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A double hit preeclampsia model results in sex-specific growth restriction patterns

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Abstract

Preeclampsia is a multifactorial pregnancy-associated disorder characterized by angiogenic dysbalance and systemic inflammation, however, animal models which combine these two pathophysiological conditions are missing. Here we introduce a novel double-hit preeclampsia mouse model which mimics the complex multifactorial conditions that are present during preeclampsia, and allows for the investigation of early consequences for the fetus. Adenoviral overexpression of soluble fms-like tyrosine kinase (sFlt-1) and lipopolysaccharide (LPS) administration at mid-gestation in pregnant mice resulted in hypertension and albuminuria comparable to that of the manifestation in humans. A metabolomics analysis revealed that preeclamptic dams have increased plasma concentrations of phosphadytilcholines. The fetuses of both sexes were growth restricted, however in males a brain-sparing effect was seen as compensation to this growth restriction. According to the plasma metabolomics, male fetuses showed changes in amino acid metabolism, while female fetuses showed pronounced alterations in lipid metabolism. Our results show that combined exposure to sFlt-1 and LPS mimics the clinical symptoms of preeclampsia and affects fetal growth in a sex-specific manner with accompanying metabolome changes.

Summary statement: Double-hit exposure to the anti-angiogenic factor sFlt-1 and the pro-inflammatory factor LPS presents a novel, comprehensive model of preeclampsia in mice, which leads to sex-specific metabolomic differences in fetuses.

Introduction

Preeclampsia is a multisystemic pregnancy-associated disorder that is identified after the 20th week of gestation with the onset of hypertension and proteinuria (Mol et al., 2016). More importantly, it is one of the most frequent complications of pregnancy, affecting 3-7% of the population (Mol et al., 2016; Rajakumar et al., 2005). In up to 60% of cases, especially in early onset pre-eclampsia, it is further complicated by fetal growth restriction (Weiler, Tong, & Palmer, 2011; Xiao, Sorensen, Williams, & Luthy, 2003). Moreover, preeclampsia and the subsequent fetal growth restriction leads to increased susceptibility of the offspring to chronic cardiometabolic diseases later in life (see Stojanovska et al., 2016 for review). In the recent years, it has become apparent that preeclampsia shares characteristics with the metabolic syndrome, at least through altered angiogenic and inflammatory markers (Salzer, Tenenbaum-Gavish, & Hod, 2015; Scioscia, 2017).

Two of the most pronounced pathophysiological mechanisms during preeclampsia are the disrupted angiogenic balance and the increased systemic inflammatory responses. Firstly, the concentrations of antiangiogenic factors are elevated in preeclamptic patients (Hertig et al., 2004); e.g., elevated levels of circulating soluble fms-like tyrosine kinase 1 (sFlt-1) have been shown to be clearly associated with the severity of preeclamptic symptoms (Park et al., 2005). Secondly, several markers of inflammation, such as tumor necrosis factor alpha, interleukin 6, C reactive protein are also increased in plasma of preeclamptic patients (Borzychowski, Sargent, & Redman, 2006). Moreover, inflammation impacts blood pressure and renal function during pregnancy, contributing to the clinical course of preeclampsia (Cotechini et al., 2014; Kalinderis et al., 2011).

In vivo models of preeclampsia are of extreme importance in clarifying the pathophysiological aspects of the disease and the evaluation of potential fetal programming mechanisms. Inflammatory models of preeclampsia, such as low dose endotoxin infusion (Faas, Schuiling, Baller, Visscher, & Bakker, 1994) or TNFa administration (Cotechini et al., 2014), have provided significant insights into kidney and placental pathophysiology during preeclampsia based on inflammatory mechanisms. Furthermore, models that involve antagonism of angiogenesis (Maynard et al., 2003; Venkatesha et al., 2006) has been widely studied in the evaluation of the maternal and fetal health (Bytautiene et al., 2013, 2011; Lu et

al., 2007; Patten et al., 2012). Altogether, these models are each based only on a single pathophysiological mechanism (McCarthy, Kingdom, Kenny, & Walsh, 2011; Sunderland, Hennessy, & Makris, 2011) that results in some of the clinical symptoms of preeclampsia, not covering the full pathophysiological spectrum that occurs during this disorder in human patients. Therefore, we aimed to develop a model that involves the interplay of both anti-angiogenesis and inflammation.

During pregnancy, perturbations in maternal health can lead to morphological and functional changes of different organ systems in the offspring leading to a demonstrable impact on the offspring's health for a lifetime (Davis et al., 2012; Kajantie, Eriksson, Osmond, Thornburg, & Barker, 2009); an effect known as *developmental programming*. For example, during early onset-preeclampsia, there is a 2- to 4- fold increased risk for fetal growth restriction (Xiao et al., 2003). Putative predisposing factors for the development of cardiometabolic diseases in the growth-restricted offspring include alterations in metabolism, the epigenome or fetal autonomic regulation (Ching et al., 2015; Jiménez-Chillarón et al., 2012; Schäffer et al., 2009). However, far too little attention has been paid on the effects of preeclampsia on the maternal and fetal metabolome, which can provide a better understanding of the relationship between early-life circumstances and later-life disease susceptibility at a metabolomics level. The current study describes a novel, double hit model of preeclampsia that closely resembles the complete clinical course. We apply this novel model to investigate the genuine role of anti-angiogenesis and inflammation in pregnant mice with regard to metabolic fetal outcomes and function.

Materials and methods

Animals and experimental procedures

C57Bl/6J mice (Charles River, France), between 9-12 weeks old, were housed in a light and temperature controlled facility (lights on from 7:00 am until 7:00 pm, 21 ^{o}C). Mouse chow diet (2186 RMH-B, AB diets) and water were provided to the animals ad libitum. Animals were timely mated overnight. When a vaginal plug was present the following day it was counted as gestational day 0.5. At gestational day 8.5, animals were randomly assigned to receive either recombinant adenovirus encoding mouse sFlt-1 (Ad-sFlt1) or empty control adenovirus (Ad-null) via retroorbital injection. At gestational day 10.5, animals received either 25 µg/kg LPS (E. coli 0111:B4, Sigma-Aldrich, St Louis, MO, USA) in the group that received AdsFlt-1 or PBS (in the group that received the Ad-null). The dosage of adenovirus and LPS have previously been established in pilot studies (data not shown). At gestational day 16.5, the pregnant animals were placed in a metabolic cage for 24 hours to collect urine and measure food and water consumption. At gestational day 18.5, blood pressure was assessed via the abdominal aorta (Datex-Ohmeda, Cardiocap/5). Placenta and fetal tissues were collected at gestational day 18.5. Tissue weights were directly recorded as fresh weight. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen (DEC number 6803).

Amplification and purification of sFlt-1 and control adenovirus

Adenovirus vector stock of Ad-null (a kind gift from U.J. Tietge, University Medical Center Groningen, Netherlands) and Ad-sFlt1 (a kind gift from S.A. Karumanchi, Beth Israel Deaconess Medical Center, Boston, MA, USA) were used for adenoviral gene delivery. Viruses were amplified in HEK 293A cells at a multiplicity of infection (MOI) of 10. Adenoviral purification was performed with a cesium chloride (CsCl) density gradient (d= 1.45 g/ml and 1.20 g/ml). Adenoviral elution was performed with DG columns (Biorad, Temse, Belgium). The concentration of plaque forming units (PFU) was analyzed with an enzyme-linked immunoassay that detects the adenoviral hexon (Adeasy viral titer kit, Agilent Technologies, Santa Clara, CA, USA). $1x10^{9}$ PFU of adenovirus expressing an empty vector (Ad-null; n=9) or mouse sFlt-1 (Ad-sFlt-1; n=9) in 100 µl PBS were injected via the retroorbital plexus on gestational day 8.5.

Plasma analysis

Maternal blood was collected on gestational day 18.5 in EDTA containing tubes (Greiner Bio-One, Kremsmünster, Austria) with a heart puncture. Within a half an hour the blood was centrifuged for 20 minutes at 1000 rpm and the plasma was stored at -80^{o}C until analysis. Fetal blood was collected by nicking the left ventricle of the heart, while the fetuses were slightly tilted in order to keep the pooled blood in the thoracic cavity while it was collected in EDTA-coated capillary tubes (Greiner Bio-One, Kremsmünster, Austria). SFlt-1 concentrations in plasma were determined using a mouse sFlt-1 ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

Plasma metabolome detection

Plasma was obtained and stored as described above. Plasma metabolome analysis was performed with Biocrates AbsoluteIDQ p180 Kit at their facility (Biocrates Life Sciences AG, Austria), as described previously (Stanley et al., 2015). In short, a commercially available direct flow injection and LC-MS/MS kit was used to analyze 188 available metabolites in plasma samples, including hexose (1), amino acids (21), biogenic amines (21), glycerophospholipids (90), sphingolipids (15) and acylcarnitines (40). Internal standards were pre-pipetted and a calibration standard mix in seven different concentrations were included in a standardized assay in 96 well plate format. Per sample 10 µl of plasma was loaded in each well. Waters Acquity BEH C8 column (75 mm × 2.1 mm, particle size of 1.7 µm) (Waters, Milford, USA) was used for chromatographic separation at 50°C using a gradient mixture of solvent A (water with 0.2% formic acid) and solvent B (acetonitrile with 0.2% formic acid) at a flow rate of 0.9 ml/min using a linear gradient. The optimized parameters included: capillary at 3.2 kV; desolvation gas flow at 1200 l/h; cone gas flow at 150 l/h; desolvation temperature at 650°C; source temperature at 150°C and cone voltage at 10 V, respectively. The samples were delivered to API4000 Qtrap® tandem mass spectrometry instrument (Applied Biosystems, Foste City, CA), using reverse phase HPLC column followed by a direct flow injection assay.

Urine analysis

Urine samples were collected by placing the pregnant dams in metabolic cages at gestational day 16.5 for 24 hours. The protein and albumin levels were determined using the

Pierce BSA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) or Assaypro Mouse Albumin Elisa kit (St. Charles, MO, USA), respectively. The concentration of total protein and albumin per sample was multiplied by the 24-hour urine volume.

Tissue preparation and histological analysis

At embryonic day 18.5, anesthetized pregnant females were killed by cervical dislocation. Embryos were dissected in PBS, and the amniotic membrane was removed from the placenta. Placentas were fixed in in 4% paraformaldehyde (PFA) for 24 hours and stored in 70% ethanol until embedded in paraffin under standard procedures.

Placental sections (7 µm) were mounted on standard slides (Engelbrecht Medizin- und Labortechnik GmbH, Edermünde, Germany). For morphological analysis, sections were stained with hematoxylin and eosin (H&E).

Morphometric analysis

Morphometric analysis of placentas and of placental compartments (labyrinth and spongiotrophoblast layer) was performed on nine serial sections of the central region of at least five placentas from each experimental group (control Ad0+PBS, n=5 and AdsFlt+LPS, n=6) with an Axiophot model microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Nikon DS-U1 camera and NIS-BR 3.1 software (Nikon, Düsseldorf, Germany). An auto-white-balance-correction was performed using ImageJ 1.51n (Wayne Rasband, Maryland, USA).

RNA isolation and gene expression analysis

Total RNA from placentas was extracted with TriReagent (Life Technologies, Carlsblad, CA, USA). RNA quality and quantity was assessed with Nanodrop 2000c (Nanodrop Technologies, Wilmington, DE). CDNA synthesis was performed on 1 µg of total RNA using M-MLV reverse transcriptase (Life technologies, Carlsblad, CA, USA), RNaseOUT (Life Technologies, Carlsblad, CA, USA), random nonamers (Sigma-Aldrich, St Louis, MO, USA). For quantitative real-time PCR, cDNA was amplified with TaqMan (Applied Biosystems, CA, USA) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, CA, USA). Primers used for RT-qPCR are listed in supplementary table 1 (see also Kühnel et al. 2017). The average expression levels of mouse *b-actin* and *Gapdh* were used as a house-keeping gene in all qPCR analysis and a standard curve method was used for quantification.

Statistical analysis

Differences between groups were calculated with the Mann-Whitney U test. Data are presented as a median and interquartile ranges, if not stated otherwise. For all statistical tests, a p-value < 0.05 was considered significant. Pearson R correlation was used to check the association between selected parameters. Sample size was determined based on alpha =0.05, power 0.90, difference considered meaningful 20% and anticipated coefficient of variation 10. Data was analyzed using GraphPad Prism 8 Software (GraphPad) for Windows.

For metabolomics data, all the analyses were performed with MetaboAnalyst 3.0 (Xia and Wishart, 2016). For row-wise normalization we chose to normalize with a reference sample (sample in the control with the least missing values) and column-wise normalization was done by log2 transformation of the data. Univariate data analysis was performed using volcano plot with fold change threshold of 1.4 and t-tests threshold of 0.1, as well as Mann-Whitney U test. Multivariate data analysis was performed with principal component analysis (PCA) and partial least squares discriminant analysis PLS-DA in order to visualize the metabolic differences between controls and double hit preeclampsia subjects (dam and fetuses). The PLS-DA model was assessed by its R2 and Q2 values to avoid the risk of overfitting and cross-validation was performed with LOOCV (leave one out cross validation) model. The variable importance in the projection (VIP) scores higher than 1.0 were considered relevant for group discrimination (Jansson et al., 2009).

Data availability

Metabolic raw data have been uploaded to the Metabolomics workbench (www.metabolomicsworkbench.org) with the data track ID: 1571, and will be available to the community after February 2019. Meanwhile the raw data is available from the authors upon request.

Results

Combined sFlt-1 and LPS exposure induces preeclampsia symptoms in pregnant dams

By this novel approach, C57Bl/6J mice were subjected to adenoviral overexpression of sFlt-1, and 48 hours later challenged with LPS. The weight gain, food and water consumption on gestational day 17.5 were not different between the groups (Supplementary Figs S1B-D). At gestational day 17.5, total urinary protein excretion (Fig. S1A), as well as of mouse-specific albumin (Fig. 1A), were significantly increased in the dams that have been exposed to the double hit of sFlt-1 and LPS. In continuation, these dams had a 2-fold increased sFlt-1 concentrations in their plasma (Fig. 1B). On gestational day 18.5, also systolic blood pressure in the pregnant dams exposed to the double hit was significantly increased in comparison to controls (Fig. 1C). Moreover, there was a positive correlation between the blood pressure values and the obtained sFlt-1 concentrations of the pregnant dams (Fig. 1D). There were no differences in the number of pups nor in the percentage of fetal resorptions between the groups (Supplementary Figs S1E, F). Together, these data show that the mid-gestation double hit exposure to sFlt-1 and LPS replicates the clinical features of human preeclampsia in pregnant dams.

Double hit preeclampsia is not accompanied by changes in placental compartment area

In order to evaluate whether the double hit exposure of pregnant dams to sFlt-1 and LPS differentially affects the placental growth or morphology, we analyzed placental sections at gestational day 18.5. We assessed total placental area as well as the different placental compartments, namely the labyrinth and the spongiotrophoblast layer. Total placental area tended to be decreased in the double hit placentas in comparison to controls (Fig. 2B, $p=0.052$). This can be attributed both to the labyrinth and the spongiotrophoblast layer, although the specific changes did not reach statistical significance (Figs 2C, D). Assessment of the labyrinth to spongiotrophoblast ratio showed no differences between the double hit placentas and control ones (Fig. 2E). Despite the overall decreased placental area, the placental morphology between the double hit preeclamptic dams and controls was unaffected.

Maternal plasma phosphatidylcholines are increased during double hit preeclampsia

Given that preeclampsia is characterized by widespread adaptations in metabolites (Kenny et al., 2010), including altered concentrations of lipids and carnitines (Kelly et al., 2017) we expected that the double hit preeclamptic dams would have a unique metabolomic profile, resembling that of the human condition. A total of 183 metabolites, including monosaccharides, amino acids and several types of lipids (acylcarnitines, sphingolipids and glycerophospholipids) were investigated by tandem mass (MS/MS) spectrometry. Metabolites that were below the lower limit of quantification (<LLOQ) were excluded (Supplementary Table 1); the remaining 141 metabolites were included in the analysis. To identify metabolomic differences between the groups, we performed an unsupervised principal component analysis (PCA) (Supplementary Fig. S2A) and a supervised partial least squares discriminant analysis (PLS-DA) (Fig. 3A). The results show that the metabolome profile of the double hit preeclamptic dams tends to cluster separately from the one of controls (Fig. 3A). The clear distinction of these groups is based on the variable importance of projection (VIP) scores obtained from each of the 141 metabolites included in the analysis and the top 15 variable compounds are listed in Supplementary Fig. S2B. A heat map representation of the top 25 modified metabolites, showed distinct metabolic differences between the groups, with the levels of a number of metabolites from the class of phosphatidylcholines (PC) being upregulated in the double hit preeclamptic dams (Fig. 3B). Furthermore, we examined the top modified metabolites with a threshold combination of fold change and t-tests. In total, 10 metabolites were significantly changed in the plasma from double hit preeclamptic dams including several long chain fatty acid phosphatidylcholines (PC) and acylcarnitine C4 (Table 1).

Fetuses exposed to double hit preeclampsia show growth restriction differences in a sex-specific manner

Considering that up to 60% of the early onset preeclamptic pregnancies (Weiler 2011) are complicated by fetal growth restriction, we assumed that our double hit preeclampsia model would also lead to impaired fetal growth. Therefore, we phenotyped body size and major organs at GD 18.5 to define the presence of as well as the type of growth restriction. Male and female fetuses from double hit preeclamptic dams weighed less in comparison to

controls (Fig. 4A). The liver weight was compromised in both sexes (Fig. 4B), while the brain was smaller only in the female fetuses that were exposed to double hit preeclampsia (Fig. 4C). In order to evaluate whether there is a brain sparing effect in our fetuses, we calculated the brain to liver ratio. This was significantly increased for the males, while no brain sparing was observed for the females exposed to the double hit preeclampsia (Fig. 4D). These data show that double hit preeclampsia results in fetal growth restriction and brain sparing is only observed in the males.

Fetal metabolome after double hit preeclampsia exposure shows sex-specific differences

To explore whether the different growth restriction patterns are associated with metabolomic changes, we analyzed the fetal plasma metabolome. The univariate analysis of log-transformed mouse fetal plasma metabolome data revealed significant sex-specific differences. The unsupervised principal component analysis (PCA) (Supplementary Fig. S3A) and the supervised partial least squares discriminant analysis (PLS-DA) (Fig. 5A) showed an overlap between the metabolic footprint of the males exposed to double hit preeclampsia and the controls. Two metabolites were significantly decreased in the plasma of the double hit preeclampsia exposed male fetuses when compared to controls, including the amino acids proline and threonine ($p < 0.05$) (Fig. 5B). There were no sex-specific differences between the groups for these metabolites (Fig. 5B). In contrast, the unsupervised multivariate analysis PCA (Supplementary Fig. 3B) and the supervised PLS-DA (Fig. 5C), revealed more obvious clustering pattern between the metabolic footprint of female fetuses exposed to double hit preeclampsia and controls. In total, 5 metabolites showed reduced levels ($p<0.05$) in the plasma from female fetuses exposed to double hit preeclampsia in comparison to controls, including phosphatidylcholines (PC ae 32:1; PC ae 42:1), acylcarnitine (C14:1) and sphingomyelins (SM C24:1; SM C24:0), althought only C14:1 and PC ae 32:1 showed sex-specific differences between the control groups (Fig. 5D).

To determine whether these sex-specific metabolomic differences are potentially associated with changes in placental nutrient transport, we evaluated the expression levels of several amino acid-, fatty acid-, and glucose transporters in the placenta. However, no differences were observed in the gene expression levels between the groups with male placentas (Figs 6A, B). On the contrary, in the female placentas, there was a significantly

decreased expression of sodium-coupled neutral amino acid transporter 1 (*Snat1*), fatty acid transporter 6 (*Fatp6*) and fatty acid binding protein 3 (*Fabp3*) in the placentas exposed to double hit preeclampsia.

Discussion

The results of this study demonstrate that a combined exposure to an anti-angiogenic (sFlt-1) and a proinflammatory (LPS) factor lead to the development of preeclampsia in mice, mimicking the human clinical course of preeclampsia. This double hit exposure leads to an increase in blood pressure, albuminuria, increased phosphatidylcholines and smaller placentas in the affected dams. Although the placental compartments were not severely compromised, fetuses were growth restricted in a sex-specific manner and showed different metabolomic footprints.

Preeclampsia is closely linked to the metabolic syndrome on several levels. Obesity and diabetes mellitus serve as known risk factors for preeclampsia (Persson, Cnattingius, Wikström, & Johansson, 2016; Weissgerber & Mudd, 2015) and increased pro-inflammatory cytokines contribute to the pathogenesis of preeclampsia (Cotechini et al., 2014; Lockwood et al., 2008; Pinheiro et al., 2013). Furthermore, preeclamptic women are at increased risk to develop cardiovascular diseases later in life (Irgens, Reisaeter, Irgens, Lie, & Lie, 2001; Wu et al., 2017). Moreover, dysbalance in angiogenesis impacts endothelial function resulting in changes that resemble preeclamptic symptoms, characterized by increased plasma sFlt-1 levels and hypertension (Lu et al., 2007; Maynard et al., 2003; Venkatesha et al., 2006). However, a combined effect of these distinct pathophysiological components to the development of preeclampsia has not been addressed thus far. Therefore, a double hit exposure to anti-angiogenic factors and low-grade inflammation is useful in employing a comprehensive *in vivo* model for preeclampsia.

Here, we reported that exposure to sFlt-1 and LPS in vivo lead to hypertension and albuminuria in the pregnant dam. Although earlier reports suggested that high-dose LPS administration may lead to a fetal loss (Kohmura, Kirikae, Kirikae, Nakano, & Sato, 2000; Silver

et al., 1995), inflammation induced by low dose LPS administration showed no effect on the number of fetuses between the groups in our study (data not shown). sFlt-1 binds to angiogenic factors such as vascular endothelial growth factor (*Vegf*) and placental growth factor (*Plgf*) resulting in endothelial dysfunction (Barleon et al., 1997; Tsatsaris et al., 2003). With regard to the impact of endothelial dysfunction on blood pressure, it has been previously shown that inhibition of endothelial protectors (such as *eNOS*) can lead to hypertension (Sander, Chavoshan, & Victor, 1999), granting a role for sFlt-1 in blood pressure regulation. In our model, we observed increased plasma sFlt-1 levels to have a positive correlation with blood pressure values. Altogether, we demonstrate that this novel double hit rodent model is very similar to the human clinical representation of preeclampsia.

Studies by Kühnel et al. (2017) using a placental-specific overexpression of human sFlt-1 in a lentiviral mouse model of preeclampsia, as one hit, led to intrauterine growth restriction (IUGR) in the fetus and resulted in lower placental weights, the same finding as observed in our double hit model. However, in the study by Kühnel et al. (2017) a smaller labyrinth as the transporting trophoblast, and the loss of glycogen cells in the junctional zone were observed. In contrast to the findings of this study, the expression of the glucose diffusion channel Cx26 is decreased, expression of one fatty acid transporter, CD36, is significantly increased and the amino acid transporters are unchanged. These differences might be due to the different mouse strains used in both studies, the continuous sFlt-1 production in the lentivirus model, or by its lower sFlt-1 concentration in our double hit model.

Derived from the clinical observation that preeclamptic patients have 4- to 8-fold increased risk in developing cardiovascular disorders later in life (Irgens et al., 2001; Wu et al., 2017), characterization of their metabolic footprint is of major interest. In preeclampsia, a change in metabolome has been reported (Benton, Ly, Vukovic, & Bainbridge, 2016; Kelly et al., 2017) with specific effects on the fatty acid metabolome, sharing similarities with other cardiovascular and idiopathic inflammatory diseases (Famularo, De Simone, Trinchieri, & Mosca, 2004; Ruiz-Núñez, Dijck-Brouwer, & Muskiet, 2016). Moreover, preeclamptic patients show increased choline levels in plasma and urine (Austdal et al., 2014; Friesen, Novak, Hasman, & Innis, 2007) most probably due to increased oxidative stress. In our model, we also report an increase of several types of long-chain fatty acids phosphatidylcholines. Although the metabolic synthesis and function of these phosphatidylcholines is yet to be elucidated,

they have been associated with peroxisomal disorders, because peroxisomes are needed for beta-oxidation of long chain phosphatidylcholines. Moreover, our results are in agreement with the metabolomics analysis of the transgenic model of preeclampsia employing catechol-O-methyl transferase knockout mice (Stanley et al., 2015). However, in their model more profound changes were reported in the metabolome including increased levels of several phosphatidylcholines, sphingomyelins, and acylcarnitines. This can be explained by the different mechanisms applied to induce the preeclampsia phenotype, where the catechol-O-methyl transferase knock-out acts via inhibition of enzymes involved in the estrogen conversion. We hence conclude that our joint intervention with sFlt-1 and LPS only increases the glycerophospholipids metabolites without affecting other classes of metabolites.

Exposure to a harsh intrauterine environment has been implicated in sex-specific consequences for the offspring later in life (Lu et al., 2007; Stark, Clifton, & Wright, 2009). Although the relative contribution of sex on the fetal size, body proportions and growth patterns (Melamed et al., 2013) is not well defined, evidence has accumulated that males have increased body weight at birth in comparison to females in uncomplicated pregnancies (Broere-Brown, Schalekamp-Timmermans, Hofman, Jaddoe, & Steegers, 2016). In addition, in humans during the first 20 weeks of pregnancy, male fetuses have higher head circumference in comparison to females, but this difference is almost non-existent as the pregnancy proceeds (Broere-Brown et al., 2016). In this context, the timing and exposure to harsh intrauterine stimuli are relevant for sex-specific outcomes. In the current study, we have shown that exposure to sFlt-1 and LPS during mid-gestational days results in smaller brains in the female fetuses. On the contrary, no weight changes were observed in the male brain, which is consistent with the observation that in humans, males have decreased growth rate of the head circumference in the last weeks of pregnancy (Broere-Brown et al., 2016), making them then less susceptible to the harsh intrauterine conditions. Data on sex-specific differences in the fetal growth responses due to preeclampsia are still limited, but a study from Stark et al. reported that female infants have significantly lower birth weight percentiles whereas males maintain normal growth (Stark et al., 2009). This is, at least in part, in accordance with our results where female fetuses show symmetrical growth restriction, while on the other hand males show brain-sparing and asymmetrical growth restriction.

Sufficient delivery of macronutrients is an important pre-requisite for optimal fetal development. Amino acids, acylcarnitines, and glycerophospholipids act as key metabolic factors for the fetus and the placenta (Alexandre-Gouabau et al., 2011). Moreover, a sudden shift in the source of energy will lead to adaptations in several metabolic processes such as fatty acid oxidation, gluconeogenesis, and ketogenesis (Cotter, Ercal, D'Avignon, Dietzen, & Crawford, 2013). In response to a hypoglycemic insult, several amino acids, including proline and threonine, serve as gluconeogenic mediators (Houin et al., 2015). Furthermore, an excess of stress hormones (Rando et al., 2016) and inflammatory cytokines (Hashizume, Yoshida, Koike, Suzuki, & Mihara, 2010) can affect the hepatic lipid catabolism and lipid metabolites severely. In our study, we reported that male and female fetuses are affected with different degrees of growth restriction and have differentially affected metabolic profiles. Whereas the males only have lower concentrations of amino acids such as proline and threonine, the females show decreased levels of certain acylcarnitines, sphingomyelins, and glycerophospholipids. This suggests that the symmetrical growth restricted female fetuses in our double hit preeclampsia model have dysbalanced fat and energy metabolism. Recently, a study was reported showing differences in the cord blood metabolome from preeclamptic neonates in comparison to controls (Jääskeläinen et al., 2018). The most affected metabolites are similar to the ones we report and they included: acylcarnitines, phosphatidylcholines and metabolites included in urea and tryptophan metabolism. However, the concentrations of these metabolites were higher in the cord blood from preeclamptic neonates and there were no clear distinction between the sexes. Moreover, it is not clear whether the collected cord blood was venous or arterial in order to distinguish between neonatal and maternal background of the plasma. In conclusion, in our double hit preeclamptic model, fetuses show metabolic differences, which are clearly sex-specific.

Finally, it is also possible that alterations of transport processes in the placenta may contribute to the observed growth-restriction phenotype. As a first step, we here measured gene expressions of several transporters in the placenta. Interestingly, we registered limited changes in the gene expression pattern of nutrient transporters in the placenta and only decreased levels of amino acid transporter (*Snat1*) and fatty acid transporters (*Fabp3* and *Fatp6*) were observed in females placentas exposed to double hit preeclampsia. In particular, it is known that these fatty acids transporters are increased in obese pregnancies (Díaz, Harris,

Rosario, Powell, & Jansson, 2015), but are quite resilient to hypoxic conditions (Jadoon, Cunningham, & McDermott, 2015). Moreover, decreased levels of *Snat1* are associated with growth restriction (Chen et al., 2015; T. Jansson, Ylvén, Wennergren, & Powell, 2002) and can be correlated to the severity of the restriction. Our findings that these transporters are down-regulated only in the female double hit preeclamptic placentas suggest that they might be involved in the mechanisms leading to the growth restriction and metabolic changes. Compatible with this, up-regulation of placental transporters may contribute to fetal overgrowth (T. Jansson et al., 2006; Segura et al., 2017). In contrast, the gene expression was not altered in the male placentas and the minor changes in the metabolic footprint of male fetuses exposed to double hit preeclampsia might be explained by increased fetal or placental consumption of certain metabolites. However, the mechanisms underlying the different metabolomics patterns in fetuses exposed to preeclampsia still remains to be fully elucidated, as here, we could only determine gene expression levels and not metabolite fluxes.

In conclusion, in this study, we present a clinically relevant mouse model that closely mimics human preeclampsia. Moreover, it results in sex-specific differences in the growth restriction pattern and the metabolomic footprint (see Fig. 7 for graphical abstract), which in turn can shed a light on the sex-specific programming effect of adult-onset disorders due to preeclampsia.

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Conflict of interest

The authors have no conflict of interest to declare.

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Table 1. Plasma metabolite differences between control and double hit preeclamptic dams. FC: fold change; PC-phosphatydilcholine; AA-diacyl; AE-acyl-alkyl; C- carnitine

Name	FC	Log2 (FC)	p-value
PC aa C34:1	0.63	-0.67	7.0299E-5
PC aa C36:3	0.64	-0.65	0.004
PC ae C34:1	0.62	-0.71	0.006
PC aa C36:5	0.50	-0.99	0.01
PC aa C36:1	0.56	-0.84	0.02
C4	0.48	-1.06	0.02
PC ae C38:3	0.62	-0.69	0.03
PC aa C38:3	0.52	-0.99	0.03
PC aa C32:1	0.52	-0.96	0.04
PC ae C36:3	0.56	-0.83	0.04

Figures

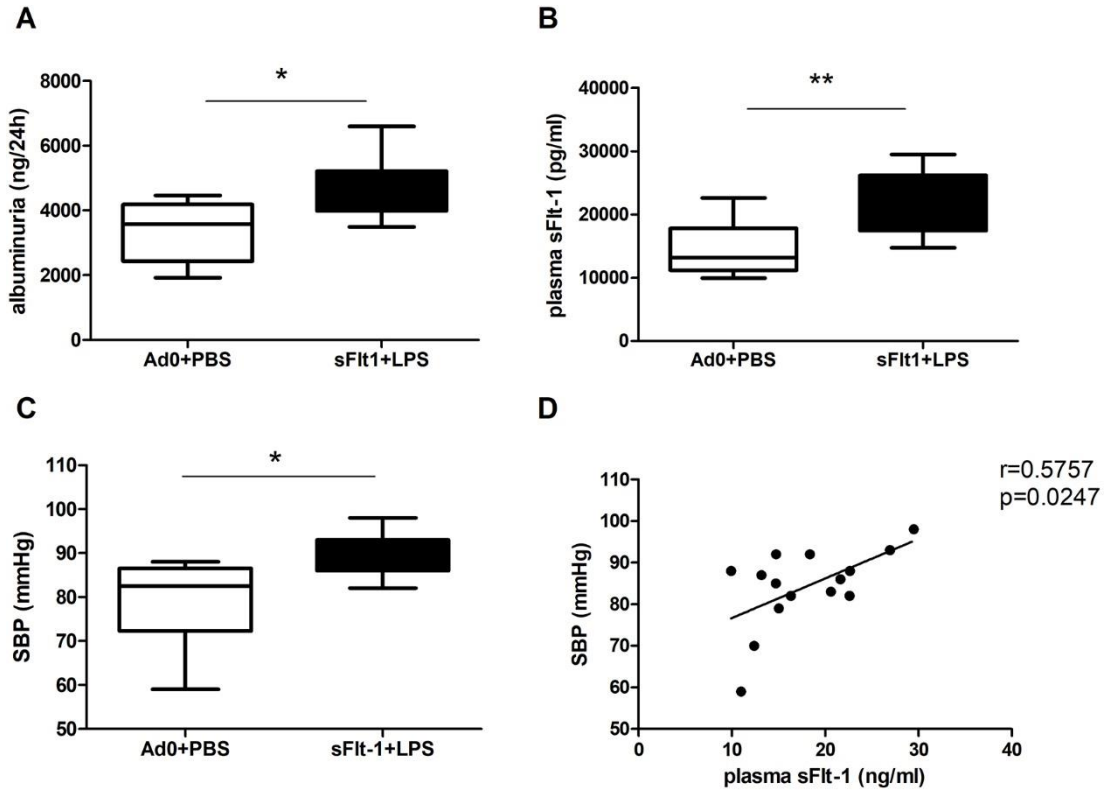

Figure 1. Double hit exposure to sFlt-1 and LPS in pregnant dams can induce preeclampsia symptoms. (A) Urine albumin concentration in 24 hour urine samples from pregnant dams at GD 17.5 (Ad0+PBS n=8; sFlt-1+PBS n=10). (B) Plasma sFlt-1 concentrations from pregnant dams at GD 18.5 (Ad0+PBS n=9; sFlt-1+PBS n=8). (C) Systolic blood pressure in pregnant dams at GD 18.5 (Ad0+PBS n=8; sFlt-1+PBS n=7). (D) Correlation between sFlt-1 plasma concentrations and systolic blood pressure in pregnant dams (r= 0,57;p= 0,02; n=15). Data are given as median and interquartile ranges (figure A, B and C), * p<0.05; **p<0.01.

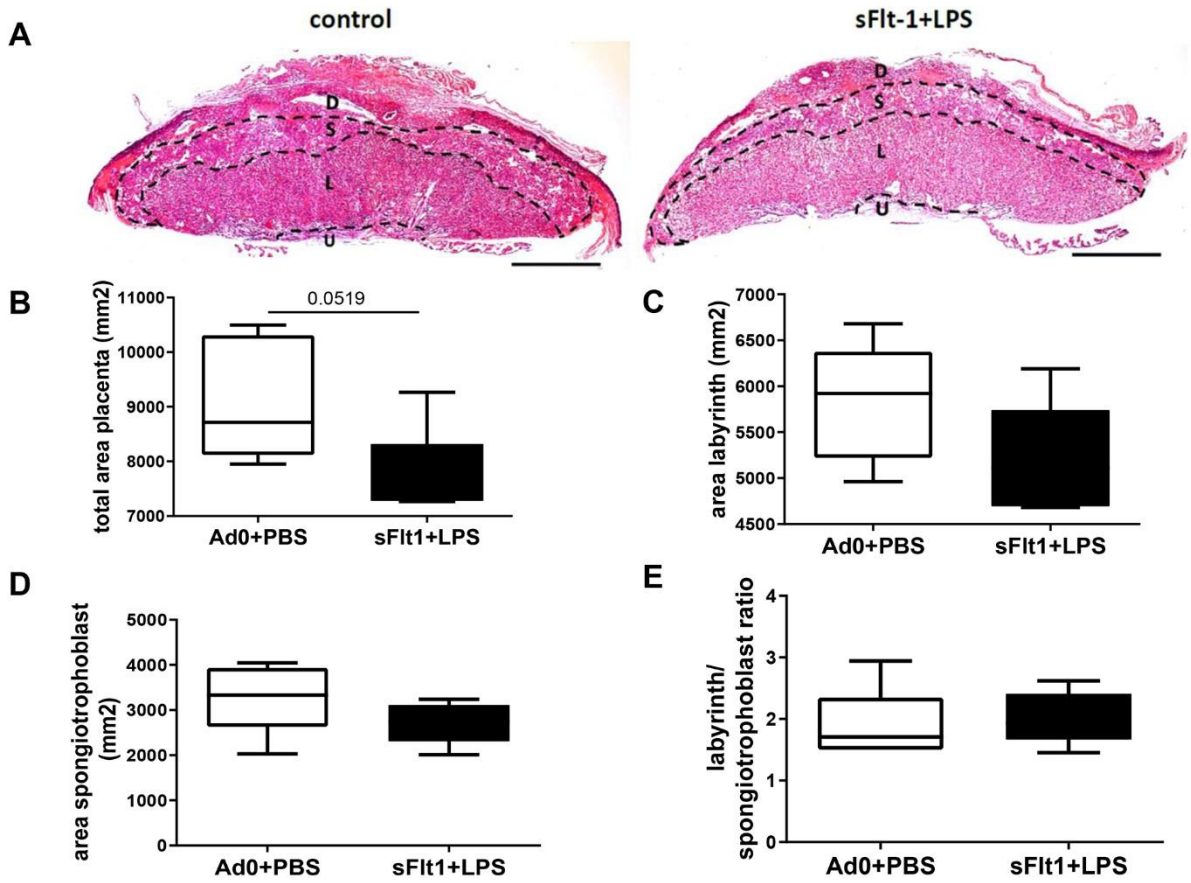

Figure 2. Placental morphology at GD 18.5 in double hit preeclampsia model. (A) Placentas were collected and 7 µm sections were stained with hematoxylin and eosin. Scale bar = 1000 µm. D= decidua, S= spongiotrophoblast layer, L= labyrinth layer, U=umbilical cord. Surface area of (B) the whole placenta, (C) the labyrinth layer and, (D) the spongiotrophoblast layer were measured in mm^2. (E) The ratio of the labyrinth area to the spongiotrophoblast area. Data given as median and interquartile ranges (Figure B-E; Ad0+PBS n=5; sFlt-1+PBS n=6; *p=0,05).

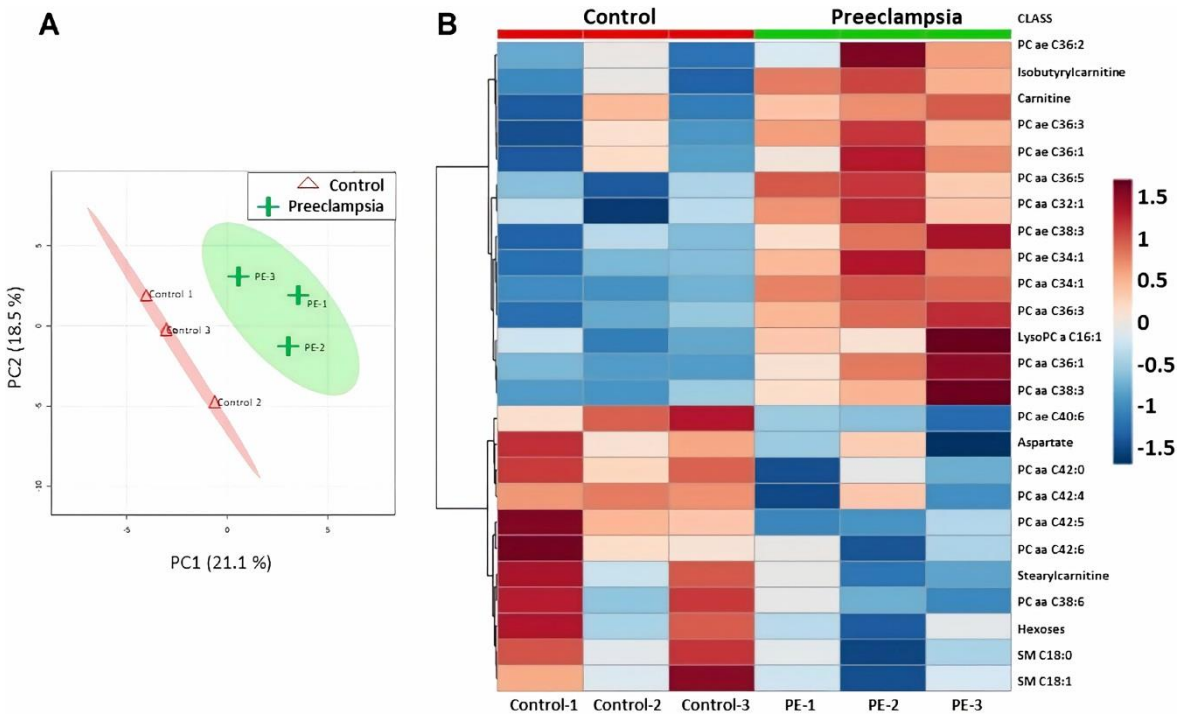

Figure 3. Maternal metabolome during double hit preeclampsia (n=3) (A) Supervised partial least discriminatory analysis PLS DA on 141 metabolites in plasma of control and double hit PE dams (R^2=0.827, Q^2=0.348) (B) Heat map representation of top 25 modified metabolites, color-coding intensity in the red spectrum shows increases of given metabolites and color intensity in the blue spectrum shows decreases of the given metabolites.

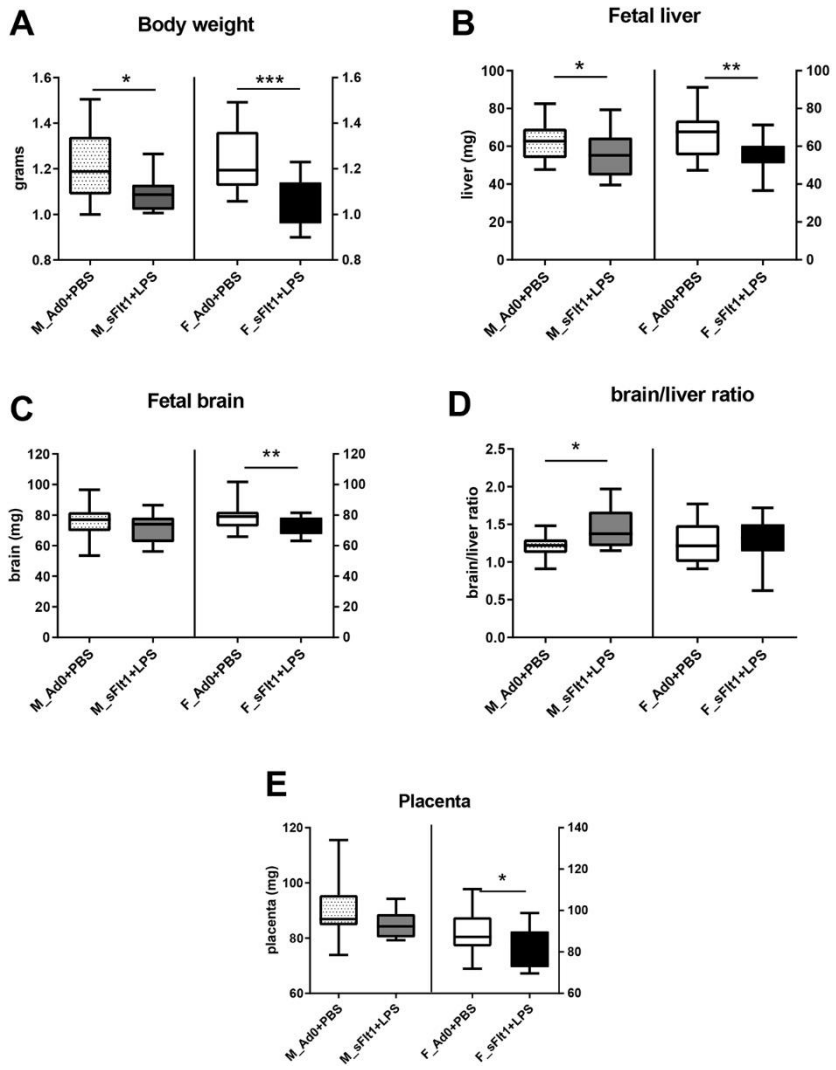

Figure 4. **Fetal characterization at GD 18 in the double hit preeclampsia model.** (A) Fetal body weight in grams, (B) fetal liver weight in grams, (C) fetal brain weight in grams, (D) brain to liver ratio, (E) placental weight. Data given as median and interquartile ranges, Ad0+PBS n=17; sFlt-1+PBS n=21, for males and Ad0+PBS n=18; sFlt-1+PBS n=19 for females; *p<0.05; **p<0.01, ***p<0.001.

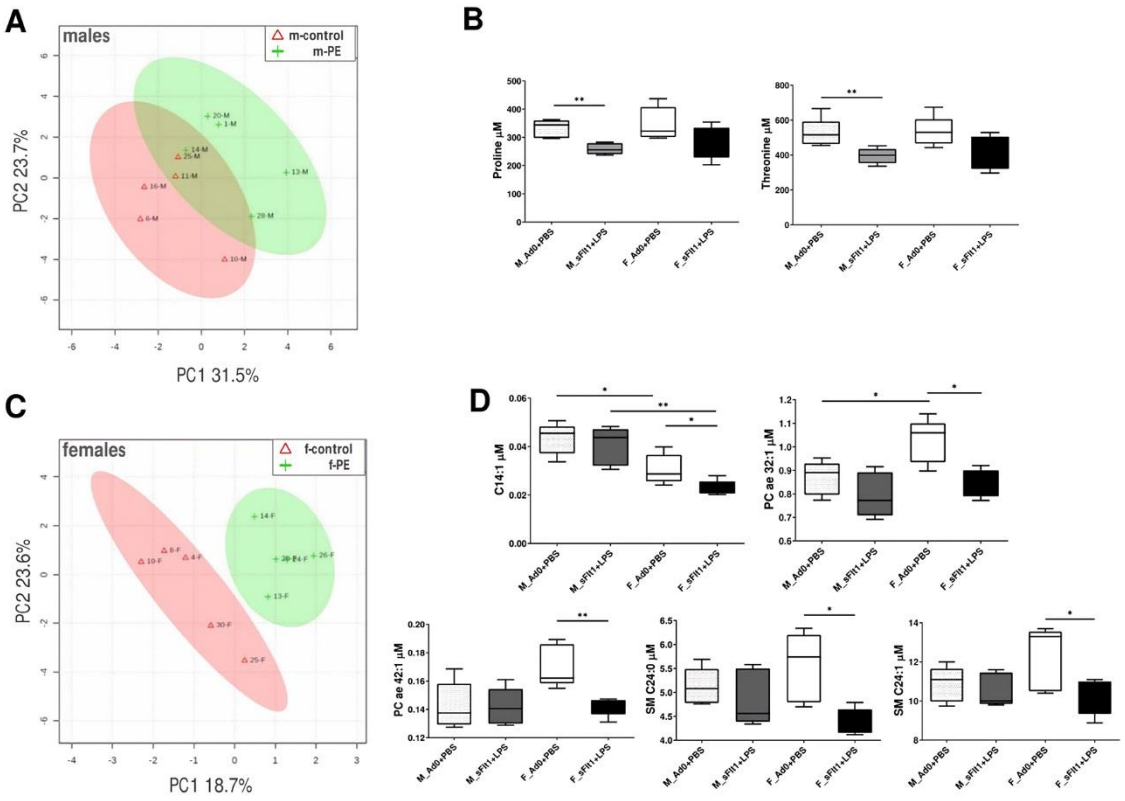

Figure 5. Fetal metabolomics are differentially affected by double hit preeclampsia. (A) Supervised partial least discriminatory analysis PLS DA on 141 metabolites in plasma of male control fetuses and double hit PE male fetuses (R^2=0.65; Q^2=0.0607), (B) Proline and threonine concentrations in male and female fetal plasma, (C) supervised partial least discriminatory analysis PLS DA on 141 metabolites in plasma of female control fetuses and double hit PE female fetuses (R^2=0.778; Q^2=0.461), (D) Plasma concentrations of changed metabolites in male and female fetal plasma. Data given as median and interquartile ranges (figure B, D), n=5 per group, * p<0.05; **p<0.01.

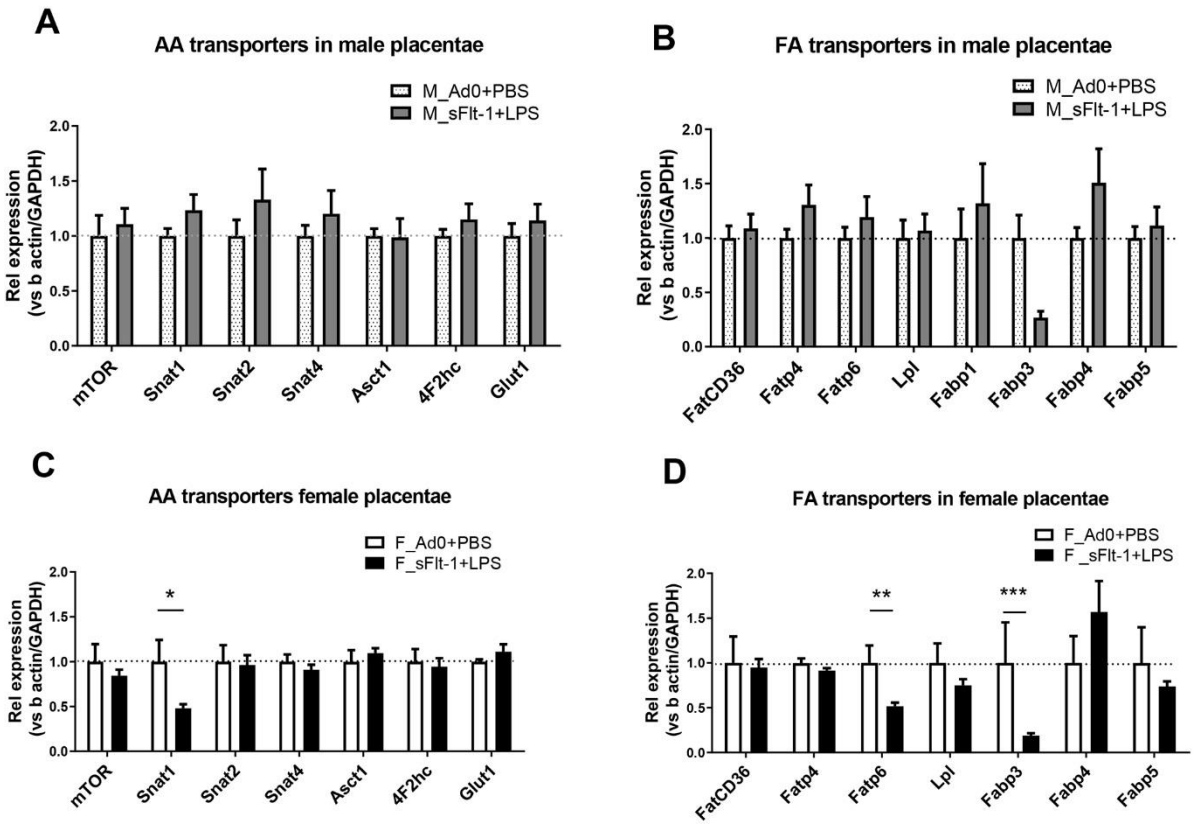

Figure 6. Gene expression analysis of important placental nutrient transporters. (A) Amino acid transporters and glucose transporter *Glut-1* and, (B) fatty acids transporters in male placentas (n=8-9), (C) amino acid transporters and *Glut-1* and, (D) fatty acid transporters in female placentas. Data given as ± SEM and number of samples per group Ad0+PBS n=8; sFlt-1+PBS n=9; *p<0.05; **p<0.01; ***p<0.001.

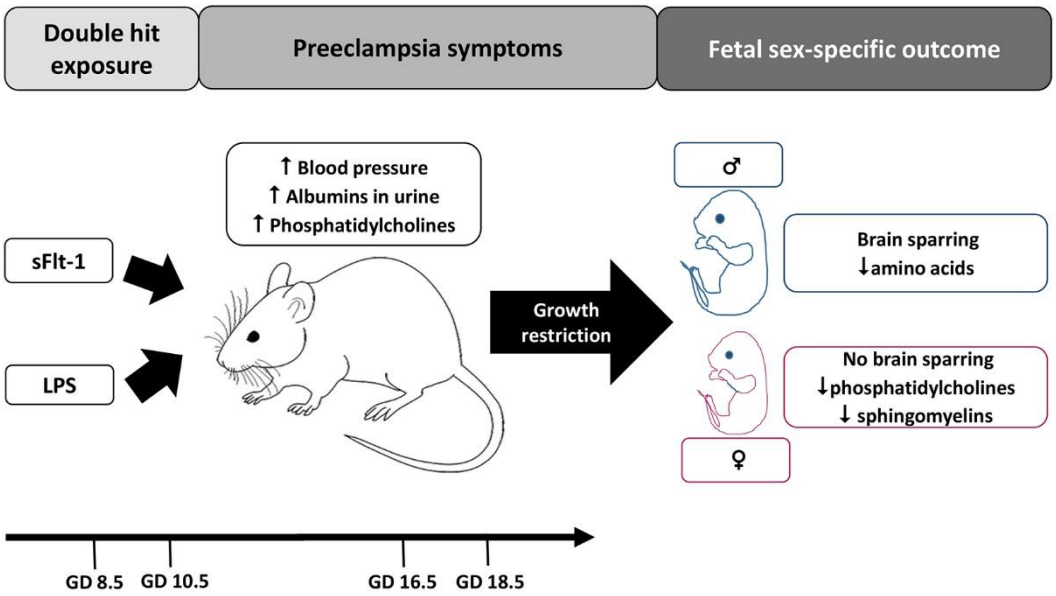

Figure 7. Graphical representation of maternal and fetal changes due to double hit experimental preeclampsia with respective timepoints.

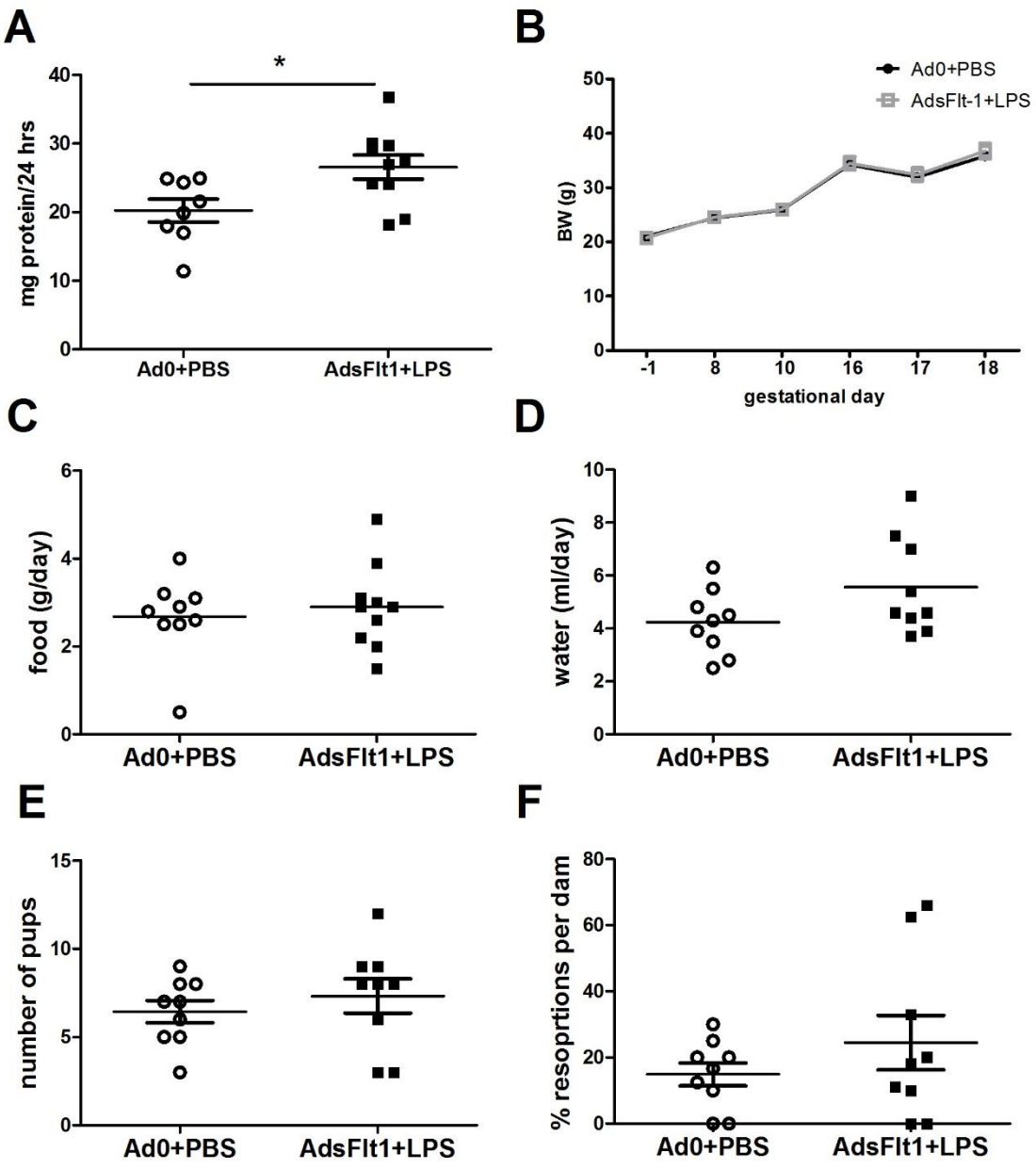

Figure S1. Maternal characteristics during double hit experimental preeclampsia (A) proteins in urine collected over 24 hours, (B) growth trajectories of pregnant dams, (C) food and (D) water consumption per day for pregnant dams, (D) number of pups and (E) and % of resorption per dam. Data given as median, *p<0.05.

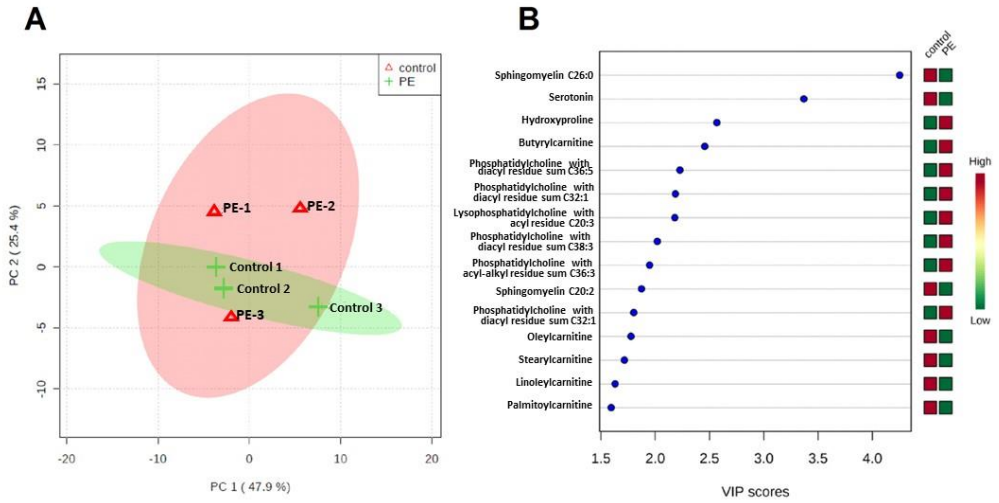

Figure S2. Metabolome characteristics of the dam (n=3), (A) PCA plot and, (B) VIP scores from supervised multivariate analysis of the dam metabolome.

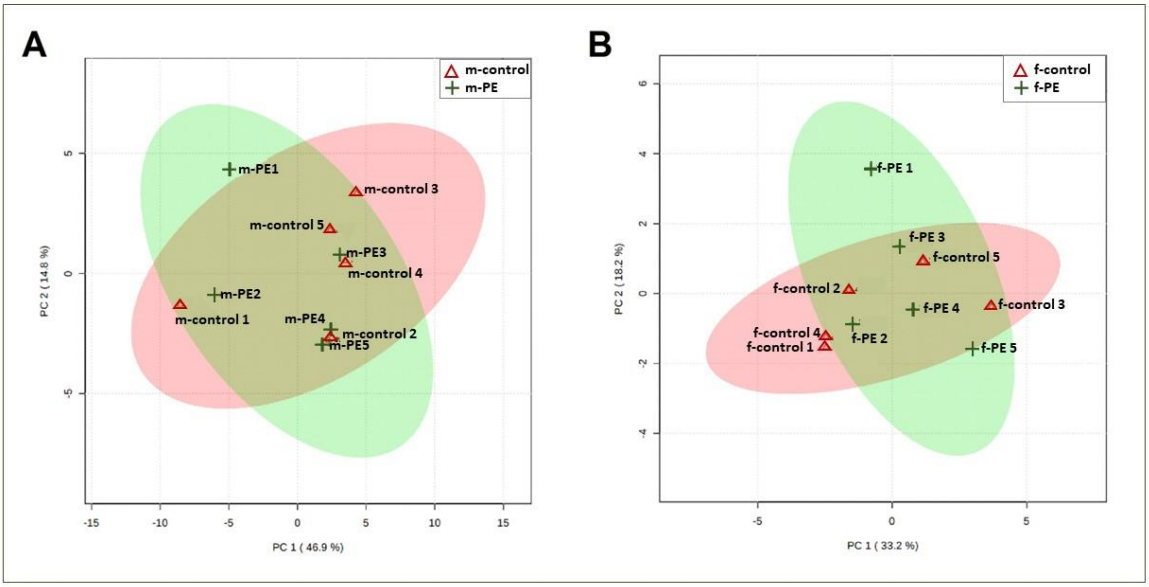

Figure S3. Metabolome characteristics of male and female fetuses exposed to double hit preeclampsia. (A) principal component analysis (PCA) plot for males and, (B) PCA plot for females.