression of transcription factors: early growth response 1 (*EGR1*), early growth response 2 (*EGR2*), Jun proto-oncogene AP-1 Transcription Factor Subunit (*JUN*) and Fos proto-oncogene AP-1 transcription factor subunit (*FOS*) (Table 3, Supplementary Table S2). Global analysis pointed to deregulation of genes involved on DNA damage with under-representation of "DNA repair" (NES = −1.63) and "G2M Checkpoint" (NES = −1.48) hallmark gene sets (Fig. 3 and Supplementary Table S3).

A sex-specific response of the blastocyst to the maternal HH diet

The effect of HH maternal diet on gene expression in blastocysts was then explored according on sex. In the C group, we identified 8 males and 7 females and in the HH group, we identified 7 males and 5 females. The gene-by-gene statistical analysis did not detect significant differential diet-induced expression in males or females.

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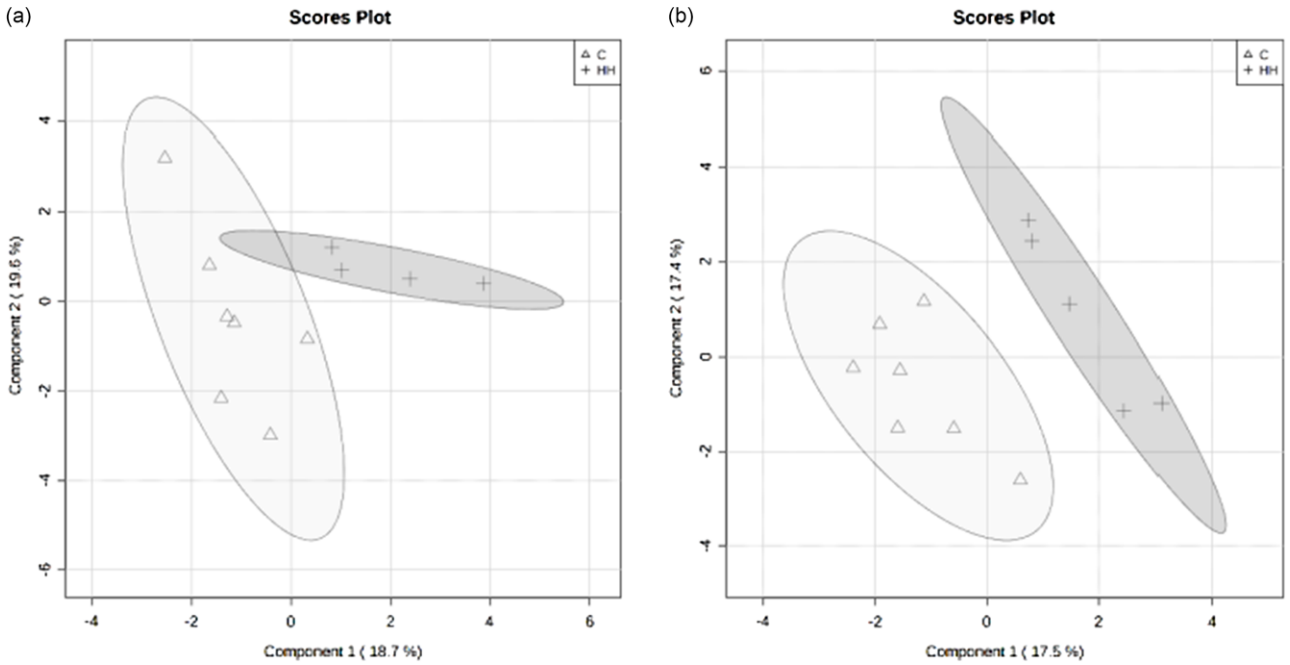

Figure 2. Multivariate statistical data analysis of C (Δ) and HH (+) uterine (***a***) and blastocoelic (***b***) fluids. Supervised partial least squares discriminant analysis of metabolites concentrations.

GSEA analysis was performed as before, using hallmark gene sets and GO terms on male or on female blastocysts data sets.

In male HH blastocysts, GSEA identified the positive enrichment of one hallmark gene set and the negative enrichment of 2 GO terms (Fig. 4a and Supplementary Table S3). These 3 groups of genes were not identified as enriched in the sex-independent analysis (Fig. 3 and Supplementary Table S3).

In female HH blastocysts, GSEA identified the enrichment of large number of gene sets: 13 hallmarks and 43 GO terms (Fig. 4b and Supplementary Table S3). Among them, 24 were under-represented (11 hallmark gene sets and 13 GO terms) (Fig. 4b and Supplementary Table S3). As in the sex-independent analysis, functional analysis identified an under-representation of metabolism gene sets such as "Fatty Acid Metabolism" (NES = −1.63), "Mtorc1 Signaling" (NES = −2.03), as well as "glycolysis" (NES = −1.52) or "cholesterol homeostasis" (NES = −1.48) hallmark gene sets in female HH blastocysts as compared to female C blastocysts (Fig. 4b and Supplementary Table S3). Functional analysis also identified under-representation of gene sets involved in transcriptional regulation response in female HH blastocysts such as "E2F targets" (NES = −1.60), "DNA repair" (NES = −1.60), "G2M Checkpoint" (NES = −1.45). Moreover, global analysis identified female-specific negative enrichment of gene sets involved in "histone ubiquitination" (NES = −2.31) gene sets and in translation such as "Ribosome Biogenesis" (NES = −2.15) and "Ribonucleoprotein complex biogenesis" (NES = −2.26) (Fig. 4b and Supplementary Table S3).

In addition, 32 gene sets were overrepresented in HH female blastocyst (2 hallmark gene sets and 30 GO terms). Of these gene sets, a large part was related to ion channel activity and to receptor signaling pathway as highlighted by the enrichment of gene set like "Ligand Gated ion Channel Activity" (NES = 2.30), "transmitter gated channel activity" (NES = 1.99), "transmembrane receptor protein tyrosine kinase activity" (NES = 1.73) and "Mitogen Activated Protein Kinase Binding" (NES = 1.82) (Fig. 4b and Supplementary Table S3).

Discussion

Objectives of this study were to decipher mechanisms involved in the sex-specific offspring programing previously observed in rabbit does fed a maternal high-fat diet.[15] Transcripts content was weakly deregulated in the oocytes of females fed a HH diet, with the affected transcripts mainly involved in meiosis and translational control. The maternal HH diet had an impact on the composition of the uterine fluid in which increased pyruvate concentrations were observed. Lipid, glucose and amino acids transport and metabolism were also altered in the preimplantation embryos with a more pronounced effect in female than in male embryos.

The high-fat diet was given to rabbit does from the time of puberty, for a total period of 8 weeks. During this period in rabbits, hormonal dependent antral follicular maturation takes place, which is characterized by oocyte growth and maturation, in interaction with the surrounding cumulus cells.[28,29] Oocyte growth and maturation have been shown to be particularly sensitive to changes in maternal nutritional and metabolic environment.[30] Indeed, it has been previously shown in the same model that high-fat diet affects follicular growth with a decrease in the number of antral follicles and conversely an increase in the number of atretic follicles.[30] During oocyte growth, transcriptional and translational activities and their post-regulations are critical for the oocyte accumulation and long-term storage of mRNA and proteins, which are essential to subsequently complete meiotic maturation and support early embryo development.[29,31] Environmental conditions during maturation can influence the pattern of transcripts in matured oocytes.[32] In rodents,[33–35] non-human primates[36] and women,[37,38] maternal metabolic dysfunctions were shown to impair meiotic resumption and oocyte gene expression. Here, in agreement with these reports, a small number of genes involved in the regulation of meiosis were differentially expressed in HH oocytes. CCNA1 is known to control female meiotic cell cycle progression by blocking metaphase to anaphase transition.[39,40] ZCWPW1, a reader of histone (H3) modifications, is required to

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Table 2. Metabolites concentration ratio of between HH and C uterine and blastocoelic fluids

Identified compound	Uterine fluid	Blastocoelic fluid
alanine	0.96	0.72*
asparagine	0.87	0.79
aspartate	nd	1.06
Beta-alanine	1.11	0.85
choline	1.12	0.77
citrate	1.11	1.01
creatine	0.88	0.94
dimethylamine	1.14	0.85
dimethylsulfone	0.62	0.61
ethanolamine	0.93	0.90
fumarate	1.25	0.85
glucose	0.99	0.50*
glutamate	nd	0.89
malonate	0.89	0.64
methionine	0.86	0.62*
o-acetylcholine	1.01	0.90
pyruvate	1.36*	0.85
succinate	1.09	0.96
tyrosine	0.89	nd
Valine	0.92	0.77
3-hydroxy-isobutyrate	0.86	0.61
2-oxoglutarate	1.03	0.90
2-phenylpropionate	1.10	0.89
3-methyl-2-oxovalerate	1.02	0.71

nd, no detection.
*indicate significant differences between HH and C within the same column ($p < 0.05$).

initiate the recombination of genetic information during meiosis.[41] In addition, transcriptomic variations observed in the present study also suggest that the HH diet affects the post-transcriptional regulation of oocyte gene expression, as several differentially expressed genes have been implicated in ribosomal processing like RARS,[42] RIOK1[43] and MRPL55.[44,45] Thus, maternal HH diet altered oocyte transcripts content of genes involved in meiosis and translational control, suggesting difficulties to achieve meiosis and to support the embryo development. Global gene set enrichment analysis, however, did not identify enrichment of functional gene sets, suggesting a limited impact of HH diet on global oocyte transcripts content. The hypothesis of weak consequences of exposure to the HH diet during the pregestational window is reinforced by a recent study in which we showed that the biometric parameters as well as hepatic and placental gene expression were unaffected in term fetuses and placentas obtained after transfer of zygotes collected from HH females into control recipients, although fine differences in fatty acid profiles were observed compared to controls.[46]

Consequences of HH maternal diet on the preimplantation embryo were first addressed through the study of its micro-environment, i.e., the uterine fluid. In mammals, uterine fluid composition is complex,[22,47] varies according to the hormonal cycle[48] and also in response to the presence of an embryo.[49] Despite numerous studies on the impact of maternal diet on offspring, the consequences of altered maternal metabolism on uterine fluid composition remains poorly explored, partly because of sampling difficulties. Uterine fluid collected during surgery in women was shown to differ in branched-chain amino acid concentrations based on the patient's healthy or unhealthy diet.[50] In mice fed a low protein diet during the preimplantation period, the concentration of branched-chain amino acids was reduced compared to that of controls in uterine fluids collected postmortem.[51] In ewes, a high protein diet was reported to induce increased ammonia and urea and decreased glucose concentrations in both oviductal and uterine fluids, also collected postmortem.[52] Post-mortem hypoxia can lead to cell death, the degradation products of which can be found in the fluids.[53] Here, uterine fluid surrounding the embryo was collected in vivo under ultrasound bio-microscopic control, as previously developed in our laboratory.[22] Using ^{1}H-NMR, we observed an impact of the HH diet on uterine fluid metabolomic profiles, more specifically on pyruvate concentration, that was increased in HH. Pyruvate is abundant in oviductal and uterine fluids in humans, mice and cows.[54] In mice embryo culture media, pyruvate is essential for the preimplantation embryo development as a nutrient and antioxidant.[55,56] Pyruvate regulates first steps of embryonic development such as the embryonic genome activation in mice[57] and regulates levels of histone modification (H3K9) during embryonic genome activation in pigs.[58] In addition, while glucose has long been considered the preferred nutrient for blastocyst compaction and cavitation, it has recently been shown in mice that exogenous pyruvate contributes to the tricarboxylic acid cycle (TCA) and represents the main source of energy for the blastocyst.[55] Thus, the increased pyruvate concentration in uterine fluid of HH could have consequences on embryo development.

The impact of the maternal HH diet on the preimplantation embryo was assessed at the blastocyst stage through the analysis of metabolic profiles of blastocoelic fluids collected in vivo using ultrasound-guided puncture.[22] The blastocoel is a fluid-filled cavity, the blastocoelic fluid resulting from both uterine fluid influx and blastocyst cells' secretion.[48] Although playing a central role in embryonic development and being in direct contact with the inner cell mass and trophectoderm, little is known about the composition and function of the blastocoel. The impact of maternal metabolism on its composition is even less well understood. Here, a decrease in amino acids' and carbohydrates' concentrations was observed in HH blastocoelic fluids. Transcriptomic profiles of HH blastocysts demonstrated changes in the expression of genes involved in nutrient transport.

Genes involved in the transport of fatty acids were affected. The global functional gene sets analysis identified an under-representation of several pathways involved in lipid/fatty acid function. First, *RBP4* was under-expressed. RBP4, is a fatty acid transporter,[59] regulated by the nutrient-sensitive kinase mTORC1,[60] associated with insulin resistance, dyslipidemia, liver steatosis, type 2 diabetes and cardiovascular dysfunction.[59] In contrast, *SCP2* was over-expressed in HH blastocysts. SCP2 is a lipid-binding protein that plays key roles a large variety of lipid trafficking and signaling.[61] SCP2 regulates lipids and fatty acids signaling pathways through lipid raft micro-domains of the plasma membrane in interaction with CAV1, which was also over-expressed in blastocysts from females exposed to high-fat diet of the present study.[62] Moreover, SCP2 is described to enhance cholesterol transfer from intracellular membranes to mitochondria.[62] *OMA1*, a zinc metalloprotease involved in the quality control system in the inner membrane of

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Table 3. Differential expressed genes in HH vs C blastocysts

Gene name	Gene Symbol	Log2FC	Adjusted *p* value
Over-expressed in HH blastocysts			
Early growth response 1	EGR1	1.22	0.0107
Early growth response 2	EGR2	1.18	0.0063
Jun proto-oncogene, AP-1 Transcription Factor Subunit	JUN	1.17	0.0236
Cyclin B1 interacting protein 1	CCNB1IP1	1.17	0.0146
Fos proto-oncogene, AP-1 transcription factor subunit	FOS	1.14	0.0330
Methionyl-tRNA synthetase 1	MARS1	0.99	0.0388
Dual specificity phosphatase 2	DUSP2	0.78	0.0006
OMA1 zinc metallopeptidase	OMA1	0.74	0.0003
Calpastatin	CAST	0.71	0.0002
Purinergic receptor P2X, ligand gated ion channel, 4	P2RX4	0.70	0.0136
ABI family, member 3	ABI3	0.59	0.0313
Sterol carrier protein 2	SCP2	0.54	0.0447
Solute carrier family 22 (organic anion transporter), member 7	SLC22A7	0.53	0.0231
Serine peptidase inhibitor, Kunitz type, 2	SPINT2	0.49	0.0388
Sperm associated antigen 4	SPAG4	0.45	0.0419
Caveolin 1	CAV1	0.42	0.0455
Under-expressed in HH blastocysts			
Solute carrier family 38, member 6	SLC38A6	−0.39	0.0290
Epoxide hydrolase 1, microsomal	EPHX1	−0.47	0.0136
Family with sequence similarity 228, member A	FAM228A	−0.49	0.0426
Family with sequence similarity 126, member A	FAM126A	−0.57	0.0356
Adenylate kinase 1	AK1	−0.60	0.0426
Protein C receptor	PROCR	−0.61	0.0356
Tumor protein p63 regulated 1	TPRG1	−0.90	0.0419
CDC42 effector protein 4	CDC42EP4	−0.86	0.0070
Phosphogluconate dehydrogenase	PGD	−1.40	0.0010
Retinol binding protein 4	RBP4	−1.60	0.0330

mitochondria, was also over-expressed.[63] OMA1 is required for mitochondrial metabolism in the blastocyst.[64] In preimplantation embryo, lipids and fatty acids metabolism serves as an energy source through fatty acid beta-oxidation and inhibition of beta-oxidation impaired blastocyst development.[65]

In addition to fatty acid metabolism impairment, both transcriptomic and metabolomics analyses revealed an alteration in glucose metabolism in HH blastocysts. Reduced glucose concentrations were observed in HH blastocoelic fluids. Glucose is essential for the morula to blastocyst transition in mouse.[55] In mouse embryo, glucose is preferentially metabolized through the pentose phosphate pathway (PPP) to provide carbon for nucleotide formation.[66] *PGD*, that converts 6-phosphogluconate into ribulose 5-P in the PPP,[67] was under-expressed in HH blastocysts. The PPP is suggested to control the signals required for the trophectoderm differentiation occurring in the blastocyst development.[55] An impairment in glucose metabolism may impede the differentiation of the trophectoderm from the blastocyst stage. Impaired glucose metabolism may interfere with trophectoderm differentiation from the blastocyst stage and participate in defects in trophoblast function subsequently observed.[15]

Amino acid trafficking and metabolism were also impacted in HH blastocyst. Transcriptomic analysis identified negative enrichment of amino acid transport terms. The amino acid transporter *SLC38A6* was under-expressed. SLC38A6 translocates small neutral amino acids, mostly glutamine and glutamate.[68] Glutamate concentrations were not altered in HH blastocoelic fluids, but alanine concentrations were decreased. Alanine is one of the most abundant amino acids in blastocoelic and uterine fluids.[22] Alanine regulates pH and is a major player in the detoxification of ammonium generated by amino acid metabolism.[69,70] Alanine can be transported from uterine fluid across the trophectoderm,[71] as well as be produced by the embryo, both by the inner cell mass and by the trophectoderm.[72] Pyruvate and glutamate can lead to the production of alanine and alpha-keto glutarate, respectively, through transamination. Thus, the observed decrease in alanine may reflect a decrease in alanine production from pyruvate to

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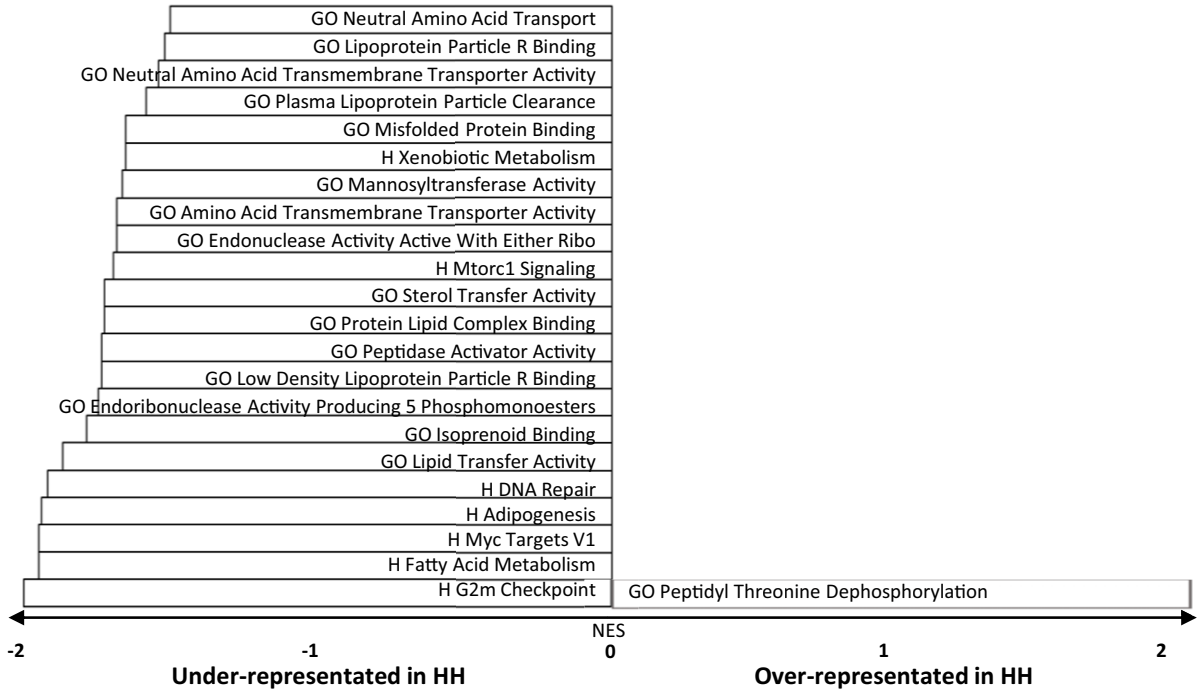

Figure 3. Enrichment analysis of blastocysts gene transcription data. Gene sets enrichment analysis was performed on hallmark gene sets (H) and GO terms (GO) database. Gene set was considered significantly enriched when FDR score was less than 0.25. Normalized enrichment score (NES) was used for the visualization. Positive and negative NES reflects respectively over-representation and under-representation in HH blastocysts.

maintain sufficient pyruvate availability for the TCA cycle. A decrease in methionine concentrations was also detected in HH blastocoelic fluids. Methionine is an essential amino acid regular, central in methylation process, protein synthesis, lipid metabolism and oxidative stress regulation.[73]

Maternal HH diet-induced impairment in lipid, glucose and amino acids in blastocysts. The HH blastocysts were mainly affected by a decrease in nutrients and their metabolisms. Consistently with these decreases in glucose and amino acids metabolism, the central nutrient-sensing signaling pathway mTORC1 was identified as under-represented in HH blastocysts. Inhibition of mTORC is essential to maintain metabolic homeostasis under metabolic stress.[74] Central to sense changes in nutrient supply in preimplantation embryo, mTORC1 signaling is promoted by maternal diabetes in rabbits[75] and repressed by low protein maternal diet in mice.[76] The key metabolic enzyme *AK1* was also shown to be under-expressed in HH blastocysts in the present work. AK1 is involved in the synthesis, equilibration and regulation of adenine nucleotides[77] and in multiple energetic and metabolic signaling processes, partially *via* the AMP-activated protein kinase (AMPK) pathway.[77] AMPK is a sensor of glucose availability and energy status, acting in coordination with mTORC1.[78] In parallel to the under-representation of the AMPK/mTORC1 pathway, an over-representation of the mitogen-activated protein kinase pathway was observed. *DUSP2* was over-expressed. DUSP2 is a phosphatase known to dephosphorylate and control subcellular localization of MAPKs, such as extracellular signal regulated p38 proteins and Jun N-terminal kinases.[79] These kinases control a variety of cellular processes including proliferation, apoptosis, differentiation and signal transduction by activating transcription factors such as c-JUN.[80] Transcription factors JUN and FOS, that, combined, form the transcription factor AP-1, were over-expressed.[81] We also identified over-expression of the transcription factors *EGR1* and *EGR2*, known to control ovarian function, embryo development and implantation.[82–84] These four transcription factors are defined as immediate early genes, known to rapidly and transiently be induced by diverse stimuli including nutrients, growth factor and stress to transduce signals to the downstream cascades involved in cell proliferation and apoptosis regulation.[85]

Programming by an altered maternal metabolic environment differs according to offspring sex.[12] Evidence for sexual dimorphism to programing stimuli prior to gonadal differentiation and the appearance of sex-related hormonal differences is emerging.[86] Differences in sex chromosome dosage emerges with embryonic genome activation (EGA) and X chromosome inactivation, at a timing varying across species.[23,87] In rabbits, major EGA occurs from the 8-cell stage and X chromosome inactivation initiates from blastocyst stage.[23] The sex chromosome transcripts' expression regulates autosomal genes' expression leading to transcriptional sexual dimorphism before implantation.[87] This transcriptional sexual dimorphism can lead to different susceptibilities to environmental stressors. Blastocyst sex-specific response to the maternal HH diet was explored here. Whereas gene-by-gene analysis did not identify differentially expressed genes, global analysis identified significant transcriptomic differences between male and female blastocysts. In female blastocysts, a large number of gene sets enrichment was induced by HH maternal diet, suggesting a coordinated response. As in the sex-independent analysis, functional pathways involved in fatty acid transport, glucose metabolism, nutrient sensing and transcriptional regulation were affected in female HH blastocysts. In addition, over-representation of gene sets involved in channel transporter and receptor signaling pathways was observed. Moreover, pathways involved in ribosomal RNA (rRNA) processing and more largely ribosome biogenesis were under-represented in female HH

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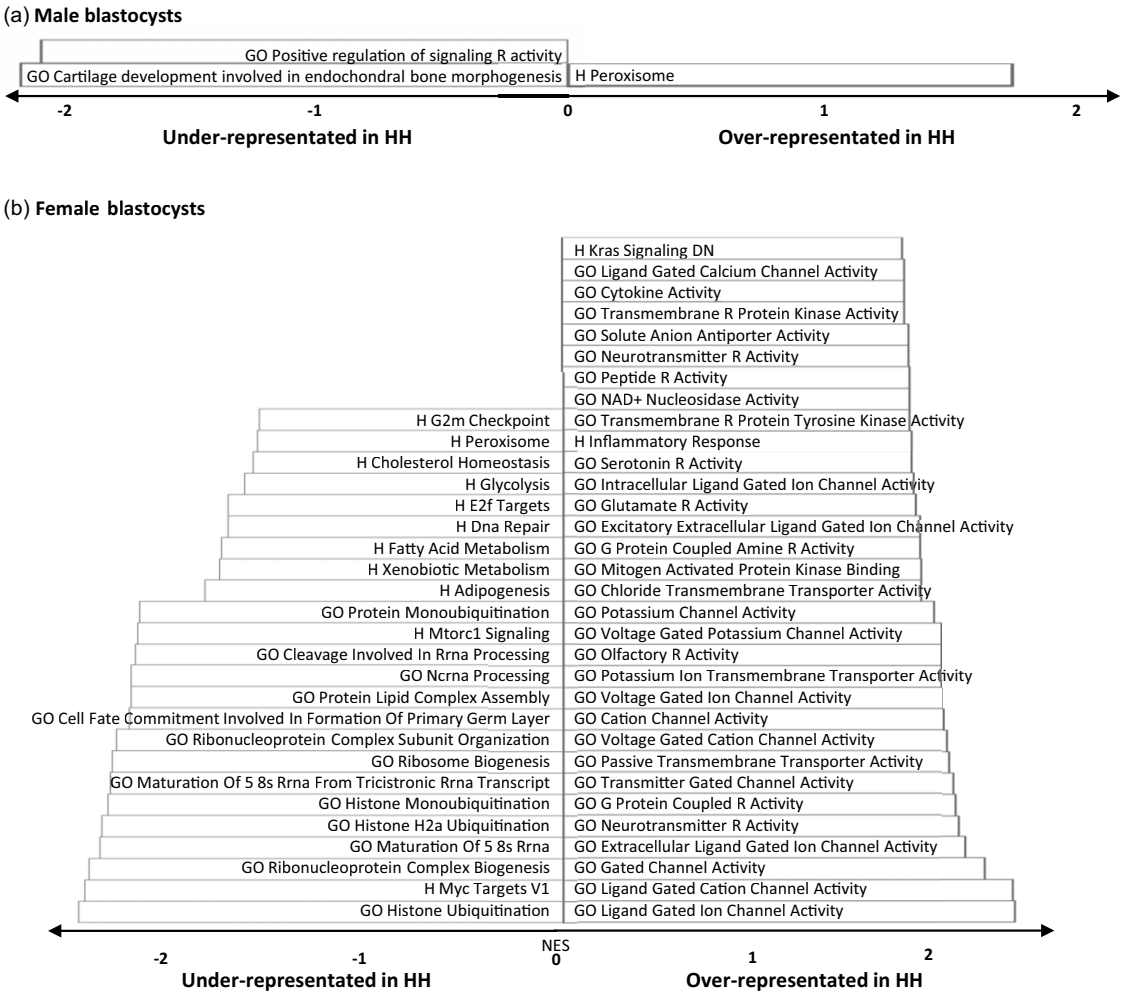

Figure 4. Enrichment analysis of gene transcription data of blastocysts according to sex. Gene sets enrichment analysis was performed on hallmark gene sets (H) and GO terms (GO) database in male (***a***) and female (***b***) blastocysts. Gene set was considered significantly enriched when FDR score was less than 0.25. Normalized enrichment score (NES) was used for the visualization. Positive and negative NES reflects respectively over-representation and under-representation in HH blastocysts.

blastocysts, suggesting an impact on translational mechanisms. Synthesis of rRNA is regulated in response to metabolic and environmental changes.[88] In mice, maternal low protein diet increases rDNA transcription and RNA per cell content in offspring via the mTORC1 signaling pathway.[76] In contrast to females, the HH maternal diet had very little impact on the transcriptome of male blastocysts, which exhibited very few gene sets enrichments. Thus, at the blastocyst stage, females seem to be more sensitive than males to the maternal HH diet. Interestingly, we have previously shown in pregnant rabbit does fed the HH diet, that at the fetal stage, males were more metabolically affected than females by the maternal diet.[15] It can be hypothesized that females, by adjusting their transcriptome in response to the maternal HH diet, were more successful in adapting to this altered environment than males.

In conclusion, the present work highlights the impact of a maternal high-fat diet on the embryo in its microenvironment. Metabolomics analyses revealed differences, notably an increased concentration of pyruvate, in the composition of uterine fluid surrounding the embryo from females on the HH diet. Thus, in the first stages of development, before the protective role of the placenta is established, the embryo is in direct contact with an altered environment. This result underlines the importance of exploring the impact of maternal metabolism alterations through in vivo exploration of uterine fluid composition in the DOHaD context. Further explorations, such as lipidomic studies, could improve our understanding of the consequences of the HH diet on the uterine fluid composition. Blastocysts that developed in this nutrient-rich environment were affected by a decrease in nutrients sensing and metabolism, partly through the mTORC pathway, that may represent protective mechanisms. The observation of a more altered transcriptome in female than in male embryos reinforces the hypothesis of the role of early sexual dimorphism in offspring programing.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S2040174423000260.

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Author contribution. Anne Couturier-Tarrade, Véronique Duranthon, and Pascale Chavatte-Palmer are contributed equally to this study.

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Competing interests. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the European regulations on animal welfare guides on the care and use of laboratory animals and has been approved by the institutional committee (Comethea, n°45 in French National register, experimental procedures n° 11/037 and n°2015042115447836_v2).

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